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TAXONOMÍA MOLECULAR Y FILOGENIA DE ESPECIES DE *TRICHURIS* PARÁSITAS DE HOSPEDADORES VERTEBRADOS

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IGNACIO DAVID RODRÍGUEZ LLORENTE, Catedrático y Director del Departamento de Microbiología y Parasitología de la Facultad de Farmacia de la Universidad de Sevilla, por la presente:

CERTIFICO: Que la Tesis Doctoral titulada: "TAXONOMÍA MOLECULAR Y FILOGENIA DE ESPECIES DE *TRICHURIS* PARÁSITAS DE HOSPEDADORES VERTEBRADOS" presentada por la Graduada Dña. **JULIA RIVERO FERNÁNDEZ** para optar al grado de Doctor, ha sido realizada en el Departamento de Microbiología y Parasitología de la Facultad de Farmacia de la Universidad de Sevilla, bajo la dirección de las Dras. Dña. CRISTINA CUTILLAS BARRIOS y Dña. ROCÍO CALLEJÓN FERNÁNDEZ, reuniendo los requisitos exigidos.

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Marilyn Monroe

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I.1.1. Características generales de los nematodos

Los nematodos o gusanos redondos son un grupo de helmintos con forma cilíndrica o filariformes, no segmentados con simetría bilateral. Comprende uno de los grupos de invertebrados más abundante en casi cualquier tipo de ambiente y hábitat, así como con la mayor diversidad de especies. Se piensa que existen más de un millón de especies de nematodos (Lambshead, 2004), sin embargo, hasta la fecha, se conocen aproximadamente 25.000 especies (Navone y col., 2017). Ocupa el tercer lugar entre los *phyla* más ricos en especies dentro del Reino Animalia (junto con Arthropoda y Mollusca). La mayoría de las especies son de vida libre y en menor proporción de vida parásita. Tienen papeles críticos en el flujo de la energía y en el ciclo de nutrientes. Es un *phylum* de gran importancia por la gran diversidad de especies parásitas que habitan en todos los grupos de vertebrados, en plantas y en invertebrados. Desde un punto de vista antropocéntrico, parasitan a las plantas, humanos, animales domésticos y salvajes, algunos causantes de enfermedades del humano, y pueden servir como indicadores de cambio ambiental. Muchas de estas especies son de importancia agrícola, sanitaria y veterinaria (Navone y col., 2017).

El tamaño de los adultos varía mucho, desde unos pocos milímetros hasta más de un metro de largo. La mayoría son incoloros o de color blanquecino. El cuerpo de los adultos está cubierto con una capa exterior conocida como cutícula. La capa intermedia es la hipodermis y la más interna es la capa muscular. Los nematodos son dioicos y presentan dimorfismo sexual. La hembra es generalmente más grande que el macho y presenta su extremo posterior acabado en punta roma o recta, mientras

que el macho presenta su extremo posterior curvo o enrollado ventralmente. El aparato reproductor masculino consta de testículo, conducto deferente, vesícula seminal y conductor eyaculador que se abre en la cloaca. También pueden presentar estructuras accesorias copuladoras como espículas, bolsas copulatrices, o ambas. El aparato reproductor femenino está compuesto por el ovario, el oviducto, receptáculo seminal, útero, que puede ser sencillo o doble, vagina y vulva. Las hembras de los nematodos pueden producir huevos (ovíparas), larvas (vivíparas) o poner huevos que contienen larvas que eclosionan inmediatamente (ovovivíparas) (Navone y col., 2017). El modo de infección es por ingestión de huevos embrionados (infectantes) o larvas enquistadas en el músculo. En algunos casos, los huevos también pueden ser inhalados y posteriormente tragados. Además, la infección también puede ocurrir por la penetración a través de la piel de larvas filariformes infectantes o transmitidas por insectos chupadores de sangre (Mahmud y col., 2017).

Los nematodos parásitos causan una gran morbilidad y mortalidad en animales y humanos en todo el mundo, además de grandes pérdidas en la producción de alimentos (Jex y col., 2013; WHO, 2022). Destacan los geohelmintos causantes de las infecciones transmitidas por el suelo (STH = "Soil-transmitted helminths") por encontrarse entre los parasitismos más comunes en todo el mundo, afectando a las comunidades más pobres y desfavorecidas (WHO, 2022).

I.1.2. Taxonomía y filogenia de los nematodos en base a estudios morfológicos, biométricos y moleculares

Tradicionalmente, los parásitos se han clasificado según sus semejanzas morfológicas y biométricas, debido a la inmediata fuente de información que proporciona la morfología externa de un parásito y la evidente comparación entre especies.

Taxonómicamente, según Chitwood y Chitwood (1937) y posteriormente según Chabaud (1974), el *phylum* Nematoda se divide en dos grandes clases: Adenophorea (portadores de glándulas) y Secernentea (secretores). Esta división está basada en las características morfológicas y en los rasgos ecológicos. La clasificación morfológica está fundada principalmente en la presencia o ausencia de fasmidios, un par de órganos glándulo-sensoriales que desembocan en cada lado de la región posterior. Incluyen formas de vida libre, parásitas de plantas, de invertebrados y de vertebrados (Navone y col., 2017). La clase Secernentea incluye las especies parásitas (Orden Ascaridida, Spirurida, Strongylida y Oxyurida) y la mayoría de los nematodos terrestres de vida libre (Orden Rhabditida y Tylenchida). La clase Adenophorea incluye las subclases Enoplia (Orden Enoplida, Dorylaimida, Mermithida, Muspiceida y Trichocephalida), que incluyen vidas parásitas y de vida libre, y, Chromadoria (Orden Aerolaimida, Desmodorida, Desmoscolecida y Monhysterida), que incluyen formas de vida libre y marinos (Navone y col., 2017).

Actualmente, esta clasificación se sigue utilizando por una gran cantidad de especialistas parasitólogos, pero, se ha llegado a la convicción de que la identificación específica de los helmintos parásitos, a partir de las características externas, establece un gran problema, debido a sus ciclos de vida, a veces complejos, que dan origen a unas formas larvarias y adultas diferentes que dificultan el establecimiento de relaciones entre distintos estados de desarrollo de las especies.

Las restricciones de observación, junto con el déficit de caracteres fenotípicos útiles para el diagnóstico, son principalmente las razones por las que algunos autores creen que los enfoques tradicionales nunca pueden describir completamente la diversidad biológica, y que los métodos moleculares en base al ADN son probablemente los únicos caminos por seguir (Blaxter, 2003).

El uso de los marcadores moleculares para realizar clasificaciones de los seres vivos se estableció gracias al impulso del "dogma central de la biología molecular" (Hershey y Chase, 1952; Watson y Crick, 1953). El principal fundamento del uso de los caracteres moleculares era la evidencia del proceso evolutivo, más directo que los morfológicos, y que eran compartidos por todos los seres vivos, y se mostraban más fácilmente medibles y comparables entre todos ellos (Zuckerkandl, 1964; Suárez-Díaz y Anaya-Muñoz, 2008). Los marcadores moleculares proporcionan un mecanismo comparativo más estandarizado y cuantificable. Además, esto se vio reforzado con la incorporación de los algoritmos computacionales a la reconstrucción filogenética (Fitch y Margoliash, 1967, 1968).

Blaxter y col. (1998) realizaron un estudio del *phylum* Nematoda basándose en las secuencias de la subunidad pequeña ribosómica (SSU), identificando cinco clados diferentes (Dorylaimida, Mermithida, Mononchida, Rhabditida y Trichinellida). Esta clasificación fue actualizada según De Ley y Blaxter (2002, 2004) utilizando datos moleculares de la misma secuencia analizada anteriormente (SSU). Los datos moleculares confirmaron la existencia de dos clados principales: Chromadorea y Enoplea, divididos a su vez en tres linajes o subclases de nematodos: Chromadoria y, Dorylaima y Enoplia. Este estudio fue completado por Meldal y col. (2007), corroborando la monofilia de los nematodos. Esta clasificación ha sido aceptada durante las últimas dos décadas, incluyendo muchos grupos parafiléticos y polifiléticos, con la clasificación de taxones no estrictamente equivalente a la del punto de vista filogenético. Desde entonces se han publicado numerosos análisis filogenéticos respaldando y rechazando varias partes de la clasificación de "De Ley y Blaxter" (Leduc y col., 2018; Ahmed y Holovachov, 2021).

Hodda (2007) proporcionó una clasificación revisada de los nematodos introduciendo un rango de superorden y un estado superior de muchos

taxones. Sin embargo, esta clasificación no fue aceptada por los taxónomos. Recientemente, se ha realizado una nueva versión basada en la evidencia molecular y morfológica actual (Hodda, 2022). Esta clasificación refleja las relaciones evolutivas dentro del *phylum*, así como las áreas significativas de incertidumbre. Incluye 3 clases (Enoplea, Dorylaimia y Chromadoria), 8 subclases, 12 superórdenes, 32 órdenes, 53 subórdenes, 101 superfamilias, 276 familias, 511 subfamilias, 3030 géneros y 28537 especies (Hodda, 2022).

Recientes estudios filogenéticos basados en marcadores únicos (Holterman y col., 2006, 2019) y múltiples (Smythe y col., 2019; Ahmed y col., 2022), apoyan la división del *phylum* en tres clases, con la divergencia basal con Enoplia, siendo hermana con el clado de Dorylaimia y Chromadoria, rechazando la clasificación anterior.

I.1.3. Uso de la técnica MALDI-TOF MS en nematodos

Tradicionalmente, la espectrometría de masas (EM) fue utilizada como una técnica analítica de química clínica, pero no fue hasta comienzos del siglo XXI, con la aparición de las técnicas de "ionización suave" cuando se consiguió analizar biomoléculas de gran tamaño usando un láser como fuente de ionización y una matriz orgánica para facilitar el proceso. De ahí el nombre MALDI-TOF MS, que obedece a las siglas "matrix-assisted laser desorption/ionization time of flight mass spectrometry" o "espectrometría de masas por ionización-desorción asistida por matriz con tiempo de vuelo". De este modo, MALDI-TOF MS es una técnica de ionización que permite el análisis de moléculas complejas como las proteínas, generando huellas dactilares de proteínas (espectros) a partir de extractos de organismos (Figura 1) (Bizzini y col., 2010). La obtención de espectros permite la creación de una base de datos espectral de referencia que se puede utilizar para identificar especies de manera sencilla, rápida y reproducible (Yssouf y col., 2014).

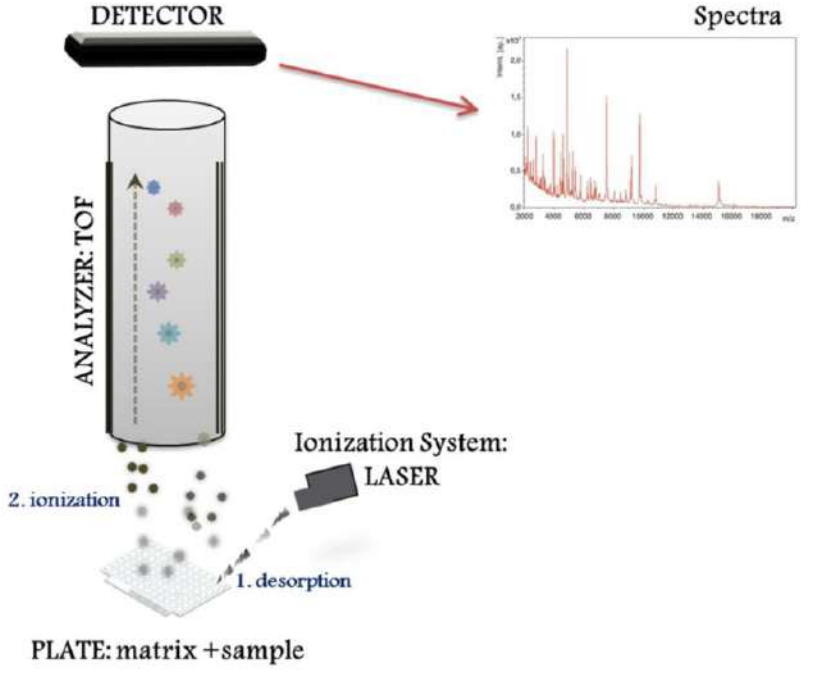

Figura 1. Esquema del modo de trabajo de un sistema MALDI-TOF MS (Torres-Sangiao y col., 2021).

La aceptación del uso del MALDI-TOF MS se atribuye a la relativa sencillez para la preparación de las muestras, la rapidez con la que se obtienen y analizan los resultados y la alta fiabilidad para la identificación específica que posee. Además, comparándola con otras técnicas moleculares, la relación coste efectividad es bastante competitiva.

La utilización de la EM comenzó en el análisis de proteínas de muestras en laboratorios de química médica (Tanaka y col., 1988; Marvin y col., 2003). En 1975, Anhalt y Fenselau (1975) propusieron por primera vez la técnica de MALDI-TOF MS como un método para caracterizar bacterias, y demostraron que diferentes especies bacterianas mostraban espectros de masas de proteínas específicas, pudiéndose utilizar para una identificación rápida. En virtud de ello, desde su descubrimiento, esta técnica ha sido introducida en la mayoría de los laboratorios de microbiología clínica

primero, en el análisis rutinario para la identificación de microorganismos, estableciéndose como método de diagnóstico de referencia, y, cada vez más, en laboratorios clínicos. Además de bacterias (Seng y col., 2009; Sandrin y col., 2013) se ha demostrado su uso para micobacterias (Clark y col., 2013; El Khéchine y col., 2011), hongos (Angeletti, 2017), y más recientemente, en virus (Sjöholm y col., 2008), protozoos (Villegas y col., 2006; Cassagne y col., 2014), artrópodos (Yssouf y col., 2016; Zurita y col., 2019) y algunos nematodos (Ahmad y col., 2012a; Millares y col., 2012; Marzano y col., 2020).

A diferencia de la bacteriología clínica, se han realizado pocas investigaciones relacionadas con la aplicación de la técnica del MALDI-TOF MS para la identificación de parásitos de importancia humana o veterinaria (Singhal y col., 2016).

Los primeros estudios utilizando esta técnica se realizaron a diferentes parásitos protozoos como *Leishmania* spp. (Cassagne y col., 2014), *Giardia* spp. (Villegas y col., 2006), *Cryptosporidium* spp. (Magnuson y col., 2000), *Trypanosoma* spp. (Avila y col., 2016), *Plasmodium* spp. (Marks y col., 2004) y *Dientamoeba* spp. (Calderaro y col., 2018).

Las infecciones causadas por los nematodos que pertenecen a las geohelmintiasis como *Ascaris lumbricoides* (ascárido), *Trichuris trichiura* (tricocéfalo), y *Ancylostoma duodenale* y *Necator americanus* (anquilostomas), representan una carga mundial considerable de morbilidad y se encuentran entre las infecciones más comunes en las poblaciones marginadas en los trópicos y subtrópicos (Hotez y col., 2008), infectando a casi el 24% de la población mundial (WHO, 2022). El diagnóstico es fundamental para un tratamiento eficaz, pero se requiere al menos una infraestructura básica de laboratorio, microscopios ópticos y técnicos de laboratorio capacitados para realizar la tarea, que podría no estar disponible en áreas remotas de los países de zonas tropicales y subtropicales. Por el contrario, en los entornos de altos recursos los

conocimientos sobre identificación microscópica de helmintos están disminuyendo en muchos laboratorios. Por esa razón, sorprende que la aplicabilidad potencial de la técnica de MALDI-TOF MS como herramienta de diagnóstico para helmintos de importancia humana y veterinaria aún no se haya evaluado, en concreto porque la técnica ha sido empleada con éxito para la identificación de parásitos nematodos de plantas (Ahmad y col., 2012b, 2014).

Fue en 2015 cuando se publicó el primer artículo sobre el MALDI-TOF MS como herramienta de diagnóstico para la detección directa de nematodos con el objetivo de encontrar un nuevo método de diagnóstico más rápido, eficaz, sencillo, automatizado y económico que las técnicas de análisis del ADN. En primer lugar, para diferenciar especies de *Dirofilaria* (Pshenichnaya y col., 2015), permitiendo a través de este método la diferenciación entre *Dirofilaria repens* y *Dirofilaria immitis*, mediante el estudio de su espectro de proteínas. Poco después, lo siguió un estudio para la identificación y diferenciación de *Trichinella* spp. con el mismo fin, de reducir tiempo y trabajo, creando además una base de datos de espectros de referencia (Mayer-Scholl y col., 2016). Ambos estudios proporcionaron evidencia de que MALDI-TOF MS podía diferenciar de manera fiable entre especies dentro de los géneros de nematodos. Hasta la fecha, se han realizado pocos estudios en parásitos nematodos, únicamente las especies citadas anteriormente, y *Ascaris* spp. (Nagorny y col., 2019), *Coronocyclus* spp. (Bredtmann y col., 2017) y *Anisakis* spp. (Marzano y col., 2020), han sido objeto de estudio para la identificación a través de la técnica de MALDI-TOF MS. De este modo, en estos estudios se ha puesto de manifiesto la utilidad de esta técnica.

I.2. GÉNERO *TRICHURIS*

I.2.1. Morfología

Las especies del género *Trichuris* son nematodos intestinales que se localizan principalmente en el ciego, y con menor frecuencia, en otras partes del intestino grueso de sus hospedadores. A los adultos pertenecientes a este género se les conoce comúnmente como "gusanos látigos", debido a su forma característica de látigo con la porción anterior del cuerpo, larga y fina, conteniendo el esófago, y la zona posterior más ancha, gruesa y corta, que contiene el aparato reproductor y el intestino (Figura 2A-B). El nombre genérico *Trichuris* se aceptó bajo la impresión de que la zona delgada del cuerpo era la zona caudal. No obstante, posteriormente, se demostró que la región delgada correspondía con el extremo anterior, y consecuentemente, se le nombró como *Trichocephalus* (*Trichos* = pelo; *kephale* = cabeza). Sin embargo, esta denominación no fue aceptada debido al principio de prioridad del Código Internacional de Nomenclatura Zoológica (ICZN), donde *Trichuris* tiene preferencia y el resto de los nombres se consideran sinónimos de éste (Gállego-Berenguer, 2013).

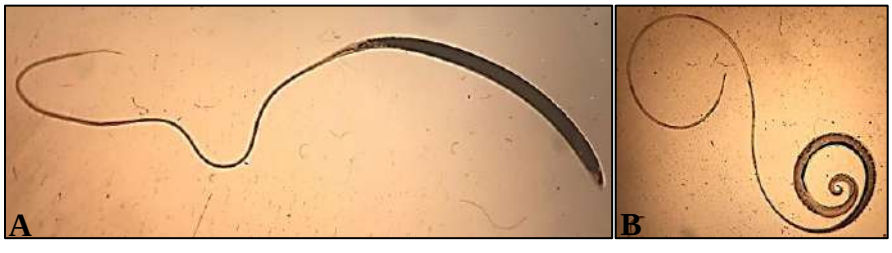

Figura 2. Hembra (A) y macho (B) pertenecientes al género *Trichuris*.

Los vermes adultos pertenecientes a este género presentan un esófago moniliforme muy largo y delgado, ocupando aproximadamente dos terceras partes de la longitud total del cuerpo (Figura 2A-B). El esófago

presenta en la parte anterior una pequeña zona muscular reducida, mientras que el resto consiste en un tubo de pared delgada rodeado por esticocitos o glándulas grandes y unicelulares (Bogitsh y col., 2005). Estos esticocitos forman una estructura conocida como esticosoma (Figura 3A). La boca es una simple apertura sin labios. El sistema excretor está ausente en ambos sexos. La superficie ventral de la región esofágica tiene una banda de poros diminutos que conducen a células glandulares y no glandulares subyacentes conocida como banda bacilar. Esta banda bacilar es típica del orden (Figura 3B). La función de estas células no es conocida, sin embargo, su ultraestructura sugiere que las células glandulares pueden desempeñar un papel en la regulación osmótica o de iones, y que las células no glandulares pueden estar implicadas en la formación de la cutícula y el almacenamiento de alimentos. Son organismos dioicos, y ambos sexos presentan una sola gónada (Skrjabin y col., 1957; Schmidt y Roberts´, 2005).

Figura 3. Esticocitos (A) y banda bacilar (B) pertenecientes a la zona anterior del adulto de *Trichuris*.

Los adultos del género *Trichuris* presentan un acusado dimorfismo sexual en la zona posterior del cuerpo. El tamaño de las hembras es un poco mayor que el de los machos. En la hembra, el extremo posterior está ligeramente curvado, ocupado por el genital y el intestino, cuyo ano es terminal (Figura 2A). El aparato reproductor presenta desde la región distal el ovario-oviducto-útero y la vagina (Figura 4A). La vagina presenta circunvoluciones marcadas en algunas especies y los huevos dispuestos en fila, finalizando en la vulva, localizada cerca de la unión de la parte anterior y posterior del cuerpo. La vulva es una protuberancia que sobresale por encima de la superficie del cuerpo y rodea la abertura de la vagina (Figura 4B). La superficie puede o no estar rodeada de espinas (según la especie), que se asemejan a las de la vaina espicular de los machos. El útero se encuentra lleno de huevos sin embrionar (Skrjabin y col., 1957; Schmidt y Roberts´, 2005).

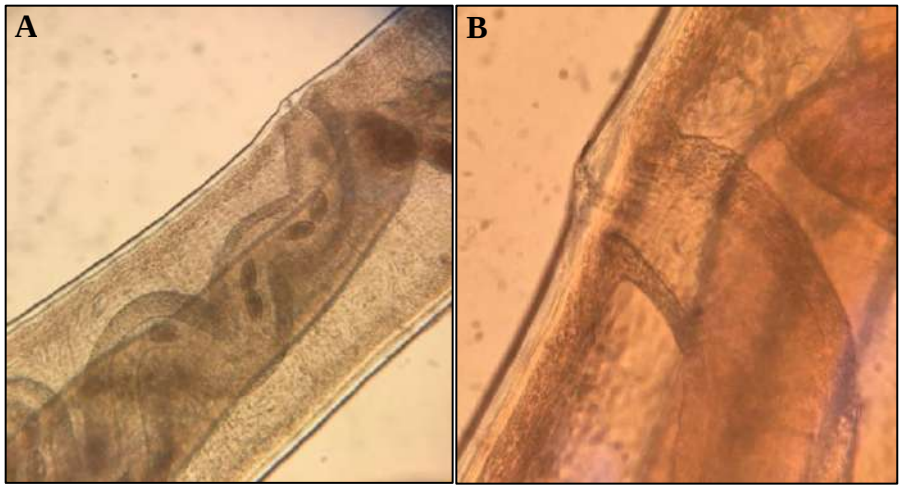

Figura 4. Hembra de *T. trichiura*. Vulva y vagina (A). Vulva (B).

El macho presenta el extremo terminal curvado ventralmente (Figura 2B y Figura 5A). El aparato genital es un tubo largo con diferentes secciones con funciones diferentes desembocando en una cloaca terminal. Únicamente presenta una espícula que está rodeada por una vaina retráctil,

conocida como vaina espicular, que está armada normalmente con unas características finas espinas cuticulares (Figura 5B). Cuando la espícula se encuentra invaginada, en ciertas especies, se aprecian un par de papilas caudales. En ambos lados de la región pericloacal está presente un grupo de pequeñas papilas (Skrjabin y col., 1957; Schmidt y Roberts´, 2005). Asimismo, algunas especies presentan la espícula fuera del tubo cloacal distal, conocido como tubo espicular (Eberhardt y col., 2019).

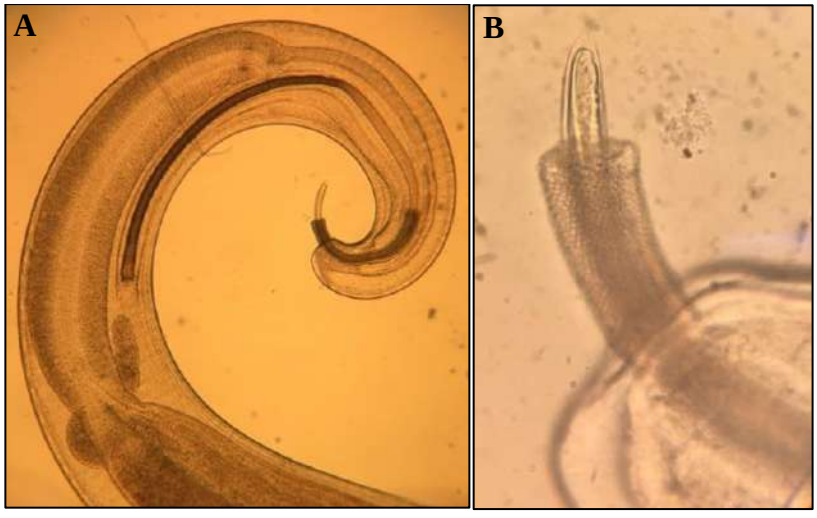

Figura 5. Macho de *T. trichiura*. Extremo posterior enrollado mostrando la espícula y la vaina espicular (A). Vaina espicular con espinas y extremo anterior de la espícula (B).

Los huevos tienen forma típica de barril o de balón de rugby, con tapones operculares prominentes en ambos polos, con apariencia de tapones mucosos (Figura 6A). Presentan además de la membrana vitelina, una cubierta triple cuya capa más externa se encuentra impregnada de bilis. Tras la oviposición, los huevos contienen en su interior un cigoto no embrionado (Beaver y col., 2003).

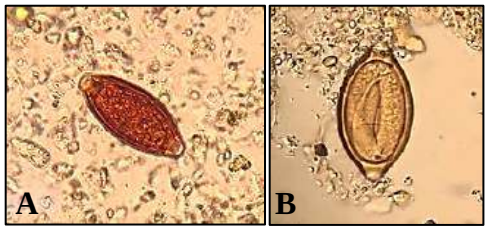

Figura 6. Huevo de *T. trichiura* sin embrionar (A). Huevo de *T. trichiura* con la larva desarrollada en el interior (fase infectante) (B).

La fase infectante es el huevo larvado (Figura 6B). El cuerpo de la larva es alargado con el extremo cefálico redondeado. La apertura bucal presenta un estilete afilado con protuberancias y el extremo caudal tiene un pequeño tubérculo que sobresale (Skrjabin y col., 1957).

Los nematodos pertenecientes al género *Trichuris* tienen una distribución cosmopolita y parasitan a un amplio espectro de hospedadores mamíferos abarcando primates y primates no humanos (PNH), roedores, rumiantes y marsupiales (Eberhardt y col., 2019).

I.2.2. Ciclo biológico

Las especies del género *Trichuris* presentan un ciclo de vida directo. Los adultos se localizan fundamentalmente en el ciego y en el colon de los hospedadores, donde viven enhebrados a la mucosa intestinal por el extremo anterior del cuerpo, y la parte posterior se encuentra en la luz del lumen. Con el extremo anterior se fijan al hospedador resistiendo los movimientos del intestino y permitiendo el paso de un fermento digestivo secretado por el verme, el cual transforma el material de la mucosa en un líquido que puede succionar el parásito (Lapage, 1976).

Las hembras comienzan la oviposición en el ciego entre 60 y 70 días después de la infección. Las hembras ponen entre 3.000 y 20.000 huevos por día. La vida útil de los vermes adultos es de aproximadamente 1 año. Los huevos no embrionados se eliminan con las heces en el suelo y ahí se

desarrollan hasta presentar la larva 1 en su interior. Estos huevos con larva 1 son ya infectantes en 15 a 30 días. Después de la ingestión de los huevos embrionados, a través de manos o alimentos contaminados del suelo, eclosionan en el intestino delgado, en el duodeno, y liberan las larvas que maduran en las criptas intestinales (Monroe, 1995). Después de un periodo de tiempo de maduración, las larvas migran al colon proximal y allí maduran a vermes adultos en un periodo de 1-3 meses, y, finalmente, se establecen como adultos en el ciego (Figura 7) (Fishman y Perrone, 1984; Monroe, 1995; Chandra y Long, 1998; Santos y col., 2005; CDC, 2013).

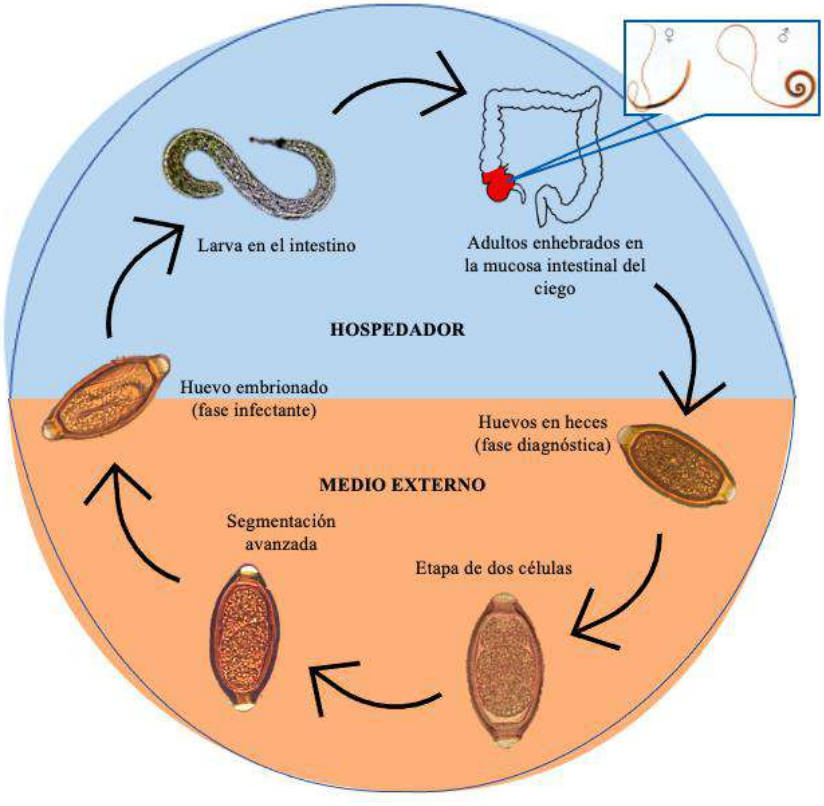

Figura 7. Esquema del ciclo biológico de *Trichuris* spp.

I.2.3. Generalidades de la parasitosis causada por *Trichuris*

I.2.3.1. Epidemiología e importancia sanitaria

La infección por especies de *Trichuris* se establece, fundamentalmente, en el ciego de los hospedadores. *T. trichiura* es responsable de la "enfermedad del gusano látigo" o más conocida como tricuriasis en los humanos. Es una enfermedad cosmopolita, es decir, afecta a hospedadores de todo el mundo, pero desarrollándose, con mayor frecuencia, en áreas con clima tropical. Constituye una de las geohelmintiasis o STH, unas de las parasitosis más comunes en el mundo, infectando a casi el 24% de la población mundial, afectando a las comunidades más desfavorecidas y pobres (WHO, 2022). *T. trichiura* es la segunda geohelmintiasis más común en los humanos, y se estima que aproximadamente 604-795 millones de personas en el mundo estén parasitadas con este parásito (CDC, 2020). Más de 267 millones de niños en edad preescolar y más de 568 millones de niños en edad escolar viven en áreas endémicas de STH y necesitan tratamiento e intervenciones preventivas o de control de la infección (Figura 8) (WHO, 2019). Su distribución geográfica se superpone a la de la ascariasis, con el que se encuentra asociado frecuentemente (Gállego-Berenguer, 2013). Estos parasitismos pueden afectar al estado nutricional del hospedador al alimentarse del contenido del intestino o de los tejidos de éste (por ejemplo, sangre), alterando la digestión o la absorción de nutrientes, provocando una respuesta inflamatoria.

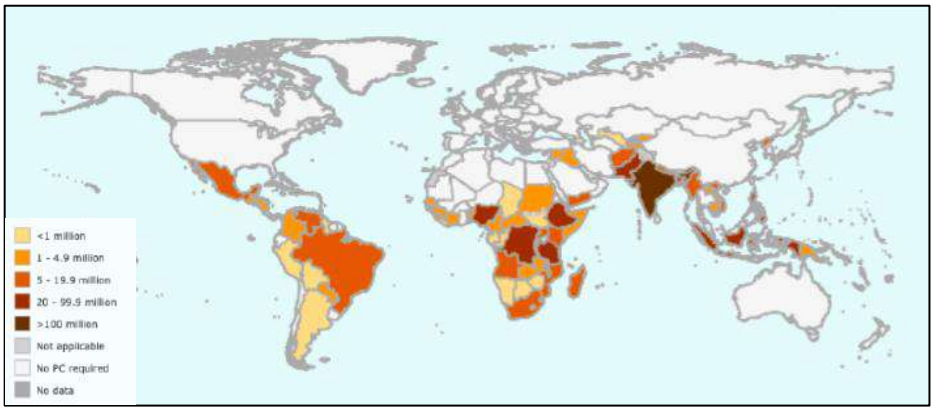

Figura 8. Prevalencia del número de niños en edad pre-escolar y escolar, que requieren quimioterapia preventiva para las STH en 2021 (WHO, 2022).

Las personas que viven en los países en vías de desarrollo, con redes de saneamientos y condiciones de hacinamiento inadecuadas, combinado con la falta de acceso a la higiene personal y los bajos niveles de educación, hacen que sean más susceptibles a esta enfermedad. La tricuriasis está asociada a lugares donde no se dispone de acceso a la higiene personal y prácticas de saneamiento adecuadas, un acceso inadecuado al agua potable, que conduce a un aumento del riesgo de infección, lo que afecta a millones de personas en poblaciones de regiones tropicales y subtropicales y, sobre todo, afecta a los niños de 5 a 15 años, que son más vulnerables por el alto riesgo de exposición que presentan, con una disminución de la intensidad y frecuencia en la edad adulta (Bethony y col., 2006; Jourdan y col., 2018).

I.2.3.2. Clínica

Las manifestaciones clínicas de la infección por STH en individuos, se correlacionan con el número de helmintos, y, por lo tanto, con el número de huevos en heces, y la intensidad de la infección.

La infección por *Trichuris* es normalmente asintomática, excepto en casos fuertes de infección. Debido a que en el ciclo de vida no se produce migración de las larvas, los síntomas clínicos están asociados con manifestaciones gastrointestinales agudas y crónicas resultantes del parasitismo por los vermes adultos en el tracto intestinal (Bethony y col., 2006).

Los adultos se encuentran principalmente en el colon de los hospedadores humanos, pero también pueden estar en el apéndice y el recto. La parte anterior del gusano está enhebrada en la mucosa del colon y, teóricamente, produciendo poco daño al hospedador, ya que la carga parasitaria, generalmente, no es elevada. Por esa razón, las infecciones leves se limitan al ciego y al colon ascendente. En infecciones graves, podemos encontrar afectación del extremo final del intestino delgado, el íleon terminal, o incluso el recto (Bethony y col., 2006).

Una carga parasitaria de menos de 100 adultos apenas causa síntomas, en cambio, los niños pequeños son particularmente más susceptibles a infecciones graves que pueden involucrar a más de 1000 adultos parásitos (Cooper y Bundy, 1987).

La inflamación en el sitio de unión por un gran número de tricocéfalos produce colitis (inflamación de la mucosa del colon). La colitis de larga duración produce un trastorno clínico semejante a la enfermedad inflamatoria intestinal, que entre sus síntomas incluye dolor abdominal crónico y diarrea, así como retraso del crecimiento, anemia de enfermedad crónica y dedos en palillo de tambor (Figura 9A) (Bundy y Cooper, 1989; Kellerman y Rakel, 2021). El origen del extraño engrosamiento de los extremos de los dedos (dedos en palillo de tambor), se debe al nervio vago que une la rama aferente de un arco reflejo, y sugiere que la afectación al íleon y el colon proximal, inervados por el nervio vago, es el responsable de este síntoma (Kitis y col., 1979). Es más, las infecciones de moderadas

a graves afectan negativamente a la función cognitiva en los niños (Nokes y col., 1992).

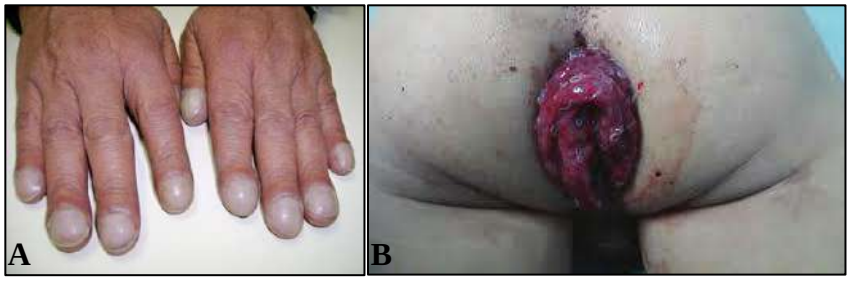

Figura 9. Imagen que muestra un paciente con los dedos en forma de palillos de tambor (A) (Desherinka, CC BY-SA 4.0, vía Wikimedia Commons) y paciente infantil con prolapso rectal (B) (Argueta, 2015) causado por *T. trichiura.*

La colitis se produce por el efecto traumático sobre el epitelio intestinal y la submucosa producido por los delgados extremos anteriores de los vermes que quedan enhebrados en la mucosa intestinal. Además, las respuestas inmunitarias Th2 a las infecciones por helmintos pueden causar patologías al hospedador y dañar órganos. Por esta razón, en infecciones parasitarias crónicas, la inmunidad mediada por los linfocitos Th2 puede mediar respuestas fibróticas patológicas (Zaccone y col., 2003). Se ha demostrado que la infección da como resultado la producción del factor de necrosis tumoral (FNT)-α por parte de las células de la lámina propia en el colon, lo que puede contribuir a la falta de apetito y la emaciación con una infección significativa (Kellerman y Rakel, 2021). También hay un aumento en la desgranulación de mastocitos. Esto sugiere que la inflamación puede ser considerada una respuesta anafiláctica tisular local (Cooper y col., 1992).

El síndrome de disentería de *Trichuris* (SDT) es una manifestación aún más grave que una fuerte infección por tricocéfalos, que se presenta con una fuerte infección en el colon que cursa con disentería crónica y

prolapso rectal. Además, puede cursar con náuseas, vómitos, diarrea mucoide, palpitaciones en los dedos de las manos, anemia por deficiencia de hierro, retraso del crecimiento y desnutrición (Bundy y Cooper, 1989; Mahmud y col., 2017).

El prolapso rectal es otro síntoma común en infecciones severas por *Trichuris* (Figura 9B). Se produce una invaginación recto-rectal, es decir, del intestino en sí mismo. Este mecanismo difiere del prolapso rectal asociado a un debilitamiento de la musculatura pélvica y por ello el prolapso rectal en la tricuriasis tiene un buen pronóstico tras la expulsión de los vermes. Además, también se han notificado con frecuencia apendicitis provocadas por la obstrucción de la luz de ese órgano a causa de los vermes (Faust, 2003).

Finalmente, con frecuencia, la respuesta inmune obtenida por la interacción parásito-hospedador, en este caso por *Trichuris*, induce una cascada de respuestas inmunológicas caracterizadas por hiper-IgE y por eosinófilos o por respuestas humorales mediadas en el intestino grueso (Lillywhite y col., 1991; King y col., 1993; Belhassen-García y col., 2014). Hay dos factores críticos asociados con la expulsión de los vermes: el genotipo inflamatorio del hospedador y la carga de infección. La interacción entre las dos partes de la respuesta inmunitaria, tanto innata como adaptativa, son responsables de la erradicación del parásito. Las células inmunitarias son las responsables de conducir la expulsión de los vermes al aumentar el recambio de las células epiteliales intestinales y la secreción de mucina. Específicamente, las citocinas IL-4 e IL-13 promueven la contracción del músculo liso acelerando la expulsión (Marillier y col., 2008; Sharba y col., 2019; Darlan y col., 2021).

I.2.3.3. Diagnóstico

El diagnóstico de infecciones por *Trichuris* spp. puede realizarse a través de técnicas convencionales o parasitológicas, por técnicas moleculares o por otras técnicas de diagnosis.

La elección de los métodos de diagnóstico más apropiados debe tener en cuenta la precisión del método, la endemicidad de las infecciones y la disponibilidad de los recursos, incluido el costo del diagnóstico. Además, las coinfecciones con otras especies parásitas son comunes en áreas endémicas, lo que dificulta el diagnóstico.

I.2.3.3.1. Técnicas convencionales o parasitológicas

La técnica más ampliamente utilizada para la detección de *Trichuris* es el examen de heces para determinar la presencia y, si es posible, la cantidad de huevos (Else y col., 2020). Cabe destacar que estos nematodos no liberan huevos a un ritmo constante, por lo tanto, es necesario una recolección de muestras fecales múltiples (3 veces en días alternos) para aumentar la sensibilidad de los métodos.

Actualmente existen varias técnicas para detectar la presencia de huevos de *Trichuris* en muestras fecales de forma cuantitativa o cualitativa. Los métodos cuantitativos tienen la ventaja de registrar el número de huevos por gramo de heces (EPG = “Eggs per gram of faeces”), una medida indirecta de la intensidad de la infección, que es un indicador directamente asociado con la morbilidad a causa de helmintos. Una de las primeras evaluaciones de diagnóstico cuantitativo de una tricuriasis antes y después de recibir tratamiento, se remonta a 1950 (Burrows, 1950), y actualmente, sigue vigente.

Durante una infección intensa, los huevos se pueden detectar a través del examen fecal directo, en el cual se coloca una gota de heces homogeneizada con solución salina fisiológica 0,9% (p/v) en un portaobjetos y, posteriormente, se observa al microscopio. Sin embargo,

esta técnica tiene una baja sensibilidad. Asimismo, existen otras técnicas para detectar el parasitismo, incluyendo la técnica de concentración de formol-éter (FECM = "Formol-eter concentration method") (Allen y Ridley, 1970; Knight y col., 1976), la técnica de concentración por flotación utilizando soluciones saturadas de azúcar, la técnica de Sheather (Gupta y Singla, 2013) o de cloruro sódico, la técnica de Willis (Euzeby, 1981), la técnica de Kato-Katz (KK) (Katz y col., 1972; Ramsan y col., 1999), la técnica de McMaster (Rossanigo y Gruner, 1991; Pereckiené y col., 2007), la técnica de FLOTAC (Cringoli, 2006; Utzinger y col., 2008), la técnica de Mini-FLOTAC (Cringoli y col., 2013), la técnica de Mini Parasep® solvent-free (SF) (Zeeshan y col., 2011; Kitvatanachai y Rhongbutsri, 2017), o la técnica de Midi-Parasep® (Zeeshan y col., 2011). En áreas endémicas, principalmente en lugares con recursos limitados, la Organización Mundial de la Salud (OMS) recomienda el uso del KK para contabilizar el EPG, y también recomiendan usar 2 gotas de muestra por portaobjetos por muestra analizada (WHO, 2002), ya que Nikolay y col. (2014) demostraron que preparar y analizar más de dos gotas de muestra de heces utilizando KK, solo aumenta ligeramente la sensibilidad para la detección de *Trichuris* (84,8% dos muestras vs. 90,5% 3 muestras). Esta técnica ha sido ampliamente utilizada debido a que tiene una moderada sensibilidad para áreas alta o moderadamente endémicas, es relativamente simple, y la mayoría de los materiales se puede reutilizar para realizar varios exámenes. Sin embargo, el examen de heces basado en el KK depende en gran medida del observador y, en infecciones de baja intensidad, carece de sensibilidad, específicamente cuando se acerca al final de la fase de eliminación (Barda y col., 2015; Utzinger y col., 2015). En los últimos años se han realizado más de 200 estudios para comparar las diferentes técnicas de diagnóstico para identificar *Trichuris*. El uso no estandarizado de las diferentes técnicas se debe a la falta de un estándar de oro para el diagnóstico de infecciones causadas por helmintos

intestinales (Levecke y col., 2009, 2011; Albonico y col., 2013). Sayasone y col. (2015) llevaron a cabo una comparación entre KK por triplicado y FECM y demostraron que para la detección de infección por *Trichuris*, FECM es menos sensible que KK. Estos datos fueron posteriormente confirmados por Funk y col. (2013), que detectaron un mayor rendimiento de la técnica KK en comparación con el método de concentración de formalina acetato de etilo y Midi Parasep®. También se comparó entre la técnica KK y McMaster y se encontró un mayor rendimiento en KK, aunque no significativo estadísticamente (Levecke y col., 2011).

La técnica FLOTAC ha sido comparada con las técnicas mencionadas anteriormente y resultó ser más sensible (Knopp y col., 2011; Nikolay y col., 2014), aunque presentaba ciertas limitaciones. En primer lugar, el procedimiento es complejo y largo, además de necesitar material adicional, que no siempre está disponible en entornos de bajos recursos. En segundo lugar, debido a las limitaciones de las técnicas de flotación, algunos desechos pueden flotar junto con los huevos, dificultando así el conteo de huevos (Cringoli y col., 2010; Knopp y col., 2011). Sin embargo, presenta la ventaja de poder realizarlo en muestras fijadas, lo que permite el almacenamiento de las heces durante algunas semanas (Cringoli y col., 2010). La técnica Mini-FLOTAC es una evolución de esta última, diseñada para ser un método más simple y de menos costo, demostrando ser una buena alternativa a KK y otros métodos directos de diagnóstico (Barda y col., 2014).

Las técnicas mencionadas anteriormente también se utilizan en países endémicos. Además, las técnicas de McMaster y, más recientemente, las de Mini-FLOTAC, también han sido recomendadas por la OMS para monitorizar la eficacia de los medicamentos (WHO, 2013).

Existe una gran limitación en las técnicas directas debido a que las muestras de heces deben ser frescas. Por lo tanto, es necesario un método de diagnóstico que permita la identificación con muestras conservadas (Fernández-Niño y col., 2015).

Hay otros métodos de diagnóstico que han evolucionado a partir del perfeccionamiento de métodos de parasitología basados en el diagnóstico a distancia. Muchas de estas innovaciones diagnósticas tienen la ventaja de ser portables, de bajo costo, fáciles de usar y que no requieren un suministro eléctrico constante.

FecPak^{G2} es una técnica de diagnóstico basada en la flotación de huevos usando una solución salina hipersaturada. Su innovación consiste en el uso de un dispositivo de alta tecnología para el estudio de la muestra. Una vez la solución se mezcla con la muestra, se llena un pocillo con ésta y se lee a través de una cámara conectada a una Tablet con internet, que recibe la imagen y se la envía a parasitólogos expertos. Una vez los expertos analizan los resultados analizando la imagen, le devuelven el resultado a la persona o técnico que procesó la muestra (Ayana y col., 2018; Rashid y col., 2018). Sin embargo, la sensibilidad de esta técnica en comparación con las técnicas convencionales es todavía considerablemente inferior (Moser y col., 2018).

Otra técnica es la tecnología lab-on-a-disk (LOD). Es una plataforma de microfluidos basada tanto en la centrifugación como en la flotación de huevos para su concentración dentro de una zona de imágenes, seguida de la captura de imágenes. Una evaluación de esta técnica mostró su éxito incluso en entornos de baja intensidad de infección (Sukas y col., 2019).

En la misma línea de introducir dispositivos electrónicos, está el diagnóstico móvil. Para ello, se utiliza un teléfono móvil inteligente como microscopio. Este método presenta una alta sensibilidad para el parásito *Trichuris*, pero las imágenes no eran de alta calidad y, a menudo, se pasaban por alto infecciones leves (Bogoch y col., 2013). Posteriormente,

se introdujo un nuevo dispositivo móvil, el microscopio portátil Newton, el cual no mostró diferencias significativas entre infecciones leves y moderadas/intensas (Bogoch y col., 2014).

Actualmente, se están desarrollando métodos de inteligencia artificial basados en el diagnóstico de muestras digitalizadas, realizando una cuantificación objetiva y automática de la infección. Este método incluye un sistema de digitalización basado en una aplicación móvil que digitaliza muestras de un microscopio utilizando un adaptador de microscopio impreso en 3D, una plataforma de telemedicina para el análisis y el etiquetado en remoto, y nuevos algoritmos de aprendizaje para evaluar y cuantificar automáticamente las infecciones parasitarias (Dacal y col., 2021).

Recientemente, también se ha desarrollado el "kubic FLOTAC microscope (KFM)", que es un microscopio digital compacto, económico, versátil y portátil, que se ha diseñado para analizar muestras fecales preparadas con Mini-FLOTAC o FLOTAC, tanto en el campo como en el laboratorio, aportando grandes ventajas por la combinación de técnicas diagnósticas sensibles, precisas y estandarizadas, como el Mini-FLOTAC, con un sistema automatizado fiable que permite la observación y cuantificación en tiempo real de estructuras parasitarias, gracias también al software de inteligencia artificial, que se encuentra bajo desarrollo (Cringoli y col., 2021).

Por otro lado, en áreas de baja endemicidad, donde las estrategias de control se acercan a la eliminación de la parasitosis y se deba construir un sistema de vigilancia robusto, se requieren herramientas de diagnóstico más sensibles y específicas. Estas técnicas pueden ser técnicas de diagnóstico molecular o serológicas más sofisticadas, aunque, mayoritariamente, más costosas (Bergquist y col., 2009, 2015; Cringoli y col., 2013).

I.2.3.3.2. Técnicas moleculares

En las últimas décadas, la aplicación de la reacción en cadena de la polimerasa (PCR = "Polymerase chain reaction") ha aumentado exponencialmente en parasitología (Taniuchi y col., 2011; Verweij y col., 2014) incluso para el diagnóstico de *Trichuris* (Liu y col., 2014). Esta técnica presenta muchas ventajas frente a los métodos microscópicos directos utilizados anteriormente: presenta una mayor sensibilidad frente a la detección del adulto parásito, especialmente con intensidades bajas de infecciones; permite diferenciar especies de *Trichuris* estrechamente relacionadas (Cutillas y col., 2009; Liu y col., 2012a; Ravasi y col., 2012; Hawash y col., 2015; Callejón y col., 2017), y a través de huevos de morfología muy similar (Liu y col., 2014); la cantidad de ADN necesaria para realizar la prueba es realmente pequeña, por lo que la recogida de muestras de heces no depende de su peso, lo que puede limitar el diagnóstico cuando se usan métodos directos. Además, los ensayos de PCR a tiempo real (RT-PCR = "Real-time polymerase chain reaction") han permitido la detección de varios parásitos dentro de la misma prueba, que es una gran ventaja en entornos donde existe el multiparasitismo (Mejia y col., 2013). Asimismo, una gran ventaja, dado que el ADN es una molécula estable, es que las muestras de ADN extraídas pueden almacenarse durante muchos años después de la recolección por si se necesitan análisis posteriores, como la investigación de patógenos o la resistencia a fármacos (Barda y col., 2015).

La principal ventaja de los ensayos por PCR radica en su mayor sensibilidad y la capacidad de detectar múltiples infecciones, aunque la PCR simple es un poco más sensible en comparación con los ensayos múltiples (Pilotte y col., 2016; Meurs y col., 2017). Incluso, el uso de la RT-PCR proporciona estimaciones de prevalencia más precisas en comparación con el microscopio y se puede multiplexar (Papaiakovou y col., 2019; Dunn y col., 2020).

Aunque el método de PCR no es imprescindible generalmente para el diagnóstico de *Trichuris*, ya que los huevos tienen una forma peculiar y se distinguen fácilmente, este método es más sensible y puede ser útil en entornos de baja prevalencia donde el principal objetivo sea la eliminación de la infección (Demeler y col., 2013).

Asimismo, la principal desventaja de la PCR es su elevado costo, ya que no solo requiere costosos instrumentos, sino también, una fuente de alimentación constante, reactivos específicos y un alto costo de mantenimiento (Barda y col., 2015).

Recientemente, se ha comercializado el primer ensayo de PCR múltiple utilizando un instrumento de RT-PCR, el ensayo Allplex™ GI-Helminth(I) (Seegene Inc., Seoul, Corea del Sur), que está acoplado a un dispositivo de extracción de ADN automatizado, MICROLAB® STARlet (Hamilton Company, Reno, NV, EE. UU.), el cual utiliza un pequeño volumen de muestra (200 µl) y permite la detección de 1 especie de protozoo y 8 de helmintos (Autier y col., 2021).

Finalmente, se ha desarrollado la tercera generación de la PCR, ddPCR ("Droplet Digital™ polymerase chain reaction"), utilizada en la cuantificación absoluta de parásitos gastrointestinales (Elmahalawy y col., 2018). Esta técnica es un refinamiento de los métodos convencionales de la PCR que se puede utilizar para directamente cuantificar y amplificar mediante la clonación el ADN, y proporciona una detección más precisa, sensible, y reproducible de patógenos de baja abundancia (Li y col., 2018). Además, ha mostrado mejores resultados frente a la RT-PCR para el género *Trichuris* (Yu y col., 2020).

I.2.3.3.3. Otras técnicas

Dentro de otras técnicas de diagnóstico, destacamos los ensayos serológicos, entre los que citaremos la detección de anticuerpos en suero y la detección de coproantígenos. Todavía estos métodos no son particularmente útiles para el diagnóstico de las geohelmintiasis. Se han realizado pruebas ELISA a muestras fecales infectadas experimentalmente con huevos de *Trichuris vulpis*, y se obtuvieron buenos resultados comparados con los métodos de flotación de heces debido a que se permitieron una detección más temprana de la infección, sin embargo, los resultados no fueron determinantes (Dopchiz y col., 2013; Elsemore y col., 2014).

Además, hay otros métodos de diagnóstico utilizados en clínicas hospitalarias, como la colonoscopia o la sigmoidoscopia, útil en infecciones graves, ya que los tricocéfalos se pueden encontrar en el recto (Mahmud y col., 2017; Ishizaki y col., 2022).

I.2.3.4. Tratamiento y control

Actualmente, las infecciones causadas por *Trichuris* son tratadas con benzimidazoles (mebendazol o albendazol) o ivermectina, siendo el mebendazol el más efectivo y, por ello, utilizado en la primera línea de tratamiento. Por otro lado, en Estados Unidos, la FDA ("Food and Drug Administration") no aprueba ni el albendazol ni la ivermectina para el tratamiento de la tricuriasis, utilizando únicamente el tratamiento con mebendazol (CDC, 2022; Viswanath y col., 2022).

Para el control de las STH, se ha introducido una estrategia central que consiste en la administración de quimioterapia preventiva a grupos de población de alto riesgo mediante una distribución de medicamentos antihelmínticos a gran escala (MDA = "Mass drug administration"), integrada con saneamiento ambiental y educación sanitaria (Gabrielli y col., 2011; Zeng y col., 2019). A nivel mundial, más de 1 billón de niños

requieren una desparasitación anual. En el año 2019, 613 millones de niños recibieron quimioterapia preventiva, 27 ciudades alcanzaron el 100% de cobertura en los niños de edad escolar y 23 ciudades consiguieron cubrir el 75% de cobertura en niños de edad escolar, adquiriendo una cobertura global del 58% (WHO, 2022). Sin embargo, en el año 2020, fueron tratados más de 436 millones de niños, lo cual corresponde al 42% de todos los niños en riesgo, inferior al año anterior. La disminución significativa en la cobertura se debe al cierre parcial durante la pandemia de COVID-19 (WHO, 2022).

Los esfuerzos de control contra la trichuriasis arrojan resultados negativos, debido a que los medicamentos disponibles muestran tasas de curación insatisfactorias y, además, las tasas de reducción de huevos son decepcionantemente bajas (Adegnika y col., 2015; Welsche y col., 2022). También, se ha observado que *T. trichiura* tiene una susceptibilidad diferente a los medicamentos según la ubicación geográfica (Hürlimann y col., 2022). Además, la administración de MDA de forma periódica puede contribuir a la aparición de resistencias (Prichard, 2017; Tinkler, 2019).

En las últimas décadas, científicos veterinarios han reportado la resistencia antihelmíntica en nematodos que infectan al ganado después del uso frecuente de benzimidazoles como parte de MDA (Kaplan y Vidyashankar, 2012). Estos fármacos se unen a los dímeros de la tubulina de los nematodos e inhiben la formación y la estabilidad de los microtúbulos. La resistencia a los benzimidazoles se caracteriza por polimorfismos de un solo nucleótido en el gen que codifica la β-tubulina que reduce la afinidad de unión, específicamente, sustituciones de aminoácidos de fenilalanina a tirosina en los codones 167 o 200 (F167Y o F200Y), y sustituciones de glutamato a alanina o leucina en el codón 198 (E198A o E198L) (Kwa y col., 1994; Silvestre y Cabaret, 2002; Ghisi y col., 2007; Prichard, 2017; Martínez-Valladares y col., 2020).

Estos medicamentos reducen la intensidad de las infecciones y tienen el potencial de ejercer la selección de genotipos portadores de mutaciones asociadas con la resistencia a los medicamentos con precisión, lo que puede resultar en el establecimiento de poblaciones de vermes resistentes (Mendes de Oliveira y col., 2022). Este hecho podría resultar en un problema de salud pública mundial si se produjera una resistencia antihelmíntica documentada durante la campaña para eliminar las geohelmintiasis, ya que no habrá opciones terapéuticas eficaces para las personas con mayor morbilidad (Tinkler, 2019).

I.3. TAXONOMÍA DEL GÉNERO *TRICHURIS*

I.3.1. Ancestro

Los humanos han estado parasitados por nematodos del género *Trichuris* durante milenios. Se han encontrado huevos de parásitos en coprolitos humanos (heces fosilizadas) de zonas arqueológicas que datan del 7100 a.C. (Araujo y col., 2008; Ledger y col., 2019, 2020) incluyendo ubicaciones en Europa y América del Norte, donde las infecciones ahora son inusuales (Gonçalves y col., 2003; Søe y col., 2018; Flammer y col., 2020; Ledger y col., 2021).

Respecto al lugar que ocupa el género *Trichuris* dentro del *phylum* Nematoda, actualmente la hipótesis filogenética más aceptada es la que establece este género dentro de la clase Enoplea, la subclase Trichocephalia, el superoden Trichinellina, la superfamilia Trichinelloidea y la familia Trichuridae, la subfamilia Trichurinae, la tribu Trichurini y la subtribu Trichurinii. Actualmente, dentro del género *Trichuris* hay descritas 107 especies (Smythe y col., 2019; Ahmed y col., 2022; Hodda, 2022).

I.3.2. Taxonomía y filogenia clásica y molecular del género *Trichuris*

La diferenciación morfológica específica de las diferentes especies del género *Trichuris* (Roederer, 1761) ha constituido un gran problema debido a que los caracteres en la mayoría de las especies comparadas están superpuestos (Gagarin, 1974). Se han evidenciado sinonimias y especies crípticas entre muchas especies de este género como consecuencia de haber sido encontradas en un hospedador diferente al hospedador habitual. Igualmente, se ha demostrado que las especies están influenciadas por los hospedadores que los albergan, dando lugar a un individuo con dimensiones morfológicas distintas (Knight, 1972). Asimismo, las variaciones morfológicas pueden manifestar adaptaciones fenotípicas de

un parásito al medio ambiente, independientemente de las diferencias genotípicas.

Diversos estudios se han llevado a cabo para clarificar la sistemática de este género, intentando determinar los caracteres morfológicos que tienen valor específico diferencial. La primera revisión de este género fue realizada por Dujardin (1845). Posteriormente, muchos autores, basándose en características exclusivamente morfológicas y biométricas, han intentado diferenciar las distintas especies de *Trichuris*, aceptando así un gran número de especies dentro de este grupo.

En primer lugar, se consideró la longitud de la espícula como el carácter más relevante para la diferenciación entre las especies (Schwart, 1926). Chandler (1930) estimó necesario añadir otros caracteres morfológicos para su confirmación. No obstante, durante estos primeros años, y debido a la falta de estudios con la mayoría de los caracteres analizados, las especies apenas pudieron ser diferenciadas unas de otras. Por ello, el principal criterio de identificación fue su hospedador. Además, varios autores estudiaron la variabilidad de algunas especies de *Trichuris* de varias regiones y hospedadores, afirmando que el hospedador ejercía influencia en las diferencias morfo-biométricas de los parásitos que albergaba (Knight, 1972, 1984; Hinks y Thomas, 1974).

Gagarin (1972) llevó a cabo un estudio morfológico y biométrico en 30 especies de *Trichuris*, y reveló un considerable grado de variabilidad en los caracteres relacionados con el sistema reproductor.

Las hembras del género *Trichuris* son más difíciles de diferenciar que los machos. La estructura de la vulva, de acuerdo con varios autores, puede utilizarse para la diferenciación de las especies. Además, puede establecerse que hay dos tipos de vulva, con o sin espinas (Kikuchi, 1974a, 1974b; Zaman, 1984; Tenora y col., 1993). Este carácter diferencial fue corroborado por Barus y col. (1978) en *Trichuris skrjabini*, *Trichuris lani*, *Trichuris globulosa* y *Trichuris ovis*, en las que analizaron la estructura

vulvar, la morfología y la presencia o no de espinas, concluyendo que se trataba de un elemento característico de especie.

Varios estudios se han llevado a cabo sobre la diferenciación de especies con caracteres morfológicos similares, como las especies *T. trichiura* y *Trichuris suis*. Los primeros estudios morfológicos realizados en adultos de estas especies revelaron que pertenecían a la misma especie (Creplin, 1825; Schwartz, 1926). Estudios posteriores confirmaron que esos dos parásitos eran especies diferentes, asumiendo que *T. trichiura* parasitaba naturalmente a los humanos y primates, y *T. suis* a los suidos (Sondak, 1948; Pavlovsky y Sondak, 1951; Ooi y col., 1993). Otros autores consideraron que los huevos y las larvas infectantes de *T. trichiura* eran significativamente más pequeños que los de *T. suis* (Dinnik, 1938). Beer (1976) no encontró diferencias visibles morfológicas entre los huevos y las larvas de estas dos especies. Se daba por sentado que el principal criterio morfológico para diferenciar *T. trichiura* y *T. suis* era la existencia de la típica papila caudal en *T. trichiura* y la ausencia de ella en *T. suis*, que habían sido observadas mediante el microscopio electrónico (Gibbons, 1986; Tenora y col., 1988). No obstante, Cutillas y col. (2009) encontraron, mediante microscopía electrónica, que esta papila caudal estaba presente en ambas especies, y, por lo tanto, no podía considerarse como característica diferencial entre ambas. Para clarificar la clasificación entre estas dos especies, estos autores complementaron el estudio morfo-biométrico con estudios moleculares. Con este fin, utilizaron una región del ADN ribosómico (ADNr), la región ITS1-5,8S-ITS2, debido a que varios estudios previos habían demostrado que los espaciadores transcritos internos (ITS) del ADNr contenían marcadores genéticos fiables para distinguir especies de nematodos estrechamente relacionadas (Chilton y col., 1995; Gasser y Hoste, 1995; Hoste y col., 1995; Oliveros y col., 2000; Cutillas y col., 2002, 2007), mientras que las regiones que codifican el ARN ribosómico (ARNr), parecen ser menos fiables para la

identificación a nivel de especies debido a su bajo nivel de divergencia de las secuencias (Zarlenga y col., 1994). Los resultados obtenidos del análisis molecular de las regiones ITS1 e ITS2 mostraron claras diferencias entre ambas especies. Sin embargo, el gen 5,8S era similar. Finalmente, concluyeron que *T. trichiura* y *T. suis* eran especies idénticas morfológicamente pero genéticamente diferentes, aunque muy cercanas (Cutillas y col., 2009).

El hecho anteriormente citado ocurría también en la diferenciación de más especies, como por ejemplo entre las especies *T. ovis* y *T. globulosa* aisladas de ovejas y cabras. Según varios autores, ambas especies eran muy similares en cuanto a la forma y tamaño del cuerpo y de los huevos (Skrjabin y col, 1957; Tenora y col., 1997). Además, presentaban muchas de las medidas, de los caracteres estudiados, superpuestas. Morfológicamente, los vermes eran similares y se encontraban ambas especies en ambos hospedadores. En virtud de ello, propusieron sinonimias entre ambas especies (Cutillas y col., 1995).

Por otro lado, otras especies, como *T. skrjabini*, pudieron ser identificadas únicamente basándose en criterios morfológicos y biométricos, a pesar de encontrarse en más de un hospedador, en este caso en camellos y cabras (Skrjabin y col., 1957; Knight, 1972; Cutillas y col., 1996). Estos hechos fueron además confirmados por estudios isoenzimáticos (Cutillas y col., 1995, 1996; Oliveros y col., 1998; Feliú y col., 2000). También, entre las especies *Trichuris muris* y *Trichuris arvicolae*, en las que además de los estudios morfo-biométricos e isoenzimáticos realizados por Feliú y col. (2000), se llevaron a cabo estudios moleculares basándose en la amplificación y secuenciación de la región ITS1-5,8S-ITS2 del ADNr, corroborando la existencia de dos especies de *Trichuris* diferentes (Cutillas y col., 2002).

La región ITS1-5,8S-ITS2 del ADNr fue utilizada también para el estudio molecular de otras especies de *Trichuris*: *T. ovis*, *T. globulosa*, *Trichuris leporis*, *T. skrjabini*, *T. muris*, *T. arvicolae* y *T. vulpis*. Los resultados mostraron que no había diferencias en las secuencias de los marcadores ITS1 y 5,8S entre *T. ovis* y *T. globulosa*. Sin embargo, se detectaron claras diferencias entre las secuencias de ITS1 entre *T. skrjabini*, *T. ovis*, *T. leporis*, *T. muris*, *T. arvicolae* y *T. vulpis*. Asimismo, el estudio comparativo de la secuencia ITS2 sugirió que el uso combinado de ambos espaciadores podría ser útil en la caracterización molecular de tricocéfalos (Cutillas y col., 1995, 2002, 2004, 2007; Oliveros y col., 2000).

Sin embargo, en los últimos años, se han evidenciado complejos de especies parasitando un mismo hospedador, o una especie parasitando varios hospedadores, dificultando la identificación entre las diferentes especies. Por esa razón, para la clasificación de las especies del género *Trichuris*, varios autores han realizado estudios paralelos basados tanto en los análisis morfológicos y biométricos como en análisis moleculares, con el objeto de esclarecer la taxonomía específica del género y así poder comparar las diferentes especies y caracterizar especies nuevas.

Por consiguiente, Callejón y col. (2010) llevaron a cabo un estudio filogeográfico de *T. muris* parasitando diferentes hospedadores de la familia Muridae y de regiones geográficas distintas mediante la amplificación y secuenciación del fragmento ITS1-5,8S-ITS2 del ADNr. Los resultados obtenidos confirmaron la presencia de polimorfismos de ADN entre las secuencias analizadas de Europa. Además, obtuvieron dos linajes geográficos y genéticos diferentes.

Hasta la fecha analizada, los ITS y el gen 5,8S eran marcadores moleculares útiles en el estudio de especies de *Trichuris* estrechamente relacionadas (Cutillas y col., 1995, 2002, 2004, 2007, 2009; Oliveros y col., 2000; Callejón y col., 2010). Sin embargo, Blouin (2002), sugirió que estas secuencias obtenidas no se deberían utilizar para realizar análisis

filogenéticos, debido a que los datos obtenidos de las secuencias de ITS no eran útiles para identificar especies crípticas potenciales a partir de un número pequeño de individuos. Dado que las secuencias del genoma mitocondrial aportaron marcadores útiles para la sistemática y la filogenia de los organismos (Avise, 1994; Hu y Gasser, 2006), Callejón y col. (2009) optaron por analizar el gen parcial de la *citocromo c-oxidasa* 1 (*cox*1) y la secuencia parcial 16S (*rrn*L) del ADN mitocondrial (ADNmt), obteniendo buenos resultados para la especie *T. skrjabini*.

En el año 2012 se realizó un estudio morfológico, biométrico y molecular comparativo de *Trichuris discolor* y *T. ovis* aislado de un mismo hospedador (*Bos taurus*) de dos regiones diferentes (España e Irán) en el caso de *T. discolor*, y de una sola región en el caso de *T. ovis* (España). Morfológicamente, las hembras de *T. discolor* de ambas regiones no presentaban diferencias, además, muchos de los parámetros medidos estaban superpuestos, aunque el tamaño difería según la región. Asimismo, cuando compararon los valores medidos entre ambas especies, las medidas obtenidas en *T. ovis* tendían a ser menores que en *T. discolor*. Este análisis no se pudo realizar con los adultos machos. Molecularmente, llevaron a cabo un estudio de la región ITS1-5,8S-ITS2 del ADNr y del gen parcial 16S del ADNmt, donde concluyeron que los marcadores utilizados eran útiles para discriminar entre diferentes especies y, además, entre diferentes poblaciones con orígenes geográficos distintos (Callejón y col., 2012).

Igualmente, Callejón y col. (2013) realizaron un estudio taxonómico molecular y filogenético del género *Trichuris*, basándose en las secuencias del gen parcial *cox*1 del ADNmt y del gen parcial 18S del ARNr, demostrando resolución a diferentes niveles, pero combinando ambos marcadores, resolvían las relaciones entre diferentes poblaciones geográficas y especies del género. El análisis de las secuencias apoyó la relación estrecha entre *Trichuris* sp. procedente de *Colobus guereza*

kikuyensis y *T. suis*, formando un clado, que a su vez estaba separado de otro clado, que agrupaba las demás secuencias de *Trichuris* de hospedadores humanos y PNH. Para confirmar dichos resultados, Cutillas y col. (2014) llevaron a cabo un estudio morfológico y biométrico de los tricocéfalos parasitando *C. g. kikuyensis*, y realizaron un estudio comparativo con datos morfo-biométricos de *T. suis* y de *T. trichiura* de PNH. En los resultados encontraron 7 variables en los machos y 3 variables en las hembras para la discriminación entre estas especies. Así, apoyados tanto con los datos moleculares (Cutillas y col., 2009; Callejón y col., 2013) como con los datos morfo-biométricos (Cutillas y col., 2014), propusieron una nueva especie para *Trichuris* sp. procedente de *C. g. kikuyensis*, nombrada, *Trichuris colobae*.

Después, Callejón y col. (2015) utilizaron por primera vez tanto el gen parcial *cox*1 como el *citocromo* b (*co*b) del ADNmt para realizar estudios taxonómicos y filogenéticos de *T. globulosa* y más especies de *Trichuris* que parasitan herbívoros. Reafirmaron que utilizando los marcadores nucleares (ITS) no podían diferenciar entre sinonimias (entre *T. ovis* y *T. globulosa*), mientras que los marcadores mitocondriales diferenciaban entre ambas especies y corroboraban la existencia de diferentes linajes de *T. ovis* procedente de ovejas de Sudáfrica que estaban estrechamente relacionadas con poblaciones de *T. globulosa* pertenecientes a camellos de Irán.

Posteriormente, en 2017, se describió una nueva especie de *Trichuris*, *Trichuris ursinus*, procedente de una especie de babuino, *Papio ursinus*, basándose tanto en estudios morfo-biométricos como moleculares. En el estudio biométrico, el análisis comparativo de los valores medios de las variables individuales entre *T. suis*, *T. trichiura* y *T. colobae*, sugirieron que *T. ursinus* constituía una nueva especie. El análisis combinado de tres marcadores moleculares (*cox*1 y *co*b del ADNmt e ITS2 del ADNr), reveló una relación hermana entre *T. colobae* y *T. ursinus*. Distintos

linajes genéticos correspondientes a las diferentes especies del género fueron apoyados por las hipótesis filogenéticas para ambos genes mitocondriales. De este modo, *T. suis*, *T. colobae* y *T. ursinus* aparecieron como un grupo hermano, y se separó del resto de especies de *Trichuris* procedentes de humanos y otras especies de primates (Callejón y col., 2017).

Asimismo, recientemente, se han descrito numerosas nuevas especies de *Trichuris* encontradas en roedores procedentes de Argentina basándose tanto en estudios morfológicos, biométricos y moleculares, utilizando marcadores del ADNr (ITS) y del ADNmt (*cox*1 y *co*b) para el análisis molecular, como por ejemplo, procedente de la familia Sigmodontinae, *Trichuris navonae*, *Trichuris bainae* y *Trichuris pardinasi* (Robles y col., 2014; Callejón y col., 2016), *Trichuris massoiai* procedente de *Holochilus chacarius* (Robles y col., 2018) o *Trichuris cutillasae* procedente de capibara (Eberhardt y col., 2019).

La sistemática del género *Trichuris* no ha podido resolverse exclusivamente con estudios morfológicos y biométricos. Por esa razón, en los últimos 20 años, se han introducido los estudios moleculares. Sin embargo, muchos estudios se han basado exclusivamente en datos genéticos, dificultando la caracterización de las especies por falta de datos. Así, muchas especies de *Trichuris* encontradas en diferentes hospedadores han sido caracterizadas únicamente de forma molecular.

Cavallero y col. (2015) utilizaron únicamente marcadores moleculares del ADNr (ITS) para caracterizar genéticamente especímenes de *Trichuris* procedentes del macaco japonés (*Macaca fuscata*) y el cercopiteco verde o tota (*Chlorocebus aethiops*), y estudiar la variación genética y la filogenia de estas secuencias y otras aisladas de otros hospedadores humanos y PNH. El análisis filogenético reveló la existencia de clados y subclados distintos, y la existencia de taxones adicionales separados, sugiriendo que los especímenes encontrados en *M. fuscata* pueden ser

distintos pero relacionados con *T. trichiura*, mientras que los especímenes encontrados en *C. aethiops* estaban en el subclado de *T. suis*. Seguidamente, analizaron la diversidad genética y la filogenia de las especies de *Trichuris* que infectaban a cinco especies diferentes de PNH mediante la secuenciación de tres genes del ADNmt (*cox*1, *co*b y *rrn*L). Los análisis filogenéticos sugirieron la presencia de dos linajes evolutivos principales. El primer linaje incluía a especies de *Trichuris* parasitadas por *C. g. kikuyensis*, *P. ursinus* y *Chlorocebus* spp., agrupándose con *T. suis*; el segundo linaje incluía a especies de *Trichuris* que parasitaban a *M. fuscata*, *Papio hamadryas* y a los humanos (Cavallero y col., 2019). Estos resultados confirman las evidencias previas de la existencia de otras especies de *Trichuris* distintas de *T. trichiura* que infectan a PNH.

En los últimos estudios, se ha utilizado el genoma mitocondrial completo para caracterizar las especies de este género. Varios autores han caracterizado los genomas mitocondriales completos de *T. trichiura* (de origen humano y de PNH) y *T. suis* (del cerdo). Estos autores, en base a los análisis moleculares y filogenéticos, apoyan la propuesta de que ambas especies son especies diferentes (Liu y col., 2012a; Hawash y col., 2015). También han sido caracterizados los genomas mitocondriales completos de las especies *T. ovis* y *T. discolor*, por su proximidad genética, y evidenciaron que ambas especies eran diferentes (Liu y col., 2012b). Además, en base a esta tecnología, se ha apoyado que la especie *T. skrjabini* es una especie distinta (Ahmad y col., 2019).

Igualmente, la secuenciación y el análisis del genoma mitocondrial completo (Figura 10), ha sido útil para la caracterización de nuevas especies y para comenzar a clarificar el complejo de especies encontradas en PNH, reconocidas con anterioridad a todas las especies de *Trichuris* encontradas en primates como *T. trichiura*. Otros autores propusieron una nueva especie de *Trichuris* procedente del langur de Francois (*Trachypithecus francoisi*). Para ello, además del genoma mitocondrial

completo, analizaron los marcadores nucleares ITS1 e ITS2 del ADNr (Liu y col., 2013), y la especie *Trichuris rhinopiptheroxella* procedente del langur chato dorado (*Rhinopithecus roxellana*) (Wang y col., 2019).

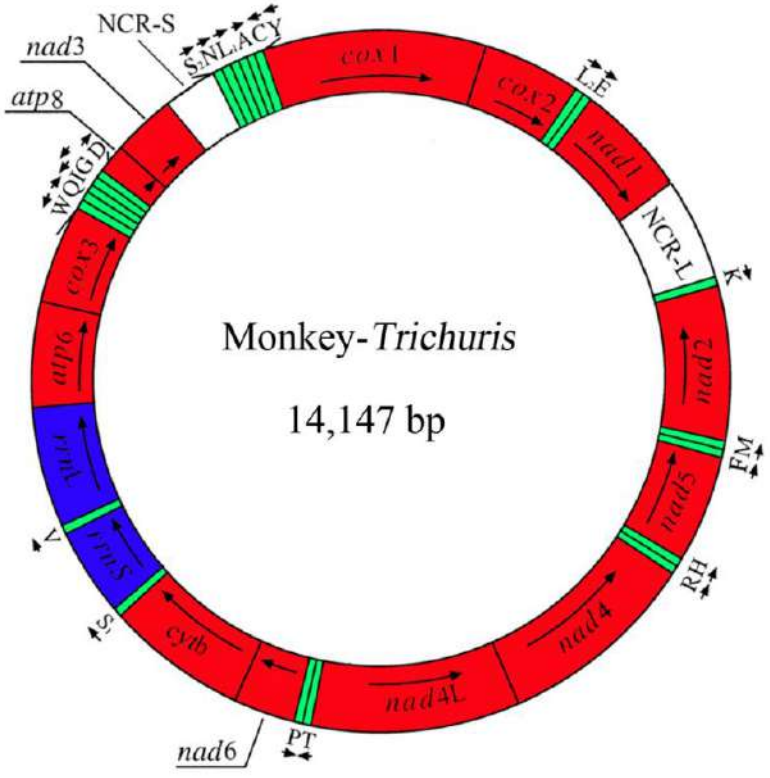

Figura 10. Estructura esquemática del genoma mitocondrial completo de *Trichuris* procedente del langur de Francois (Liu y col., 2013).

Finalmente, Doyle y col. (2022) realizaron un análisis del genoma completo de tricocéfalos aislados de humanos y PNH, junto con muestras antiguas conservadas de depósitos arqueológicos utilizando "Whole-Genome Sequencing". Los resultados revelaron una relación genética cercana entre las muestras de *Trichuris* de humanos de Uganda y babuinos, pero con regiones que diferían dependiendo del hospedador. Además, los tricocéfalos obtenidos de humanos y PNH eran genéticamente muy distintos del grupo de los colobos.

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CAPÍTULO II. OBJETIVOS

II. OBJETIVOS

II.1. OBJETIVOS GENERALES

La presente Tesis Doctoral ha tenido como objetivo principal el estudio taxonómico y filogenético de especies del género *Trichuris* de distintos hospedadores vertebrados, mayoritariamente en PNH, y otros animales, que habitan en diferentes parques zoológicos dentro del territorio español, con el fin de clarificar la complicada taxonomía actual de este grupo de nematodos y aportar nuevos datos para su lucha y control. Para ello se han utilizado técnicas morfológicas, biométricas, moleculares y la técnica proteómica MALDI-TOF MS.

II.2. OBJETIVOS ESPECÍFICOS

Los objetivos específicos han sido:

1. Estudio taxonómico

Realizar un estudio taxonómico del género *Trichuris*. Este estudio se ha llevado a cabo en tres fases:

- Mediante técnicas clásicas, atendiendo a caracteres morfológicos y biométricos de especies encontradas en distintos hospedadores, y mediante morfometría geométrica.
- Mediante técnicas moleculares basadas en la amplificación, secuenciación y análisis de las regiones del ADNr (ITS1 e ITS2), del ADNmt (*cox*1 y *co*b) y del ARNr (*rrn*L), además de secuenciar el genoma mitocondrial completo de *Trichuris* de varios hospedadores.
- Mediante la técnica MALDI-TOF MS, con el fin de poner a punto la técnica mediante la obtención de espectros únicos y característicos para las diferentes especies de *Trichuris* estudiadas, y obtener una base de datos de referencia con estas especies y, así, valorar su importancia diagnóstica.

2. Estudio filogenético

- Establecer las relaciones filogenéticas entre las diferentes especies del género *Trichuris* evaluando la utilidad de los marcadores moleculares utilizados individualmente y concatenados.
- Determinar los valores de similitud de las especies de *Trichuris* basándonos en las secuencias obtenidas a partir de los marcadores moleculares utilizados.

CAPÍTULO III. RESULTADOS

III. RESULTADOS

III.1. RESUMEN DE LOS RESULTADOS

En la presente Tesis Doctoral se ha realizado un estudio taxonómico y filogenético basado en datos morfológicos, biométricos, moleculares y proteómicos de distintas especies del género *Trichuris* parásitas de distintos hospedadores vertebrados mediante la amplificación, secuenciación y el análisis de genes y fragmentos del ADN mitocondrial y ribosómico, respectivamente, y mediante la técnica proteómica MALDI-TOF MS.

III.1.1. Taxonomía y filogenia del género *Trichuris*: Estudios morfológicos, biométricos y moleculares

Debido al complejo de especies que están presentes en los humanos y PNH y a la falta de datos sobre especificidad de hospedador, se decidió realizar un estudio para actualizar la información sobre datos morfológicos, biométricos, de caracterización molecular y filogenéticos de *T. trichiura* en humanos y PNH. En base a esto, se recolectaron tanto muestras de heces como de adultos de *Trichuris* de cinco hospedadores diferentes de PNH de parques zoológicos de España.

Por otro lado, con el fin de analizar otras especies de *Trichuris* de diferentes hospedadores vertebrados que se encuentran en parques zoológicos de España, se ha llevado a cabo un estudio morfológico, biométrico, de caracterización molecular y filogenético de una población de *Trichuris* aislada de un puercoespín crestado (*Hystrix cristata*). Y finalmente, se han realizado análisis de caracterización molecular y filogenéticos de una población de *Trichuris* sp. procedente del camello bactriano (*Camelus bactrianus*).

III.1.1.1. *Trichuris* spp. procedente de *Macaca sylvanus* (García-Sánchez y col., 2019; Rivero y col., 2020, 2021a, 2021b).

El estudio se llevó a cabo en 65 ejemplares adultos (32 hembras y 33 machos) de *Trichuris* aislados de la especie *M. sylvanus* (macaco de Gibraltar o macaco de Berbería) (Figura 11) procedente del Zoo de Castellar situado en Castellar de la Frontera (Cádiz, España).

Figura 11. Imagen del hospedador *M. sylvanus* (macaco de Gibraltar o macaco de Berbería) con una cría (Valavanidis y Vlachogiani, 2011).

En primer lugar, se realizó un estudio morfológico de los individuos aislados de *M. sylvanus* llevando a cabo una descripción tanto de los machos como de las hembras de esta población (Rivero y col., 2020). Asimismo, se realizó un estudio biométrico analizando 15 parámetros en los machos y 13 parámetros en las hembras, ya definidos previamente por otros autores (García-Sánchez y col., 2019). Se encontraron dos tipos de huevos diferentes en las heces del macaco (Rivero y col., 2020).

En segundo lugar, se utilizó la técnica de morfometría geométrica para analizar la población de *Trichuris* aislada de *M. sylvanus* (Rivero y col., 2020). Los estudios moleculares posteriores, revelaron dos linajes genéticos diferentes, dentro de la población analizada. Por esta razón, se volvieron a realizar los estudios morfo-biométricos diferenciando ambos linajes, resultando, con la prueba t de Student, que los valores analizados no tenían diferencias significativas entre ambas poblaciones detectadas.

Las medidas utilizadas para los análisis fueron: la longitud total del cuerpo, la longitud del esófago (LE), la longitud de la parte posterior del cuerpo (LP), la relación LE/LP, el ancho del cuerpo, la longitud de la espícula y la longitud de la vaina espicular para los machos y, la longitud total del cuerpo, la LE, la LP, la relación LE/LP, el ancho del cuerpo y el diámetro de la vulva para las hembras.

Del mismo modo, se llevó a cabo el mapa de factores de ambos linajes, y también diferenciados en machos y hembras. Así, las pruebas t de Student mostraron que varias de las medidas eran significativas para realizar los análisis morfo-biométricos. En el mapa de factores de los machos, ambas poblaciones estaban ligeramente superpuestas, mientras que, en las hembras, las poblaciones aparecían superpuestas completamente (Rivero y col., 2020).

Los resultados obtenidos mediante morfometría geométrica mostraron que las poblaciones con linajes genéticos distintos encontradas en *Trichuris* sp. de *M. sylvanus*, no podían ser diferenciadas utilizando esta técnica (Rivero y col., 2020).

Posteriormente, se llevó a cabo un estudio de caracterización molecular y filogenético utilizando las secuencias obtenidas en el estudio junto con otras secuencias obtenidas de la base de datos de GenBank de otras especies de *Trichuris* de hospedadores humanos y PNH. Para ello, se amplificaron y secuenciaron los fragmentos ITS1 e ITS2 del ADNr, los genes parciales *cox*1 y *co*b del ADNmt y, el gen parcial *rrn*L del ARNr (Rivero y col., 2020, 2021b).

La longitud total de las secuencias de ITS1 obtenidas fueron de 586 – 587 pares de bases (pb) y se obtuvieron 6 haplotipos diferentes (Rivero y col., 2021b). La longitud total de las secuencias de ITS2 obtenidas fueron de 514 – 587 pb, y se obtuvieron 8 haplotipos diferentes (Rivero y col., 2020, 2021b). La variabilidad observada en las secuencias de ITS2 eran

consecuencia de ≪microsatélites≫, unas secuencias repetitivas de nucleótidos.

Por otro lado, la longitud total de las secuencias del gen parcial *cox*1 fue de 370 pb, y se revelaron 4 haplotipos diferentes, uno mayoritario, representando a la mayoría de las secuencias analizadas (87,7%), y los otros tres minoritarios (12,3%), en cuanto al número de secuencias analizadas. La longitud total de las secuencias del gen parcial *co*b fue de 520 pb y se revelaron 5 haplotipos diferentes (Rivero y col., 2020, 2021b), donde uno era el mayoritario, y los otros cuatro minoritarios. La longitud total de las secuencias del gen parcial *rrn*L fue 386 – 387 pb, y se revelaron 5 haplotipos diferentes siguiendo el mismo patrón que los marcadores mitocondriales previamente analizados.

Los árboles filogenéticos obtenidos para cada marcador molecular individual o de forma concatenada tenían en común dos clados fuertemente soportados, que fueron nombrados como clado 1 o "Linaje *T. suis*" y clado 2 o "Linaje *T. trichiura*". El clado 1 incluía las especies *T. colobae*, *T. ursinus* y *T. suis*, y el clado 2 incluía las especies *T. trichiura* y el resto de las especies de *Trichuris* procedentes de humanos y PNH (Figura 12).

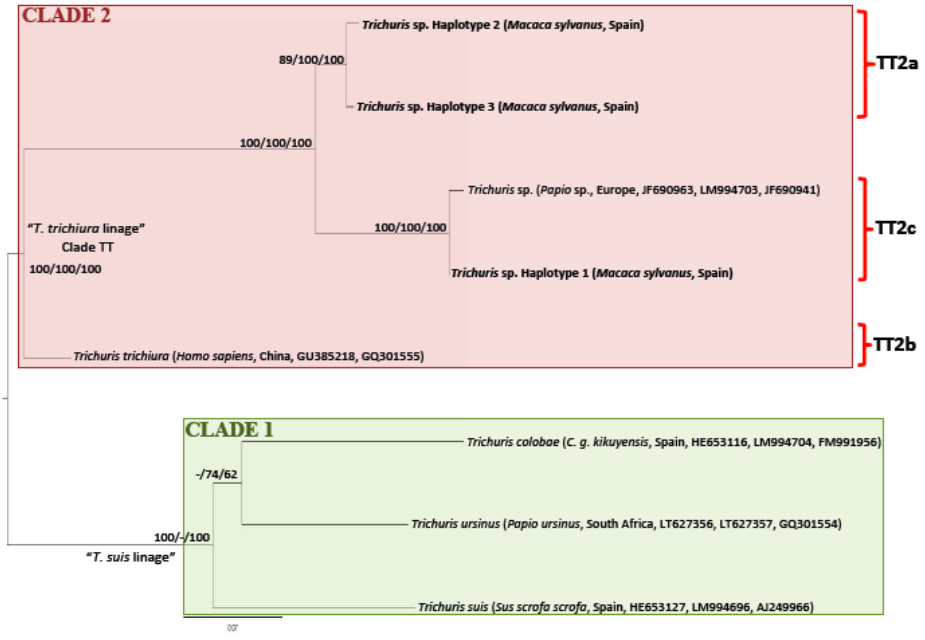

Figura 12. Árbol filogenético inferido de especies de *Trichuris* basado en el análisis de los marcadores mitocondriales (*cox*1 y *co*b) y ribosómico (ITS2) usando el método de inferencia bayesiana (IB). Están listadas primero las probabilidades posteriores bayesianas (PPB), seguidas de los valores obtenidos por el método de máxima parsimonia (MP), y finalmente por los valores de máxima verosimilitud (MV), para frecuencias de clado superiores al 60% (Rivero y col., 2020).

Los árboles filogenéticos inferidos de los marcadores ribosómicos individuales y concatenados (ITS1 e ITS2), mostraron que todas las secuencias de los adultos de *Trichuris* sp. de *M. sylvanus* estaban en el "Linaje *T. trichiura*", pero sin llegar a resolver relaciones filogenéticas atendiendo a su especificidad hospedadora o la influencia de la procedencia geográfica. No obstante, las secuencias de *Trichuris* sp. de *M. sylvanus* de España aparecían en diferentes subclados dentro del clado "Linaje *T. trichiura*". Excepcionalmente, varios individuos de *T. trichiura* de hospedadores humanos y PNH, procedentes en su mayoría de Camerún, estaban dispuestos en el clado 1 o "Linaje *T. suis*".

El cálculo de los porcentajes de similitud intrapoblacional observados en el conjunto de datos ribosómicos concatenados (ITS1 e ITS2) osciló entre el 95% y el 100% (Rivero y col., 2021b). Los porcentajes de similitud intrapoblacional en el "Linaje *T. trichiura*" oscilaron entre 94,4 – 100% y en el "Linaje *T. suis*" entre 85,1 – 100%. La similitud entre ambos linajes osciló entre 74 – 78,6% (Rivero y col., 2020).

A diferencia de las regiones ribosómicas, los genes parciales *cox*1 y *co*b mostraron dos linajes genéticos claramente diferenciados dentro de la población de *Trichuris* sp. de *M. sylvanus* de España, donde un linaje genético correspondía con el haplotipo mayoritario, y el otro linaje genético con los haplotipos minoritarios, a excepción de un haplotipo para el marcador molecular *cox*1, que corresponde únicamente con una secuencia analizada y difería en pocas pb con el haplotipo mayoritario. Además, en el clado 2, se obtuvieron 4 subclados altamente soportados, donde el linaje del haplotipo mayoritario, de la población de *Trichuris* sp. de *M. sylvanus*, formaba un subclado con *T. trichiura* procedente de *Homo sapiens* africano y de *Trichuris* sp. procedente de *P. hamadryas* y de *M. fuscata* de Europa ambos, mientras que el otro linaje, de los haplotipos minoritarios, formaban un subclado diferente sin más especies. Los otros dos subclados estaban formados, en primer lugar, por *T. trichiura* de *H. sapiens* procedente de Asia y *Trichuris* sp. de *Papio anubis* procedente de EE. UU. Y, el segundo, por diferentes haplotipos de *Trichuris* sp. de *M. fuscata* procedente de Europa.

La similitud intrapoblacional obtenida del marcador molecular *cox*1, osciló entre 83,6 – 100%. La similitud observada entre los 4 subclados osciló entre 79,1 – 87%. Y la similitud obtenida entre los dos clados principales osciló entre el 73,4% y el 80,6% (Rivero y col., 2020, 2021b). Asimismo, la similitud intrapoblacional obtenida del marcador molecular *co*b, osciló entre 84,2 – 100%. Del mismo modo, los datos obtenidos para este gen fueron similares al gen *cox*1. Finalmente, la similitud

intrapoblacional en el marcador molecular *rrn*L, osciló entre 91,3 – 98,9% y la similitud obtenida entre los dos clados principales osciló entre 68,5 – 82,7% (Rivero y col., 2021b).

Así, para profundizar en la diferencia entre los dos linajes genéticos encontrados en la población de *Trichuris* sp. procedente de *M. sylvanus* se realizó un estudio molecular y filogenético comparativo de los genomas mitocondriales completos (Rivero y col., 2021a).

Las secuencias obtenidas del genoma mitocondrial completo de las muestras analizadas, TMF31 (adulto de *T. trichiura* procedente de *M. sylvanus* perteneciente al haplotipo mayoritario) y TMM5 (adulto de *Trichuris* sp. procedente de *M. sylvanus* perteneciente al haplotipo minoritario), fueron 14.091 y 14.047 pb, respectivamente (Figura 13). Estos genomas mitocondriales completos contenían 37 genes, donde 13 eran genes que codifican proteínas (PCG = "protein-coding genes"), 22 genes de ARN de transferencia (ARNt), y dos genes del ARNr. Igualmente, contenían dos regiones ricas en AT, que no codificaban.

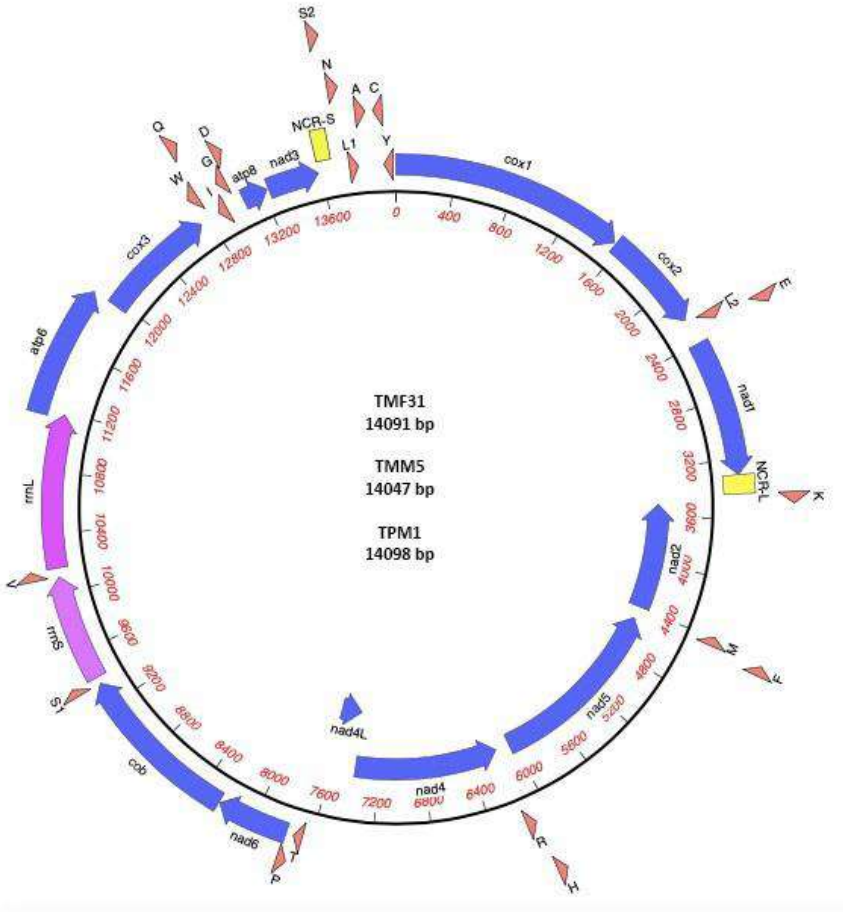

Figura 13. Estructura esquemática del genoma mitocondrial completo de *Trichuris* procedente de *M. sylvanus* y *Papio papio* (Rivero y col., 2021a).

Entre las secuencias TMF31 y TMM5 había serias diferencias, donde 22 genes de los 37 eran diferentes en longitud. El gen con mayor variación genética fue el gen *atp*8, y el más conservado fue el gen *rrn*S. Sin embargo, entre todos los PCG, el más conservado fue el gen *cox*1. Las diferencias de nucleótidos y aminoácidos entre TMF31 y TMM5 fueron del 18,7% y del 14,5%, respectivamente.

III.1.1.2. *T. trichiura* procedente de *P. papio* (Rivero y col., 2021a, 2021b)

Se llevó a cabo el estudio de tres especímenes adultos y de 9 lotes de 40 – 200 huevos de *Trichuris* aislados de *P. papio* (papión de Guinea) (Figura 14) procedentes del Parque de la Naturaleza de Cabárceno (Santander, España). El estudio morfológico y biométrico de los adultos reveló características morfológicas y biométricas similares a la especie *T. trichiura*.

Figura 14. Imagen del hospedador *P. papio* (papión de Guinea), perteneciente a D. Michal Sloviak (2013).

Por otro lado, se realizaron estudios moleculares y filogenéticos mediante el análisis de marcadores moleculares del ADNr (ITS1 e ITS2), del ADNmt (*cox*1 y *co*b) y del ARNr (*rrn*L). Así, en los alineamientos, además de las secuencias obtenidas en el presente trabajo, se introdujeron, de forma comparativa, otras secuencias del género *Trichuris* procedentes de la base de datos de GenBank.

La longitud total de las secuencias de ITS1 e ITS2 obtenidas fueron de 588 – 607 y 602 – 611 pb, y se obtuvieron 2 haplotipos y 5 haplotipos diferentes, respectivamente. La similitud intrapoblacional de estas secuencias fue del 98%. En las secuencias de los marcadores ribosómicos se encontraron ≪microsatélites≫.

La longitud total de las secuencias de *cox*1, *co*b y *rrn*L resultantes fueron de 370, 520 y 387 pb, y los haplotipos obtenidos fueron 1, 4 y 1, respectivamente.

Los árboles filogenéticos se realizaron en base a las secuencias obtenidas para los marcadores ribosómicos y mitocondriales, tanto individuales como concatenados, confirmando la presencia de dos clados principales: el clado 1 o "Linaje *T. suis*" y el clado 2 o "Linaje *T. trichiura*". Todas las secuencias obtenidas de *T. trichiura* de *P. papio* quedaban agrupadas dentro del subclado 2c, en el clado mayoritario, ya que es el que presentaba un mayor número de especies hospedadoras diferentes de humanos y PNH.

Por otra parte, se llevó a cabo la amplificación, secuenciación y análisis del genoma mitocondrial completo de *T. trichiura* procedente de *P. papio*, y se comparó con ambos genomas mitocondriales analizados previamente procedentes de *Trichuris* sp. de *M. sylvanus* (Rivero y col., 2021a).

La secuencia obtenida del genoma mitocondrial completo del adulto de *T. trichiura* procedente de *P. papio*, TPM1, fue de 14.089 pb. Este genoma mitocondrial completo contenía 37 genes: 13 eran PCG, 22 genes de ARNt, y dos genes del ARNr. Además, contenían dos regiones ricas en AT, que no codificaban.

Entre las secuencias TPM1 y TMF31 había ligeras diferencias. Las secuencias eran idénticas en todos los codones de iniciación y terminación y longitudes de genes, excepto para un gen de ARNt (ARNt-ser), que presentó un nucleótido más en TPM1. Del mismo modo, entre las secuencias TPM1 y TMF31, y TMM5, 22 genes de los 37 eran diferentes en longitud. Igualmente, que lo señalado anteriormente, el gen con mayor variación genética fue el gen *atp*8, y el más conservado fue el gen *rrn*S. No obstante, entre todos los genes que codifican proteínas, el más conservado fue el gen *cox*1.

Los genomas mitocondriales completos obtenidos del haplotipo mayoritario de *Trichuris* sp. de *M. sylvanus* (TMF31) y de *Trichuris* sp. de *P. papio* (TPM1), fueron similares, con escasas diferencias de nucleótidos y aminoácidos (0,25 y 0,41%, respectivamente). En cambio, entre ambas secuencias y el haplotipo minoritario de *Trichuris* sp. de *M. sylvanus* (TMM5) las diferencias fueron superiores (18,7 y 14,6%, respectivamente).

Consecuentemente, se realizó el estudio comparativo de los genomas mitocondriales completos obtenidos y de todas las secuencias de *Trichuris* disponibles en la base de datos de GenBank, procedentes de PNH y humanos (Rivero y col., 2021a). Para ello, se analizó la diversidad de nucleótidos utilizando los 13 PCG y los dos genes del ARNr. Los genes con la diversidad nucleotídica más baja fueron los pertenecientes al ARNr (*rrn*L y *rrn*S) y el gen *cox*1. Asimismo, la secuencia más variable fue la de *Trichuris* de *Trachypithecus francoisi*.

El árbol filogenético inferido estaba dividido en 4 clados diferentes, donde *Trichuris* sp. de *T. francoisi* apareció separado del resto de los clados. Estos resultados concordaban con los datos de diversidad nucleotídica. Dentro de los tres clados restantes, el primer clado correspondía con secuencias de *Trichuris* sp. de *H. sapiens* y *P. anubis* de Asia, Japón y EE. UU. El segundo clado estaba compuesto por la secuencia TMM5 procedente de *M. sylvanus*, y el tercer clado, correspondía con secuencias de *T. trichiura* de humanos y PNH (*M. sylvanus* (TMF31), *P. papio* (TPM1) y *P. hamadryas*) de Europa y África (Figura 15).

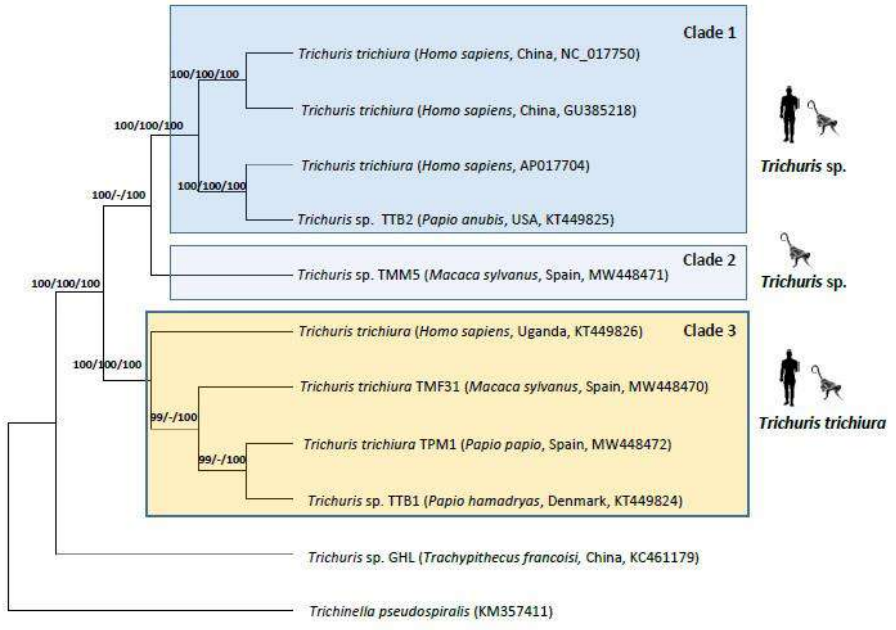

Figura 15. Árbol filogenético basado en las secuencias de nucleótidos concatenadas de 13 PCG y dos ARNr de especies de *Trichuris* de humanos y PNH mediante IB. En primer lugar, se enumeran los valores de soporte de rama obtenidos mediante el método MV, seguidos de los valores obtenidos mediante el método MP y los valores de PPB, para frecuencias de clado superiores al 60% (Rivero y col., 2021a).

Del mismo modo, se realizó un análisis filogenético entre nematodos de la clase Enoplea, concatenando 12 PCG (el *atp*8 no se pudo añadir debido a que no está presente en todos los nematodos del orden). Los resultados obtenidos revelaron congruencia con el análisis filogenético anterior. Así, la monofilia del género *Trichuris* estaba fuertemente apoyada. Asimismo, los análisis reflejaron una clara distinción entre *T. trichiura* y *Trichuris* sp. de humanos y PNH, respectivamente, con respecto a *T. suis*, *T. ovis* y *T. discolor* de herbívoros y *T. muris* de roedores, y agrupando a estos miembros de *Trichuris* con especies del orden Trichinellida, pero excluyendo a los miembros de Dorylaimida y Mermithida (Figura 16).

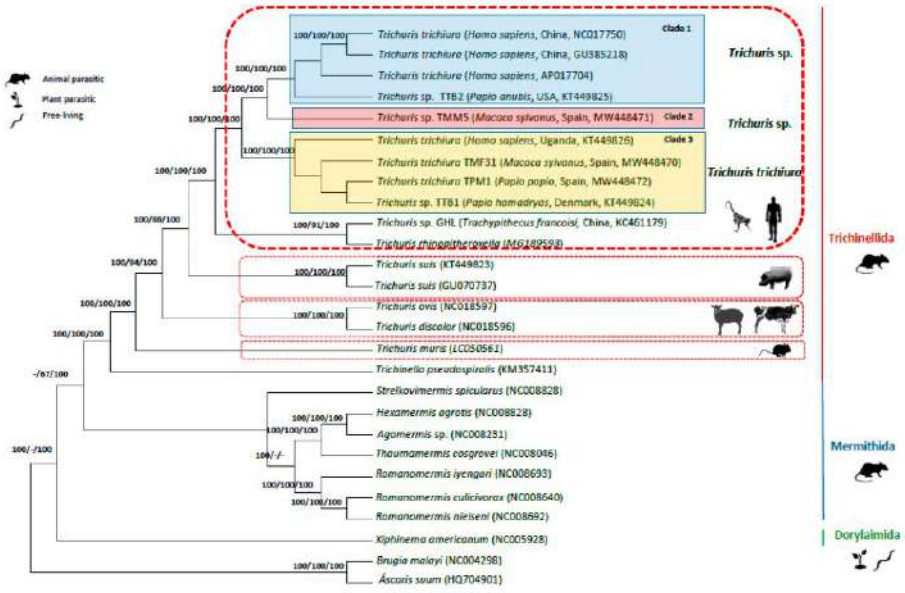

Figura 16. Árbol filogenético de nematodos enoplidos basado en secuencias de aminoácidos concatenadas de 12 PCG (excepto el gen *atp*8) usando el método de IB. Se enumeran en primer lugar los valores de soporte de rama obtenidos mediante el método MV, seguidos de los valores obtenidos mediante el método MP y los valores de PPB, para frecuencias de clado superiores al 60% (Rivero y col., 2021a).

III.1.1.3. *Trichuris* sp. procedentes de diferentes PNH (Rivero y col., 2021a, 2021b)

Se realizó un estudio de caracterización molecular y filogenético de especies de *Trichuris* de diferentes PNH a través del análisis de individuos adultos y de huevos encontrados en heces. Se obtuvieron 5 lotes de huevos (40 – 65 huevos por lote) de *Trichuris* sp. aislados de *C. aethiops* (cercopiteco verde o tota) (Figura 17A) procedente de Selwo aventura (Málaga, España), 1 lote de huevos (50 huevos) de *Trichuris* sp. aislado de *Erythrocebus patas* (mono patas) (Figura 17B) procedente del Zoo de Barcelona (Barcelona, España) y 5 adultos de *T. colobae* aislado de *C. g. kikuyensis* (colobo kikuyu) (Figura 17C) procedente de Bioparc Fuengirola (Málaga, España) (Rivero y col., 2021b).

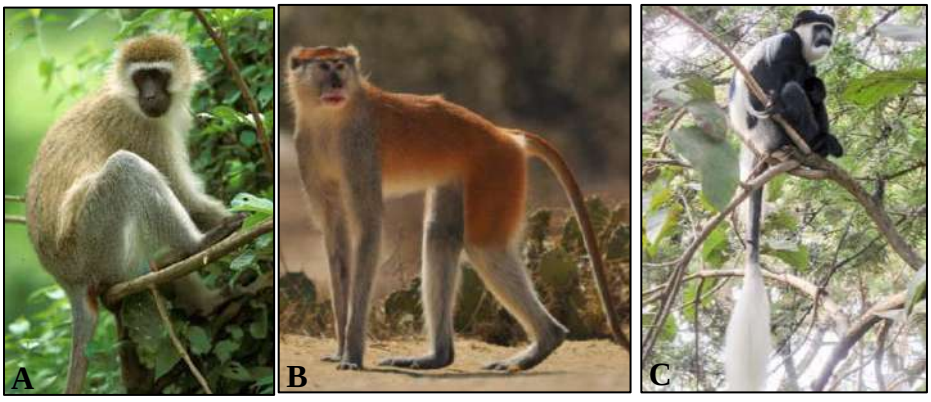

Figura 17. Imágenes de los hospedadores *C. aethiops* (A) (imagen tomada por D. William M. Ciesla, perteneciente a Forest Health Management International, Bugwood.org), *E. patas* (B) (imagen tomada por D. Arno Niehof, 2014, perteneciente a Observation.org) y *C. g. kikuyensis* (C) (imagen tomada por D. Arthur Grosset, 2016).

Se amplificaron y secuenciaron las regiones del ADNr (ITS1 e ITS2) y del ADNmt (*cox*1 y *co*b) de las muestras de huevos de *Trichuris* de *C. aethiops* y *E. patas* y el gen parcial *rrn*L de *T. colobae* (analizado morfo-biométricamente y molecularmente en estudios previos, a excepción del gen parcial *rrn*L). El gen parcial *rrn*L no pudo secuenciarse en las otras poblaciones debido a problemas de amplificación.

De las muestras de *Trichuris* procedentes de *C. aethiops* se obtuvieron 2 y 5 haplotipos diferentes, con una longitud de secuencia entre 595-601 y 556-580 pb para los marcadores moleculares ITS1 e ITS2, respectivamente. Asimismo, se obtuvo 1 haplotipo para cada marcador mitocondrial, con una longitud de secuencias de 370 pb para el gen parcial *cox*1 y 522 pb para el gen parcial *co*b.

Para las muestras procedentes de *Trichuris* aisladas de *E. patas* se obtuvo 1 haplotipo de cada marcador molecular analizado, y la longitud de las secuencias fue de 593 pb para la región ITS1, 436 pb para la región ITS2, 332 pb para el gen parcial *cox*1 y 520 pb para el gen parcial *co*b.

Finalmente, de los adultos analizados de *T. colobae* se obtuvieron 5 haplotipos diferentes para el marcador molecular *rrn*L, con una longitud de secuencia de 395 pb.

III.1.1.4. Estudio comparativo (morfológico, biométrico y molecular) de los resultados obtenidos de las especies de *Trichuris* aisladas de diferentes PNH (García-Sánchez y col., 2019; Rivero y col., 2021a, 2021b)

En la presente Tesis Doctoral se ha llevado a cabo un estudio comparativo basado en datos morfo-biométricos entre diferentes poblaciones de adultos de *Trichuris* spp. procedentes de PNH, así como con otras poblaciones de *T. trichiura* de otros hospedadores analizados en estudios previos. Asimismo, se ha realizado una redescripción de la especie *T. trichiura* (Rivero y col., 2021b).

En primer lugar, se realizó un estudio biométrico comparativo de la especie de *Trichuris* aislada de *M. sylvanus*, utilizando 30 ejemplares adultos (15 machos y 15 hembras), con distintas especies del género *Trichuris* aisladas de tres especies de PNH que residían en cautividad (*T. colobae*, *T. ursinus*, *T. trichiura* aislados de *C. g. kikuyensis*, *P. ursinus* y *Pan troglodytes*, respectivamente) y una especie de cerdo (*T. suis* aislado de *Sus scrofa domestica*), publicados previamente. Este estudio se llevó a cabo mediante microscopía óptica (García-Sánchez y col., 2019). La prueba t de Student mostró que varias medidas eran significativas para los análisis morfométricos posteriores: ancho máximo de la región posterior del cuerpo, longitud de la espícula, longitud máxima de la vaina espicular, distancia entre la parte posterior del testículo y el extremo posterior del cuerpo y la longitud del tubo cloacal distal en los machos y, la anchura máxima de la región posterior del cuerpo y la longitud de la zona muscular del esófago en las hembras.

Seguidamente, se realizó el análisis morfo-biométrico comparativo utilizando la técnica de morfometría geométrica entre las especies de *Trichuris* analizadas previamente (García-Sánchez y col., 2019). Con las variables de análisis utilizadas (descritas con anterioridad), se obtuvo un primer componente principal (PC1) que contribuía con el 64% en la variación general en los machos y en un 61% en las hembras. Se crearon mapas de factores para los adultos, tanto para los machos como para las hembras individualmente. En el mapa de factores de los machos se reflejó claramente la diferencia de tamaño entre las diferentes especies de *Trichuris* analizadas, mostrando un tamaño más grande en los machos procedentes del macaco de Gibraltar. Además, las poblaciones de machos estaban bien agrupadas, sin áreas superpuestas entre ellas. En consecuencia, cada población aparecía separada una de otra a excepción de los adultos de *Trichuris* procedentes de macaco y cerdo, que mostraron un solapamiento parcial, pero sin comprometer la correcta identificación de las comunidades (Figura 18).

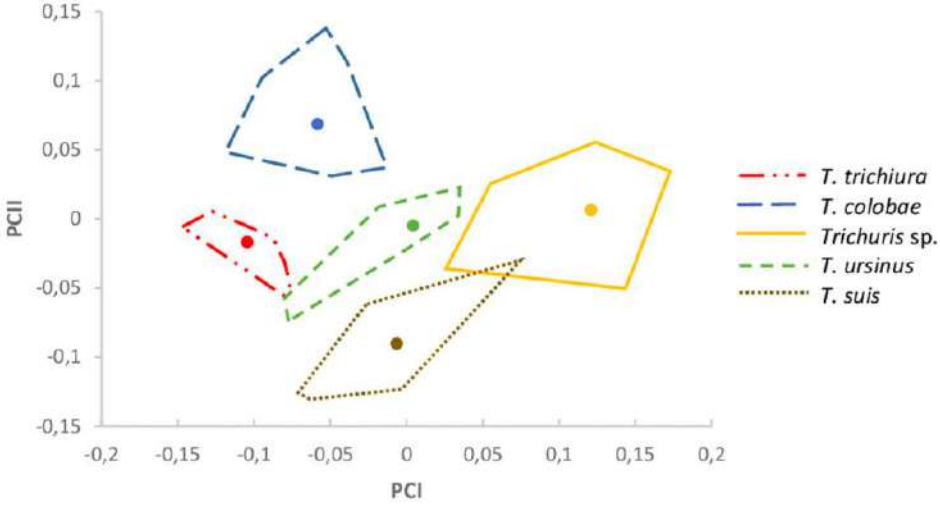

Figura 18. Mapa factorial correspondiente con machos del género *Trichuris* (García-Sánchez y col., 2019).

Por otro lado, el mapa factorial de las hembras, no mostraba diferencias de tamaño global en las cinco especies. Se distinguieron dos zonas, una estaba formada por los adultos de *Trichuris* procedentes de los cuatro hospedadores primates, y la segunda, se componía únicamente de los adultos de *Trichuris* procedentes del cerdo. Dentro de la zona de los adultos de *Trichuris* de primates, mostraban una amplia área de superposición que no permitía una específica identificación de cada población de las hembras. Por lo tanto, las hembras de *T. suis* constituyeron la excepción, ya que formaban un grupo independiente de la comunidad de adultos de *Trichuris* de primates, sin superposición entre ellos (Figura 19) (García-Sánchez y col., 2019).

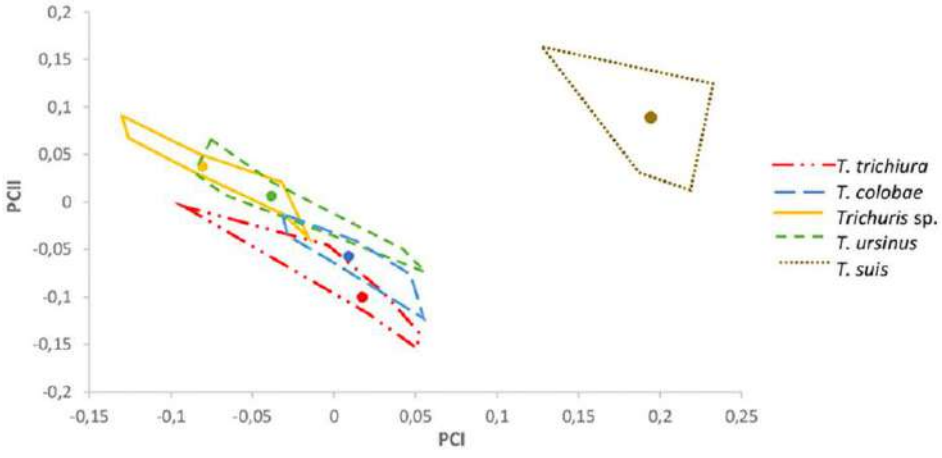

Figura 19. Mapa factorial correspondiente con hembras del género *Trichuris* (García-Sánchez y col., 2019).

Del mismo modo, se evaluó el grado de similitud entre las poblaciones de tricocéfalos, separadas por sexo, a través del cálculo de la distancia de Mahalanobis. Se observaron mayores distancias entre adultos de *Trichuris* de primates y *T. suis*, y además las distancias fueron mayores entre las hembras que entre los machos, revelando que el fenotipo de los tricocéfalos de *T. suis* frente a los de *Trichuris* de primates, son más

divergentes en las hembras que en los machos (García-Sánchez y col., 2019).

Los resultados obtenidos gracias a la técnica de morfometría geométrica demostraron que es una herramienta útil para diferenciar entre diferentes especies de machos de *Trichuris*. No obstante, dicha técnica aplicada a las hembras no parecía aportar información adicional, ya que todas las combinaciones de parámetros biométricos obtenidas mostraron resultados similares (García-Sánchez y col., 2019).

Para poder realizar el análisis filogenético, en los alineamientos, además de las secuencias obtenidas en los trabajos, se compararon con otras secuencias del género *Trichuris* disponibles en la base de datos de GenBank. Los análisis filogenéticos se realizaron basándose en las secuencias de los marcadores moleculares analizados, tanto individuales como de forma concatenada (ITS1, ITS2, *cox*1, *co*b y *rrn*L), confirmando la presencia de dos clados principales: el clado 1 o "Linaje *T. suis*" y el clado 2 o "Linaje *T. trichiura*".

El árbol filogenético de los marcadores ribosómicos concatenados reveló la existencia de dos clados principales, descritos anteriormente, que estaban fuertemente soportados. Dentro del clado 1 se obtuvieron cuatro subclados diferentes, y dentro del clado 2 se obtuvieron seis clados diferentes. De los seis clados obtenidos, cinco de ellos mostraron una alta homología (94 – 99%), quedando un subclado, formado por la secuencia de *Trichuris* sp. de *T. francoisi*, separado del resto.

Asimismo, examinando las secuencias de los marcadores ribosómicos, los porcentajes de similitud entre las secuencias de *Trichuris* sp. de *P. papio*, *C. aethiops* y *E. patas* obtenidas en el presente trabajo y las demás secuencias de *Trichuris* de primates y PNH, fueron muy elevados (94 – 100%). Además, cuando se compararon los dos clados principales, el porcentaje de similitud fue menor (90 – 92%).

Los árboles filogenéticos de los marcadores mitocondriales mostraron topologías similares. En el clado de "Linaje *T. trichiura*" se observaron 4 subclados principales, donde, en el subclado 2c, quedaron agrupadas las secuencias de *Trichuris* procedentes de *P. papio, C. aethiops* y *E. patas* junto con las secuencias de *T. trichiura* de *H. sapiens* de Uganda y *Trichuris* sp. procedentes de otros primates de África y Europa. Sin embargo, como se ha mencionado previamente, las secuencias de *Trichuris* sp. de *M. sylvanus* quedaban separadas en dos subclados diferentes, unos haplotipos se relacionaban con el subclado 2c y los otros en el subclado 2a.

Las secuencias de los marcadores mitocondriales de *Trichuris* sp. de *P. papio, C. aethiops, E. patas* y el haplotipo mayoritario de *M. sylvanus*, obtenidas en el presente trabajo, mostraron porcentajes de similitud muy elevados dentro del subclado 2c (92,1 – 100%). En cambio, el porcentaje de similitud entre los dos clados principales fue del 68% al 82%.

El árbol filogenético obtenido de la concatenación de los marcadores mitocondriales (*cox*1 y *co*b) reveló el subclado 2c como el principal dentro del clado "Linaje *T. trichiura*", ya que agrupaba a la mayoría de las especies de *Trichuris* que parasitaban a humanos procedentes de África y diferentes PNH de África y Europa.

Además, se realizó el árbol filogenético concatenado de marcadores ribosómicos (ITS1 e ITS2) y mitocondriales (*cox*1 y *co*b), a excepción del marcador *rrn*L, debido a la falta de datos en paralelo, en el cual, el clado de "Linaje *T. trichiura*" se dividió en tres subclados, donde el subclado 2c era el más representativo incluyendo la mayor variedad de *Trichuris* de diferentes hospedadores (humanos y PNH) (Figura 20).

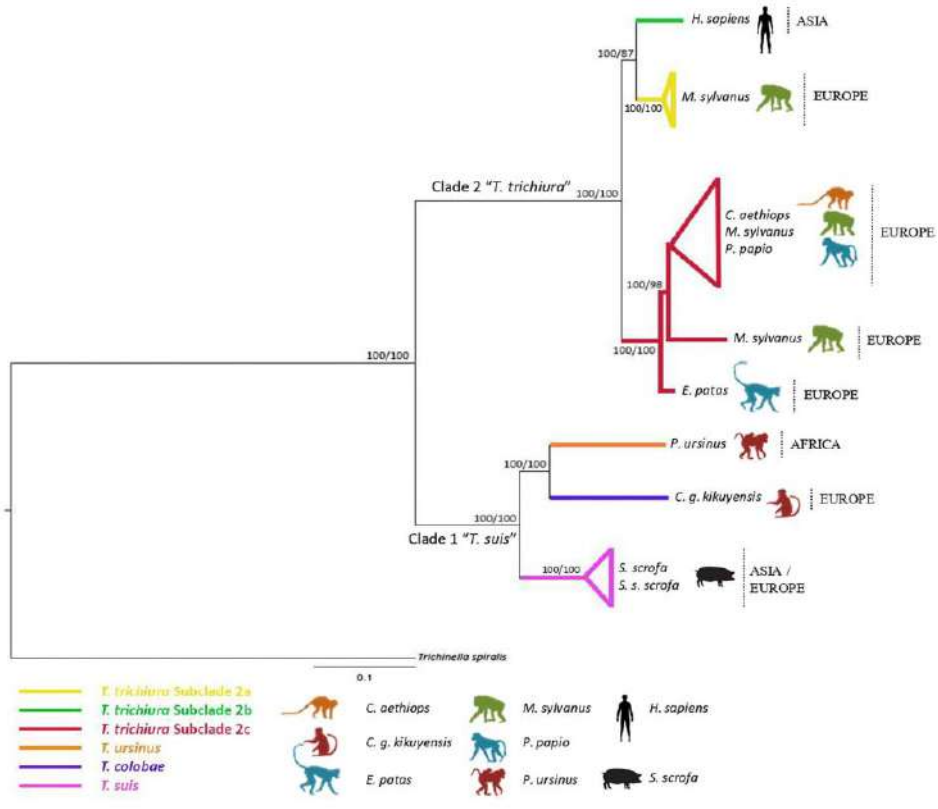

Figura 20. Árbol filogenético de especies de *Trichuris* basado en el análisis combinado del ADNmt (*cox*1 y *co*b) y del ADNr (ITS1 e ITS2) inferido utilizando el método de IB. Los valores de soporte de rama obtenidos mediante el método MV se enumeran primero, seguidos de los valores de PPB, para frecuencias de clado superiores al 60% (Rivero y col., 2021b).

Asimismo, fue analizada la diversidad genética de los diferentes marcadores dentro del clado 2 o "Linaje *T. trichiura*", revelando una diversidad haplotípica de 1,0 para los ITS y 0,95 – 0,93 para los marcadores mitocondriales.

Además, se analizó la saturación de sustitución de los nucleótidos, revelando que las regiones de ITS estaban saturadas. En cambio, los genes mitocondriales no estaban saturados, pero el marcador *rrn*L presentaba una saturación menor que la de los ITS, y mayor que la de los marcadores mitocondriales (*cox*1 y *co*b), proporcionando una mayor resolución,

debido a que presenta una menor variabilidad intraespecífica (Rivero y col., 2021b).

III.1.1.5. *Trichuris* sp. aislado de *H. cristata* (Rivero y col., 2022a)

Se ha llevado a cabo un estudio morfológico, biométrico, de caracterización molecular y filogenético de una población de 30 especímenes adultos (15 machos y 15 hembras) de *Trichuris* aislados de un puercoespín crestado (*H. cristata*) (Figura 21) procedente de Bioparc Fuengirola en Málaga, España (Rivero y col., 2022a).

Figura 21. Imagen del hospedador *H. cristata* (puercoespín crestado) (Beiträge, CC BY-SA 3.0, vía Wikimedia Commons).

En primer lugar, se ha realizado un estudio morfológico y biométrico, así como una descripción detallada de los caracteres morfológicos de los machos y de las hembras analizadas. Del mismo modo, se ha realizado un estudio comparativo entre esta población de *Trichuris* sp. de *H. cristata* y otras especies del género *Trichuris* procedentes de diferentes especies de puercoespines: *Trichuris landak* aislada de *Hystrix javanica*, *Trichuris hystricis* aislada de *H. cristata* e *Hystrix indica*, *Trichuris thrichomysi* aislada de *Thrichomysi apereoides*, *Trichuris infundibulum* aislada de *H. cristata*, *Trichuris metami* aislada de *Atelerix* o *Aethehinus* y *Trichuris lenkorani* aislada de *Hystrix hirsutrirostris*.

Para realizar el análisis molecular, se han utilizado tanto marcadores ribosómicos como mitocondriales (ITS1 del ADNr y los genes parciales *cox*1, *co*b del ADNmt y el gen parcial *rrn*L del ARNr), de 30 individuos (15 machos y 15 hembras), donde se han obtenido dos haplotipos diferentes (THC1 y THC2).

La longitud total de las secuencias de ITS1 obtenidas fue de 550 pb para THC1 y 552 pb para THC2; para el marcador molecular *cox*1 fue de 370 pb y para la *co*b 520 pb en ambos haplotipos, y para el *rrn*L fue 360 pb para THC1 y 373 pb para THC2.

Para realizar el estudio filogenético, se ha llevado a cabo un análisis de las secuencias de *Trichuris* obtenidas en el presente trabajo, junto con otras secuencias de *Trichuris* obtenidas de la base de datos de GenBank.

El árbol filogenético del marcador ITS1 mostró dos clados principales. Sin embargo, estos clados no presentaban altos valores de soporte de rama. El porcentaje de similitud intrapoblacional de *Trichuris* procedente de *H. cristata* para este marcador fue del 99,9%. Así, entre las secuencias estudiadas, el máximo porcentaje de similitud fue entre ambos haplotipos y *T. vulpis* (87,03%), y el mínimo fue con la especie *Trichuris myocastoris* (80,6 – 80,9%).

Los árboles filogenéticos de los marcadores mitocondriales, tanto individuales como concatenados, confirmaron la existencia de dos grupos principales. El primer grupo estaba formado por especies de *Trichuris* procedentes de herbívoros, y el segundo grupo, por especies de *Trichuris* procedentes de omnívoros. Dentro de esos dos grupos se observaron 5 clados con porcentajes de similitud similares. El clado 1 incluía especies de *Trichuris* procedentes de *H. cristata*; el clado 2, especies de *Trichuris* de diferentes roedores y cánidos; el clado 3 incluía especies de *Trichuris* de humanos y PNH; el clado 4 incluía especies de *Trichuris* procedentes de suidos y PNH (*T. suis*, *T. ursinus* y *T. colobae*); y finalmente, el clado

5 incluía especies de *Trichuris* que parasitan a herbívoros (*T. ovis*, *T. discolor*, *T. skrjabini* y *T. leporis*).

El árbol filogenético del ADNmt (*cox*1 y *co*b) y del ARNr (*rrn*L) concatenado, reveló un linaje monofilético para el género *Trichuris*. Los cinco clados principales estaban fuertemente soportados. Las secuencias de *Trichuris* de *H. cristata* obtenidas en el presente trabajo se agrupaban en el clado 1, separadas del resto de las secuencias. Sin embargo, se observa una relación hermana entre el clado 1 y el clado 2 fuertemente soportada. Además, ambos clados, mostraron una relación filogenética más estrecha con los clados 3 y 4 respecto al clado 5.

El porcentaje de similitud entre los dos haplotipos encontrados en la población de *Trichuris* sp. procedente de *H. cristata* fue del 86,3% para el marcador molecular *cox*1, 90,1%, para la *co*b, 91,2% y para el *rrn*L. En el estudio del gen parcial *cox*1, la secuencia THC1 mostraba el máximo porcentaje de similitud con la secuencia de *Trichuris* sp. de *H. cristata* procedente de Italia (TIS1) (99,7%), mientras que comparándolo con el haplotipo THC2, la similitud era menor (86,6%). El mínimo porcentaje de similitud se obtuvo con la secuencia de *Trichuris* sp. procedente de *T. francoisi*. Asimismo, en las secuencias analizadas del gen parcial *co*b, el máximo porcentaje de similitud fue entre el haplotipo THC2 y la secuencia de *Trichuris* sp. de *H. cristata* procedente de Italia (TIS8) con un 91,2%, mientras que con el haplotipo THC1 el porcentaje fue del 89,1%. Para el marcador *rrn*L, las secuencias THC1 y THC2 obtuvieron un máximo porcentaje de similitud con las secuencias de *Trichuris discolor* (82,8%) y *T. suis* (83,1%), respectivamente.

Finalmente, se realizó el árbol filogenético concatenado tanto de los marcadores mitocondriales (*cox*1, *co*b y *rrn*L) como del marcador ribosómico (ITS1) y se obtuvieron 5 clados fuertemente soportados por el método de MV, en cambio, el método de IB no resolvía adecuadamente a nivel de especies (Figura 22). Dentro de estos clados, el clado 1

correspondiendo a *Trichuris* de *H. cristata* del presente trabajo se agrupaba con el clado de *T. muris*, mientras que los clados de *Trichuris* de primates se agruparon, *T. suis* y *T. colobae*, y *T. discolor* y *T. ovis* también.

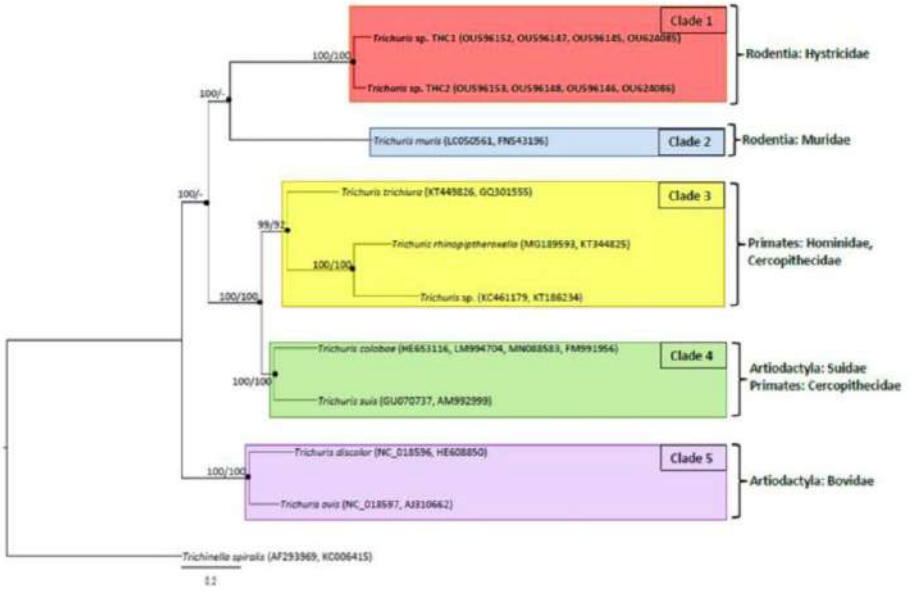

Figura 22. Árbol filogenético de especies de *Trichuris* basado en el análisis combinado del ADNmt (*cox*1 y *co*b), ARNr (*rrn*L) y el ADNr (ITS1) inferido utilizando el método de MV. Los valores de soporte de rama obtenidos mediante el método MV se enumeran primero, seguidos de los valores de PPB, para frecuencias de clado superiores al 60% (Rivero y col., 2022a).

III.1.1.6. *Trichuris* sp. aislado de *C. bactrianus* (Rivero y col., 2023)

Se ha llevado a cabo un análisis molecular de una población de *Trichuris* sp. aislada de camello bactriano (*C. bactrianus*) (Figura 23) que procedía del Parque de la Naturaleza de Cabárceno, situado en Santander, España. Se obtuvieron cuatro especímenes diferentes llevándose a cabo la caracterización molecular, así como estudios filogenéticos comparativos con especies de *Tricuhris* parásitas de diferentes hospedadores. Igualmente, fueron amplificados, secuenciados y analizados los marcadores moleculares del ADNr (ITS1 e ITS2), del ADNmt (*cox*1 y *co*b) y del ARNr (*rrn*L) (Rivero y col., 2023).

Figura 23. Imagen del hospedador *C. bactrianus* (camello bactriano) con una cría (imagen tomada en el año 2015, perteneciente a parquecabarceno.com).

Para realizar el alineamiento de los distintos conjuntos de datos, se utilizaron las secuencias obtenidas en el presente trabajo además de otras secuencias obtenidas de la base de datos de GenBank.

En todos los árboles filogenéticos inferidos, utilizando los marcadores moleculares tanto individuales como de forma concatenada, las cuatro secuencias de *Trichuris* sp. de *C. bactrianus* objeto de estudio formaron un clado con altos valores de soporte de rama. Debido a que no todas las secuencias de los marcadores analizados estaban disponibles en GenBank, no se pudo obtener un conjunto de datos de todos los marcadores concatenados. Por esa razón, el marcador *rrn*L, debido a la falta de secuencias, y el marcador ITS2, debido a la presencia de más secuencias, se analizaron también por separado.

El árbol filogenético obtenido del conjunto de datos del marcador ITS2 reveló un árbol sin valores soportados para inferir la relación filogenética entre las especies de *Trichuris*. Sin embargo, los resultados si soportaban

valores para delimitar entre especies. Así, las poblaciones de *Trichuris* de *C. bactrianus* procedentes de España y República Checa se agruparon, y estaban más relacionados con *T. leporis*. En cambio, estas secuencias no estaban relacionadas con las secuencias de *T. globulosa* de *C. dromedarius*. Además, este árbol concuerda con los datos de similitud entre las secuencias, donde el máximo porcentaje de la población de *Trichuris* procedentes de *C. bactrianus* fue con la secuencia de *Trichuris* sp. de *C. bactrianus* de República Checa (99,8 – 99,9%). Sin embargo, se obtuvieron valores de porcentajes de similitud similares entre la población de *Trichuris* procedentes de *C. bactrianus* y las secuencias de *T. skrjabini* (95,6 – 95,7%), *Trichuris* sp. de *Ovis aries* (95,3%) y *T. leporis* (95,5 – 95,6%).

El conjunto de datos obtenido de la concatenación de las secuencias de ITS1 e ITS2 infirió un árbol filogenético con cuatro clados principales: el clado 1 incluía las secuencias de *Trichuris* procedentes de humanos, PNH y suidos; el clado 2 incluía especies de *Trichuris* procedentes de cánidos y roedores; el clado 3 incluía especies de *Trichuris* encontradas en camellos, ovejas, cabras y liebres; y el clado 4, especies de *Trichuris* encontradas en vacas, cabras, ovejas y roedores. Los clados estaban fuertemente soportados por ambos métodos analizados (MV y IB), exceptuando el clado 4, que obtuvo valores menores al 65% por el método de MV. La inferencia filogenética de *Trichuris* procedente de *C. bactrianus* con respecto a otras especies de *Trichuris*, reveló un grupo hermano que incluía a *T. skrjabini*, *Trichuris* sp. procedente de *Ovis* spp. y *T. leporis*, soportado con altos valores. Estos datos estaban apoyados por los porcentajes de similitud obtenidos para el marcador molecular ITS1, donde las secuencias de la población de *Trichuris* procedentes de *C. bactrianus* obtuvo los valores máximos de similitud con las especies de ese mismo clado descrito previamente (94,2 – 95,4%).

La inferencia filogenética obtenida del marcador molecular *rrn*L no resolvía bien debido a que los clados estaban en politomía. Aun así, las secuencias de *Trichuris* obtenidas de *C. bactrianus* formaban un clado sólido y hermano a *T. skrjabini*. Asimismo, el máximo porcentaje de similitud de la población de *Trichuris* procedentes de *C. bactrianus* fue con la secuencia de *T. skrjabini* (88,8 – 89%).

El árbol filogenético inferido del conjunto de datos concatenados de los marcadores mitocondriales (*cox*1 y *co*b), estaban en congruencia con los árboles obtenidos de los marcadores ribosómicos, ya que, mostraron los cuatro clados principales citados previamente, además de un quinto clado que incluía las especies de *Trichuris* procedentes de *H. cristata*. Las especies de *Trichuris* encontradas en camélidos se separaron en diferentes clados; la población de *Trichuris* procedente de *C. bactrianus* de España se relacionó con *T. skrjabini* dentro del clado 3, mientras que la población de *Trichuris* de *C. bactrianus* de Italia estaba más relacionado con las secuencias de *T. ovis* dentro del clado 4 y, éstas a su vez, más relacionadas con *Trichuris* sp. de *Addax nosamaculatus* procedente de Italia y *T. globulosa* procedente de *C. dromedarius* de Irán. Ambos clados que incluían especies de *Trichuris* que parasitaban especies herbívoras, fueron los más relacionados filogenéticamente, quedando el resto de clados más separados. Asimismo, estos datos concuerdan con los resultantes en los estudios de similitud para ambos marcadores moleculares.

Finalmente, se obtuvo el árbol filogenético inferido de la concatenación de los marcadores ribosómicos (ITS1 e ITS2) y los mitocondriales (*cox*1 y *co*b) analizados, el cual mostró cuatro clados principales de acuerdo con los marcadores individuales analizados previamente. Las secuencias de *Trichuris* obtenidas de *C. bactrianus*, obtenidas en el presente trabajo, se agruparon en el mismo clado, y mostró una relación hermana con *T. skrjabini* con altos valores de soporte de rama, separado de los demás clados (Figura 24).

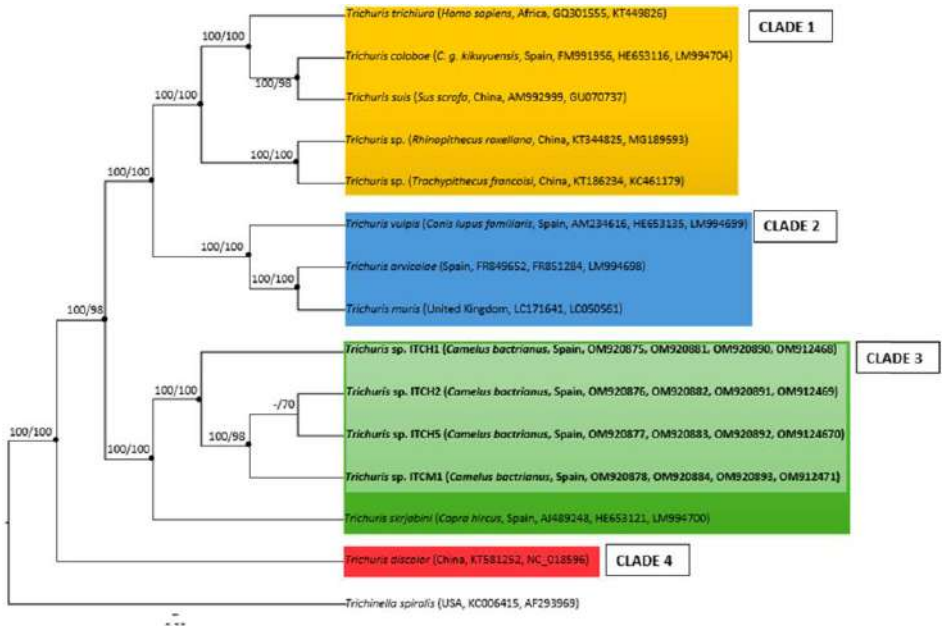

Figura 24. Árbol filogenético de especies de *Trichuris* basado en el análisis combinado del ADNmt (*cox*1 y *cob*) y el ADNr (ITS1 e ITS2) inferido utilizando el método de IB. Los valores de soporte de rama obtenidos mediante el método MV se enumeran primero, seguidos de los valores de PPB, para frecuencias de clado superiores al 60% (Rivero y col., 2023).

III.1.2. Aplicación de la técnica de diagnóstico MALDI-TOF para la identificación de diferentes especies de *Trichuris* (Rivero y col., 2022b)

Se ha llevado a cabo un estudio proteómico de distintas especies del género *Trichuris* mediante la técnica de diagnóstico MALDI-TOF MS.

El estudio se ha realizado en dos partes diferentes. La primera parte consistió en la puesta a punto de la técnica de extracción de proteínas y en la creación de una base de datos. Para ello, se utilizó la especie *T. suis* y se analizaron 5 vermes adultos, dividiendo el cuerpo en dos partes; la parte anterior ("esófago": mayoritariamente constituida por el esófago) y la parte posterior ("intestino": constituida por el intestino y por las gónadas). Así, cinco esófagos y tres intestinos fueron el material utilizado para generar la base de datos de referencia. Del mismo modo, para verificar

que los adultos utilizados eran de la especie *T. suis*, se amplificó, secuenció y analizó el gen parcial *cox*1 del ADNmt frente a secuencias previamente depositadas en BLASTn, revelando aproximadamente un 100% de identidad entre la especie *T. suis* y las secuencias obtenidas en el presente estudio.

Utilizando el protocolo de extracción de proteínas y preparación descrito en el Anexo I, Material y Métodos, para las muestras de adultos de *Trichuris*, conseguimos obtener espectros de referencia de alta calidad, con una alta reproducibilidad e intensidad espectral. Ambas partes del verme mostraron espectros similares, con perfiles de alta intensidad y picos fuertes, localizados en el mismo rango.

Para la creación de los espectros de referencia, el total de los espectros obtenidos fueron analizados previamente con el software que incluye la base de datos comercial (con especies de bacterias y hongos), obteniéndose valores logarítmicos (LSV = "Log-score values") < 1,7. Posteriormente, se realizó un análisis con el software conteniendo la base de datos comercial y la base de datos interna y reveló la correcta identificación de la especie *T. suis* en todas las muestras analizadas. Sin embargo, no fue posible diferenciar entre esófago e intestino de las mismas muestras de *T. suis*. Los LSV observados para cada muestra eran mayores cuando eran comparados con su correspondiente parte del cuerpo, pero se identificaban correctamente con ambas partes. Los LSV oscilaron entre 2,10 y 2,82.

Para validar los datos obtenidos, se realizó una prueba ciega con un total de 20 adulos de *Trichuris*. Para ello, de la misma manera, se dividieron los vermes en dos partes y se analizaron a través de la técnica de MALDI-TOF MS. A excepción de dos muestras de esófagos, que no se extrajeron las proteínas correctamente, el 100% de las muestras restantes fueron correctamente identificadas.

En la segunda parte del estudio, se analizaron cuatro especies más del género *Trichuris*. Para realizar este estudio, como anteriormente se habían analizado las dos partes del verme y ambas daban resultados correctos y similares, se precisó utilizar la parte anterior o del esófago para el análisis con la técnica del MALDI-TOF MS, y utilizar la parte posterior para estudios moleculares o estudios posteriores.

Para proporcionar una correcta identificación, fueron amplificadas y secuenciadas 19 muestras utilizando el gen parcial *co*b del ADNmt y fueron comprobadas con secuencias previamente depositadas en BLASTn, mostrando valores de identidad de 98,8 – 100%. El protocolo preliminar usado para la especie de *T. suis* proporcionó espectros de alta calidad para cada especie de *Trichuris* analizada (Figura 25) (*Trichuris* sp. procedente de *H. cristata*, *T. trichiura*, *T. vulpis* y *T. ovis*). Asimismo, la base de datos interna fue actualizada con estas cuatro especies de *Trichuris* y 16 nuevos espectros de referencia (*Trichuris* sp. de *H. cristata* (*n* = 5), *T. vulpis* (*n* = 2), *T. ovis* (*n* = 4), and *T. trichiura* (*n* = 5)).

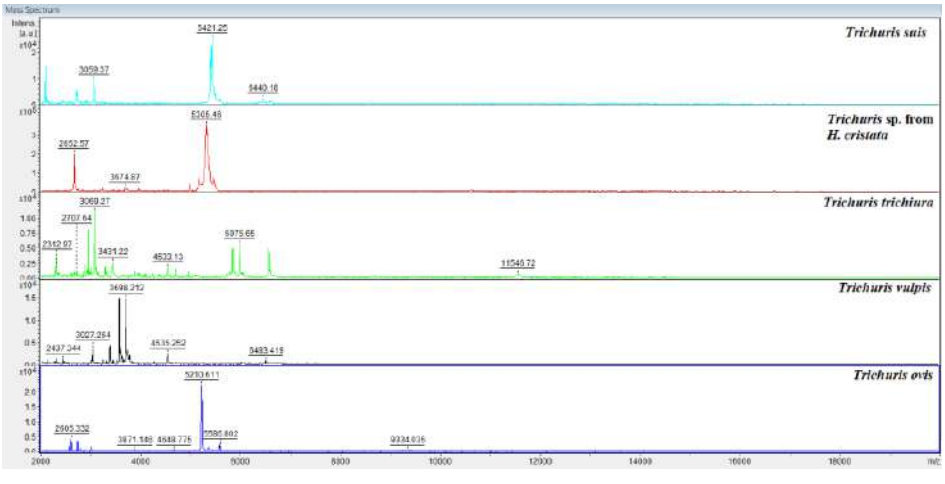

Figura 25. Espectros obtenidos para el género *Trichuris* mediante el uso de la técnica MALDI-TOF MS.

Para validar los espectros introducidos en la base de datos de referencia, se llevó a cabo una prueba ciega con 79 especímenes. Los resultados confirmaron la correcta identificación del 100% de las muestras con LSV > 1,70. Para los valores de LSV ≥ 2,00, los porcentajes fueron del 72,2% para *Trichuris* sp. de *H. cristata*, del 87,5% para *T. vulpis*, del 100% para *T. ovis* y del 88,9% para *T. trichiura*.

Posteriormente, se obtuvo un dendrograma con los espectros de referencia de las cinco especies estudiadas, y se pudo observar que las cinco especies estaban claramente separadas y, agrupadas aquéllas que pertenecían a la misma especie en un mismo clado.

Finalmente, utilizando la parte posterior de los adultos se realizó un estudio molecular mediante la amplificación y secuenciación del gen parcial *co*b del ADNmt obtenido en el estudio de las diferentes especies del género *Trichuris* analizadas.

El árbol filogenético obtenido reveló 5 clados principales, correspondiendo cada clado con una especie diferente del género *Trichuris*. Todos los clados estaban fuertemente soportados. Además, los clados de *T. trichiura* y *T. suis* estaban más relacionados entre ellos, pero únicamente por el método de IB. Ambos clados a su vez estaban estrechamente relacionados con el clado de *T. vulpis*, y todos a su vez estaban más relacionados con *Trichuris* sp. de *H. cristata*, quedando el clado de *T. ovis* separado de ellos.

La similitud intraespecífica de todas las muestras de la misma especie estudiada daba valores cercanos al 100%, a excepción de la población de *Trichuris* sp. de *H. cristata* que mostró dos grupos diferentes. El mínimo porcentaje de similitud nucleotídica y aminoacídica entre las especies estudiadas fue entre *T. trichiura* y *Trichuris* sp. de *H. cristata* (68% y 65,3%, respectivamente), y el máximo porcentaje de similitud nucleotídica fue entre *T. ovis* y *Trichuris* sp. de *H. cristata* (73,6%) y aminoacídica entre *T. trichiura* y *T. suis* (76,3%).

III.2. PUBLICACIONES

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Differentiation of *Trichuris* species using a morphometric approach

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ABSTRACT

Trichuris trichiura is a nematode considered as the whipworm present in humans and primates. The systematics of the genus *Trichuris* is complex. Morphological studies of *Trichuris* isolated from primates and humans conclude that the species infecting these hosts is the same. Furthermore, numerous molecular studies have been carried out so far to discriminate parasite species from humans and Non-Human Primates using molecular techniques, but these studies were not performed in combination with a parallel morphological study. The hypothesised existence of more species of *Trichuris* in primates opens the possibility to revise the zoonotic potential and host specificity of *T. trichiura* and other putative new species of whipworms.

In the present work, a study of *Trichuris* Roederer, 1761 (Nematoda:Trichuridae) parasitizing *C. g. kikuyensis*, *P. ursinus*, *Macaca sylvanus*, *Pan troglodytes*, and *Sus scrofa domestica* has been carried out using modern morphometric techniques in order to differentiate populations of *Trichuris* isolated from four species of captive NHP from different geographical regions, and swine, respectively.

The results obtained revealed strong support for geometrical morphometrics as a useful tool to differentiate male *Trichuris* populations. Therefore, morphometrics in combination with other techniques, such as molecular biology analyses, ought to be applied to further the differentiation of male populations.

On the other hand, morphometrics applied to female *Trichuris* species does not seem to contribute new information as all the measurements combinations of obtained from females always showed similar results.

1. Introduction

Trichuris species are nematodes parasitizing the caecum of different hosts. The specific differentiation of the genus *Trichuris* has been the subject of a long-ranging controversy. Several morphological studies have been reported (see Cutillas et al., 2014), since Dujardin (1845) reviewed the genus for the first time. Chandler (1930) and Knight (1971) found that the spicule length is the most dependable character for *Trichuris* species differentiation. Nevertheless, the spicule length of *Trichuris trichiura* and that of *Trichuris suis* overlaps. Furthermore, Spakulová and Lýsek (1981) found that the male and female of *T. suis* from abattoir pigs differed significantly from *T. suis* from wild boars.

Morphometric characters of nematode parasites such as body length, body width, oesophagus length, spicule length, etc can vary according to a variety of environmental factors, including host. Thus, Knight (1984) reported that the morphological characteristics of *Trichuris ovis* were affected by the host to a greater extent than by geographical area.

Several features, such as the presence/absence of the spicule tube, the shape and distribution of the spines of the spicule sheath, length of the spicule and the cloacal tube, the shape of the proximal and distal cloacal tube, and the vulvar morphology, along with classic morphometric characteristics have been found to have a high discriminatory value to differentiate *Trichuris* species (Correa et al., 1992; Suriano and Navone, 1994; Robles et al., 2006, 2014; Callejón et al., 2017). Furthermore, some studies (Kikuchi, 1974a, 1974b; Tenora et al., 1993, 1997; Robles et al., 2006; Cutillas et al., 2009, 2014; Callejón et al., 2017) have used scanning electron microscopy (SEM) as a useful diagnostic tool. *Trichuris* species have been described with a narrow range of anatomic and biometric characteristics, and they have been insufficiently compared with their congeneric species (Robles et al., 2014).

Cutillas et al. (2014) proposed a new species, *Trichuris colobae*, for a Non-Human Primate (NHP), based on different parameters that significantly discriminated *T. suis* and *T. trichiura* to *T. colobae* from *Colobus guereza kikuyensis*. Furthermore, Callejón et al. (2017) reported, in

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a morphological and morphometric study, that *Trichuris ursinus* from another NHP (*Papio ursinus*) differed significantly from *T. trichiura* (nine different characters) and *T. colobae* (six different characters). Also, *T. ursinus* shows features close to *T. suis* (only three different characters). *Trichuris* specimens were measured according to parameters reported by Spakulová and Lýsek (1981), Suriano and Navone (1994) and Robles et al. (2006) who summarized the parameters used in recent years.

Traditional parasitological research on *T. trichiura* from humans and NHPs has focused on differentiating it from *T. suis* found in pigs (Beer, 1976; Ooi et al., 1993; Cutillas et al., 2009; Nissen et al., 2012; Liu et al., 2012). Morphological studies of *Trichuris* isolated from primates and humans conclude that the species infecting these hosts is the same, although slight morphological variations are distinguishable when scanning electron microscopy is used (Ooi et al., 1993). Nevertheless, these studies were based on a few morphological characters such as the total length or spicule length, etc., but not on discriminative analysis of several significant morpho-biometric parameters using statistical tests. Thus, numerous molecular studies have been carried out so far to discriminate parasite species from humans and NHPs using molecular techniques, but these studies were not performed in combination with a parallel morphological study. Ravasi et al. (2012) suggested the need for morphological analysis of *Trichuris* sp. adult worms collected from *P. ursinus* (Chacma baboon) from South Africa to determine whether the genetic lineages correspond to different morphological species. In this sense, Callejón et al. (2017) reported in a morphological and morphometric study that *T. ursinus* from Chacma baboon (*P. ursinus*) differed significantly from *T. trichiura* (nine different characters) and *T. colobae* (six different characters) and showed features close to *T. suis*.

The systematics of the genus *Trichuris* is complex. Although morphological variation has been quantified by modern morphometric techniques in parasite as arthropods (Zurita et al., 2019; Santillán-Guayasamín et al., 2017) and helminths (Ashrafi et al., 2015; Valero et al., 2018), it has never been applied to *Trichuris* species.

Our findings suggest the need for morphometric analyses of adult *Trichuris* species of different NHPs to determine whether the genetic lineages correspond to different species and whether there are significant morphological features and biometric data that can be used to distinguish them from *T. suis* and *T. trichiura*. Thus, in the present work, a study of *Trichuris* Roederer, 1761 (Nematoda: Trichuridae) parasitizing *C. g. kikuyensis*, *P. ursinus*, *Macaca sylvanus*, *Pan troglodytes*, and *Sus scrofa domestica* has been carried out using modern morphometric techniques in order to differentiate populations of *Trichuris* isolated from four species of captive NHP from different geographical regions, and swine, respectively.

2. Material and methods

2.1. Collection of samples

120 adult *Trichuris* sp. specimens (60 males and 60 females) were collected from the caecum of adult primates (*M. sylvanus*, *P. troglodytes*, *C.g. kikuyensis* and *P. ursinus*) and 30 adults from pigs, thoroughly washed with saline solution of 0.9% sodium chloride, and subsequently stored in 70% ethanol.

30 adults (15 males and 15 females) of *Trichuris* sp. were collected from the caecum of *M. sylvanus* died in the zoo of Castellar (Cádiz, Spain). Genetic analysis are unpublished.

30 adults (15 males and 15 females) of *T. ursinus* were collected from Chacma baboons (*P. ursinus*) from the Cape Peninsula, South Africa. These specimens were obtained through the assistance of colleagues (see Acknowledgements). Genetic data are published (Callejón et al., 2017).

Adults of *T. colobae* were collected from the caecum of a primate (*C. g. kikuyensis*) died in the Zoo of Fuengirola (Málaga, Spain). Genetic data are published (Cutillas et al., 2014).

Adults of *T. suis* were collected from the caecum of swine (*S. s. domestica*) slaughtered at abattoirs in different locations in the provinces of Seville and Huelva (Spain). Genetic data are published (Cutillas et al., 2009).

Adults of *T. trichiura* were collected from the caecum of chimpanzees died in the zoo in Barcelona (Spain). Genetic data are published (Cutillas et al., 2009).

2.2. Morphological studies and metric data processing

Morphobiometric data of *T. suis* and *T. trichiura* from the chimpanzee were those cited by Cutillas et al. (2009). The identification of these species was carried out in accordance with previous studies (Oliveros et al., 2000; Cutillas et al., 2002, 2014, 2004; 2007; Callejón et al., 2017).

The collected specimens of *Trichuris* sp. were measured. The measurements considered in this work are the most representative reported by Spakulová and Lysek (1981), Suriano and Navone (1994) and Robles et al. (2006). In addition, a comparative study of morpho-biometric data of five *Trichuris* species was performed. Descriptive univariate statistics based on mean values, standard deviation and range for all parameters were determined for male and female populations (Callejón et al., 2017). The Student's t-test ($P < 0.001$) was used to test the equality of means for each variable. Statistical analysis was performed using Microsoft Excel 5.0 (Feliú et al., 2000). Thus, biometric characters of *Trichuris* sp. were compared between different hosts and the most significant parameters were assayed for a morphometric study (Tables 1 and 2).

Morphological variation is quantified by geometrical morphometrics (Rohlf and Marcus, 1993), a technique offering an estimate of size by which different axes of growth are integrated into a single variable (the "centroid size"; Bookstein, 1989). The estimate of size is contained in a single variable reflecting variation in many directions, as many as there are landmarks under study, and shape is defined as their relative positions after correction for size, position and orientation. With these informative data, and the corresponding software freely available to conduct complex analyses, significant biological and epidemiological features can be quantified more accurately (Dujardin, 2008). Current statistical techniques in morphometrics make it possible to test the null hypothesis of conspecific populations being simply the allometric extension of each other, provided a common allometric trend is identifiable (Rohlf and Marcus, 1993).

Multivariate analyses were applied to calculate the phenotypic variations among whipworm adults, using size-free canonical discriminant analysis on the covariance of log-transformed measurements to assess phenotypic variations between the samples. These analyses are applied to exclude the effect of within-group ontogenetic variations by reducing the effect of each character on the first pooled within-group principal component (a multivariate size estimator) (Dos Reis et al., 1990). The principal component analysis (PCA) is used to summarize most of the variations in a multivariate dataset in a few dimensions (Dujardin and Le Pont, 2004). The resulting "allometry-free", or size-free, variables were submitted to a canonical variate analysis (CVA), and Mahalanobis distances were derived (Mahalanobis, 1936). The Mahalanobis distance is a statistical technique that can be used to measure how distant a point is from the center of a multivariate normal distribution, i.e. in the present analysis, the degree of similarity between whipworm populations was assessed through pairwise Mahalanobis distances. Phenotypic analysis of whipworm adults was conducted using various modules of the CLIC package version 97 (Dujardin and Slice, 2007; Dujardin et al., 2010), which is freely available at http://mome-clic.com, and BAC v.2 software (Dujardin, 2002; Valero et al., 2009), both used for multivariate analyses of the morphometric data. Furthermore, Mahalanobis distances were calculated using CLIC software and tested by nonparametric permutation tests with 1,000 iterations each.

The results were statistically significant when $P < 0.05$. The

Table 1
Biometric data of *Trichuris* adult male groups: *T. suis* isolated from *S. s. domestica*, *T. trichiura* isolated from *P. troglodytes*, *T. colobae* from *C. g. kikuyensis*, *T. ursinus* from *P. ursinus* and *Trichuris* sp. from *M. sylvanus*. † Significant differences between *T. suis* (Cutillas et al., 2009), *T. trichiura* (Cutillas et al., 2009), *T. colobae* (Cutillas et al., 2014) and *T. ursinus* (Callejón et al., 2017) compared to *Trichuris* sp. from *M. sylvanus* (P < 0.001).

	T. trichiura from *Pan troglodytes* (Cutillas et al., 2009)				*T. colobae* from *C. g. kikuyensis* Cutillas et al. (2014)				*Trichuris* sp. from *Macaca sylvanus* (Present study)				*Trichuris ursinus* from *Papio ursinus* (Callejón et al., 2017)				*T. suis* from *Sus scrofa domestica* (Cutillas et al., 2009)			
	MAX	MIN	Б	σ	MAX	MIN	Б	σ	MAX	MIN	Б	σ	MAX	MIN	Б	σ	MAX	MIN	Б	σ
M1	3.60	3.20	3.38	0.12	4.10	2.70	3.45	0.37	3.90	3.00	3.45	0.25	4.10	3.20	3.70	0.24	5.00	3.50	4.04 †	0.40
M2	2.30	1.80	2.08	0.13	3.50	2.10	2.64	0.37	2.80	1.90	2.19	0.27	2.70	2.10	2.43	0.19	3.30	2.30	2.61	0.29
LP	1.40	1.20	1.30	0.05	1.20	0.60	0.81 †	0.31	1.70	0.80	1.25	0.22	1.40	1.10	1.27	0.07	1.70	1.20	1.43	0.12
M3	0.31	0.09	0.14	0.07	0.10	0.07	0.08 †	0.01	0.18	0.12	0.14	0.02	0.20	0.15	0.17 †	0.01	0.23	0.16	0.20 †	0.02
M4	0.60	0.33	0.09 †	0.47	0.52	0.23	0.40 †	0.09	0.74	0.49	0.61	0.07	0.80	0.53	0.62	0.07	0.94	0.76	0.87 †	0.06
M5	0.32	0.15	0.22 †	0.05	0.26	0.13	0.20 †	0.04	0.49	0.25	0.37	0.06	0.46	0.30	0.38	0.03	0.40	0.28	0.35	0.04
M6	0.42	0.40	0.41 †	0.01	1.80	0.60	0.99 †	0.42	0.64	0.33	0.49	0.09	0.64	0.30	0.42	0.09	0.88	0.50	0.66	0.12
M7	0.88	0.59	0.67 †	0.09	3.95	2.33	3.36 †	0.44	1.57	1.10	1.33	0.14	2.29	1.10	1.71 †	0.35	1.76	1.00	1.43	0.27
M8	2.22	1.61	1.94 †	0.26	2.07	1.48	1.64 †	0.17	3.23	2.23	2.65	0.23	2.29	1.90	2.10 †	0.12	2.57	2.05	2.35 †	0.15
M9	0.22	0.16	0.21 †	0.02	0.65	0.26	0.49 †	0.09	1.23	0.53	0.93	0.20	0.69	0.23	0.54 †	0.14	0.76	0.16	0.39 †	0.19
M10	0.05	0.02	0.04 †	0.01	0.05	0.02	0.04 †	0.01	0.08	0.04	0.06	0.01	0.10	0.06	0.08 †	0.02	0.10	0.04	0.05	0.02
M11	0.06	0.03	0.04 †	0.01	0.06	0.03	0.04 †	0.01	0.08	0.04	0.06	0.01	0.10	0.05	0.09	0.14	0.08	0.06	0.07 †	0.01
M12	0.12	0.04	0.08	0.11	0.12	0.04	0.08	0.11	0.09	0.06	0.07	0.01	0.10	0.06	0.07	0.01	0.23	0.06	0.13 †	0.01
M13	2.98	1.32	2.14 †	0.47	2.98	1.32	2.14 †	0.47	5.19	2.90	4.06	0.58	2.34	1.55	1.84 †	0.43	2.34	1.99	2.15 †	0.14
M14	2.90	2.15	2.45 †	0.20	2.46	2.15	2.45	0.33	2.34	1.32	1.97	0.29	2.67	1.44	1.86	0.33	5.51	3.63	3.86 †	0.25
M15	3.29	2.2	2.87 †	0.28	3.06	2.55	2.87 †	0.28	2.75	1.55	2.20	0.36	3.79	2.77	3.18 †	0.27	4.92	3.42	4.31 †	0.54

M1 = Total body length of adult worm; M2 = Length of esophageal region of body; LP = Length of posterior region of body; M3 = Width of esophageal region of body; M4 = Maximum width of posterior region of body (thickness); M5 = Body width in the place of junction of esophagus and the intestine; M6 = Distance from the head end to beginning of bacillary stripes; M7 = Length of bacillary stripes; M8 = Length of spicule; M9 = Maximum length of spicule sheath; M10 = Width of proximal end of spicule; M11 = Width of spicule sheath at the tail end of body; M12 = Maximum width of spicule sheath; M13 = Distance between posterior part of testis and tail end of body; M14 = Length of ejaculatory duct; M15 = Length of distal cloacal tube. Б = Arithmetic mean. σ = Standard deviation.

following non-redundant measurements (one measurement is not included in another) used for whipworm adults were: M4, M8 and M9 for males; ratio: F2/LP, F4 and F9 for females.

3. Results

The Student's T test showed a number of measurements to be significant for subsequent morphometric analyses: maximum width of posterior region of the body (thickness) (M4), length of spicule (M8), maximum length of spicule sheath (M9), distance between posterior part of testis and tail end of body (M13) and length of distal cloacal tube (M15) in males, and maximum width of posterior region of body (thickness) (F4) and length of the muscular zone of the oesophagus (F13) in females. All these measurements are presented in millimeters (Tables 1 and 2).

The study of the influence of the host species on the adult size was carried out by PCA in *Trichuris* spp. from *M. sylvanus*, *P. troglodytes*, *C. g. kikuyensis*, *P. ursinus* and *S. s. domestica*. Different parameters were included according to the significant value obtained by the Student's T test. Therefore, three measurements for each population: M4, M8 and M9 for males, and F2/LP, F4 and F9 for females were used. *Trichuris* spp. variables from the primates and pigs all correlated significantly with PC1, contributing 64% to the overall variation in males and 61% in females. The resulting factor maps for male and female populations

Table 2
Biometric data of *Trichuris* adult female groups: *T. suis* isolated from *Sus scrofa domestica*, *T. trichiura* isolated from *P. troglodytes*, *T. colobae* from *C. g. kikuyensis*, *T. ursinus* from *P. ursinus* and *Trichuris* sp. from *Macaca sylvanus*. † Significant differences between *T. suis* (Cutillas et al., 2009), *T. trichiura* (Cutillas et al., 2009), *T. colobae* (Cutillas et al., 2014) and *T. ursinus* compared to *Trichuris* sp. from *M. sylvanus* (P < 0.001).

	T. trichiura from *Pan troglodytes* (Cutillas et al., 2009)				*T. colobae* from *C. g. kikuyensis* (Cutillas et al., 2014)				*Trichuris sp.* from *Macaca sylvanus* (Present study)				*Trichuris ursinus* from *Papio ursinus* (Callejón et al. (2017))				*T. suis* from *Sus scrofa* domestica (Cutillas et al., 2009)			
	MAX	MIN	Б	σ	MAX	MIN	Б	σ	MAX	MIN	Б	σ	MAX	MIN	Б	σ	MAX	MIN	Б	σ
F1	4.20	2.00	3.34	0.78	5.20	4.10	4.60 †	0.37	3.80	3.00	3.41	0.25	4.90	3.00	3.80	0.55	5.70	3.60	4.50 †	0.70
F2	3.30	1.30	2.53	0.68	3.80	3.00	3.34 †	0.31	2.60	1.80	2.19	0.23	3.70	2.00	2.60	0.50	4.10	2.70	3.30 †	0.41
LP	1.00	0.60	0.81 †	0.14	1.40	1.20	1.22	0.14	1.40	0.90	1.21	0.16	1.50	1.00	1.21	0.15	1.60	0.90	1.15	0.32
F3	0.19	0.01	0.11	0.05	0.15	0.08	0.11 †	0.02	0.18	0.13	0.15	0.01	0.19	0.15	0.17 †	0.02	0.24	0.16	0.20	0.06
F4	0.64	0.40	0.45 †	0.08	0.77	0.46	0.60 †	0.09	0.81	0.64	0.72	0.05	0.89	0.54	0.68	0.08	1.04	0.77	0.89 †	0.11
F5	0.23	0.13	0.17 †	0.03	0.31	0.16	0.23 †	0.04	0.48	0.36	0.42	0.03	0.45	0.35	0.40	0.02	0.46	0.24	0.32 †	0.08
F6	0.64	0.48	0.56	0.11	1.03	0.51	0.82 †	0.18	0.76	0.42	0.50	0.09	0.62	0.42	0.52	0.07	0.66	0.52	0.61 †	0.05
F7	0.95	0.36	0.65	0.30	4.92	2.90	3.50 †	0.79	1.71	0.90	1.44	0.21	2.00	1.29	1.70	0.23	1.92	1.45	1.69	0.19
F8	1.29	0.05	0.83	0.40	1.65	0.95	1.29 †	0.29	1.99	0.73	1.12	0.35	2.81	1.88	2.26 †	0.33	1.80	0.75	1.33	0.34
F9	0.11	0.03	0.05	0.03	0.08	0.05	0.07	0.01	0.09	0.02	0.05	0.02	0.12	0.06	0.08 †	0.02	1.00	0.60	0.80 †	0.14
F10	0.24	0.11	0.61	0.06	0.39	0.21	0.29	0.05	0.33	0.15	0.25	0.05	0.38	0.03	0.18	0.10	0.28	0.22	0.24	0.03
F11	0.22	0.20	0.21 †	0.01	0.45	0.20	0.32 †	0.08	0.84	0.40	0.61	0.14	0.75	0.10	0.47	0.22	0.65	0.48	0.60	0.05
F12	0.53	0.32	0.43	0.15	1.90	0.93	1.49 †	0.30	0.48	0.19	0.30	0.09	1.71	0.50	1.04 †	0.35	1.42	0.65	0.99 †	0.31
F13	0.67	0.65	0.66 †	0.01	1.20	0.50	0.79 †	0.27	0.14	0.05	0.11	0.04	1.32	0.77	0.99 †	0.20	1.20	0.72	0.92 †	0.18

F1 = Total body length of adult worm; F2 = Length of oesophageal region of body; LP = Length of posterior region of body; F3 = Width of esophageal region of body; F4 = Maximum width of posterior region of body (thickness); F5 = Body width in the place of junction of oesophagus and the intestine; F6 = Distance from the head end to beginning of bacillary stripes; F7 = Length of bacillary stripes; F8 = Length of vagina; F9 = Diameter of vulva turned over the surface of body; F10 = Distance of vulva from place of junction of oesophagus and the intestine; F11 = Distance of posterior loop of uterus from tail end of body; F12 = Distance of tail end of body and posterior fold of seminal receptacle; F13 = Length of muscular zone of the oesophagus. Б = Arithmetic mean. σ = Standard deviation.

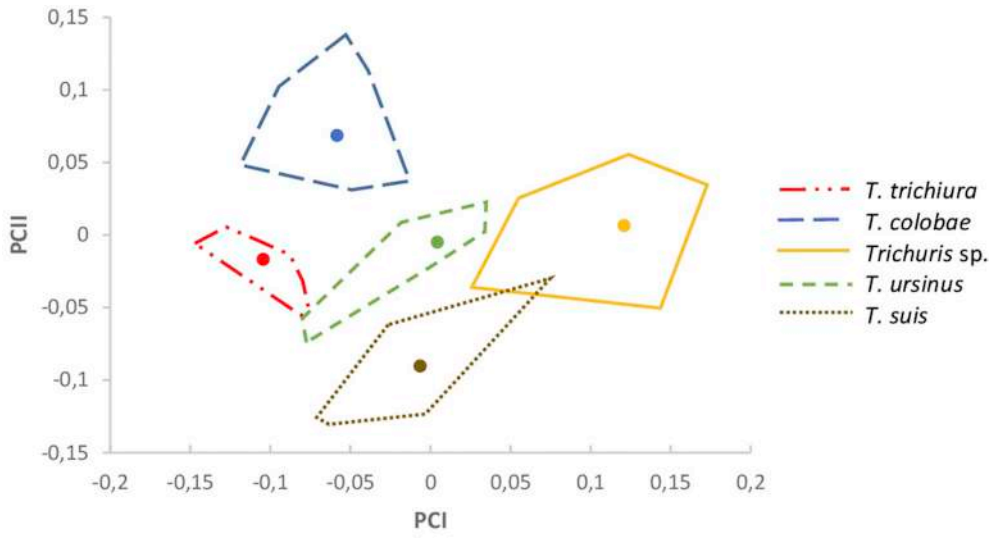

Fig. 1. Factor map corresponding to adult *Trichuris* sp. males derived from different host primate species (*Macaca sylvanus*, *Pan troglodytes*, *Colobus guereza kikuyensis*, *Papio ursinus*) and the pig (*Sus scrofa domestica*) from zoos and abattoirs, respectively, in Spain. Samples are projected onto the first (PC1, 64%) and second (PC2, 29%) principal components. Each group is represented by its perimeter. Circles represent the centroid in each community.

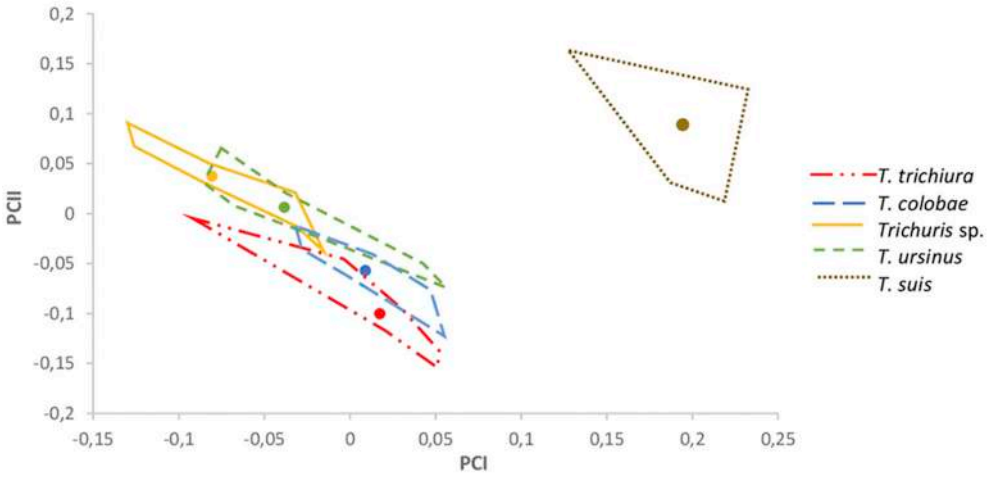

Fig. 2. Factor map corresponding to adult *Trichuris* sp. females derived from four different host primate species (*Macaca sylvanus*, *Pan troglodytes*, *Colobus guereza kikuyensis*, *Papio ursinus*) and the pig (*Sus scrofa domestica*) from zoos and abattoirs, respectively, in Spain. Samples are projected onto the first (PC1, 61%) and second (PC2, 35%) principal components. Each group is represented by its perimeter. Circles represent the centroid in each community.

of *Trichuris* sp. adults are represented in Fig. 1 and Fig. 2, respectively.

On the one hand, the first factor map clearly illustrates the global size differences in the male *Trichuris* sp. populations analyzed, showing a larger size in males collected from macaques (Fig. 1). All of the adult male communities are well grouped in the factor map, with a lack of overlapping areas between them. Therefore, each population appeared separate from each other. Only parasites from macaques and pigs showed a partial overlap but with no inconvenience in the identification of the communities. Furthermore, the CS differences in males between three groups (*T. trichiura* vs *Trichuris* sp, *T. trichiura* vs *T. colobae* and *Trichuris* sp vs *T. colobae*) presented the highest values.

On the other hand, the female factor map does not show global size differences in four of the five *Trichuris* sp. populations. Thus, two zones could be distinguished: one zone was made up of the four primate communities, while the other zone consists only of pigs. The primates show a wide overlap area that does not allow a clear identification of each adult female population, although the CS differences in females between three groups (*T. trichiura* vs *Trichuris* sp, *T. trichiura* vs *T. ursinus* and *Trichuris* sp vs *T. colobae*) presented clear differences. Therefore, the exception are adult *Trichuris* sp. females collected from pigs making up an independent group from the primate communities and with no overlap between them, only a bigger size in female adults retrieved from pigs being patent (Fig. 2).

The degree of similarity between whipworm populations was assessed through pairwise Mahalanobis distances. These distances were

Table 3
Mahalanobis distances between *Trichuris* adult male groups: *T. suis* isolated from *S. s. domestica*, *T. trichiura* isolated from *P. troglodytes*, *T. colobae* from *C. g. kikuyensis*, *T. ursinus* from *P. ursinus* and *Trichuris sp.* from *M. sylvanus*.

	T. trichiura	*T. colobae*	*Trichuris* sp.	*T. ursinus*	*T. suis*
T. trichiura	0.00				
T. colobae	3.05	0.00			
Trichuris sp.	3.53	2.34	0.00		
T. ursinus	1.82	1.24	2.58	0.00	
T. suis	1.56	3.58	4.80	2.46	0.00

Table 4
Mahalanobis distances between *Trichuris* adult female groups: *T. suis* isolated from *S. s. domestica*, *T. trichiura* isolated from *P. troglodytes*, *T. colobae* from *C. g. kikuyensis*, *T. ursinus* from *P. ursinus* and *Trichuris sp.* from *M. sylvanus*.

	T. trichiura	*T. colobae*	*Trichuris* sp.	*T. ursinus*	*T. suis*
T. trichiura	0.00				
T. colobae	2.10	0.00			
Trichuris sp.	5.10	3.10	0.00		
T. ursinus	3.73	1.79	1.38	0.00	
T. suis	6.01	7.24	8.77	7.73	0.00

calculated comparing males with each other (Table 3), respectively, comparing females with each other (Table 4). When comparing males of *Trichuris* species from NHPs vs *Trichuris* species from pigs, larger distances were detected than in the inter-NHPs comparison. Similarly, when comparing females of *Trichuris* species from NHPs vs *Trichuris* species from pigs, larger distances were detected than in the inter-NHPs comparison. In the inter-NHPs comparison in males of *Trichuris* larger distances were detected *T. trichiura* vs *Trichuris* sp and *T. trichiura* vs *T. colobae* were compared. Similarly, in the inter-NHPs comparison in females of *Trichuris*, larger distances were detected *T. trichiura* vs *Trichuris* sp, *T. trichiura* vs *T. ursinus* and *Trichuris* sp vs *T. colobae* were compared. In general, larger distances between females than between males were detected when Trichuris species from NHPs vs Trichuris species from pigs where compared, revealing that the phenotype of T. suis vs NHP whipworm species is more divergent in females than in males. These results agree with the above-mentioned analysis obtained in Figs. 1 and 2.

4. Discussion

The systematics of the genus *Trichuris* Roederer, 1761 is controversial at species level. Different authors have cited synonymies (Oliveros et al., 2000), cryptic species (Callejón et al., 2012) or new species (Cutillas et al., 2014; Robles et al., 2014; Callejón et al., 2017). Many of these studies have been based on morphometric and molecular data since Dujardin (1845) reviewed the genus for the first time.

Furthermore, we have carried out the first morphometric study applied to adult *Trichuris* species derived from different host primates and the pig, in order to explore complementary methods of morphological phenotypic characterization. Previous studies demonstrated the difficulty in discriminating populations of the genus *Trichuris* from primates (Cutillas et al., 2009, 2014; Callejón et al., 2017) as it usually contains cryptic species. In view of these problems, an additional method was required to elucidate different species. The present study demonstrates that morphometrics is a useful tool to shed light on this topic.

As for adult male *Trichuris*, we achieved the differentiation of all of the communities, obtaining well defined different areas for each population. These results are in agreement with the molecular biology analysis applied to the adult male samples that allow the identification of different species of *Trichuris* (Cutillas et al., 2009, 2014; Callejón et al., 2017). The results confirm that each community of male *Trichuris* sp. adults possesses its own morphological identity.

The size of female *Trichuris* populations does not follow a host-dependent pattern, probably due to the absence of representative measurements. Only the females retrieved from pigs presented a larger size than the females collected from primates (Fig. 2). All combinations of measurements considered always led to similar factor maps, with wide overlap areas between them (data not shown). These results agree with those reported previously (Cutillas et al., 2009, 2014; Callejón et al., 2017), in which the differentiation of female *Trichuris* was not achieved. Females of trichurids are more difficult to differentiate than males, and some authors suggested that the structure of the vulva could be used for species differentiation (Chandler, 1930; Ooi et al., 1993; Barus et al., 1978; Rickard and Bishop, 1991; Gibbons, 1986). Tenora et al. (1993) stated that there are two different types of vulva in trichurid females: with and without spines. *T. suis* belongs to the group of species possessing the vulvar region equipped with cuticle spines present of various forms and different numbers. Furthermore, Spakulová (1994) found that the combination of seven metric characters distinguished the whipworms from pigs from those of humans.

According to different reports mentioned above, the main gaps in the systematics of the genus *Trichuris* are:

1. The absence of comparative morpho-biometric data using multiple parameters, statistical tests (e.g. the Student's Test, $P < 0.001$) and geometrical morphometrics applied to the taxonomic study of different species of *Trichuris*.
2. The reports about different genetic lineages in *T. trichiura* and species of this genus parasitizing NHPs are not supported by morpho-biometric studies. Thus, future research priorities should include morphological, morphometric and molecular analyses of individuals of *Trichuris* sp., in order to compare data.

These issues can be addressed by means of:

1. A classical taxonomic study based on the analysis of 15–20 biometric parameters and statistical tests, along with geometrical morphometrics based on Principal Component analysis of different species of *Trichuris* isolated from different hosts and geographic areas involving the four continents: Europe, Africa, America and Asia.
2. The amplification and sequencing of nuclear and mitochondrial genes (genome) of these *Trichuris* species.
3. The phylogenetic and phylogeographic study based on whole-genome and through analysis of concatenated sequences obtained from these species from different geographical localities, to shed light on the degree of divergence with respect to *T. trichiura*.
4. The comparative study of sequences to obtain new pharmacological targets in therapy for autoimmune diseases such as Crohn's disease, as well as new treatments of the parasitism by these species in livestock.

5. Conclusions

The results obtained revealed strong support for geometrical morphometrics as a useful tool to differentiate male *Trichuris* populations. Therefore, morphometrics in combination with other techniques, such as molecular biology analyses, ought to be applied to further the differentiation of male populations.

On the other hand, morphometrics applied to female *Trichuris* species does not seem to contribute new information as all the measurement combinations of obtained from females always showed similar results. The application of other techniques to differentiate populations is warranted given the difficulty in finding representative measurements in females.

Combining genetic and morphometric analyses seem to be the key factor to enable the differentiation of *Trichuris* sp. in the near future.

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Conflicts of interest

The authors declare no conflicts of interest.

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RESEARCH ARTICLE Open Access

Trichuris trichiura isolated from *Macaca sylvanus*: morphological, biometrical, and molecular study

Julia Rivero, Ángela María García-Sánchez, Antonio Zurita, Cristina Cutillas* and Rocío Callejón

Abstract

Background: Recent studies have reported the existence of a *Trichuris* species complex parasitizing primate. Nevertheless, the genetic and evolutionary relationship between *Trichuris* spp. parasitizing humans and Non-Human Primates (NHP) is poorly understood. The hypothesised existence of different species of *Trichuris* in primates opens the possibility to evaluate these primates as reservoir hosts of human trichuriasis and other putative new species of whipworms.

Results: In this paper, we carried out a morphological, biometrical and molecular study of *Trichuris* population parasitizing *Macaca sylvanus* from Spain based on traditional morpho-biometrical methods, PCA analysis and ribosomal (ITS2) and mitochondrial (*cox*1 and *cob*) DNA sequencing. Morphological results revealed that *Trichuris* sp. from *M. sylvanus* is *Trichuris trichiura*. Ribosomal datasets revealed that phylogenetic relationships of populations of *Trichuris* sp. from *M. sylvanus* were unresolved. The phylogeny inferred on mitochondrial datasets (partitioned and concatenated) revealed similar topologies; Thus, phylogenetic trees supported the existence of clear molecular differentiation between individuals of *Trichuris* sp. from *M. sylvanus* appearing in two different subclades.

Conclusions: Based on morphological parameters, biometrical measurements, and molecular sequence analysis, we conclude that the whipworms isolated from *M. sylvanus* were *T. trichiura*. Further, the evolutionary relationship showed that these worms belonged to two genotypes within the *T. trichiura* lineage. Since *T. trichiura* is of public health importance, it is important to carry out further studies to improve the understanding of its hosts range, evolution and phylogeography.

Keywords: Barbary macaque, *Macaca sylvanus*, Mitochondrial DNA, *Trichuris trichiura*, Zoonosis

Background

Trichuris species are nematodes belonging to Order Trichocephalida (Class Enoplea) and they parasitize the caecum of different hosts. For many years, *Trichuris trichiura* Linnaeus, 1771 was considered as the whipworm present in humans and Non-Human-Primates (NHP). Until now, it is known that several whipworm species are able to parasitize humans: *T. trichiura* (human whipworm), *Trichuris suis* Schrank, 1788 (pig whipworm) and *Trichuris vulpis* Froelich, 1789 (dog whipworm), but only *T. trichiura* has been considered for many years to be the specific whipworm of primates.

Whipworms' genetic and evolutionary relationship between human and NHP is poorly understood. Moreover, given the phenotypic plasticity of these parasites themselves: host-induced variation, lack of morphological characteristics, and overlap between species in morphological characteristics, it is very difficult to distinguish among closely related *Trichuris* species [1–4].

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Traditionally, the research on *T. trichiura* from humans and NHP had its main objective on differentiating this species from *T. suis* found in pigs [5–9]. Morphological studies of *Trichuris* isolated from primates and humans concluded that the species infecting these hosts is the same, despite slight morphological variations observed using scanning electron microscopy [6]. However, these studies were based on a few morphological features such as the total length or spicule length, but not on discriminative analysis of many morpho-biometrical significant parameters using statistical tests. Ravasi et al. [10] carried out a study to discriminate parasite species from human and NHP using exclusively molecular techniques. Thus, these authors [10] suggested the need for morphological analysis of *Trichuris* sp. adult collected from *Papio ursinus* (Chacma baboon) from South Africa to determine whether the genetic lineages corresponded with different morphological species. It seems to be a pattern of infection with different *Trichuris* species infecting host species, thus, some authors [11] concluded that it would be necessary to apply multiple genetic markers to *Trichuris* collected from humans and NHP from sympatric areas and worldwide locations. This would clarify parasite transmission routes between these primates allowing the implementation of appropriate control and prevention measures [11].

Hawash et al. [12] suggested the existence of a *Trichuris* species complex in primates and pigs based on complete mitochondrial genome analysis. Recently, Cutillas et al. [13] proposed a new species, *Trichuris colobae,* from *Colobus guereza kikuyensis,* which is distinguished from *T. suis* from pigs and *T. trichiura* from humans. Afterward, Callejón et al. [14] reported a morpho-biometric study showing the new species *Trichuris ursinus* from another NHP (*P. ursinus*) that differed significantly from *T. trichiura* (nine different characters) and *T. colobae* (six different characters). Furthermore, *T. ursinus* showed features close to *T. suis* (only three different characters). In all these studies, *Trichuris* specimens were measured according to parameters reported by Spakulová and Lýsek [15], Suriano and Navone [16] and Robles et al. [17], who summarized the morpho-biometric parameters used in recent years.

The Barbary macaque or Gibraltar macaque (*Macaca sylvanus*) is the only member of this genus found outside Asia, distributed in Africa, North of the Sahara Desert, and the only NHP to occur in Europe (Rock of Gibraltar) [18, 19]. In early times, it was widespread throughout North Africa from Libya to Morocco, but its current distribution is limited to patches of forest and scrub in Algeria and Morocco [19–22]. A long-established introduced Barbary macaque population lives in Gibraltar [23–25].

To date, morphological and molecular studies of the *Trichuris* populations of *M. sylvanus* have not been carried out. Hence, the main objective of this work is to determine the morphologic, biometric and molecular features of *Trichuris* sp. parasitizing *M. sylvanus* from Zoo Castellar (Spain) in order to: (i) identify at species level adult parasites of these specimens and (ii) to evaluate genetic variation and evolutionary relationships between *Trichuris* spp. from NHP and humans.

Results

Morphological and biometrical description

General

The anterior part of the body is long, narrow, tapered and whip-like; the posterior part of the body broad and handle-like. The cuticle has a fine transversal striation. The bacillary band is located laterally in the anterior portion of the body.

Male

The proximal cloacal tube is wide and continued with the distal cloacal tube that contains the spicule, which projected into the anterior portion of the body in a spicule tube (Figs. 1a and 2a). The spicule was observed to have two more chitinized extreme zones and a lighter central part (Figs. 1a and 2a). Spicule sheath was cylindrical without a distal bulb (Figs. 1c and 2a-b) and with triangular spines distributed from the proximal to distal portion (Figs. 1c and 2b). The testis ends near the union of the ejaculator duct and intestine. The cloaca with anus subterminal with one pair of paracloacal papillae not ornamented (Figs. 1b and 2c) was observed when the spicule sheath was invaginated. No cluster of papillae was observed.

Female

A non-protrusive vulva without ornamentation was located at esophagus-intestinal junction level (Figs. 3a-b and 2d) and a short and straight vagina continuing with circumvolutions (Figs. 3a-b and2d) without papillae. The anus was subterminal (Figs. 3b and 2e). In addition, two different types of eggs were found in feces of macaque (Figs. 2f-g) which measurements ranged 60–50.6 × 38.4–23.9 μm.

Morphological results revealed that the *Trichuris* populations of *M. sylvanus* in Spain correspond to *T. trichiura*.

The comparative morphological study with other males of *Trichuris* species from primates revealed a typical spicule in *Trichuris* sp. from macaque presenting a central clear part (Fig. 1a, arrowed, 2a) that was not present in that of *T. colobae* and *T. ursinus* (Fig. 1d and g-h). In addition, the cluster of typical papillae was only present in *T. colobae* (Figs. 1e-f). Females of these species appeared to have a non-protrusive vulva (*Trichuris* sp. and *T. ursinus*) (Fig. 3b, e and h) and a vulva like a crater with papillae in *T. colobae* (Figs. 3D-E). The vagina was very long and straight in *T. ursinus* (Fig. 3g)

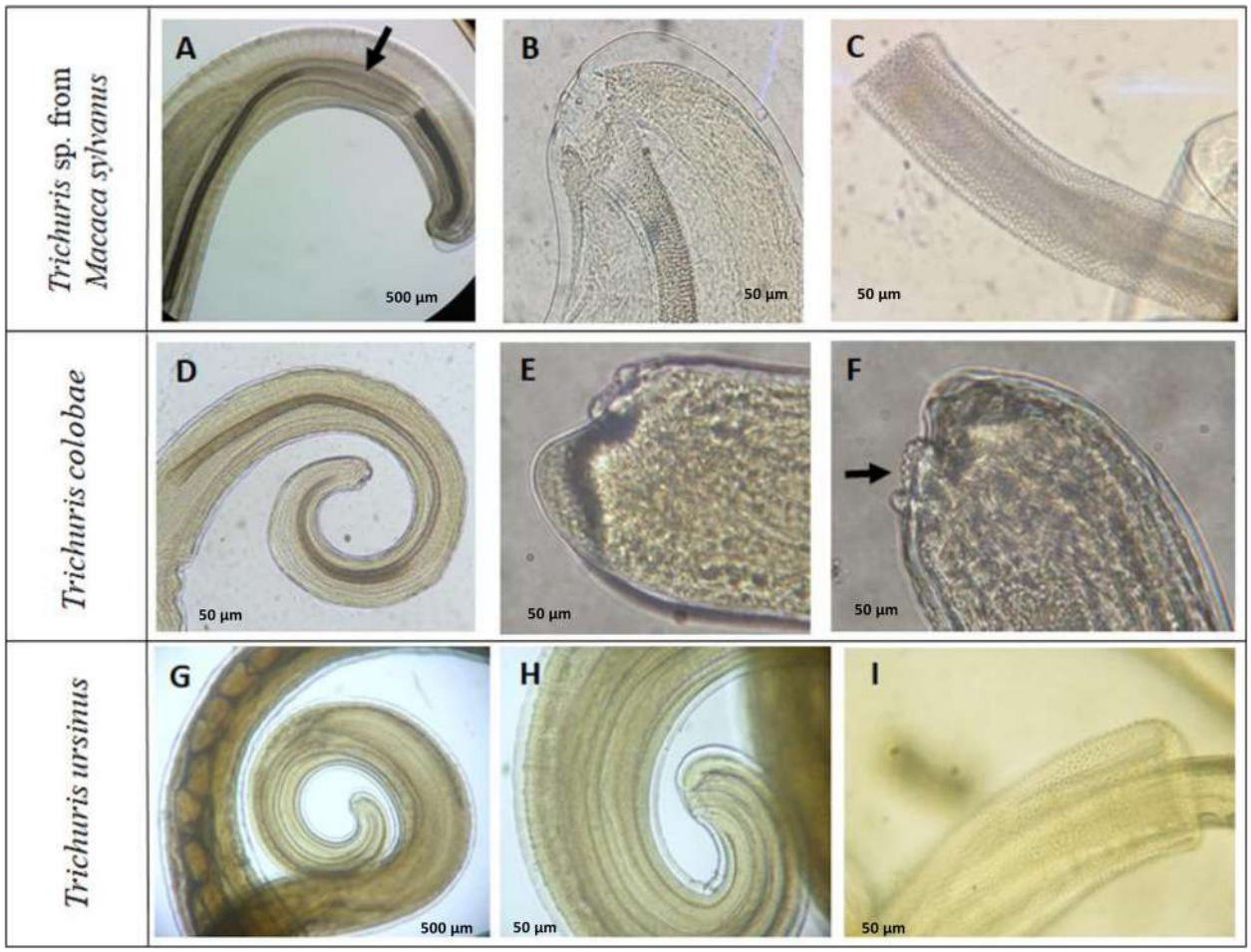

Fig. 1 Morphology of males of *Trichuris* sp. from primates. *Trichuris* sp. from *M. sylvanus* (**a**-**c**), *Trichuris colobae* [13] (**d**-**f**) and *Trichuris ursinus* [14] (**g**-**i**). **a**-**b** Posterior end showing spicule (arrowed) and spicule sheath. **c** posterior end, spiny spicule sheath and cloaca. **d** Posterior end with spicule invaginated. **e**-**f** Posterior end showing cluster of papillae (arrowed). **g**-**h**: Posterior end with spicule invaginated. **i** spiny spicule sheath

but appeared with circumvolutions in *Trichuris* sp. from macaque (Figs. 2d, 3a-b).

The preliminary biometrical study carried out in males and females of *Trichuris* sp. from *M. sylvanus* revealed data of 15 different parameters (see Additional files 1 and 2).

The Student's t test was carried out considering the two different populations according to the two observed genetic lineages: TT2a and TT2c. *P* values for all measurements were higher than 0.05, thus, no any significant differences between both populations were detected (Tables 1 and 2).

The resulting factor maps for male and female populations of *Trichuris* sp. adults are represented in Fig. 4a and b, respectively. Both male populations slightly overlapped while the female communities appeared 100% overlapped. Males factor map was PC1: 57% and PC2: 35%, while females factor map was PC1: 81% and PC2: 18%.

Annotation and features of ribosomal and mitochondrial genomes

The ITS2 region was amplified from the genomic DNA of 13 *Trichuris* sp. specimens from *M. sylvanus* from Spain revealing 7 haplotypes. These sequences were 587 base pairs (bp) in length and their G + C content ranged from 66.1–66.6%. *Cox*1 mitochondrial DNA (mtDNA) partial gene was sequenced from 43 individuals revealing the presence of 4 haplotypes. *Cox*1 sequences were 370 bp in length and their G + C content ranged from 38 to 39.2%. The *cob* partial gene was amplified from 13 specimens of *Trichuris* sp. revealing the presence of 4 haplotypes. The sequences (520 bp) revealed a G + C content ranging from 30.2 to 31.0%.

The datasets generated and analyzed during the current study are available in the GenBank™, EMBL and DDBJ repository, [Accession numbers: LR130781–4, LR132031–4, LR535742, LR535746–51] (Table 3).

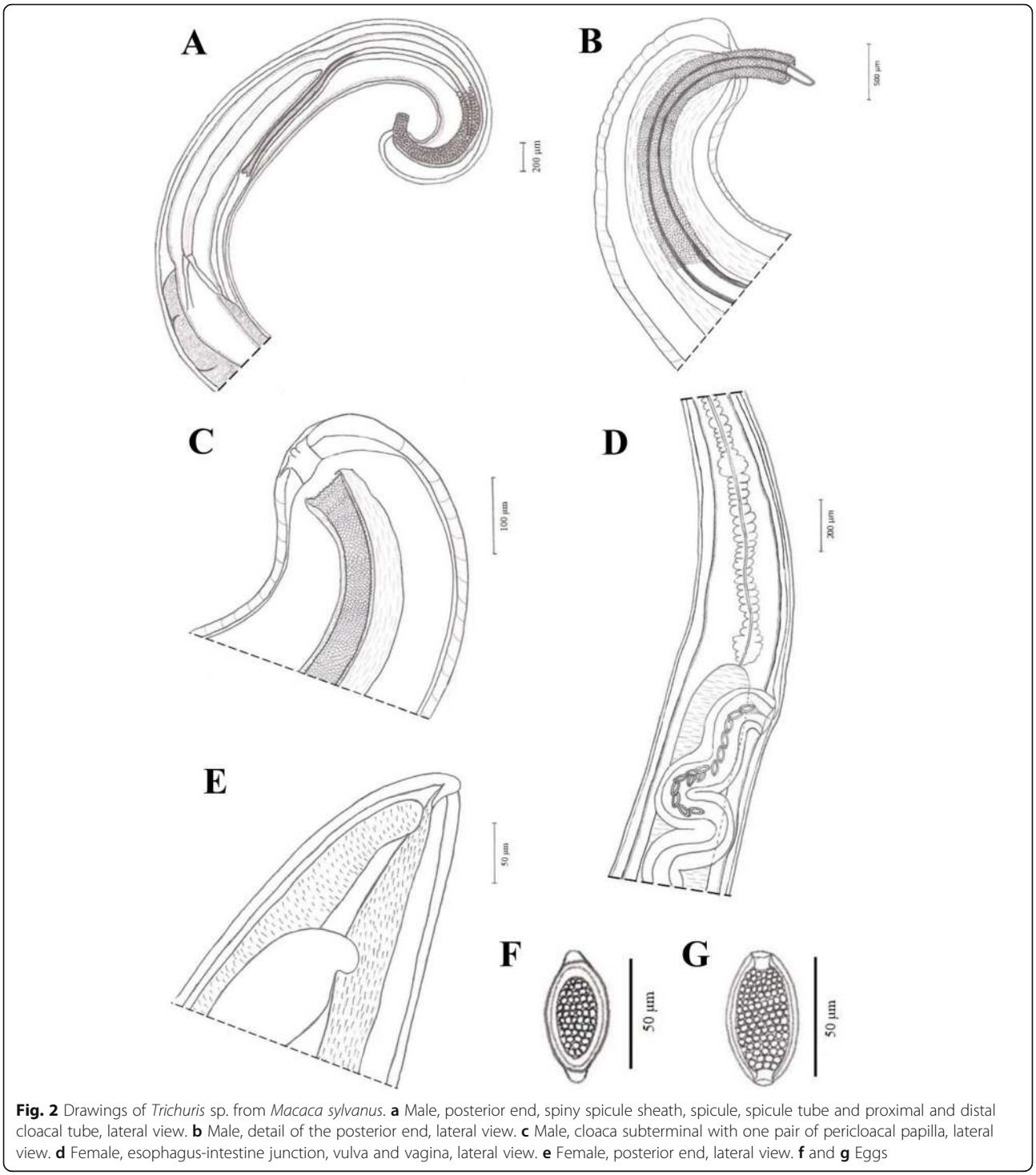

Fig. 2 Drawings of *Trichuris* sp. from *Macaca sylvanus*. **a** Male, posterior end, spiny spicule sheath, spicule, spicule tube and proximal and distal cloacal tube, lateral view. **b** Male, detail of the posterior end, lateral view. **c** Male, cloaca subterminal with one pair of pericloacal papilla, lateral view. **d** Female, esophagus-intestine junction, vulva and vagina, lateral view. **e** Female, posterior end, lateral view. **f** and **g** Eggs

Phylogenetic analysis

All trees (*cox*1, *co*b and ITS2) obtained by ML, MP and BI for *Trichuris* sp. revealed two highly supported phylogenetic clades (observed in a previous study) [26] that we named: "*T. trichiura* lineage" and "*T. suis* lineage" (Fig. 5 and Table 4). Clade 1 ("*T. suis* lineage") included *T. colobae, T. ursinus* and *T. suis* and Clade 2 ("*T. trichiura* lineage") included *T. trichiura* and *Trichuris* sp. from NHP corresponding to the genus *Macaca*, *Papio* and *Chlorocebus*.

The alignment of 29 ITS2 ribosomal DNA (rDNA) sequences of *Trichuris* species from human, swine and NHP from different geographic origins yielded a dataset of 584 characters. The phylogenetic tree inferred on

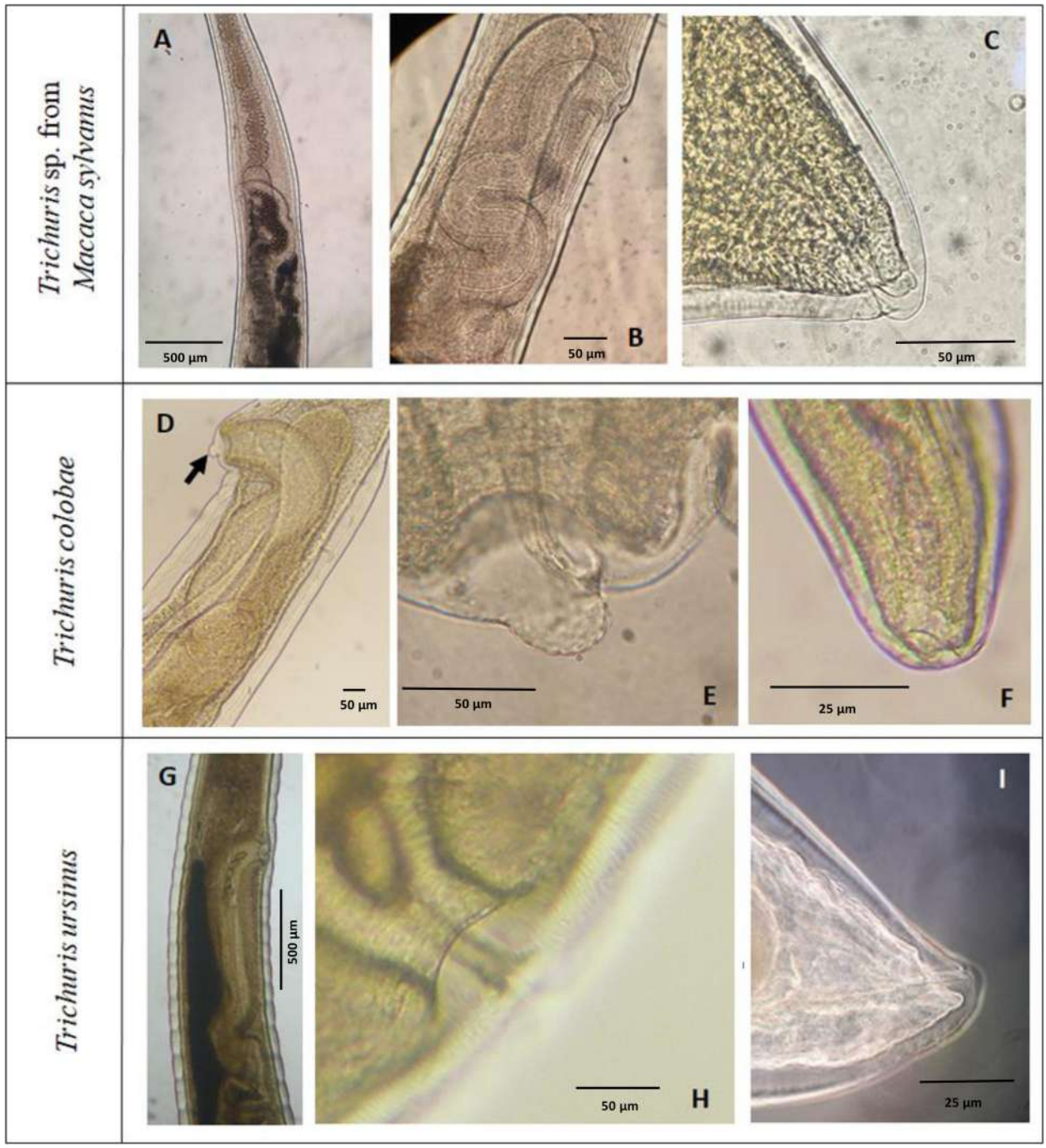

Fig. 3 Morphology of females of *Trichuris* sp. from primates. Morphology of females of *Trichuris* sp. from primates. *Trichuris* sp. from *M. sylvanus* (**a**-**c**), *Trichuris colobae* [13] (**d**-**f**) and *Trichuris ursinus* [14] (**g**-**i**). **a** Stychocites, esophagus-intestine junction and vagina. **b** Vulva and vagina. **c** Anus subterminal. **d** Vagina and like-crater vulva (arrowed). **e** Vulva. **f** Anus. **g** Vagina and vulva. **h** Vulva. **i** Anus

DatasetITS2 (Fig. 5) placed *Trichuris* spp. from *M. sylvanus* within "*T. trichiura* lineage" (Clade 2) without any pattern of distribution according to the host species or geographical origin. Nevertheless, *Trichuris* sp. populations from genus *Macaca* from Asia (*M. leonina*, *Macaca fuscata* and *M. mulatta*) clustered together and separated from *Trichuris* sp. from *M. sylvanus* from Europe (Spain) (Fig. 5). In addition, populations of *Trichuris* sp. from *M. sylvanus* appeared in different groups, out of which the haplotypes H1, H2, H5 and H6 clustered together (95% ML BV, 95% BPP), H3 and H4 clustered to the rest of the populations of *Trichuris* spp.

Table 1 Biometrical and morphological data of 33 males of *Trichuris* sp. isolated from *M. sylvanus*. TM = *Trichuris* male. EL/BL = Esophagus length/Body length. Б: standard deviation. X: arithmetic mean. Min: Minimum value. Max: Maximum value. All measurements are in millimetres

Lineage TT2a	Total length	Esophagus length	Body length	Ratio EL/BL	Wide body	Spicule length	Spicular length sheath	Spicular tube	Proximal cloacal tube	Pericloacal papillae	Spicular sheath with spines	Distal bulb	Cluster with papillae
TM3	38	24	14	1.71	0.64	2.46	0.86	Present	Present	Not visible	Present	Not present	Not present
TM5	39	24	15	1.60	0.75		0.84	Present	Present	Not visible	Present	Not present	Not present
TM8	41	28	13	2.15	0.74	2.87	0.91	Present	Present	Not visible	Present	Not present	Not present
TM18	31	19	12	1.58	0.63	2.77	0.97	Present	Present	Present	Present	Not present	Not present
MIN	31	19	12	1.58	0.63	2.46	0.84						
MAX	41	28	15	2.15	0.75	2.87	0.97						
X	37.25	23.75	13.50	1.76	0.69	2.70	0.90						
Б	4.35	3.69	1.29	0.27	0.06	0.21	0.06						
Lineage TT2c													
TM1	35	22	13	1.69	0.69	2.75	0.87	Present	Present	Not visible	Present	Not present	Not present
TM2	34	23	11	2.09	0.67	2.56	0.90	Present	Present	Not visible	Present	Not present	Not present
TM4	36	22	14	1.57	0.63	2.82	0.81	Present	Present	Not visible	Present	Not present	Not present
TM6	32	21	11	1.91	0.66	2.46	0.73	Present	Present	Not visible	Present	Not present	Not present
TM7	31	20	11	1.82	0.50	2.33	1.12	Present	Present	Present	Present	Not present	Not present
TM9	34	22	12	1.83	0.54	2.55	1.01	Present	Present	Present	Present	Not present	Not present
TM10	35	21	14	1.50	0.63	2.61	0.70	Present	Present	Not visible	Present	Not present	Not present
TM11	35	22	13	1.69	0.59	2.51	0.47	Present	Present	Present	Present	Not present	Not present
TM12	35.50	24.50	11	2.23	0.67	2.48	1.02	Present	Present	Present	Present	Not present	Not present
TM13	37	24	13	1.85	0.7	2.73	0.96	Present	Present	Not visible	Present	Not present	Not present
TM14	39	29	10	2.90	0.61	2.66	0.85	Present	Present	Not visible	Present	Not present	Not present
TM15	27	16	11	1.45	0.47	2.16	0.91	Present	Present	Not visible	Present	Not present	Not present
TM16	36	23	13	1.77	0.57	2.71	1.16	Present	Present	Not visible	Present	Not present	Not present
TM17	32	21	11	1.91	0.65	2.53	0.77	Present	Present	Not visible	Present	Not present	Not present
TM19	35	23	12	1.92	0.62	2.64	0.86	Present	Present	Not visible	Present	Not present	Not present
TM20	33	21	12	1.75	0.65	2.63	0.95	Present	Present	Not visible	Present	Not present	Not present
TM21	32	21	11	1.91	0.63	2.88	0.75	Present	Present	Not visible	Present	Not present	Not present
TM22	35	21	14	1.50	0.65	2.68	0.64	Present	Present	Not visible	Present	Not	Not

Table 1 Biometrical and morphological data of 33 males of *Trichuris* sp. isolated from *M. sylvanus*. TM = *Trichuris* male. EL/BL = Esophagus length/Body length. Ƃ: standard deviation. X: arithmetic mean. Min: Minimum value. Max: Maximum value. All measurements are in millimetres *(Continued)*

Lineage TT2a	Total length	Esophagus length	Body length	Ratio EL/BL	Wide body	Spicule length	Spicular length sheath	Spicular tube	Proximal cloacal tube	Pericloacal papillae	Spicular sheath with spines	Distal bulb	Cluster with papillae
												present	present
TM23	33	21	12	1.75	0.67	2	0.63	Present	Present	Not visible	Present	Not present	Not present
TM24	31	20	11	1.82	0.69	2.55	0.79	Present	Present	Not visible	Present	Not present	Not present
TM25	32	20.5	11.50	1.78	0.67	2.77	0.88	Present	Present	Not visible	Present	Not present	Not present
TM26	34	23	11	2.09	0.66	2.39	1.10	Present	Present	Present	Present	Not present	Not present
TM27	30	20	10	2.00	0.5	2.19	1.01	Present	Present	Present	Present	Not present	Not present
TM28			11.50		0.57	2.81	0.88	Present	Present	Not visible	Present	Not present	Not present
TM29	30	19	11	1.73	0.53	2.22	0.73	Present	Present	Not visible	Present	Not present	Not present
TM30	34	23	11	2.09	0.62	2.53	0.57	Present	Present	Not visible	Present	Not present	Not present
TM31	31	20	11	1.82	0.56	2.39	0.90	Present	Present	Not visible	Present	Not present	Not present
TM32	34	22	12	1.83	0.7	2.71	0.94	Present	Present	Present	Present	Not present	Not present
TM33	35	23	12	1.92	0.59	2.46	0.87	Present	Present	Not visible	Present	Not present	Not present
MIN	27	16	10	1.45	0.47	2	0.47						
MAX	39	29	14	2.90	0.70	2.88	1.16						
X	33.48	21.71	11.70	1.88	0.61	2.54	0.85						
Ƃ	2.50	2.22	1.12	0.28	0.06	0.21	0.16						

from humans and NHP and separated from H7 (Fig. 5). Exceptionally, one individual of *T. trichiura* from *Homo sapiens* from Cameroon and one individual of *Trichuris* sp. from *M. silenus* from the Czech Republic were included within "*T. suis* lineage" (Clade 1). The DatasetITS2 provided moderate phylogenetic resolution among most of *Trichuris* taxa regardless of inference method (Fig. 5).

The phylogeny inferred on mitochondrial datasets (partitioned and concatenated) revealed similar topologies; therefore, we assumed the concatenated tree based on mitochondrial datasets (*cox*1 and *cob*) to be the most representative (Fig. 6; Additional files 3 and 4). The concatenated dataset included 746 aligned positions and 22 taxa, including outgroups. ML, MP and BI methods showed congruence between each other revealing two main clades (corresponding with "*T. suis* lineage" and "*T. trichiura* lineage") and respect to the sister-group relationships between *Trichuris* spp. from NHP, humans and pigs (Fig. 6 and Table 4). Four different highly supported subclades were observed within "*T. trichiura* lineage" (Clade 2): subclade TT2a including: *Trichuris* sp. from *M. sylvanus* from Spain (haplotypes 2 and 3); subclade TT2b: *T. trichiura* from *H. sapiens* from China and *Trichuris* sp. from *Papio anubis* from the USA; subclade TT2c: *Trichuris* sp. from *M. sylvanus* from Spain (majority haplotype 1), *T. trichiura* from *H. sapiens* from Uganda, *Trichuris* sp. from *Papio hamadryas* from Europe and two haplotypes of *Trichuris* sp. from *M. fuscata* from Europe; subclade TT2d: five haplotypes of *Trichuris* sp. from *M. fuscata* from Europe (Fig. 6). The phylogenetic topology revealed a sister relationship between subclades TT2a and TT2b and between subclades TT2c and TT2d with high bootstrap and BPP values (Fig. 6 and Table 4).

The multiple alignments of 33 *cox*1 nucleotide sequences (including outgroups) yielded a dataset of 301 characters. Population from *M. sylvanus* was represented by 4 haplotypes, out of which, haplotypes 3 and 4 clustered together with *Trichuris* sp. from *H. sapiens* from

Table 2 Biometrical and morphological data of 32 females of *Trichuris* sp. isolated from *M. sylvanus*. TF = *Trichuris* female. Б: standard deviation. X: arithmetic mean. Min: Minimum value. Max: Maximum value. All measurements are in millimetres

Lineage TT2a	Total length	Esophagus length	Body length	Ratio EL/BL	Wide body	Vulvar diameter	Vulva non protusive	Vulva with papillae or ornamentation	Vagina with circumvolutions	Anus subterminal
TF10	27	17	10	1.70	0.6	0.07	Present	No	Present	Present
TF27	35	22	13	1.69	0.66	0.09	Present	No	Present	Present
TF30	36	24	12	2.00	0.69	0.07	Present	No	Present	Present
TF32	32	22	10	2.20	0.61	0.08	Present	No	Present	Present
MIN	27	17	10	1.69	0.60	0.07				
MAX	36	24	13	2.20	0.69	0.09				
X	32.50	21	11.33	1.91	0.64	0.08				
Б	4.04	2.99	1.50	0.25	0.04	0.01				
Lineage TT2c										
TF1	33	23	10	2.30	0.69	0.06	Present	No	Present	Present
TF2	35	23	12	1.92	0.67	0.05	Present	No	Present	Present
TF3	33	21	11	1.91	0.7	0.06	Present	No	Present	Present
TF4	35	22	13	1.69	0.75	0.05	Present	No	Present	Present
TF5	32	21	11	1.91	0.7	0.06	Present	No	Present	Present
TF6	32.50	21	11.50	1.83	0.76	0.06	Present	No	Present	Present
TF7	37	25.50	11.50	2.22	0.72	0.05	Present	No	Present	Present
TF8	36	22	14	1.57	0.52	0.05	Present	No	Present	Present
TF9	33	22	11	2.00	0.69	0.05	Present	No	Present	Present
TF11	37	26	11	2.36	0.74	0.05	Present	No	Present	Present
TF12	32	21	11	1.91	0.66	0.06	Present	No	Present	Present
TF13	32	22	10	2.20	0.57	0.05	Present	No	Present	Present
TF14	37	24	13	1.85	0.71	0.06	Present	No	Present	Present
TF15	38	25	13	1.92	0.65	0.06	Present	No	Present	Present
TF16	31	21	10	2.10	0.63	0.05	Present	No	Present	Present
TF17	34	22	12	1.83	0.71	0.05	Present	No	Present	Present
TF18	34	22	12	1.83	0.71	0.05	Present	No	Present	Present
TF19	33	22	11	2.00	0.73		Present	No	Present	Present
TF20	34	22	12	1.83	0.59	0.06	Present	No	Present	Present
TF21	33	23	10	2.30	0.83	0.05	Present	No	Present	Present
TF22	34	24	10	2.40	0.69	0.06	Present	No	Present	Present
TF23	30	20	10	2.00	0.62	0.07	Present	No	Present	Present
TF24	36	23	13	1.77	0.69	0.05	Present	No	Present	Present
TF25	32	21	11	1.91	0.77	0.05	Present	No	Present	Present
TF26	37	26	11	2.36	0.71	0.06	Present	No	Present	Present
TF28	34	23	11	2.09	0.7	0.06	Present	No	Present	Present
TF29	37	26	11	2.36	0.79	0.06	Present	No	Present	Present
TF31	35	24	11	2.18	0.61	0.07	Present	No	Present	Present
MIN	30	20	10	1.57	0.52	0.05				
MAX	38	26	14	2.40	0.83	0.07				
X	34.15	22.78	11.40	2.02	0.69	0.06				
Б	2.10	1.72	1.09	0.23	0.07	0.01				

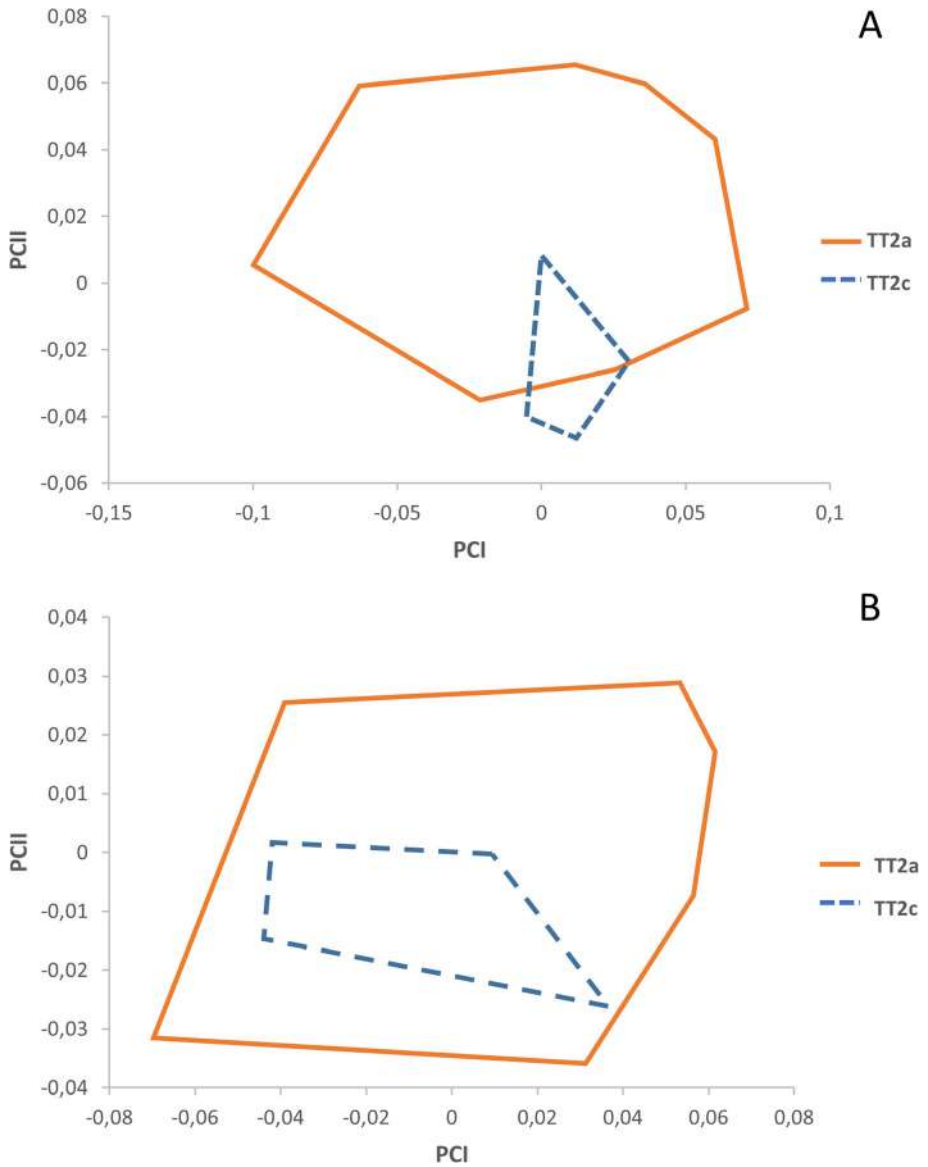

Fig. 4 Factor maps corresponding to adult *Trichuris* sp. Samples are projected onto the first (PC1) and second (PC2) principal components. Each group is represented by its perimeter. **a** Males factor map. PC1: 57% and PC2: 35%. **b** Females factor map. PC1: 81% and PC2: 18%

the Czech Republic (subclade TT2a) and separated from the main haplotype 1 and haplotype 2 (subclade TT2c) (Additional file 3 and Table 4).

The multiple alignments of 27 *cob* nucleotide sequences (including outgroups) yielded a dataset of 520 characters. Within Clade 2, population from *M. sylvanus* was represented by 4 haplotypes, out of which, haplotypes 2, 3 and 4 clustered together within subclades TT2a whereas haplotype 1 appeared within subclade TT2c (see Additional file 4).

The concatenated mitochondrial and ribosomal sequences included 1463 aligned positions and 8 taxa (outgroups not included). Phylogenetic trees again supported the existence of the two main evolutionary lineages previously recognized and the existence of clear differentiation between individuals of *Trichuris* sp. from *M. sylvanus* separated in two different subclades (TT2a and TT2c) (see Additional file 5 and Table 4).

Comparative sequence analysis

The range of intra-population similarity of *Trichuris* sp. from *M. sylvanus* based on ITS2 rDNA sequences (seven haplotypes) was 99.6–100%. The similarity obtained within "*T. trichiura* lineage" ranged from 94.4 to 100% while within "*T. suis* lineage" this value ranged from 85.1 to 100%. When we compared ITS2 sequences corresponding

Table 3 Sequences of *Trichuris* spp. and outgroups species obtained from GenBank and used for phylogenetic analysis

Species	Host species/Family	Geographical origin	Marker	Accession number
Trichuris colobae	*Colobus guereza kikuyensis*/ Cercopithecidae	Spain (Europe)	***Cox*1**	HE653116 HE653118
Trichuris sp.	*Colobus guereza kikuyensis*/Cercopithecidae	Czech Republic (Europe)		JF690968
	Homo sapiens/Hominidae	Czech Republic (Europe)		JF690962
	Macaca fuscata/ Cercopithecidae	Italy (Europe)		MK762905 MK762906 MK762908 MK762909 MK762915 MK762919 MK762920 MK762921
	Macaca sylvanus/ Cercopithecidae	**Spain** (Europe)		**LR130781[a]** **LR130782[a]** **LR130783[a]** **LR130784[a]**
	Papio anubis/ Cercopithecidae	USA (North America)		KT449825
	Papio hamadryas/ Cercopithecidae	Czech Republic (Europe)		JF690963
	Papio hamadryas/ Cercopithecidae	Denmark (Europe)		KT449824
Trichuris trichiura	*Homo sapiens*/Hominidae	China (Asia)		GU385218
		Japan (Asia)		AP017704
		Uganda (Africa)		KT449826
Trichuris suis	*Sus scrofa domestica*/Suidae	Spain (Europe)		HE653124
		Denmark (Europe)		KT449822
		Uganda (Africa)		KT449823
		China (Asia)		GU070737 HQ204208 HQ204209 HQ204210
	Sus scrofa scrofa/Suidae	Spain (Europe)		HE653127
Trichuris ursinus	*Papio ursinus*/ Cercopithecidae	South Africa (Africa)		LT627353
Trichinella spiralis		USA (North America)		AF293969
Trichinella pseudospiralis		Australia (Oceania)		KM357411
Trichuris colobae	*Colobus guereza kikuyensis*/ Cercopithecidae	Spain (Europe)	***Cob***	LM994704
Trichuris sp.	*Macaca fuscata*/ Cercopithecidae	Italy (Europe)		MK914550 MK914551 MK914554 MK914555 MK914556 MK914557 MK914560
	Macaca sylvanus/ Cercopithecidae	**Spain** (Europe)		**LR132031[a]** **LR132032[a]** **LR132033[a]** **LR132034[a]**
	Papio anubis/ Cercopithecidae	USA (North America)		KT449825
	Papio hamadryas/ Cercopithecidae	Denmark (Europe)		KT449824
	Papio sp. /Cercopithecidae	Spain (Europe)		LM994703
Trichuris trichiura	*Homo sapiens*/Hominidae	China (Asia)		GU385218
		Uganda (Africa)		KT449826
Trichuris suis	*Sus scrofa domestica*/Suidae	China (Asia)		GU070737
		Denmark (Europe)		KT449822

Table 3 Sequences of *Trichuris* spp. and outgroups species obtained from GenBank and used for phylogenetic analysis *(Continued)*

Species	Host species/Family	Geographical origin	Marker	Accession number
		Uganda (Africa)		KT449823
	Sus scrofa scrofa/Suidae	Spain (Europe)		LM994696
Trichuris ursinus	*Papio ursinus*/ Cercopithecidae	South Africa (Africa)		LT627357 LT627358 LT627359 LT627360
Trichinella spiralis		USA (North America)		NC_002681
Trichinella pseudospiralis		Australia (Oceania)		KM357411
Trichuris colobae	*Colobus guereza kikuyensis*/ Cercopithecidae	Spain (Europe)	**ITS2**	FM991956
Trichuris trichiura	*Macaca fuscata*/ Cercopithecidae	China (Asia) Japan (Asia)		AM992987 AB586133
	Homo sapiens	Cameroon (Africa)		GQ301555
Trichuris sp.	*Chlorocebus aethiops*/ Cercopithecidae	Tanzania (Africa)		JF690949 JF690950
	Homo sapiens/Hominidae	Czech Republic (Europe)		JF690940
	Macaca leonina/ Cercopithecidae	China (Asia)		MH390365 KT344828
	Macaca mulatta/ Cercopithecidae	China (Asia)		MH390367 MH390369
	Macaca silenus/ Cercopithecidae	Czech Republic (Europe)		JF690945
	Macaca sylvanus/ Cercopithecidae	**Spain** (Europe)		**LR535742**[a] **LR535746**[a] **LR535747**[a] **LR535748**[a] **LR535749**[a] **LR535750**[a] **LR535751**[a]
	Papio anubis/ Cercopithecidae	Czech Republic (Europe)		JF690942
	Papio hamadryas/ Cercopithecidae	Czech Republic (Europe)		JF690941
	Papio hamadryas ursinus/ Cercopithecidae	South Africa (Africa)		GQ301551 GQ301552 GQ301553
	Pan troglodytes/Hominidae	Netherlands (Europe)		JF690948
Trichuris suis	*Sus scrofa*	Slovakia (Europe)		JF690951
		Tanzania (Africa)		JN181811
	Sus scrofa domestica	Spain (Europe)		AJ249966
Trichuris ursinus	*Papio hamadryas ursinus*/ Cercopithecidae	South Africa (Africa)		GQ301554

[a]Access number of this study

with both lineages, the similarity observed ranged from 74 to 78.6%. Within "*T. trichiura* lineage", the minimum similarity observed between *Trichuris* populations from different species of genus *Macaca* corresponded to *Trichuris* sp. from *M. sylvanus* from Spain and *M. fuscata* from Japan, and the maximum value of similarity was obtained when we compared *Trichuris* sp. from *M. sylvanus* and *M. leonina* from China (95.9 -99.4% respectively) (Fig. 5).

The *cox*1 sequences (four haplotypes) of *Trichuris* sp. obtained from *M. sylvanus* from Spain showed an intraspecific similarity of 83.6–100%. Thus, haplotype 1 was the main haplotype with 37 individuals showing the same *cox*1. In addition, haplotypes H1 and H2 showed a similarity percentage from 99.3 to 100% with respect to *Trichuris* sp. of *M. fuscata* from Europe and *Trichuris* sp. of *P. hamadryas* from the Czech Republic, respectively. The similarity observed within these subclades ranged from 95.6% (TT2b) to 100% (TT2a and TT2c). On the other hand, the similarity observed between these subclades ranged from 79.1% (when we compared TT2b with TT2c) to 87.0% (when we compared TT2c with TT2d). Furthermore, the similarity observed when

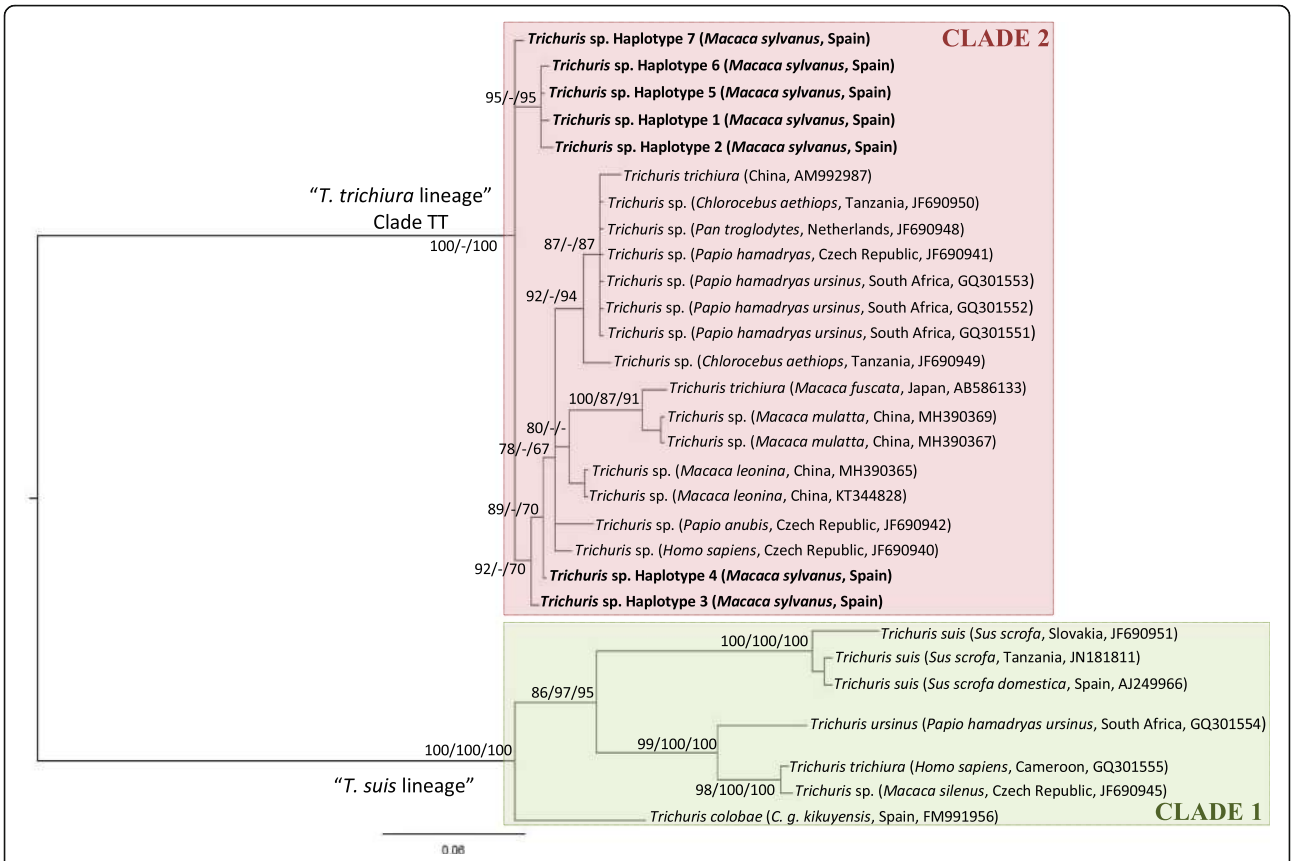

Fig. 5 Phylogenetic tree of *Trichuris* species based on ITS2 (rDNA) inferred using Bayesian Inference. Bayesian Posterior Probabilities of clades are listed first, followed by Maximum Parsimony and Maximum Likelihood bootstrap values, respectively, for clade frequencies exceeding 65%

we compared populations of *Trichuris* spp. from human and NHP with *T. suis*, *T. colobae* and *T. ursinus,* ranged from 73.4 to 80.6% (Table 5).

The 13 *co*b sequences of *Trichuris* sp. revealed the existence of four different haplotypes corresponding to two different lineages. The intra-specific similarity between those haplotypes ranged from 84.2 to 100%, corresponding to the lowest values observed when haplotype 1 was compared with haplotypes 2, 3 and 4. The *co*b sequences similarity observed within and between subclades revealed similar results that those obtained by *cox*1 sequences (Table 6).

Discussion

Morphological results revealed that the whipworm isolated from *M. sylvanus* is *T. trichiura*. Thus, in agreement with Cutillas et al. [7], Zaman [27] and Tenora et al. [28], the males of this species showed a pair of typical paracloacal papillae. Nevertheless, this is not in agreement with Ooi et al. [6] who reported the existence of a pair of paracloacal papillae associated to a cluster of small papillae not only in *T. trichiura* from human but in males of *T. trichiura* from *M. fuscata* and *Papio papio* and, furthermore, they reported females showing everted vagina covered with sharply pointed spines. We did not observe this type of vagina in *T. trichiura* from *M. sylvanus*. On the other hand, the comparative morphological study carried out on *Trichuris* species from other host primates (*C. guereza* and *P. ursinus*) revealed clear differences in respect to *Trichuris* sp. from *M. sylvanus.* Thus, this can be differentiated from *T. colobae* by the presence of a typical subterminal paracloacal papillae but not associated to a cluster of small papillae and a different spicule to that of *T, colobae* and *T. ursinus*, while females presented a non-everted vagina with a non-ornamented vulva. From a biometrical point of view, a preliminary study [29], based on modern morphometric approach, revealed that the analysis based on three measurements of males (maximum width of the posterior region of the body [thickness, M4], length of the spicule [M8], maximum length of the spicule sheath [M9], clearly illustrates globalized differences in the population of *Trichuris* sp. from *M. sylvanus* showing larger values of the males collected from the macaques with respect to *T. trichiura* from chimpanzees [7, 29]. The occurrence of different biometrical measurements in the same species was explained by Nissen et al. [9] as phenotypic adaptations [1]. This fact was also reported

Table 4 Monophyly based on different markers (ITS2, *cox*1, and *cob*) of selected group based on different combinations of datasets and inference methods

	Nuclear region ITS2	*Cox*1 mt gene	*Cob* mt gene	Mitochondrial genes (*cox*1 + *cob*)	Mitochondrial and nuclear markers (*cox*1, *cob* and ITS2)
Clade 1	100/100/100	76/–/93	93/69/99	92/61/100	100/–/100
Clade 2	100/–/100	73/98/80	92/–/100	90/–/100	100/100/100
Subclade TT2a	–	88/99/84	92/100/100	96/100/100	89/100/100
Subclade TT2b	–	98/100/93	92/99/99	98/100/96	–
Subclade TT2c	–	98/100/92	68/100/89	96/–/100	100/100/100
Subclade TT2d	–	–/100/–	92/100/99	100/100/100	–
Subclade TT2a + TT2b	–	–	100/96/99	70/96/84	–
Subclade TT2a + TT2b + TT2c	–	–	69/100/68	–	–
Subclade TT2c + TT2d	–	88/96/86	–	74/–/82	–
Trichuris colobae	–	88/100/100	–	–	–
Trichuris ursinus	–	–	100/100/100	–	–
Trichuris suis	100/100/100	–/–/100	95/99/100	98/100/100	–
Trichuris suis subclade 1a	–	100/100/96	91/–/63	88/100/100	–
Trichuris suis Subclade 1b	–	87/98/82	–	–/99/70	–
Trichuris colobae + *Trichuris ursinus*	–	–	–/64/–	76/–/86	–/74/62

ML Maximum Likelihood bootstrap, *MP* Maximum Parsimony bootstrap, *BPP* Bayesian Posterior Probability. Clade 2: *Trichuris trichiura* lineage; Clade 1: *Trichuris suis* lineage; subclade TT2a: *Trichuris* sp. from *M. sylvanus* (haplotypes 2, 3 and 4), *Trichuris* sp. from *H. sapiens* from Czech Republic; subclade TT2b: *Trichuris* sp. from *H. sapiens* and *P. anubis* from Asia and USA; subclade TT2c: *Trichuris* sp. from *M. sylvanus* (haplotypes 1 and 2), *H. sapiens* from Uganda, *P. hamadryas* from Denmark and Czech Republic, subclade TT2d*: Trichuris* sp. from *M. fuscata* from Europe

by Cutillas et al. [7, 13]. Furthermore, the existence of different types of eggs of *Trichuris* sp. in the same host has been previously reported [30].

On the other hand, molecular analyses based on mtDNA revealed the existence of two different genotypes corresponding to two different lineages within "*T. trichiura* lineage" that did not correlate with two different morphospecies. Nevertheless, we must be cautious since the number of individuals from one of the populations was very low. This fact would agree with Ghai et al. [31] who found that the host range of *Trichuris* sp. varies depending on the taxonomic group, with some groups showing host specificity and others showing host generality [31]. For this reason, these authors observed that one group was specific to humans, another one had an intermediate host range, and an additional group could infect all primates sampled, including humans. Furthermore, Ravasi et al. [10] found two different genotypes of *Trichuris* sp. from *P. ursinus* from two different geographical locations, but they did not carry out a morphological study to characterize different morphospecies. This morphological study was carried out by Callejón et al. [14] in one of these populations and they described the new species named *T. ursinus* related with *T. suis* lineage.

The combination of certain nuclear and mitochondrial markers could be considered as a useful taxonomic tool in order to infer phylogenetic relationships within *Trichuris* genus. The phylogeny of *Trichuris* spp. from humans and NHP inferred on ribosomal and mitochondrial datasets reported the existence of two main clades previously cited by different authors [10, 14, 32–34]. The similarity between different clades based on DatasetITS2 ("*T. trichiura* lineages" and "*T. suis* lineage") showed clearly lower value (74–78.6% suggesting that *Trichuris* population of *M. sylvanus* could be considered as *T. trichiura* attending to the intra-population similarity observed) [32].

In addition, phylogenetic relationships within Clade 2 based on ribosomal datasets revealed that phylogenetic relationships of populations of *Trichuris* sp. from *M. sylvanus* were unresolved. Furthermore, *Trichuris* spp. isolated from genus *Macaca* (*M. fuscata*, *M. leonina* and *M. mulatta*) clustered within the same clade separated of *Trichuris* population from *M. sylvanus*. This fact could be explained since *M. sylvanus* is the unique macaque primate extant African representative, all other species being Asiatic suggesting a co-evolutionary process together with the host [35].

The phylogeny inferred on mitochondrial datasets revealed *Trichuris* sp. from *M. sylvanus* (Spain) is separated into two different subclades: TT2a (minority haplotype) and TT2c (majority haplotype). Subclade TT2c is considered the most frequent subclade observed in *Trichuris* spp. from NHP and humans. "*T. trichiura* lineage" included a species complex with hypothetical

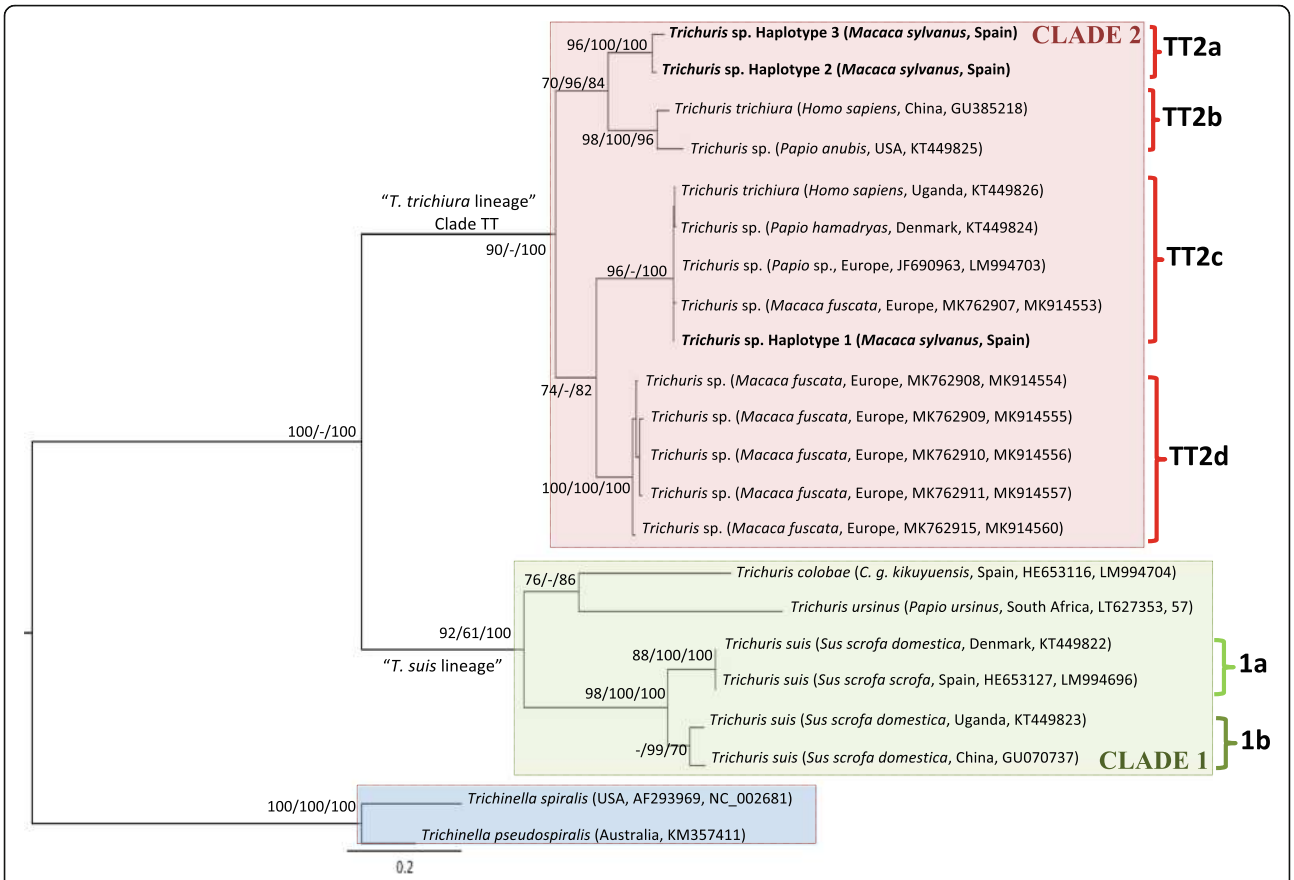

Fig. 6 Phylogenetic tree of *Trichuris* species based on combined analysis of mitochondrial DNA (*cox*1 and *cob*) inferred using Bayesian Inference. Bayesian Posterior Probabilities of clades are listed first, followed by Maximum Parsimony and Maximum Likelihood bootstrap values, respectively, for clade frequencies exceeding 60%

Table 5 Intra-specific and inter-specific similarity observed in *cox*1 partial sequences in *Trichuris* species isolated from different hosts

***Cox*1**								
	***Trichuris* spp. (Subclade TT2a)**	***Trichuris* spp. (Subclade TT2b)**	***Trichuris spp.* (Subclade TT2c)**	***Trichuris* spp. (Subclade TT2d) Subclade. *M. fuscata***	***T. suis* (Subclade 1b)**	***T. suis* (Subclade 1a)**	***T. colobae***	***T. ursinus***
Trichuris spp. (Subclade TT2a)	98–100							
Trichuris spp. (Subclade TT2b)	80.1–82.3	95.6–98.6						
Trichuris spp. (Subclade TT2c)	83.4–84.1	79.1–82.1	99.3–100					
Trichuris spp. (Subclade TT2d) Subclade. *M. fuscata*	84.1–86.4	81.7–83.4	85.4–87.0	97.7–99				
T. suis Spain (Subclade 1a)	78.4–79.2	78.4–80.6	78.1–78.7	77.4–79.7	99.7–100			
T. suis China (Subclade 1b)	77–78.1	76.7–79.5	77.1–78.7	75.4–78.4	90.9–92	95.3–99.5		
T. colobae	75.6–76.1	76.2–78.9	76.4–77.4	76.7–78.7	78.4–78.9	77.8–79.2	99.7–100	
T. ursinus	74.8–75.6	73.4–74.2	75.1–76.1	74.8–75.4	81.2–81.4	81.4–83.9	80.4–80.9	100

Subclade TT2a: *Macaca sylvanus* from Spain, *Homo sapiens* from Czech Republic,. Subclade TT2b: *H. sapiens* from China and Japan, *P. anubis* from USA. Subclade TT2c: *M. sylvanus* from Spain, *Macaca fuscata* from Italy, *H. sapiens* from Uganda and *P. hamadryas* from Denmark and Czech Republic. Subclade TT2d: *M. fuscata* from Italy. Subclade 1a: *Sus scrofa domestica* from Denmark and Spain and *S. s. scrofa* from Spain. Subclade 1b: *S. s. domestica* from China

Table 6 Intra-specific and inter-specific similarity observed in *cob* partial sequences in *Trichuris* species isolated from different host

cob								
	***Trichuris* spp. (Subclade TT2a)**	***Trichuris* spp. (Subclade TT2b)**	***Trichuris* spp. (Subclade TT2c)**	***Trichuris* spp. (subclade TT2d) *Macaca fuscata* from Europe**	***T. suis* (Subclade 1b)**	***T. suis* (Subclade 1a)**	***T. colobae***	***T. ursinus***
Trichuris spp. (Subclade TT2a)	97.1–100							
Trichuris spp. (Subclade TT2b)	81.7–88.1	93.9						
Trichuris spp. (Subclade TT2c)	83.8–84.4	84–84.7	99.1–99.8					
Trichuris spp. (subclade TT2d) *Macaca fuscata* from Europe	84.7–86.7	86–87.6	86–88.2	98.7–100				
T. suis (Subclade 1b)	74.7–75.8	73.8–74.2	72.4–72.8	73–74.2	90–100			
T. suis (Subclade 1a)	72.9–73.8	73.3–74.4	72.1–73.9	73.5–75.1	71.4–72.9	–		
T. colobae	70–71.6	72.3	73.3–74.2	73.5–73.9	77.4	78.7	–	
T. ursinus	72.1–72.3	72.7–73.3	73.2–74.2	73.5–74.2	75.9–79.1	77.4–77.8	78.5–78-6	99.25–99.8

Subclade TT2a: *Macaca sylvanus* from Spain. Subclade TT2b: *Homo sapiens* from China, *P. anubis* from USA. Subclade TT2c: *M. sylvanus* from Spain, *Macaca fuscata* from Italy, *Papio* sp. from Spain and *P. hamadryas* from Denmark. Subclade TT2d: *M. fuscata* from Italy. Subclade 1a: *Sus scrofa domestica* from Uganda and Denmark and *S.s. scrofa* from Spain. Subclade 1b: *S. s. domestica* from China

sibling/cryptic species. In this last lineage, and based on *cox*1 partial gene sequences, *Trichuris* sp. from *M. sylvanus* appeared distributed in two different subclades according to an African or European origin of *T. trichiura* from *H. sapiens*. This phylogenetic pattern of distribution could suggest that different populations are circulating, although samples were taken from the same host. Hawash et al. [36] found no difference between *T. trichiura* from humans and *Trichuris* from NHP in Uganda, and he indicated a specific African parasite origin, which would then has been transmitted to Asia and South America suggesting that *Trichuris* in humans represents an heirloom parasite. We observed that most individuals of *Trichuris* sp. from *M. sylvanus* clustered with *T. trichiura* from *H. sapiens* from Uganda (Africa) and only a few individuals clustered with *Trichuris* sp. of *H. sapiens* from the Czech Republic (Europe). Since only one reference from Africa is used, further molecular studies would be carried out to clarify if there are a specific African parasite origin and a posterior transmission to Europe and Asia.

In agreement with this study, similar results were observed on *Trichuris* sp. from *M. fuscata* [34]. Besides, this population showed two potentially distinct entities of *Trichuris* present in two different subclades: subclade TT2d (analogous to subclade MF reported by Cavallero et al. [34]) and subclade TT2c. These authors suggested the possibility of two different sources of infection for Japanese macaques corresponding with two *Trichuris* taxa. Within Clade 2, subclades TT2a, TT2b and TT2c correspond to taxonomic species able to infect primates and humans without strict host specificity. These results agree with Doležalová et al. [37] revealing the existence of *Trichuris* spp., which are shared by humans and several NHP (baboons and macaques).

Despite the fact that there seems to be a pattern of infection with different *Trichuris* species infecting particular host species, the existence of more species of *Trichuris* in primates opens up the possibility of studying the zoonotic potential of different hosts harboring *T. trichiura* and/or other putative new species of whipworms [31].

In addition, it would be necessary to carry out further morphological and molecular studies of *Trichuris* populations parasitizing NHP from different geographical origins to improve taxonomy and clarify different *Trichuris* species in primates, and to know if the diversity of *Trichuris* spp. parasitizing NHP is due to a host specific process, or if these species share different primate hosts, as well as, to evaluate these primates as reservoir hosts of human trichuriasis.

Conclusions

The morphological, biometrical, and molecular results showed that adults of *Trichuris* sp. from *M. sylvanus* were *T. trichiura*. Molecular analyses revealed the existence of two different genotypes corresponding to two different lineages within "*T. trichiura* lineage" that did not correspond to different morphospecies.

Methods

Isolation of material

Sixty-five adults (32 females and 33 males) of *Trichuris* sp. were collected from the caecum of a male Barbary macaque (*M. sylvanus*), which had died of natural causes, from the Zoo Castellar (Cádiz, Spain). This macaque male was 15-year-old, and it was born in the Zoo Castellar (Cádiz, Spain) and it was in captivity in contact with other individuals of the same species but without contact with others non-human primates' species. It was in contact with the animal keepers and veterinary from the zoo. The parasitic evaluations revealed the presence of *Trichuris* eggs in the feces for many years and the anthelmintic treatment was mebendazole (10 mg/kg for 3–4 days). A pulmonary pathology was the cause of the natural death.

We previously received consent from the Zoo Castellar to collect these samples. Worms were washed extensively in 0.9% saline solution to remove remains of the host, then, frozen at − 20 °C or preserved in 70% ethanol for morphological, biometrical, and molecular analysis. Posteriorly, worms were cleared with glycerine/alcohol or acetic acid for morphological studies.

Morphological studies

Species identification was performed according to previous studies [7, 13, 14]. Morphological examinations were carried out as described by Oliveros et al. [38] and Skrjabin et al. [39]. A comparative study of morphological data of *T. trichiura* (present study), *T. colobae* [7, 13] and *T. ursinus* [14] was carried out.

Biometrical analysis of *Trichuris* specimens was carried out according to parameters reported by Spakulová and Lýsek [15], Suriano and Navone [16] and Robles et al. [17]. Subsequently, a biometrical study was carried out using those measurements that are significant in the differentiation of *Trichuris* species and reported previously by García-Sánchez et al. [29]. Descriptive univariate statistics (mean values, standard deviations, and range) for all parameters were determined for all individuals of *Trichuris* sp. from *M. sylvanus*.

We carried out many of the most common tests (including mean, standard deviation, Student's t) using spreadsheet of Microsoft *Excel*. The Student's t assess was used to test the equality of means for each variable in both lineages. The following non-redundant measurements (one measurement is not included in another) used for whipworm adults were: Total length, esophagus length (EL), body length (BL), ratio EL/BL, wide body, spicule length, spicule length sheath for male; total length, esophagus length, body length, ratio EL/BL, wide body and vulvar diameter for females [29]. It was considered a value statistically significant when $P < 0.05$. Biometric characters of *Trichuris* sp. from *M. sylvanus* were compared and assayed for a geometric morphometric analysis. Multivariate analyses were used to calculate the phenotypic variations between *Trichuris* specimens, using size-free canonical discriminant analyses on the covariance of log-transformed measurements. These analyses were applied to exclude the effect of within-group ontogenetic variations by reducing the effect of each character on the first-pooled, within-group, principal component (a multivariate size estimator) [39]. The principal component analysis (PCA) was used to summarize most of the variations in a multivariate dataset in a few dimensions. The multivariate analyses of the morphometric data were carried out by using BAC v.2 software [29, 40, 41].

DNA amplification and sequencing

Genomic DNA from 43 individual was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Each nematode was placed in a sterile 1.5 ml Eppendorf tube and a pestle was used to facilitate the mechanic rupture of the cuticle. The genomic DNA was extracted from the complete body. Quality of extractions was assessed using 0.8% agarose gel electrophoresis infused with SYBR® Safe DNA gel stain.

All molecular markers sequenced in the present study (*cox*1 and *co*b, mtDNA and ITS2 rDNA) were amplified by polymerase chain reaction (PCR) using a thermal cycler (Eppendorf AG; Hamburg, Germany). PCR mix, PCR conditions and PCR primers are summarized in the Supporting information (see Additional file 6). The PCR products were checked on SYBR® Safe stained 2% Tris-Borate-EDTA (TBE) agarose gels and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified PCR products were concentrated and sequenced in both directions using same primers used for PCR by Stab Vida (Portugal).

Phylogenetic analysis

rDNA (ITS2) and mtDNA (*cox*1 and *co*b) sequences were aligned using the MUSCLE alignment method [26] included in MEGA, version 7.0 [42]. For comparison, additional ribosomal and mitochondrial sequences from *Trichuris* infecting human, NHP and pigs from different geographical regions from the National Centre for Biotechnology Information (NCBI) GenBank™ database were included in the alignments. *Trichinella spiralis* and *Trichinella pseudospiralis* were used as outgroups for mitochondrial datasets (Table 3). No outgroups were used to infer phylogenetic trees based on ITS region because the sequences of nucleotides are not sufficiently conserved for there to be a reasonably unambiguous match. Nevertheless, sequences of *Trichuris* spp. from *Macaca silenus* and *Macaca fascicularis* from the Czech

Republic were not included in the phylogenetic analysis due to errors found in these sequences. Moreover, *cox*1 sequences of *Trichuris* spp. from *Macaca mulatta* and *Macaca leonina* were not considered for phylogenetic analysis because they did not correspond with the partial fragment of *cox*1 gene analyzed in the present study; however, they were included in the phylogenetic analysis based on the ITS2 sequence (Table 3).

Phylogenetic analysis was performed by Maximum Parsimony (MP) algorithm using MEGA 7 [42], Maximum Likelihood (ML) using the PHYML package from Guindon and Gascuel [43] and Bayesian Inference (BI) using MrBayes, version 3.2.6 [44]. Each dataset was analyzed separately from each other, and both mitochondrial and ribosomal datasets were combined into a total evidence dataset. jModeltest was employed to compute the best partitioning scheme, as well as the best nucleotide substitution models for each partition [45]. Models of evolution were chosen for subsequent analysis according to the Akaike Information Criterion [46]. The concatenated dataset was partitioned by gene and models for individual genes within partitions were those selected by jModeltest. For ML inference, best-fit nucleotide substitution models included general time reversible (GTR) model with gamma-distributed rate variation and a proportion of invariable sites (GTR + I (*cox*1)), GTR model with gamma-distributed rate variation and a proportion of invariable sites (GTR + I + G (*cob*)) and GTR model with gamma-distributed rate (GTR + G (ITS2)). Support for the topology was examined using bootstrapping (heuristic option) [47] over 1000 replications to assess the relative reliability of clades. The Bayesian posterior probabilities (BPP) comprise the percentage converted for BI; the standard deviation of split frequencies used to determine whether the number of generations completed was enough. Models selected by jModeltest for BI were nst = 6 with inv. rates (*cox*1), nst = 6 with invgamma rates (*cob*) and nst = 6 with gamma rates (ITS2). BI analysis was run for ten million generations, and the tree was sampled every 500 generations. Trees from the first million generations were discarded based on an assessment of convergence. Burn-in was determined empirically by examination of the log likelihood values of the chains. After eliminating the first million trees as "burn-in", we constructed a 50% majority-rule consensus tree, with nodal values representing the probability (posterior probability) that the recovered clades exist, given the aligned sequence data. We accepted a clade in the Bayesian tree at around 70% posterior probability.

The number of base differences per sequence with respect to the sequences under investigation was evaluated using the number of differences method of MEGA 7 to assess the similarity among all marker sequences of all specimens analyzed in the present study and other *Trichuris* species.

Since molecular analysis showed two different genetic lineages in *Trichuris* sp. from macaque, we carried out a posterior biometrical study based on those measurements and the method previously used considering the two different lineages observed in *Trichuris* sp. from *M. sylvanus*:

- Lineage TT2a: Individuals showing the minority genetic lineage.
- Lineage TT2c: Individuals showing the main genetic lineage.

Thus, the Student's t test was used to test the equality of means for each variable in both lineages and biometric characters of *Trichuris* sp. from both lineages were compared and assayed for a geometric morphometric analysis.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-020-02661-4.

Additional file 1. Biometrical data of 15 males of *Trichuris* sp. isolated from *M. sylvanus*.

Additional file 2. Biometrical data of 15 females of *Trichuris* sp. isolated from *M. sylvanus*.

Additional file 3. Phylogenetic tree of *Trichuris* species based on *cox*1 mtDNA sequences inferred using Bayesian method. Bayesian Posterior Probabilities of clades are listed first, followed by Maximum Parsimony and Maximum Likelihood bootstrap values, respectively, for clade frequencies exceeding 60%.

Additional file 4. Phylogenetic tree of *Trichuris* species based on *cob* mtDNA sequences inferred using Bayesian method. Bayesian Posterior Probabilities of clades are listed first, followed by Maximum Parsimony and Maximum Likelihood bootstrap values, respectively, for clade frequencies exceeding 60%.

Additional file 5. Phylogenetic tree of *Trichuris* species based on combined analysis of mitochondrial DNA (*cox*1 and *cob*) and nuclear ribosomal DNA (ITS2) inferred using Bayesian Inference. Bayesian Posterior Probabilities of clades are listed first, followed by Maximum Parsimony and Maximum Likelihood bootstrap values, respectively, for clade frequencies exceeding 65%.

Additional file 6. PCR mix, primers and conditions used for each molecular marker sequenced.

Abbreviations

BI: Bayesian inference; bp: base pairs; BPP: Bayesian posterior probabilities; BV: Bootstrap value; *cob*: *cytochrome* b; *cox*1: cytochrome *c-oxidase* subunit 1; DNA: Deoxyribonucleic acid; ITS: Internal transcribed spacer; ML: Maximum likelihood; mm: millimeters; MP: Maximum parsimony; mtDNA: mitochondrial DNA; NHP: Non-human primates; PCA: Principal component analysis; PCR: Polymerase chain reaction; rDNA: ribosomal DNA

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Authors' contributions
Analyzed the data: JRF RCF AGS. Conceived, sampled, and designed the experiments: CCB. Contributed reagents/materials/analysis tools: JRF RCF AZC CCB AGS. First drafted the paper: JRF RCF AZC CCB. Performed the experiments: JRF RCF AGS, AZC. Wrote the paper: JRF RCF AZC CCB. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets generated and analyzed during the current study are available in the GenBank™, EMBL and DDBJ repository, [Accession numbers: LR130781–4, LR132031–4, LR535742, LR535746–51] (Table 3).

Ethics approval and consent to participate
Not applicable. This study does not require approval by an ethics committee. *Macaca sylvanus*, from which *Trichuris* specimens were collected from their caeca *post-mortem*, died of natural death. The specimen was handled and housed in a zoo in strict accordance with good animal practices.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Complete Mitochondrial Genome of *Trichuris trichiura* from *Macaca sylvanus* and *Papio papio*

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Abstract: Trichuriasis is among the most prevalent worldwide parasitism caused by helminths. For many years, *Trichuris* spp. have been described with a relatively narrow range of both morphological and biometrical features. The use of the complete mitochondrial genome (mitogenome) is an alternative and powerful molecular method for inferring phylogenies. Here, we present an overview of the contributions of mitogenome for *Trichuris* spp. from human and non-human primates. In addition, we carry out structural and phylogenetic comparative analyses with genomes of *Trichuris* species available in public datasets. The complete mt genomes of *Trichuris trichiura* and *Trichuris* sp. from *Macaca sylvanus* and *T. trichiura* from *Papio papio* are 14,091 bp, 14,047 bp and 14,089 bp in length, respectively. The three mt genomes are circular and consist of 37 genes—13 PCGs (*cox*1–3, *nad*1–6, *nad*4L, *atp*6, *atp*8 and *cob*), 22 transfer RNA genes (tRNAs), and two rRNAs (*rrn*L and *rrn*S). The molecular evidence presented here supports the hypothesis that *T. trichiura* de *M. sylvanus* (TMF31) and *T. trichiura* de *P. papio* (TPM1) were similar but genetically different with respect to *Trichuris* sp. from macaques (TMM5). The phylogenetic study also supported the evolution of the different *Trichuris* species. In conclusion, we suggest the existence of two cryptic species parasitizing *M. sylvanus*.

Keywords: complete mitochondrial genome; *Trichuris trichiura*; phylogeny; whipworms

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1. Introduction

Soil-transmitted helminths (STHs) (*Ascaris lumbricoides* (Ascariasis), *Trichuris trichiura* (Trichuriasis)) and hookworms (Ancylostomiasis/Necatoriasis) are widely distributed in tropical and subtropical areas, with the greatest numbers occurring in sub-Saharan Africa, the Americas, China, and East Asia [1]. In areas with favorable climatic and environmental conditions, poor access to potable water supply, sanitation, and hygiene resources, the transmission of these parasites is higher [2]. Recent data show that about 1.5 billion people are estimated to be affected by STHs worldwide [1].

Although Trichuriasis is a seriously neglected disease, fortunately, in recent years hereditary studies are increasing as well as related methodologies that provide new opportunities for the discovery of novel intervention strategies, with major implications for improving animal and human health and welfare globally [3]. In addition, the implications of genomic studies could also be very relevant in relation to the search for new treatments for immunopathological diseases in humans [4–9]. For example, it has been reported that infections of human patients suffering from immunological disorders (such as Crohn's disease) suppress clinical symptoms significantly with pig-*Trichuris* eggs [6,10–12].

The nematode mitogenome (complete mitochondrial genome) has several practical strengths as a phylogenetic marker, and has yielded well-supported results for clades, which were not well resolved using other approaches [13]. The whole genome and transcriptome studies have contributed to our understanding of deep node nematode phylogeny but have been limited by low taxon sampling (which is also biased toward some

parasitic groups) and by the technical challenges inherent in obtaining a large quantity of genetic information from a single nematode with a small body size. In such cases, the use of mitogenome sequences is one alternative that has been widely applied to many nematode branches where relationships were unclear [13].

Nematode mitogenomes are like those of other animals in many respects, but have a few unusual features including high variation in conservation of gene order across major branches and the occasional presence of multiple chromosomes [13]. The nematode mitogenome is usually a single, circular molecule ranging in size from 12 to 22 kb and containing 36 (sometimes 37) genes—12 (or 13) protein coding genes (PCGs) (*cox*1–*cox*3, *cyt*b, *nad*1-*nad*6, *nad*4L, *atp*6, and rarely *atp*8), two ribosomal RNA (rRNA) (*rrn*L and *rrn*S), and 22 transfer RNA genes (tRNAs). The *atp*8 gene, which is found in most other metazoan mitogenomes (except the parasitic Platyhelminthes clade Neodermata) [14], is usually absent in nematode mitogenomes, although it does appear in the order Trichinellida (*Trichinella* spp. and *Trichuris* spp.) [13,15–20]. Furthermore, within class Enoplea, members of order Trichinellida (*Trichinell*a spp., *Trichuris* spp.) show a substantial gene rearrangement even among closely related species, while members of Chromadorea show far less rearrangement in theirs mitogenomes [21].

Kern et al. [13] concluded that the mitochondrial genome is a useful tool for nematode phylogenetic because the diversity within nematode mitogenome architecture, its variable rate of gene rearrangement, and the representation of nearly every kind of lifestyle and habitat ecology within nematodes make this phylum an exciting area for addressing questions about mitogenome evolution.

Currently, the complete mt genome of several species of whipworms has been sequenced— *Trichuris trichiura* from humans and baboons [3,19]; *Trichuris* sp. from the Endangered François' Leaf-Monkey [17]; *Trichuris rhinopiptheroxella* from the endangered golden snub-nosed monkey [20]; *Trichuris suis* from pig [3,19]; *Trichuris ovis* from antelope [16]; *Trichuris discolor* from wild yak [16]; *Trichuris skrjabini* from sheep [22]; and *Trichuris muris* (LC050561, unpublished). This fact, and considering the hypothesis that *Trichuris* infecting primates represent a complex of cryptic species with some species capable of infecting both humans and non-human primates (NHPs), reflects the need to delve into the mt genomes of *Trichuris* spp. of NHPs compared to *T. trichiura* from humans.

Thus, the aim of the present study was to sequence the complete mt genome of *Trichuris trichiura* isolated from different NHPs. In addition, we carry out structural and phylogenetic comparative analyses with genomes of species of genus *Trichuris* available in public datasets.

2. Materials and Methods

2.1. Ethics Statement

This study does not require approval by an ethics committee. *Macaca sylvanus*, from which *Trichuris* specimens were collected from their caeca post-mortem. *Trichuris* specimens, recovered from the feces of one *Papio papio* after anthelmintic treatment, were handled and housed in a zoo in strict accordance with good animal practices.

2.2. Parasites, DNA Extraction and Genotyping of Worms

Specimen *Trichuris* worms were recollected from a Guinea baboon (*P. papio*) at Parque de la Naturaleza de Cabárceno (Cantabria, Spain), from a stool sample after anthelmintic treatment, and a Barbary macaque (*M. sylvanus*) in Castellar Zoo (Cádiz, Spain), from its caeca post-mortem. Adult *Trichuris* were recovered and washed in physiological saline, identified morphologically, and then, stored at −20 °C until use. Total genomic DNA was isolated from three individual worms according to the manufacturer's protocol the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Düsseldorf, Germany),used to extract the genomic DNA. Quality of extractions was assessed using 0.8% agarose gel electrophoresis infused with SYBR® Safe DNA gel stain (Thermo Fisher Scientific, Waltham, MA, USA)

2.3. Mitochondrial Genome Amplification and Sequencing

For long-range PCR amplification and next generation sequencing (NGS), different primate derived *Trichuris* specimens were chosen. One Guinea baboon worm (TPM1) and two Barbary macaque worms (TMF31 and TMM5) were chosen based on their distinct haplotypes identified in previous studies by sequencing the partial gene of mtDNA (*cox*1, *co*b and *rrn*L) and rDNA (ITS1 and ITS2) [23]. To obtain the complete mt genome, firstly, we used the primers designed by Hawash et al. [19] to amplify the *Trichuris* sp. from baboon (TTB1) and *T. trichiura* from humans from Uganda (TTHUG) genomes in two overlapping fragments (~8 and ~6 kbp), Nevertheless, we only could amplify the second fragment (~6 kbp), from *rrn*L to *nad*1. Then, new primers (MS1F and MS1R) were designed to amplify the other fragment using Primer3 from *nad*1 to *rrn*L (http://bioinfo.ut.ee/primer3-0.4.0/ (accessed on 1 February 2021)). PCR mix, PCR conditions and PCR primers are summarized in the Supplementary materials (Table S1). The PCR products were checked on SYBR® Safe stained 0.8% Tris-Borate-EDTA (TBE) agarose gels and bands were eluted and purified from the agarose gel using the QWizard SV Gel and PCR Clean Up System Kit (Promega, Madison, WI, USA). Once purified, the samples were concentrated and measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Stab Vida (Lisbon, Portugal) sequenced the mt genomes. The PCR products were used for library construction using Illumina Nextera XT library preparation kit (San Diego, CA, USA) and the generated DNA fragments were sequenced in the Illumina MiSeq platform (San Diego, CA, USA), using 300 bp paired-end sequencing reads.

2.4. Assembly, Annotation, and Genome Sequence Analyses

Sequences were assembled and analyzed using MacVector package v17.5.4 (Oxford Molecular Group, Waterbeach, Cambridge, UK). The identity of the sequences was made using BLAST by comparison with other sequences available in GenBank database. Genome annotation was performed using the pipeline MITOS web server (http://mitos.bioinf.uni-leipzig.de/index.py (accessed on 1 February 2021)) [24] using the mitochondrial invertebrate genetic code, and MacVector and protein-encoding genes of *T. trichiura*. The majority of the tRNA genes were identified using the ARWEN tool (http://130.235.244.92/ARWEN/ (accessed on 1 February 2021)) to get rRNA [25] and tRNAScan-SE web server (http://trna.ucsc.edu/tRNAscan-SE/ (accessed on 1 February 2021)) [26], but the remaining tRNA genes were recognized manually by comparison.

The genomes were compared with *T. trichiura* and *Trichuris* sp. Sequences—*T. trichiura* from humans (unknown geographical location) (AP017704), *T. trichiura* from humans in China (NC_017750), *T. trichiura* from humans in China (GU385218), *T. trichiura* from humans in Uganda (KT449826), *T. trichiura* from *P. anubis* in USA (KT449825), *T. trichiura* from *P. hamadryas* in Denmark (KT449824) and *Trichuris* sp. from *T. francoisi* in China (KC461179). All protein-coding genes (PCGs) and ribosomal RNA genes (rRNAs) sequences were individually extracted, and a nucleotide data set was generated by concatenated sequences (all PCGs and rRNAs genes). This data set was used to estimate genetic distances using MEGA X v.10.1.8 (Penn State, PA, USA) [27]. The genomes of *Trichuris* from humans and NHP were used to calculate the < Nucleotide diversity (π) using a sliding window of 100 bp with 25 bp steps implemented in DnaSP v.6 [28].

2.5. Phylogenetic Analysis

For the phylogenetic analyses, two different data sets were generated. The first included nucleotides sequences, with the 13 PCGs (*cox*1–3, *nad*1–6, *nad*4L, *atp*6, *atp*8 and *co*b) and the two rRNAs (*rrn*L and *rrn*S). The data set was aligned using MEGA X v.10.1.8 [27] and concatenated for *Trichuris* sp. and *T. trichiura* species from baboons and humans. We used as an outgroup *Trichinella pseudospiralis* (Table 1).

Table 1. Sequences analyzed in the first data set for phylogenetic analyses.

Species	Host Species/Geographical Origin	GenBank Accession Number
Trichuris trichiura	*Macaca sylvanus*/Spain	MW448470
Trichuris sp.	*Macaca sylvanus*/Spain	MW448471
Trichuris trichiura	*Papio papio*/Spain	MW448472
Trichuris trichiura	*Homo sapiens*/(unknown geographical location)	AP017704
Trichuris trichiura	*Homo sapiens*/China	NC_017750
Trichuris trichiura	*Homo sapiens*/China	GU385218
Trichuris trichiura	*Homo sapiens*/Uganda	KT449826
Trichuris sp.	*Papio anubis*/USA	KT449825
Trichuris sp.	*Papio hamadryas*/Denmark	KT449824
Trichuris sp.	*Trachypithecus francoisi*/China	KC461179
[1] *Trichinella pseudospiralis*	*Coragypus atratus/USA*	KM357411

[1] Used as an outgroup.

Another data set was generated using amino acid sequences inferred from the 12 PCGs. The sequences were aligned using MEGA X v.10.1.8 [27] and then concatenated excluding *atp*8 (because it is not present in the mitochondrial genome of all the species of nematodes except in *Trichinella* and *Trichuris* species). The data set, using the sequences previously used and all the sequences available of the complete mt genome of *Trichuris* spp. and with those of 9 other enoplid nematodes, using *Brugia malayi* and *Ascaris suum* as the outgroups, was generated (Table 2). The ambiguous regions of the alignment were excluded using Gblocks Server v.0.91b (http://phylogeny.lirmm.fr/phylo_cgi/one_task.cgi?task_type=gblocks (accessed on 1 February 2021)) with the default settings being used to select the option of less strict conservation of flanking positions [29,30].

Table 2. Sequences analyzed in the second data set for phylogenetic analyses.

Species	Host Species/ Geographical Origin	Order	GenBank Accession Number
Trichuris trichiura	*Macaca sylvanus*/Spain	Trichinellida	MW448470
Trichuris sp.	*Macaca sylvanus*/Spain	Trichinellida	MW448471
Trichuris trichiura	*Papio papio*/Spain	Trichinellida	MW448472
Trichuris trichiura	*Homo sapiens*/(unknown geographical location)	Trichinellida	AP017704
Trichuris trichiura	*Homo sapiens*/China	Trichinellida	NC_017750
Trichuris trichiura	*Homo sapiens*/China	Trichinellida	GU385218
Trichuris trichiura	*Homo sapiens*/Uganda	Trichinellida	KT449826
Trichuris sp.	*Papio anubis*/USA	Trichinellida	KT449825
Trichuris sp.	*Papio hamadryas*/Denmark	Trichinellida	KT449824
Trichuris sp.	*Trachypithecus francoisi*/China	Trichinellida	KC461179
Trichuris rhinopiptheroxella	*Rhinopithecus roxellana*/China	Trichinellida	MG189593
Trichuris ovis	*Addax nasomaculatus*/China	Trichinellida	NC_018597
Trichuris discolor	*Bos grunniens mutus*/China	Trichinellida	NC_018596
Trichuris muris	- /United Kingdom	Trichinellida	LC050561
Trichuris suis	*Sus scrofa*/Uganda	Trichinellida	KT449823
Trichuris suis	*Sus scrofa*/China	Trichinellida	GU070737
Trichinella pseudospiralis	*Coragypus atratus/USA*	Trichinellida	KM357411
Xiphinema americanum	*Plant ectoparasite*	Dorylaimida	NC_005928
Hexamermis agrotis	-	Mermithida	NC_008828
Agamermis sp.	-	Mermithida	NC_008231
Romanomermis culicivorax	-	Mermithida	NC_008640

Table 2. *Cont.*

Species	Host Species/Geographical Origin	Order	GenBank Accession Number
Romanomermis iyengari	-	Mermithida	NC_008693
Romanomermis nielseni	-	Mermithida	NC_008692
Strelkovimermis spiculatus	-	Mermithida	NC_008047
Thaumamermis cosgrovei	-	Mermithida	NC_008046
[1]*Brugia malayi*	-	Rhabditida	NC_004298
[1]*Ascaris suum*	-	Rhabditida	HQ704901

[1] Used as an outgroup.

For phylogenetic re-constructions we used three methods—Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inferences (BI). The ML tree was generated using PHYML package [31,32], and for the MP tree we used MEGA X v.10.1.8 [27] and for BI we used MrBayes v.3.2.6. [33]. To resolve the best-fit substitution model for the nucleotide data set we employed jModelTest [34] and ProtTest 3.4 for the amino acid data set. Models of evolution were defined according to the Akaike Information Criterion [35,36]. For the nucleotide data set, GTR + I + G model, with rate variation along the length of the alignment (+ G) and allowing for a proportion of invariant sites (+ I) was selected, and for the amino acid data set, the MtArt + I + G + F model, with residue frequencies estimated from the data (+ F) was chosen. Support for the topology was examined using bootstrapping (heuristic option) [37] over 1000 replications to assess the relative reliability of clades. The commands used in MrBayes for BI were *nst* = mixed. The standard deviation of split frequencies was used to determine whether the number of generations completed was sufficient; the chain was sampled every 500 generations and each dataset was run for 10 million generations. Trees from the first million generations were discarded based on an assessment of convergence. Burn-in was determined empirically by examination of the log likelihood values of the chains. The Bayesian posterior probabilities (BPPs) comprise the percentage converted.

3. Results

3.1. Annotation and Features of Mitochondrial Genomes

The complete mtDNA sequences of the primate worms TMF31, TMM5 and TPM1 were 14,091, 14,047 and 14,089 bp in length, respectively (GenBank accession nos. MW448470-2) (Figure 1). The mt genomes contained 37 genes—13 PCGs (*cox*1–3, *nad*1–6, *nad*4L, *atp*6, *atp*8 and *cob*), 22 transfer RNA genes (tRNAs), and two rRNAs (*rrn*L and *rrn*S) (Table 3). All genes are transcribed from the heavy strand, except four PCGs (*nad*2, *nad*4, *nad*4L and *nad*5) and 10 tRNA (tRNA-Met, tRNA-Phe, tRNA-His, tRNA-Arg, tRNA-Pro, tRNA-Trp, tRNA-Ile, tRNA-Gly, tRNA-Cys, and tRNA-Tyr) that are transcribed from the light strand.

The mt genomes of *Trichuris* sp. contain an AT-rich region consisting of two non-coding regions (NCRs), including a long non-coding region (NCR-L) and short non-coding region (NCR-S). The nucleotide composition (%) is summarized in Table S3. The content of A + T is 69.4%, 68% and 69.3% for TMH31, TMM5 and TPM1, respectively.

Between TMF31 and TPM1 genomes there were slight differences. The sequences were identical in terms of all initiation and termination codons and gene lengths, except for tRNA-ser (S2), which presented one nucleotide more in TPM1. In addition, between TMF31 and TMM5, 22 genes of 37 were different in length.

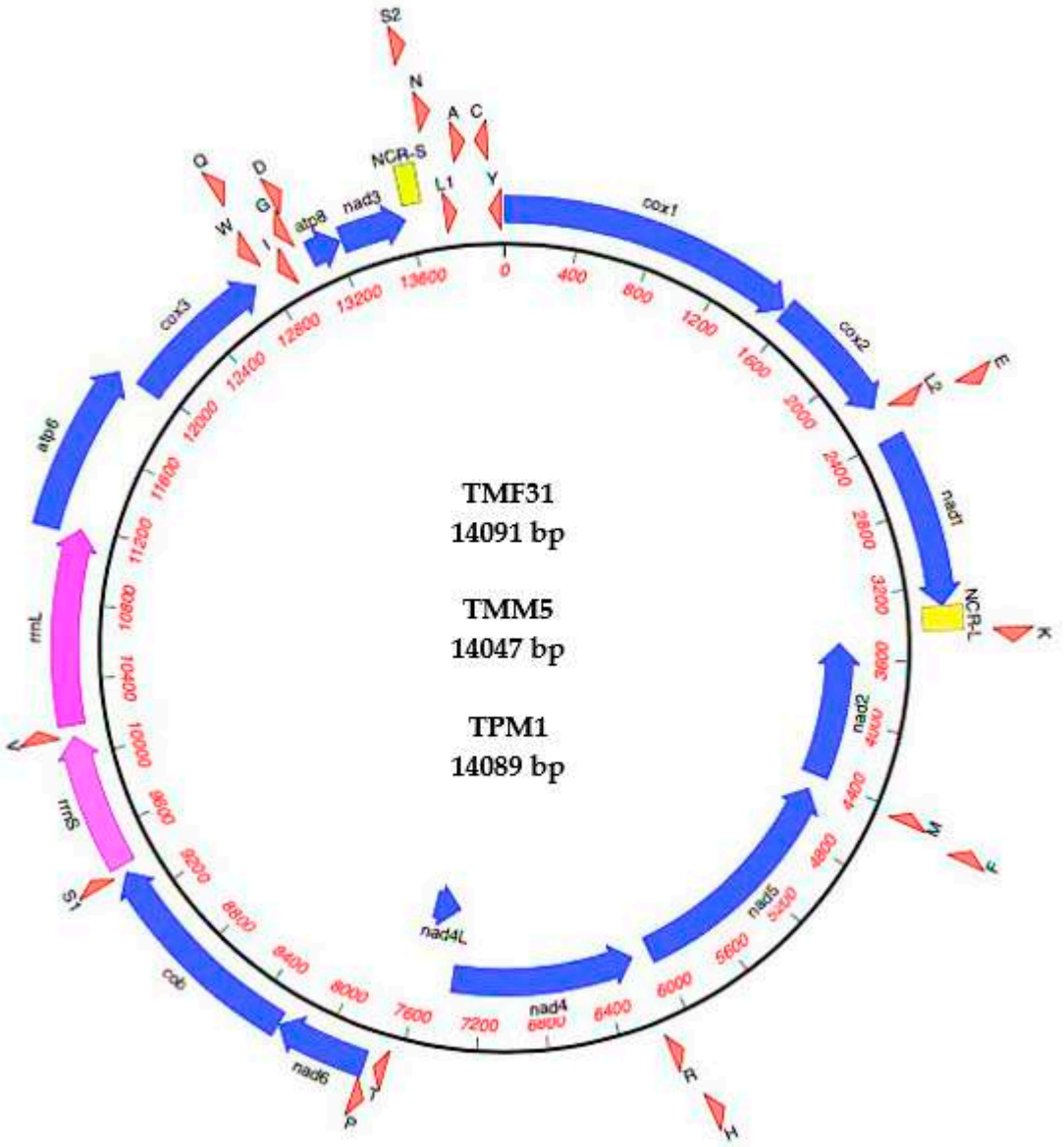

Figure 1. Mitochondrial genome structure of *Trichuris trichiura* from Barbary macaque (TMF31) and from Guinea baboon (TPM1), and *Trichuris* sp. from Barbary macaque (TMM5). Genes were represented to standard nomenclature, but tRNAs were represented using one-letter amino acid codes, with numbers differentiating each of the two leucine- and serine-specifying tRNAs. NCR-L refers to large non-coding region and NCR-S to small non-coding region.

The start/stop codons for some PCGs differed between TMM5 and the genomes previously cited (TMF31 and TPM1), which were similar. Codon usage analyses of mt genomes showed that three start and seven termination codons were different (Table 3). For instance, the starting codon for TMF31 and TPM1 is ATA for the *nad*1 gene, while it reads ATG in the TMM5 genome; for the *nad*4 gene, the starting codon is ATG in TMF31 and TPM1, while being ATA in TMM5, and in the *atp*8 gene, ATT is the start codon in TMF31 and TPM1, while it reads ATA in TMM5. In the *cox*1 gene, TAA is the termination codon in TMF31 and TPM1, whereas it is TAG in TMM5; in *cox*2 gene, TAG is the stop codon in TMF31 and TPM1, while being TAA in TMM5; in *nad*1, TAG is the stop codon in TMF31 and TPM1, but is TAA in TMM5; in *nad*2 gene, TAA is the stop codon in TMF31 and TPM1, and is TAG in TMM5; the stop codon in the *nad*5 gene is TAG for TMF31 and TPM1 and TAA for TMM5; in the *nad*4L and *nad*6 genes, TAA is the stop codon in TMF31 and TPM1 while it reads TAG in TMM5, and in *cox*3 gene TAA is the stop codon for TMF31 and

TPM1 and TAG for TMM5. There are overlaps between *rrn*L and *atp*6 in TMF31, between *rrn*L, *atp*6 and *cox*3 in TMM5, and between *rrn*L and *atp*6 in TPM1.

Table 3. Mitochondrial genomes of *Trichuris* from *Macaca sylvanus* (TMF31, TMM5) and *Papio papio* (TPM1). Protein coding, transfer RNA (tRNA), and ribosomal RNA (rRNA) genes with lengths in nucleotides (nt) are given. The lengths are not identical, and differences are given in parentheses (TMM5/TPM1), likewise for the initiation and termination codons.

Genes	Positions			Lengths	Codons		Strand
	TMF31	TMM5	TPM1	nt	Initiation	Termination	
*cox*1	1–1545	1–1545	1–1545	1545	ATG	TAA (TAG)	+
*cox*2	1558–2232	1556–2230	1558–2232	675	ATG	TAG (TAA)	+
tRNA-leu (L_2)	2255–2317	2252–2311	2255–2317	63 (60)			+
tRNA-glu (E)	2324–2384	2320–2377	2324–2384	61 (58)			+
*nad*1	2406–3305	2401–3300	2406–3305	900	ATA (ATG)	TAG (TAA)	+
Non-coding region (NCR-L)	3306–3435	3303–3442	3306–3430				
tRNA-lys (K)	3436–3501	3441–3502	3431–3496	66 (62)			+
*nad*2	3499–4395	3505–4401	3494–4390	897	ATA	TAA (TAG)	–
tRNA-met (M)	4396–4456	4402–4462	4391–4451	61			–
tRNA-phe (F)	4451–4507	4457–4513	4446–4502	57			–
*nad*5	4499–6055	4520–6067	4494–6050	1557 (1553)	ATA	TAG (TAA)	–
tRNA-his (H)	6049–6106	6065–6118	6044–6101	58 (54)			–
tRNA-arg (R)	6108–6171	6115–6181	6103–6166	64 (67)			–
*nad*4	6176–7396	6183–7394	6171–7391	1221 (1212)	ATG (ATA)	TAA	–
nad4L	7419–7631	7425–7673	7414–7626	213 (249)	ATA	TAA (TAG)	–
tRNA-thr (T)	7672–7729	7679–7736	7667–7724	58			+
tRNA-pro (P)	7729–7787	7736–7795	7724–7782	59 (60)			–
*nad*6	7780–8256	7788–8264	7775–8251	477	ATT	TAA (TAG)	+
cob	8263–9369	8272–9378	8258–9364	1107	ATG	TAG	+
tRNA-ser (S1)	9368–9420	9377–9426	9363–9415	53 (50)			+
*rrn*S	9413–10116	9419–10112	9408–10,111	704 (694)			+
tRNA-val (V)	10,118–10,174	10,114–10,170	10,113–10,169	57			+
*rrn*L	10,176–11,184	10,170–11,180	10,171–11,179	1009 (1011)			+
*atp*6	11,155–11,967	11,151–11,990	11,150–11,962	813 (840)	ATG	TAA	+
*cox*3	11,973–12,746	11,965–12,738	11,968–12,741	774	ATG	TAA (TAG)	+
tRNA-trp (W)	12,759–12,821	12,743–12,805	12,754–12,816	63			–
tRNA-gln (Q)	12,825–12,880	12,807–12,862	12,820–12,875	56			+
tRNA-Ile (I)	12,883–12,943	12,864–12,925	12,878–12,938	61 (62)			–
tRNA-gly (G)	12,957–13,013	12,934–12,989	12,952–13,008	57 (56)			–
tRNA-asp (D)	13,020–13,077	12,996–13,060	13,015–13,072	58 (65)			+
*atp*8	13,066–13,233	13,042–13,209	13,061–13,228	168	ATT (ATA)	TAG	+
*nad*3	13,243–13,584	13,219–13,560	13,238–13,579	342	ATT	TAA	+
Non-coding region NCR-S)	13,585–13,676	13,561–13,659	13,580–13,672				
tRNA-ser (S2)	13,677–13,726	13,660–13,709	13,673–13,723	50 (50/51)			+
tRNA-asn (N)	13,727–13,781	13,710–13,763	13,724–13,778	55 (54)			+
tRNA-leu (L1)	13,789–13,848	13,770–13,835	13,786–13,845	60 (66)			+
tRNA-ala (A)	13,860–13,917	13,842–13,897	13,857–13,914	58 (56)			+
tRNA-cys (C)	13,960–14,013	13,924–13,976	13,957–14,010	54 (53)			–
tRNA-tyr (Y)	14,014–14,074	13,977–14,038	14,011–14,071	61 (62)			–
Total length	14,091	14,047	14,089				

3.2. Comparative Sequence Analyses

Genetic distances between worms for individual PCGs and rRNAs genes are found in Table S2. The genetic distances between the mt genomes of *Trichuris* spp. in primates and humans are given in Table 4. The gene with highest genetic variation was the *atp*8 gene, and the most conserved was the *rrn*S gene, however between all PCGs, *cox*1 was the most conserved gene. Among the mt genomes obtained in this study, nucleotide and amino acid differences between TMF31 and TPM1 were 0.25% and 0.41%, and between TMF31 and TMM5 they were 18.7% and 14.5%, respectively. Within all sequences of *Trichuris* spp. studied, the mt genome of *T. francoisi* was the most variable (with a nucleotide difference of 27.1–28.6% and an amino acid difference of 26.8–28.2%) relative to the other worms from baboons and humans.

Table 4. Pairwise genetic and protein distances between the different complete mt genomes for different *Trichuris trichiura* and *Trichuris* sp. in different primates and human hosts at different countries. The nucleotide distances are given below the diagonal and the amino acid distances above the diagonal.

	TMF31	**TPM1**	**TMM5**	**AP017704 *T. trichiura H. sapiens* (Unknown Geographical Location)**	**NC_017750 *T. trichiura H. sapiens* China**	**GU385218 *T. trichiura H. sapiens* China**	**KT2449826 *T. trichiura H. sapiens* Uganda**	**KT449825 *Trichuris* sp. TTB2 *P. anubis* USA**	**KT449824 *Trichuris* sp. *P. hamadryas* Denmark**	**KC461179 *Trichuris* sp. GHL *T. francoisi* China**
TMF31		0.41	14.5	14.7	14.8	14.8	0.6	14.6	0.49	26.9
TPM1	0.25		14.6	14.8	14.9	14.9	0.7	14.6	0.34	26.8
TMM5	18.7	18.7		10.9	11.1	11.1	14.5	10.3	14.6	28.2
AP017704 *T. trichiura H. sapiens* (unknown geographical location)	18.7	18.7	15.7		4.68	4.68	14.7	3.47	14.9	28.3
NC_017750 *T. trichiura H. sapiens* China	18.6	18.6	15.9	6.51		0	14.8	4.66	14.9	27.8
GU385218 *T. trichiura H. sapiens* China	18.6	18.6	15.9	6.51	0		14.8	4.66	14.9	27.8
KT2449826 *T. trichiura H. sapiens* Uganda	0.4	0.47	18.8	18.7	18.6	18.6		14.5	0.78	26.8
KT449825 *Trichuris* sp. TTB2 *P. anubis* USA	18.8	18.8	15.8	4.7	6.48	6.48	18.8		14.7	28.2
KT449824 *Trichuris* sp. *P. hamadryas* Denmark	0.34	0.28	18.8	18.7	18.6	18.6	0.55	18.8		26.8
KC461179 *Trichuris* sp. GHL *T. francoisi* China	27.1	27.1	28.4	28.3	28	28	27.1	28.6	27.2	

Among the *Trichuris* genome dataset, the nucleotide diversity was analyzed using the sliding window approach for the 13 PCGs and the two rRNA genes. The number of polymorphic sites was 5108 and the nucleotide diversity was 0.166 (Figure 2). The genes with lowest nucleotide diversity were rRNAs (*rrn*L and *rrn*S) and *cox*1.

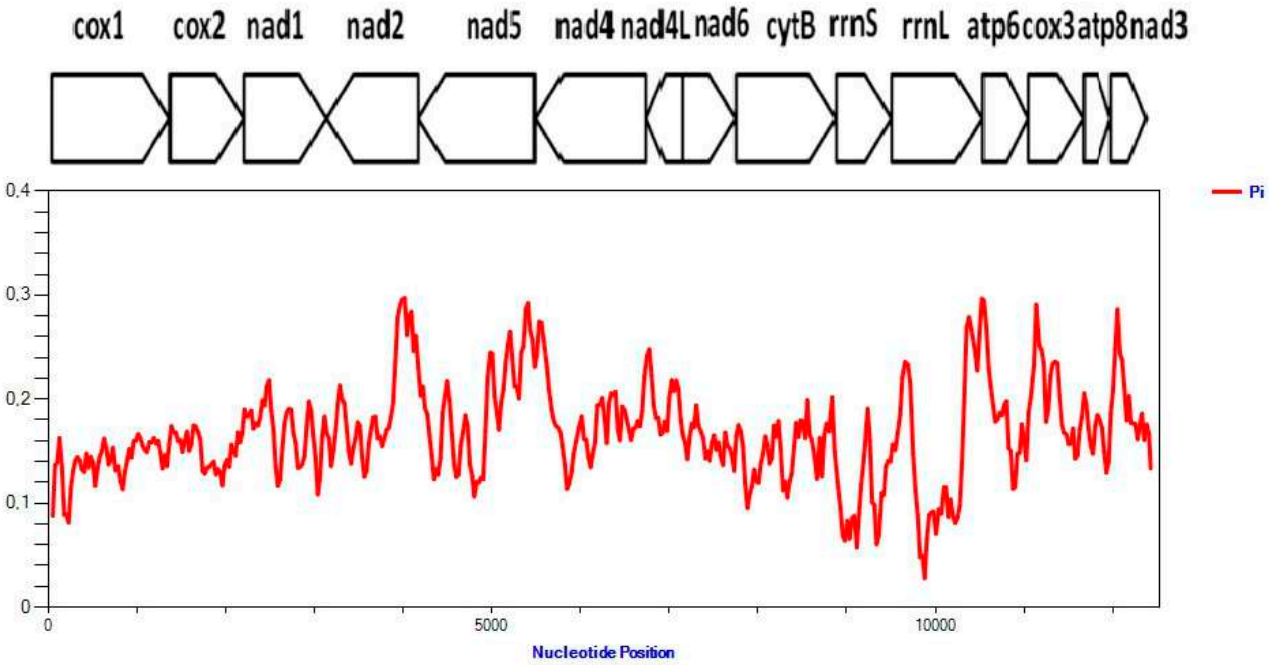

Figure 2. Nucleotide diversity (π) for all protein-coding genes (PCGs) and ribosomal rRNA (*rrn*S and *rrn*L) measured using a sliding window of 100 bp with 25 bp steps. The aligned dataset for *Trichuris* in primates (baboons, humans and Francois' leaf monkey).

3.3. Phylogenetic Analyses

The phylogenetics analyses of nucleotide sequence datasets with partial genome (the 13 PCGs (*cox*1–3, *nad*1–6, *nad*4L, *atp*6, *atp*8 and *cyt*b) and the two rRNAs (*rrn*L and *rrn*S)) and with mt genome complete datasets reflecting similar tree topologies by the three methods studied (ML, MP and BI) (Figure 3). Within *Trichuris* populations from humans and NHPs, *Trichuris* sp. from *T. francoisi* appeared separated of the other sequences. Within this last group, there are three main clades. The first clade (clade 1) is composed of sequences of *Trichuris* sp. from *H. sapiens* and *P. anubis* from Asia, Japan and USA, the clade 2 is composed of a sequence of *Trichuris* sp. from *M. sylvanus*, and the clade 3 corresponded with *T. trichiura* from humans and NHPs (*M. sylvanus*, *P. papio* and *P. hamadryas)* from Europe and Africa.

Phylogenetic trees inferred from a concatenated amino acid sequence dataset of 12 PCGs among selected enoplid nematodes, using the chromadorean nematode, *B. malayi* (NC_004298) and *A. suum* (HQ704901) as outgroups, revealed congruence with phylogenetics analyses of nucleotide sequence datasets with the 13 PCGs and the two rRNAs (*rrn*L and *rrn*S), and complete mt genomes. Thus, phylogenetics analyses of all 12 genes showed a general pattern with very strong support for clades near the terminals of the tree (Figure 4). Thus, clade 1, clade 2 and clade 3 for *Trichuris* spp. from humans and NHPs, and the monophyly of the genus *Trichuris,* are very strongly supported (100% ML BV, 100% MP BV and 100% BPP). There are no marked differences in support values between the 12 gene datasets analyzed using nucleotide versus amino acid sequences.

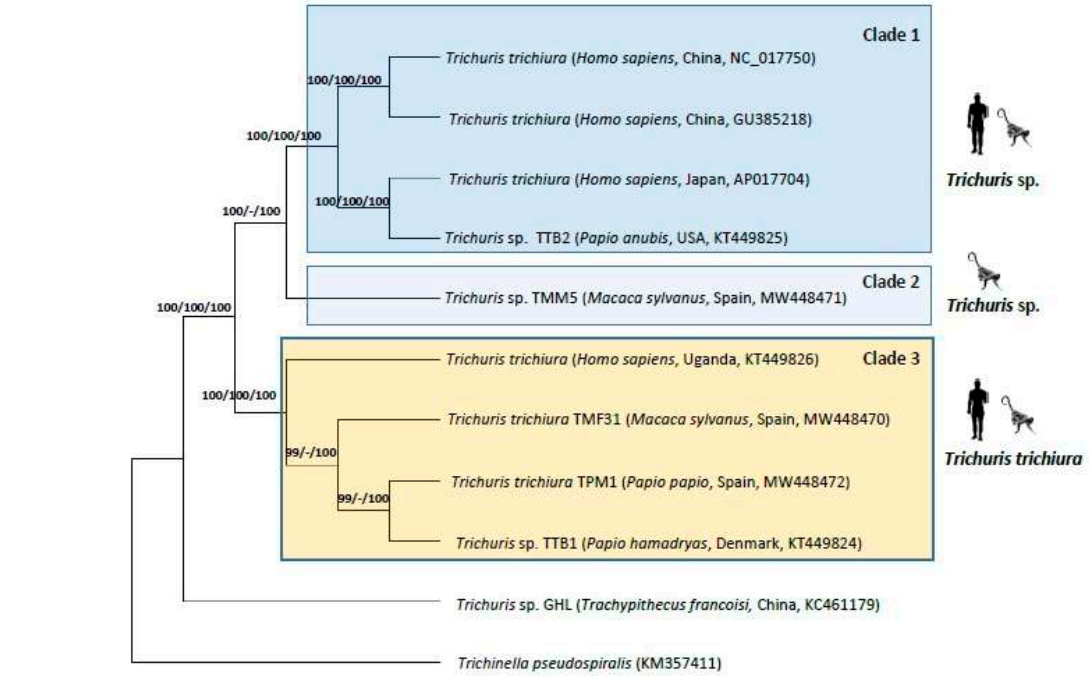

Figure 3. Phylogenetic tree based on concatenated nucleotide sequences of 13 PCGs and two rRNA of *Trichuris* from baboons, humans and françois' leaf monkey using *Trichinella pseudospiralis* as an outgroup, inferred using Bayesian Inference (BI). Maximum Likelihood (ML) bootstrap values of clades are listed first, followed by Maximum Parsimony (MP) and by Bayesian Posterior Probabilities (BPP), for clade frequencies exceeding 60%.

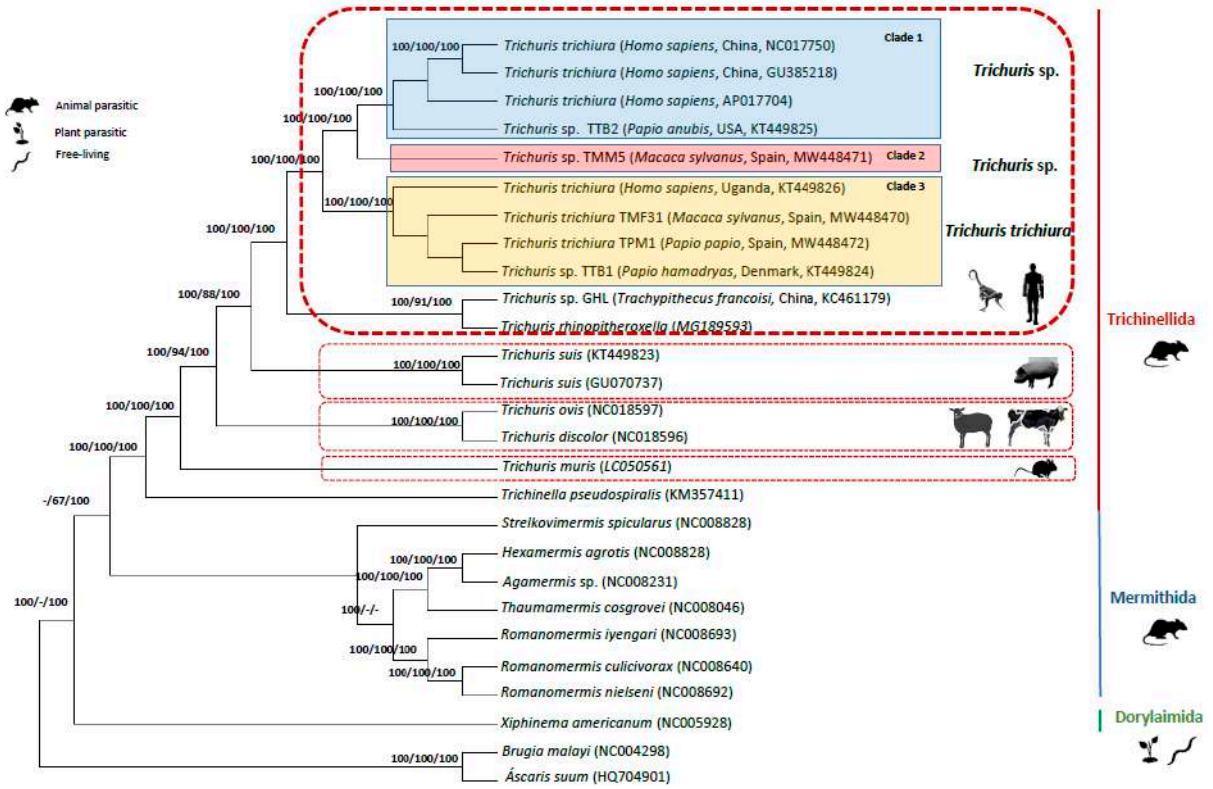

Figure 4. Phylogenetic tree among enoplid nematodes based on concatenated amino acid sequences of 12 PCGs (except for *atp*8 gene) by Bayesian Inference (BI) using *Brugia malayi* and *Ascaris suum* as the outgroups. Maximum Likelihood (ML) bootstrap values of clades are listed first, followed by Maximum Parsimony (MP) and Bayesian Posterior Probabilities, for clade frequencies exceeding 60%.

The phylogenetic analyses reflected the clear distinctiveness between *T. trichiura* and *Trichuris* sp. from humans and NHPs with respect to *T. suis* from suids, *T. ovis* and *T. discolor* from herbivorous and *T. muris* from rodents and grouping these members of *Trichuris* with *T. pseudospiralis* (order Trichinellida) with absolute support (100% ML BV, MP BV 100% and 100% BPP), but excluding the members of Dorylaimida and Mermithida (Figure 4).

4. Discussion

Previous studies have reported the hypothesis that a complex whipworm species exists in primates, suggesting that different *Trichuris* species infect primates and humans [3,19,23,38–41].

In the present study, we resolved the complete mt genome sequences of two haplotypes of *Trichuris* isolated from *M. sylvanus* and one complete mt genome of *Trichuris* from *P. papio*. The size of the complete mt genomes were within the range reported for the Trichuridae family (ranging from 13,904 bp (*T. discolor*) to 14,521 bp (*T. suis*)) [3,16,17,19,22]. The complete mtDNA is a circular molecule and encodes 37 genes—13 PCGs (*atp*6, *atp*8, *cox*1–3, *co*b, *nad*1–6 and *nad*4L), two rRNAs (*rrn*S and *rrn*L), and 22 for tRNAs.

The initiation codons (ATG, ATA and ATT) agreed with those reported in the pig-derived and human-derived *Trichuris*. [16,18,20], while *T. ovis* and *T. discolor* also employed TTG [3,16,19], and *T. skrjabini* sequence used only two start codons (ATG and ATA) [22]. Two termination codons (TAA and TAG) were used as stop codons in all the four species of Trichuridae family (*T. suis*, *T. trichiura*, *T. ovis* and *T. discolor*) except *T. skrjabini*, which possesses another stop codon, TGA [3,16,17,19,22]. The sequences of this study were in accordance with the most related species (primates derived *Trichuris*) [3,19].

The complete mt genomes of one of the haplotypes of *Trichuris* from macaques (TMF31) and that from baboon (TPM1) were genetically similar, with a difference in nucleotide and amino acid sequence of 0.25% and 0.41%, and having 14,091 and 14,098 bp, respectively, and both genomes being similar with that of *T. trichiura* from humans from Uganda, which was 14,079 bp in length, and with TTB1 (13,984 bp in length) from *Trichuris* sp. from *P. hamadryas* [19], with a sequence variation of 0.28–0.47%. In contrast, the complete mt genome of the other haplotype from macaques (TMM5) was 14,047 bp, like human *T. trichiura* from China that was 14,046 bp in length [3], showing a nucleotide difference of 15.9%. Moreover, the mt genome of *Trichuris* sp. from *P. anubis* (USA) was 14,009 bp [19], with a nucleotide difference to TMM5 of 15.8%. A substantial level of nucleotide difference was detected between TMF31 (Clade 3) with respect to *Trichuris* sp. included in clades 1 and 2 (15.8–18.8%). Comparison of the nucleotide and amino acid sequences between TMF31 and TMM5 was 18.7% and 14.5%, respectively. The sequence variation detected in the 13 PCGs between TMF31 and TMM5 was 20.3% (nucleotide sequence) and 34.4% (amino acid sequence). The percentage of dissimilarity observed between the different sequences corresponded to previously cited ranges within different nematode species. Thus, Hawash et al. [19] suggested the presence of two *Trichuris* species with a difference in nucleotide and amino acid sequences of around 18.8% and 14.6%, respectively, of *Trichuris* from Ugandan humans and Chinese humans. Furthermore, *T. suis* and *T. trichiura* presented an amino acid difference of 33.3–39.2% [19], *T. trichiura* from human and *Trichuris* sp. from leaf monkey was 29.4% [17], *T. ovis* and *T. discolor* ranged from 11.0–33.9% [16], 11.7% between *Wucheria bancrofti* and *B. malayi* [42], 10.3% between *Chabertia ovina* and *C. erschowi* [43], 4–18% between different *Trichinella* spp. [18] and 4.12% between *Ancylostoma duodenale* and *Ancylostoma caninum* [44]. Blouin [45] indicated that the difference in mt genome sequences between closely related species was normally 10–20%. Thus, the molecular evidence presented in the present manuscript supports the hypothesis that the haplotypes TMF31 and TMM5 of *Trichuris* from *M. sylvanus* display differences in amino acid and nucleotide sequences that are within the range of those previously reported by different authors to consider them as different species.

On the other hand, the complete mt genomes of *Trichuris* spp. from different hosts were evaluated to clarify the evolutionary relationships. Considering that complete mt

genome sequences are maternally inherited and mutate at a rapid rate related to nuclear genes, theses markers could prove useful for evolutionary and phylogenetic analyses [46].

Phylogenetics trees inferred from both datasets (sequences dataset of 12 PCGs among enoplid nematodes and nucleotide sequence datasets with the 13 PCGs and the two rRNAs (*rrn*L and *rrn*S)) showed congruence with a high support for the differentiation between *Trichuris* spp. and the different clades within *Trichuris* populations parasitizing humans and NHPs (clade 1, clade 2 and clade 3). Furthermore, the monophyly of the genus *Trichuris* was strongly supported and grouping these species of *Trichuris* (traditionally named as Trichocephalida) with *T. pseuospirallis* (Trichinellida,) with the exclusion of the members of the Dorylaimida and Mermithida (Figure 4). Similar results were reported by Liu et al. [3,16] who characterized the complete mitochondrial genomes of several whipworms and compared them with other enoplid nematodes.

The *atp*8 gene is present in mitogenomes of the Order Trichinellida (*Trichuris* and *Trichinella* species), but it is usually absent in mitogenomes of members of the phylum Nematoda [3,13,15–19]. These authors reported the lack of *atp*8 in the mtDNA of Chromadorean nematodes (traditionally named as Class Secernentea) and it could be derived, within that lineage, after the divergence of Chromadorean nematodes from other groups. Furthermore, phylogenetic inferences confirm the clear relationships between Trichinellida and Mermithida correlating both groups with animal parasites of nematodes separated of Dorylaimida, corresponding to free-living nematodes and plant parasitic nematodes [47].

The phylogenetic study also supported the evolution of the different *Trichuris* species showing *T. trichiura* (clade 3), to be closer to *Trichuris* spp. (clade 1 and clade 2) parasitizing humans and NHPs. As reported by previous authors [19,23,38,39,41,48], *Trichuris* populations parasitizing humans and NHPs showed a species complex in primates. In addition, the high difference in nucleotide and amino acid sequences between *T. trichiura* (clade 3) and the minority population of *Trichuris* sp. from *M. sylvanus* (clade 2) and *Trichuris* sp. from humans and NHPs from China, Japan, and USA (clade 1) was confirmed in the phylogenetic trees with strong support for the different clades. Thus, based on our results, we suggest the existence of two cryptic species parasitizing *M. sylvanus*. *Trichuris* genus is a likely candidate to containing cryptic species as it has a wide geographical distribution and infects several host species [49]. As revealed by recent studies, there is more than one taxon capable of infecting humans and other primates, including individuals in captivity, suggesting that *T. trichiura* should be considered a complex species that includes different cryptic units [19]. In addition, and based on morphobiometric and molecular parameters, new species of *Trichuris* have been described in primates, such as *T. rhinopiptheroxella* [20], that was found in the golden snub-nosed monkey (*Rhinopithecus roxellana*), *Trichuris colobae* from *Colobus guereza kikuyensis* [50], and *Trichuris ursinus* from *Papio ursinus* [51]. As a result, it has been confirmed that *T. trichiura* and other *Trichuris* species are present in humans and NHPs.

This fact has two main epidemiological repercussions—(i) the zoonotic potential of *Trichuris* spp. of NHPs for humans. This is particularly important when humans and NHPs are living in proximity, as is becoming increasingly common with human encroachment into habitats where NHPs accessing gardens and farms in search of food and has significant implications for both human health and wildlife conservation [52]. This implies that in communities where access to NHPs is common, simple public health measures should be encouraged, including thorough handwashing with soap (particularly for children) and rising and cooking of vegetables, and (ii) the presence of different cryptic species might also be very important for implementation of appropriate control strategies. Different control strategies for *Opistorchirs viverrini* have been identified due to the existence of different cryptic species based on the different fecundity as measured by eggs/g/worm [53].

5. Conclusions

In the present study, based on complete mt genome analyses, the molecular data suggested two distinct species in whipworms isolated from *M. sylvanus*. The whipworms

from *P. papio*, TPM1, and TMF31 from *M. sylvanus* are *T. trichiura* since the sequences were within the majority clade, with sequences from Ugandan humans and *P. hamadryas* (Europe)-derived *Trichuris*. Further, we suggested the existence of two cryptic species parasitizing *M. sylvanus*. Moreover, a major source of mitochondrial markers is given and may be used as the basis of subsequent epidemiological research, to clarify appropriate control measures and transmission routes.

Supplementary Materials: The following are available online at https://www.mdpi.com/2075-1729/11/2/126/s1, Table S1: PCR mix, primers and conditions used for each molecular marker sequenced in the present study, Table S2: Pairwise nucleotide and amino acid distances for the different 13 PCGs and RNAs (*rrn*S and *rrn*L) genes for *Trichuris* species from different primates and human hosts. Nucleotide genetics distances are given below the diagonal and amino acid genetic distances above the diagonal, Table S3: Nucleotide composition (%) of the mt genomes studied.

Author Contributions: Conceptualization, C.C. and R.C.; methodology, J.R. and R.C.; software, J.R.; validation, C.C., J.R. and R.C.; formal analyses, J.R. and R.C.; investigation, C.C., J.R. and C.R.; writing—original draft preparation, C.C., J.R. and R.C.; writing—review and editing, C.C., J.R. and R.C.; supervision, C.C.; project administration, C.C.; funding acquisition, C.C. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are openly available in GenBank database (GenBank accession nos. MW448470-2).

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Conflicts of Interest: The authors declare no conflict of interest.

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Trichuris trichiura (Linnaeus, 1771) From Human and Non-human Primates: Morphology, Biometry, Host Specificity, Molecular Characterization, and Phylogeny

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Human trichuriasis is a Neglected Tropical Disease, which affects hundreds of millions of persons worldwide. Several studies have reported that non-human primates (NHP) represent important reservoirs for several known zoonotic infectious diseases. In this context, *Trichuris* infections have been found in a range of NHP species living in natural habitats, including colobus monkeys, macaques, baboons, and chimpanzees. To date, the systematics of the genus *Trichuris* parasitizing humans and NHP is unclear. During many years, *Trichuris trichiura* was considered as the whipworm present in humans and primates. Subsequently, molecular studies suggested that *Trichuris* spp. in humans and NHP represent several species that differ in host specificity. This work examines the current knowledge of *T. trichiura* and its relationship to whipworm parasites in other primate host species. A phylogenetic hypothesis, based on three mitochondrial genes (*cytochrome c oxidase* subunit 1, *cytochrome* b, and large subunit rRNA-encoding gene) and two fragments of ribosomal DNA (Internal Transcribed Spacer 1 and 2), allowed us to define a complex of populations of *T. trichiura* hosting in a large variety of NHP species, in addition to humans. These populations were divided into four phylogenetic groups with a different degree of host specificity. From these data, we carry out a new morphological and biometrical description of the populations of *Trichuris* based on data cited by other authors as well as those provided in this study. The presence of *T. trichiura* is analyzed in several NHP species in captivity from different garden zoos as possible reservoir of trichuriasis for humans. This study contributes to clarify questions that lead to identification of new taxa and will determine parasite transmission routes between these primates, allowing the implementation of appropriate control and prevention measures.

Keywords: *Trichuris trichiura*, non-human primates, ribosomal DNA, mitochondrial DNA, zoonoses

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INTRODUCTION

Worldwide, ∼1.5 billion people, nearly 24% of the world's population, are infected with soil-borne helminths. Soil-borne helminthiasis is widely distributed in tropical and subtropical areas, especially in sub-Saharan Africa, The Americas, China, and East Asia. More than 267 million preschoolers and more than 568 million school-age children live in areas with

severe transmission of these parasites and need treatment and preventive interventions. The main species of soil-borne helminths that infect humans are *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), and *Necator americanus* and *Ancylostoma duodenale* (hookworms) (1).

T. trichiura, is the etiological agent of the parasitic disease known as "trichuriasis," which is considered as a Neglected Tropical Disease. *T. trichiura* is the second most common helminth in humans, and Trichuriasis has a worldwide geographical distribution. The prevalence is higher in places with warm and humid weather, where there is a lack of basic sanitation services. Between 30 and 80% of cases are recorded in children, who suffer the greatest parasitic burden and those with the most significant symptoms (2). Transmission of this parasite occurs after ingestion of embryonated eggs. These eggs can enter new hosts through contaminated hands, food, soil, and water. Then these hatch in the intestine, where L1 larvae are released. Larvae penetrate the epithelial layer of the large intestine and grow to adult stage. After mating, the non-embryonated eggs are released from the females and again reach the environment through the host's feces.

Up to date, whipworms isolated from humans and other primates have traditionally been regarded as *T. trichiura* (3–5), while those recovered from pigs and wild boars are known as *Trichuris suis* (6, 7). It is well-known that differentiation between closely related species of *Trichuris* is very difficult due to the phenotypic plasticity of the organisms themselves; host-induced variation, paucity of morphological features, and overlapping morphological characteristics that occur among species (8–11). Thus, many studies on *Trichuris* have focused on the morphological and molecular differentiation of *T. trichiura* and *T. suis*, which are molecularly different but morphologically similar (7, 12–14).

On the other hand, the relationship between *Trichuris* from humans and non-human primates (NHP) in terms of genetic and evolutionary aspects is poorly understood. In recent years, some publications addressed the question of whether *Trichuris* species are shared between humans and NHP or whether there are different species. The genus *Trichuris* is likely a candidate to harbor cryptic species as it has a wide geographical distribution and infects several host species (15). As revealed by recent studies, there is more than one taxon capable of infecting humans and other primates, including individuals in captivity, suggesting that *T. trichiura* should be considered a complex species that includes different cryptic units (16, 17). In addition, based on morpho-biometric and molecular parameters, new species of *Trichuris* have been described in primates, such as *Trichuris rhinopiptheroxella* (18) that was found in the golden snub-nosed monkey (*Rhinopithecus roxellana*), *Trichuris colobae* from *Colobus guereza kikuyuensis* (19), and *Trichuris ursinus* from *Papio ursinus* (20). Therefore, these studies confirmed that *T. trichiura* is not the only whipworm found in primates.

Currently, the systematics of the genus *Trichuris* shows significant gaps. This is due to two main reasons: (i) the lack of comparative morpho-biometric data through the use of multiple parameters and statistical tests applied to the taxonomic study of these species and (ii) the paucity of published research on the genetics of the different *Trichuris* species in humans, NHP, and pigs. Nowadays, researchers have not yet managed to finally establish the degree of divergence between the different genetic lineages that appear to exist in *Trichuris* species parasitizing these hosts.

In this paper, we carried out an update of the morphological and biometric characteristics of *T. trichiura* isolated from human and NHP. Besides, the molecular phylogenetic relationships between these populations are analyzed based on molecular data (mitochondrial and nuclear markers). Furthermore, some phylogenetic hypotheses were inferred for *Trichuris* spp. to shed light on the degree of divergence between different genetic lineages. In addition, the presence of *T. trichiura* was analyzed in several NHP species in captivity from different garden zoos as possible reservoir of trichuriasis for humans.

MATERIALS AND METHODS

Ethics Statement

This study does not require approval by an ethics committee. *Macaca sylvanus* and *C. g. kikuyensis*, from which *Trichuris* specimens were collected from their caeca *postmortem*, died of natural death. The specimens were handled and housed in a zoo in strict accordance with good animal practices. The other specimens and eggs of *Trichuris* sp. were recovered from the feces after routine anthelmintic treatment.

Isolation of Material

In this study, we sampled *Trichuris*'s adults and eggs of a total of five NHP host species, including the Barbary macaque (*M. sylvanus*), vervet monkey (*Chlorocebus aethiops*), patas monkey (*Erythrocebus patas*), Guinea baboon (*Papio papio*), and black and white colobus (*C. g. kikuyuensis*) from the Zoo Castellar (Cádiz, Spain), Selwo Aventura (Málaga, Spain), Zoo Barcelona (Barcelona, Spain), Parque de la Naturaleza de Cabárceno (Cantabria, Spain), and Bioparc Fuengirola (Málaga, Spain), respectively (**Table 1**).

Only three adult whipworm specimens (two females and one male) were collected from Guinea baboon, sixty-five adults (32 females and 33 males) from a male Barbary macaque (17, 21) and five adults from a *C. g. kikuyuensis*. Adult's worms were washed separately in saline solution (0.9% w/v), then frozen at −20°C until posterior studies. Whipworm's eggs were isolated from feces of all NHP species. The sequences successfully obtained of different molecular markers are summarized in the **Table 1**.

The fecal eggs were concentrated using a Sheather's sugar solution (22) and then embryonated at 32°C for 3–4 weeks added with potassium dichromate 0.2% w/v solution to give humidity to the medium and prevent fungal and bacterial growth (23). Subsequently, the worms were measured and genomic DNA, from both worms and eggs, was extracted.

Morphological Study

Three adult whipworms from Guinea baboon were identified according to previous studies (7, 19, 20). We carried out morphological studies as described Oliveros et al. (24) and Skrjabin et al. (25). *Trichuris* specimens were measured according

TABLE 1 | DNA obtained from the samples.

Zoo gardens	Hosts species		N° adults/eggs analyzed	*Trichuris* species	Haplotypes obtained					Number of base pairs/G+C				
	Scientific name	Common name			*cox*1	*cob*	*rrn*L	ITS1	ITS2	*cox*1	*cob*	*rrn*L	ITS1	ITS2
Zoo Castellar	*Macaca sylvanus*	Barbary macaque	43 adults	*T. trichiura*	4 H (*)	5 H (*)	5 H	6 H	8 H (*)	370/38–39.2	520/30.2–31	386–387/29.7–31.3	586–597/63.1–64.3	514–587/63.7–65.2
Selwo Aventura	*Chlorocebus aethiops*	Vervet monkey	5 sample batches of eggs (40–65 eggs/batch)		1 H	1 H	-	2 H	5 H	370/38.6	522/30.1	–	594–601/61.4–62.3	556–580/62.1–64.1
Zoo Barcelona	*Erythrocebus patas*	Patas monkey	1 sample batch of eggs		1 H	1 H	-	1 H	1 H	332/38	520/29.6	–	593/64.1	436/65.6
Parque de la Naturaleza de Cabarceno	*Papio papio*	Guinea baboon	3 adults and 9 sample batches of eggs (40–200 eggs/batch)		1 H	4 H	1 H	2 H	5 H	370/38.6	520/29.6–30.8	387/30	588–607/61.4–64.3	602–611/62.8–63.2
Bioparc Fuengirola	*Colobus guereza kikuyuensis*	Black and white colobus	5 adults	*T. colobae*	-	-	5 H	-	-	-	-	395/30.1–30.9	-	-

H, haplotype; (*) analyzed by Rivero et al. (17).

to parameters reported by Spakulová and Lýsek (26), Suriano and Navone (27), and Robles et al. (28), and a comparative study was carried out with *T. trichiura*'s specimens previously analyzed biometrically and molecularly (7, 14, 17, 21).

Molecular Study

PCR and Sequencing of Specimens

Genomic DNA from adult worms and a pack of 45–200 isolated eggs were extracted using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Quality of extractions was assessed using 0.8% agarose gel electrophoresis infused with SYBR® Safe DNA gel stain.

All molecular markers sequenced in the present study [*cox*1, *co*b and *rrn*L mitochondrial DNA (mtDNA) and ITS1 and ITS2 ribosomal DNA (rDNA)] were amplified using the polymerase chain reaction (PCR) by a thermal cycler (Eppendorf AG; Hamburg, Germany). PCR mix, PCR conditions, and PCR primers are summarized in the **Supplementary Table 1**.

The PCR products were checked on SYBR® Safe stained with 2% w/v Tris–borate–EDTA (TBE) agarose gels. Bands were eluted and purified from the agarose gel using the QWizard SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, U.S.A.). Once purified and concentrated, the products were sequenced by Stab Vida (Lisbon, Portugal).

Phylogenetic Analysis

To assess the similarity among all marker sequences of *Trichuris* sp. obtained in the present study and other *Trichuris* species, the number of nucleotide differences per sequence was analyzed using Compute Pairwise Distances based on the number of differences method of MEGA v7.0 (29).

To obtain a nucleotide sequence alignment file, the MUSCLE alignment method was used in MEGA v7.0 (29). Additional sequences from the National Center for Biotechnology Information (NCBI) GenBank® database were incorporated into the alignments (**Supplementary Table 2**).

Assessment of nucleotide substitution saturation, an indicator of whether the genetic marker is useful, was performed using DAMBE v5.3.32 (30, 31). Saturation was based on the values of Iss (index of substitution saturation) and Iss.c (critical Iss value), where Iss < Iss.c indicated that the genetic marker was not saturated and vice versa. Haplotype diversity (h) and nucleotide diversity (π) were calculated using DnaSP v6.12.03 (32).

All phylogenetic trees were inferred based on nucleotide data and obtained by two methods: Maximum Likelihood (ML) and Bayesian Inference (BI). PHYML package was used to generate ML trees (33), and MrBayes v3.2.6 to generate BI (34). jModelTest was employed to resolve the best-fit substitution model for the parasite data (35). Models of evolution were selected for subsequent analysis according to the Akaike Information Criterion (36). To examine the dataset containing the concatenation of four markers used (ITS1, ITS2, *cox*1, and *co*b), analyses based on BI were partitioned by gene and models for individual genes within partitions were those selected by jModelTest. For ML inference, best-fit nucleotide substitution models included the general time-reversible (GTR) model with gamma-distributed rate variation (G) and a proportion of

invariable sites (I), GTR + G (ITS1 and ITS2), GTR + I + G (*cox1*), GTR + I + G (*cob*), and GTR + G (*rrn*L). Support for the topology was examined using bootstrapping (heuristic option) (37) over 1,000 replications to assess the relative reliability of clades. The commands used in MrBayes for BI were *nst* = 6 with gamma rates (ITS1, ITS2, and *rrn*L), *nst* = 6 with invgamma rates (*cox*1 and *cob*), and *nst* = mixed (concatenated phylogenetic trees). The standard deviation of split frequencies was used to determine whether the number of generations completed was sufficient; the chain was sampled every 500 generations, and each dataset was run for 10 million generations. Trees from the first million generations were discarded based on an assessment of convergence. Burn-in was determined empirically by examination of the log likelihood values of the chains. The Bayesian Posterior Probabilities (BPP) comprise the percentage converted.

RESULTS

Molecular Analysis

Annotation and Features of Ribosomal and Mitochondrial Genomes

The successfully sequenced specimens, the length of the different sequences, the content of G + C%, and haplotypes of ribosomal and mitochondrial markers analyzed at the present study are shown in **Table 1**. Different repetitive nucleotide sequences, termed ≪ microsatellites ≫, were found in the ITS2 sequences of *Trichuris* sp. from human and different NHP. Thus, Poly (GCA), Poly (CGA), and Poly (GCG) were observed in positions 280, 302, and 344, respectively. Furthermore, Poly (GCA) and Poly (GGC) were found in the ITS1 sequences in positions 77 and 203, respectively. Also, a common area to all species of *Trichuris* was observed in positions 280 (GATCTGGGTGT) and 286 (GCCGCCGGTT) in this ITS1 sequence.

Nucleotide sequence data reported in this study were deposited at the GenBank™, EMBL, and DDBJ databases, and the accession numbers are available in **Supplementary Table 2**.

Phylogenetic Analysis

All phylogenetic trees based on ribosomal and mitochondrial markers (partitioned and concatenated) confirmed the existence of two main clades: clade 1: "*Trichuris suis*" and clade 2: "*Trichuris trichiura*" (**Figures 1–3** and **Supplementary Figures 1–3**).

The alignment of 46 ITS1 and 79 ITS2 rDNA sequences of *Trichuris* species yielded a dataset of 876 and 863 characters, respectively. Based on ITS1 and ITS2 sequences, the concatenated phylogenetic tree revealed the existence of two highly supported phylogenetic groups within clade 2 "*T. trichiura*": One group corresponded to *T. trichiura* lineage (100% ML and 100% BPP) (**Supplementary Table 3**) clustering *Trichuris* sp. from different hosts and geographical regions in six subclades (100% ML and 100% BPP) that included *Trichuris* sp. from *Trachypithecus francoisi* (**Figure 1**). The other 5 subclades (except *Trichuris* sp. from *T. francoisi*) showed a high homology each other, ranging 94.65–99.65% (**Supplementary Table 4**).

The phylogeny inferred on mitochondrial datasets (partitioned and concatenated) revealed similar topologies (**Figure 2** and **Supplementary Figures 1–3**). Thus, four main clades were observed in "*T. trichiura* lineage" where *Trichuris* sp. from *P. papio*, *C. aethiops*, and *E. patas* clustered together in the subclade named 2c with *T. trichiura* from *Homo sapiens* from Uganda and *Trichuris* sp. from other hosts from Africa and Europe (**Figure 2** and **Supplementary Figures 1, 2**).

The multiple alignments of 48 *cox*1 nucleotide sequences (including outgroups) yielded a dataset of 296 characters. The phylogenetic tree based on *cox*1 showed *Trichuris* from *E. patas*, *C. aethiops*, and *P. papio* clustering in subclade 2c (European and African origin) and related with *Macaca fuscata* (subclade 2d). This marker did not resolve subclade 2a (Asian and USA origin) appearing in polytomy (**Supplementary Figure 1** and **Supplementary Table 3**).

The multiple alignments of 47 *cob* nucleotide sequences (including outgroups) yielded a dataset of 444 characters. The phylogenetic tree based on *cob* was in congruence with *rrn*L phylogenetic inferences of *T. trichiura* lineage. Nevertheless, the sister relationship between subclades 2a, 2b, and 2d was not supported (**Supplementary Figure 2** and **Supplementary Table 3**).

The *rrn*L dataset included 358 aligned positions and 76 taxa, including outgroups. ML and BI methods showed congruence between each other revealing two main clades ("*T. suis* lineage" and "*T. trichiura* lineage") and respect to the sister-group relationships between *Trichuris* spp. from NHP, humans and pigs (**Supplementary Figure 3** and **Supplementary Table 3**). Within clade 2 "*T. trichiura* lineage," phylogenetic trees confirmed the existence of four different subclades highly supported clustering subclade 2b: *T. trichiura* from *H. sapiens* from China and *Papio anubis* from the USA and subclade 2a including the minority haplotype of *T. trichiura* from *M. sylvanus* from Spain; subclade 2c: *T. trichiura* from *P. papio*, *Chlorocebus sabaeus* and *M. sylvanus* from Spain, *H. sapiens* from Uganda, *Papio hamadryas* from Europe, and two haplotypes of *Trichuris* sp. from *M. fuscata* from Europe; and subclade 2d: *T. trichiura* from *M. fuscata* from Europe (**Supplementary Figure 3**). Curiously, the minority haplotype of *M. sylvanus* (subclade 2a) and *M. fuscata* (subclade 2d) (European origin) appeared related with those from Asia and USA (subclade 2b) (**Supplementary Figure 3** and **Supplementary Table 3**). In addition, a sister relationship between *T. trichiura* and *Trichuris* sp. from *T. francoisi* was observed, both species within "*T. trichiura* lineage" (clade 2).

The concatenated dataset of mitochondrial gene (*cox*1 and *cob*) sequences (**Figure 2**) revealed the subclade 2c as the main one clustering the majority of *T. trichiura* parasitizing African humans and different NHP from Africa and Europe, showing a sister relationship between 2c and 2d and besides between 2a and 2b (Asian and USA origins) (**Figure 2**).

The concatenated dataset of ribosomal (ITS1 and ITS2) and mitochondrial (*cox*1 and *cob*) gene sequences included 2,479 aligned sites and only 13 taxa, since only we could concatenate sequences of the same individual. Phylogenetic analyses of this dataset yielded a tree with branches strongly supported. Thus, the *T. trichiura* population was separated in only three different

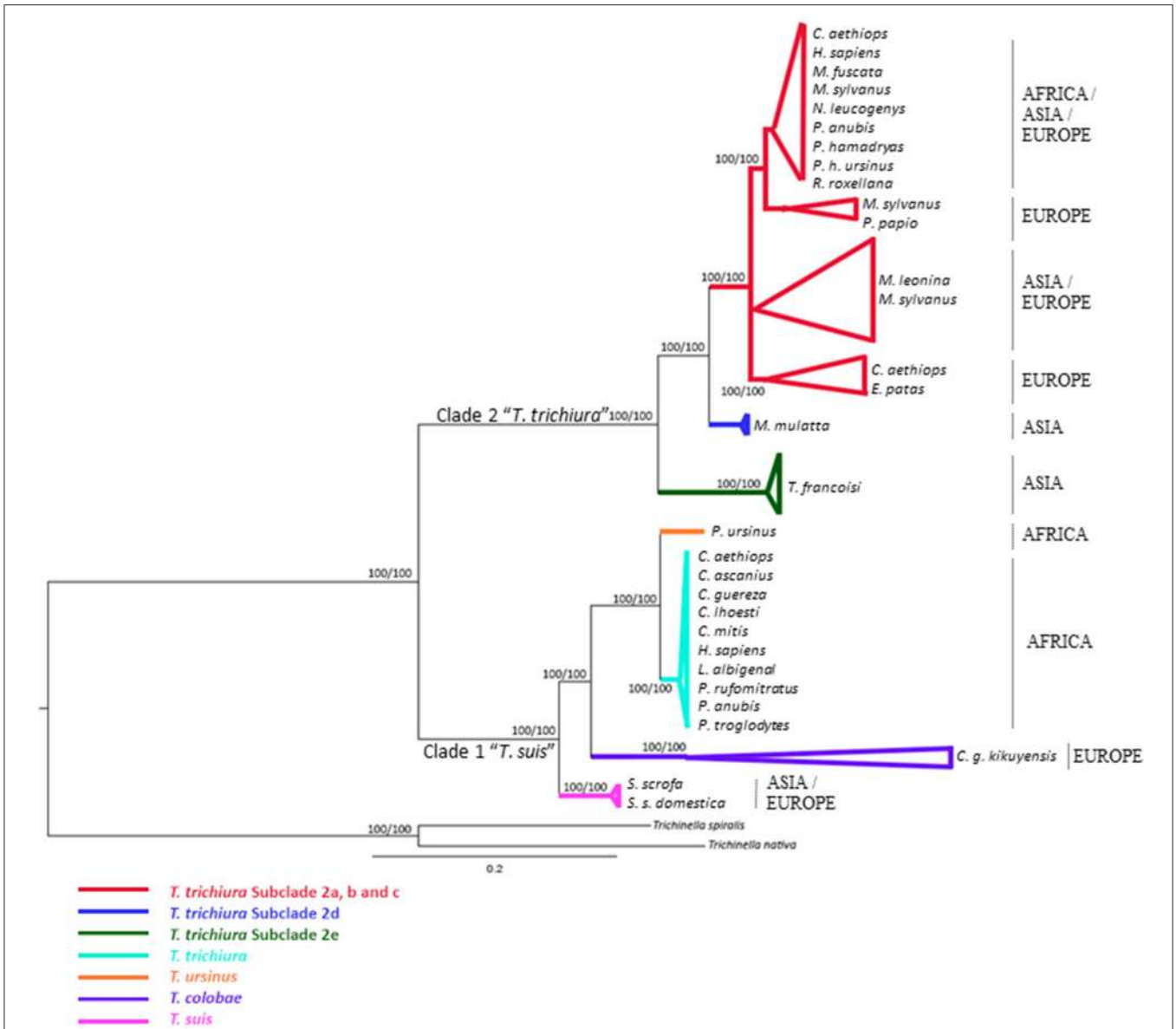

FIGURE 1 | Phylogenetic tree of *Trichuris* species based on combined analysis of ribosomal DNA (ITS1 and ITS2) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

subclades, the subclade 2d not being represented due to the absence of sequences in all the markers. Subclade 2c was the most representative subclade including populations from a high variety of host species (**Figure 3** and **Supplementary Table 3**).

The phylogenetic inferences revealed that the populations of *Trichuris* from *P. papio*, *C. aethiops*, and *E. patas* analyzed in the present study clustered mainly in the subclade of *T. trichiura* (subclade 2c).

Comparative Sequence Analysis

In order to analyze the intraspecific and interspecific similarity in *T. trichiura* and between *T. trichiura* and *Trichuris* spp. parasitizing NHP as well as *T. suis*, we carried out a comparative study considering the different clades and subclades previously described for *Trichuris* spp. hosting humans, NHP, and swine (**Supplementary Tables 4–7**).

Thus, by examining ITS1 and ITS2 sequences, specimens obtained from *P. papio*, *C. aethiops*, and *E. patas* from Spain revealed a high similarity with populations of *T. trichiura* corresponding to subclades 2a, 2b, and 2c and subclade *Macaca mulatta* from different geographical origins (94.65 to 100%) (**Supplementary Table 4**). Further, the similarity was lower when clade 2 (*T. trichiura* lineage) and clade 1 (*T. suis*) were compared (90.62–92.23%).

Mitochondrial sequences (*cox*1 and *cob*) from *T. trichiura* from *P. papio*, *C. aethiops*, and *E. patas* from Spain showed the

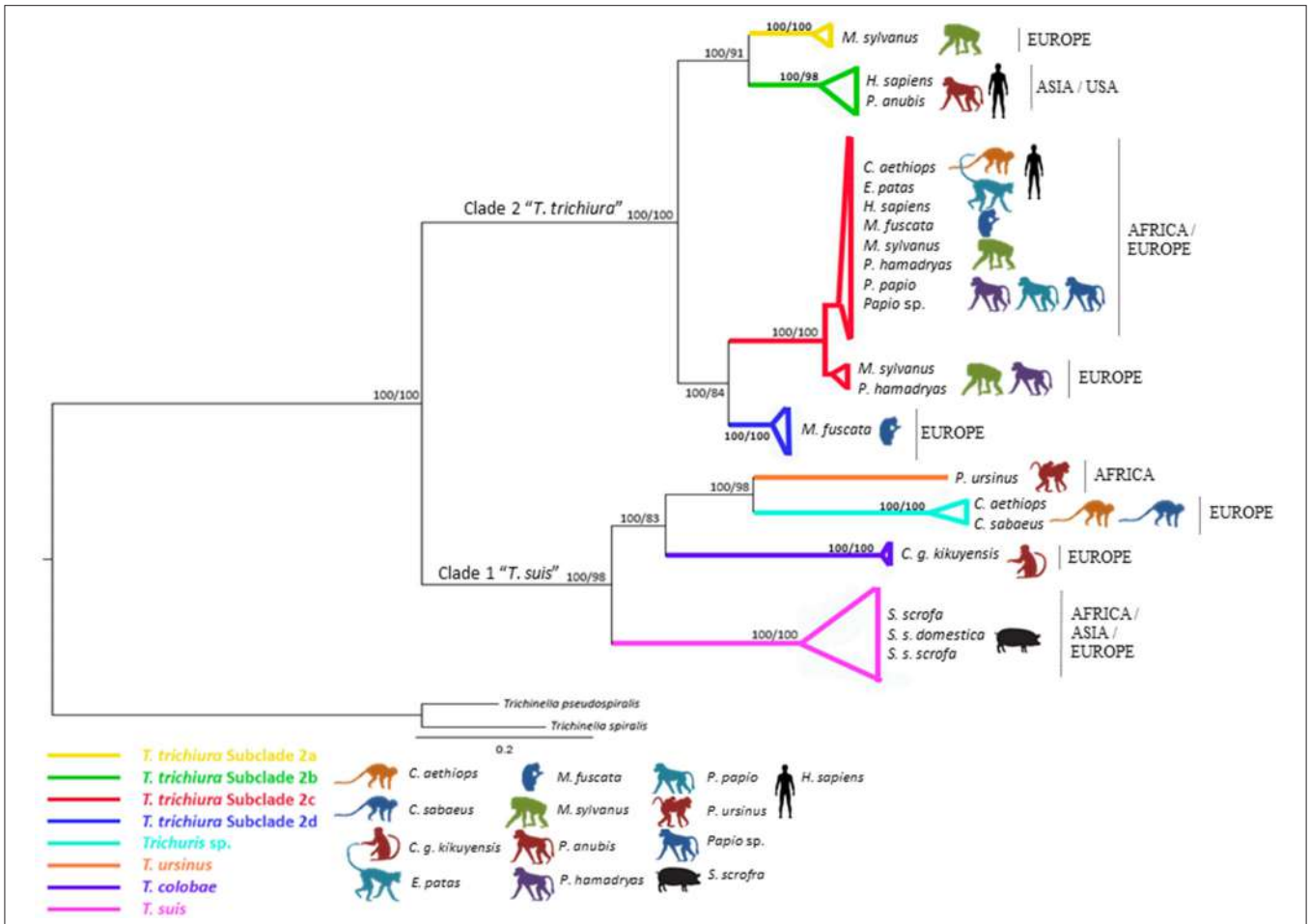

FIGURE 2 | Phylogenetic tree of *Trichuris* species based on combined analysis of mitochondrial DNA (*cox*1 and *cob*) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

highest intraspecific similarity within subclade 2c (97.3 to 100% and 92.12 to 100%, respectively) (**Supplementary Tables 5, 6**). For *rrn*L sequences, similar results were observed for *T. trichiura* from *M. sylvanus* and *P. papio* (97.77–100%). However, based on three mitochondrial markers, *T. trichiura* from *M. sylvanus* showed two different lineages corresponding to subclades 2a and 2c (**Supplementary Table 7**). Based on three mitochondrial markers, the similarity between clade 2 and *T. suis* ranged from 68.47 to 82.68%, values lower than those shown within clade 2.

Analysis of genetic diversity for clade 2 based on ITS and mitochondrial sequences revealed a haplotype diversity of 1.0 and 0.95–0.93, respectively (**Table 2**). In addition, nucleotide diversity based on ITS and *rrn*L sequences was 0.05; nevertheless, mitochondrial genes (*cox*1 and *cob* genes) revealed a higher nucleotide diversity (0.09–0.11) with the maximum values for *cox*1 (0.11) (**Table 2**). Within clade 2 (subclades 2a, 2b, 2c, and 2d), haplotype diversity of mitochondrial genes ranged 0.71–1.0 for *cox*1, 0.67–1.0 for *cob*, and 0.88–1.0 for *rrn*L with the maximum values for subclade 2a. Nucleotide diversity of mitochondrial genes ranged 0.01–0.04 for *cox*1 and *cob* with the maximum values for subclade 2b, and the value for *rrn*L was 0.01 (**Table 2**).

The analysis of nucleotide substitution saturation based on nuclear markers showed Iss > Iss.c ($P < 0.005$), indicating that ITS regions were saturated. Nevertheless, mitochondrial genes (*cox*1, *cob*, and *rrn*L) were not saturated (Iss < Iss.c, $P < 0.005$). Genetic diversity measures for all markers are summarized in **Table 2**.

Update Morphological Description of *T. trichiura* (Linnaeus, 1771)

Taxonomic summary

- Class Enoplea (Order Trichocephalida) (12, 38).
- Host: *H. sapiens* (14), *Pan troglodytes* (7), *M. sylvanus* (17, 21), *P. papio* (present study).

General: This is a parasite with a filiform anterior part and a broad and handle-like posterior part. The thin anterior portion of the parasite displays 2 types of cuticle patterns. One side is distinctly striated with transverse grooves and the other side

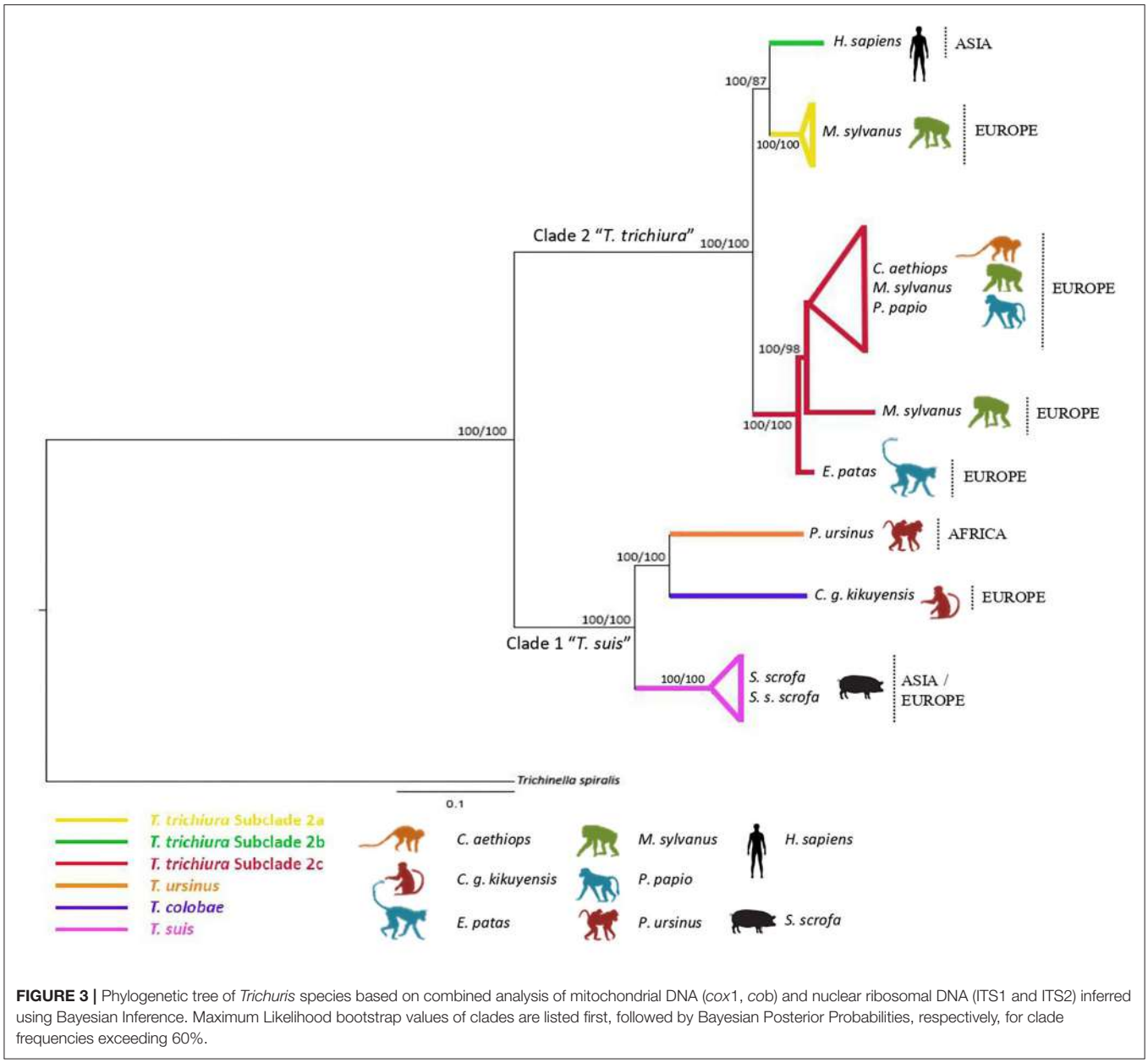

FIGURE 3 | Phylogenetic tree of *Trichuris* species based on combined analysis of mitochondrial DNA (*cox*1, *cob*) and nuclear ribosomal DNA (ITS1 and ITS2) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

in a finely tuberculate band under higher magnification reveals small circular elevated bodies, which are evenly distributed (**Figure 4A**). The ventral side of the anterior part of the body is slightly tapered toward the cephalic end which presents a broad longitudinal elongated "bacillary band" showing typical cuticle inflations (**Figure 4B**). The mouth is surrounded by a group of cephalic papillae arranged in two circles (an inner circle and a lateral circle) with a conspicuous organ, the stylet (**Figure 4C**), protruding from the middle portion of the mouth cavity. Adults present a moniliform esophagus constituted with a short muscular zone and a long stichosome with 1 row of stichocytes (**Figure 4D**), and 1 pair of conspicuous cells at esophagus-intestinal junction level (**Figures 4D**, **5A,B**). The site where the esophagus transforms into the intestine corresponds to the place of transition of the thin anterior part into the thick posterior and shows typical glandular cells at this point (**Figures 4D**, **5A,B**).

Male: The body is 25–49.3 mm long. The ratio between anterior and posterior body length is 2.6–4:1. The thin anterior part is 1:1.4–1.7 of the entire length of the body. The length of the esophagus is 18–28.9 mm. The width of the body in the esophageal end is 0.15–0.49 mm, and the maximum width (in the posterior part of the body) is 0.39–0.74 mm (**Table 3**). Posterior end of the body ventrally incurved (**Figures 4E–G**).

The genital apparatus of the male is a long tube whose sections differ from each other in structure bearing different functions (**Figure 5A**). The first section of the genital apparatus is the testis, which is very long and strongly convoluted, beginning

TABLE 2 | Summary of genetic measures for *cox*1, *cob*, *rrn*L genes, and ITS region sequences.

	Subclade 2a			Subclade 2b			Subclade 2c			Subclade 2d			*Trichuris trichiura* complex (Clade 2)			
	***cox*1**	**cob**	***rrn*L**	***cox*1**	**cob**	***rrn*L**	***cox*1**	**cob**	***rrn*L**	***cox*1**	**cob**	***rrn*L**	***cox*1**	**cob**	***rrn*L**	**ITS**
Number of haplotypes/number of sequences	3/3	3/3	2/2	3/4	2/3	10/13	5/13	14/18	15/29	6/6	4/5	5/6	17/26	23/29	31/50	21/22
Haplotype diversity	1.0	1.0	1.0	0.83	0.67	0.95	0.71	0.95	0.88	1.0	0.90	0.93	0.93	0.98	0.95	1.0
Nucleotide diversity	0.02	0.02	0.01	0.04	0.04	0.01	0.01	0.02	0.01	0.02	0.01	0.01	0.11	0.09	0.05	0.05
Nucleotide saturation*	-	-	-	-	-	-	-	-	-	-	-	-	No	No	No	Yes

**Substitution saturation test for partial cox1, cob, rrnL genes, and ITS sequences of Trichuris trichiura (Clade 2). "Yes" indicates that most of this sites have already been changed before (Iss>Iss.c), indicating nucleotide saturation.*

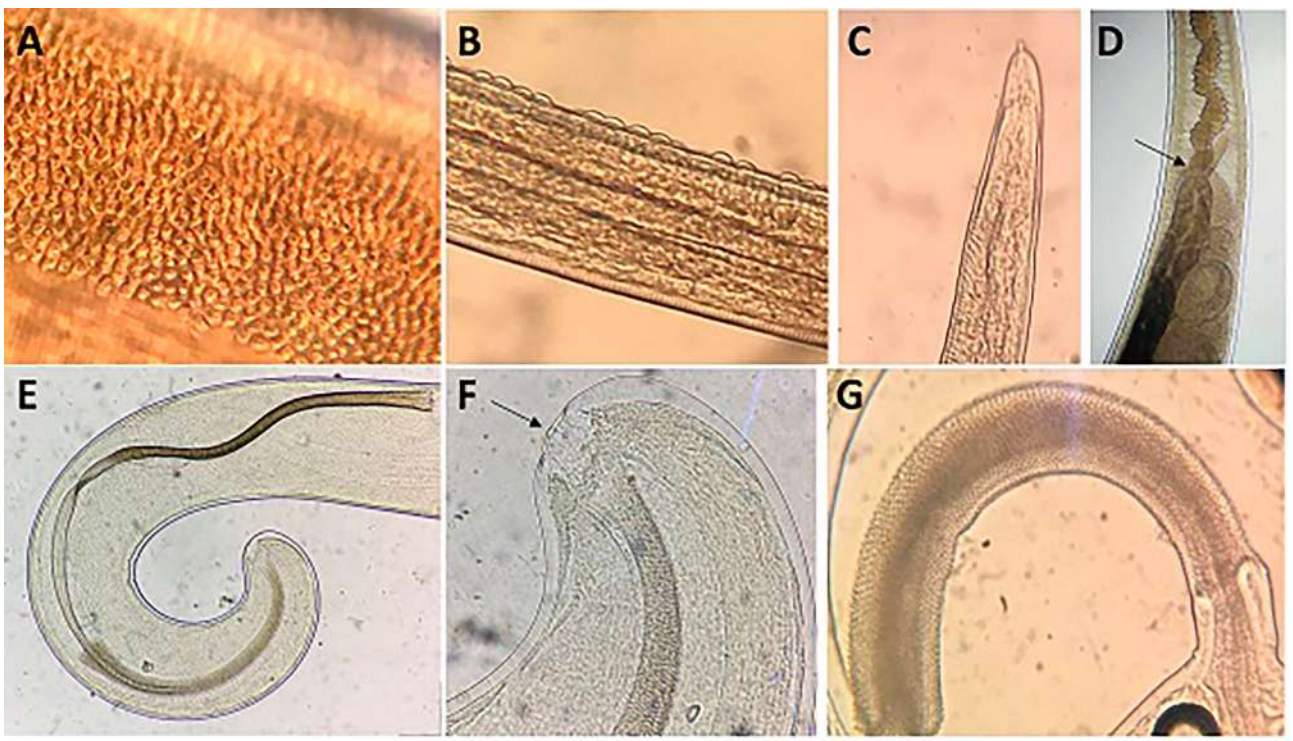

FIGURE 4 | Morphology of males of *T. trichiura*. **(A)** Tuberculate band. **(B)** Bacillary band showing typical cuticular inflations. **(C)** The mouth with the stylet. **(D)** Stichosome with 1 row of stichocytes and 1 pair of conspicuous cells at esophagus–intestinal junction level (arrowed). **(E)** Spicule. **(F)** Papillae pericloacal. **(G)** Spicule sheath.

in the posterior part of the male body, directed anteriorly, and lying along the long axis of the body terminating at a short distance from the transition of the esophagus into the intestine (**Figure 5A**). The testis ends near the union of the ejaculator conduct and intestine. The testis is followed by the vas deferens which at first runs somewhat anteriorly along the intestine, and then, at the level of the esophageal end and somewhat short of it, describes a convolution, turns backward, and terminates in a small constriction, connecting it with the following section of the genital apparatus, the seminal vesicle. The seminal vesicle runs parallel to the intestine but does not describe sharp convolutions and via a narrow tube with thick muscular walls joins the ejaculatory duct, which is 1.07–2.34 mm long (**Figure 5A**). The ejaculatory duct joins the intestine to form the cloaca, which opens at the posterior end of the male body. The cloaca with anus subterminal and one pair of paracloacal papilla not ornamented (**Figure 4F**) was observed when the spicule sheath was invaginated. No cluster of papillae was observed. The proximal cloacal tube is wide and continued with the distal cloacal tube (1.10–2.75 mm) that contains the spicule which projects into the anterior portion of the body in a spicule tube (**Figure 5A**). There is only one spicule, which is elongated with a pointed tip. This spicule presents two chitinized extreme zones and a light central part and is 1.61–3.81 mm long

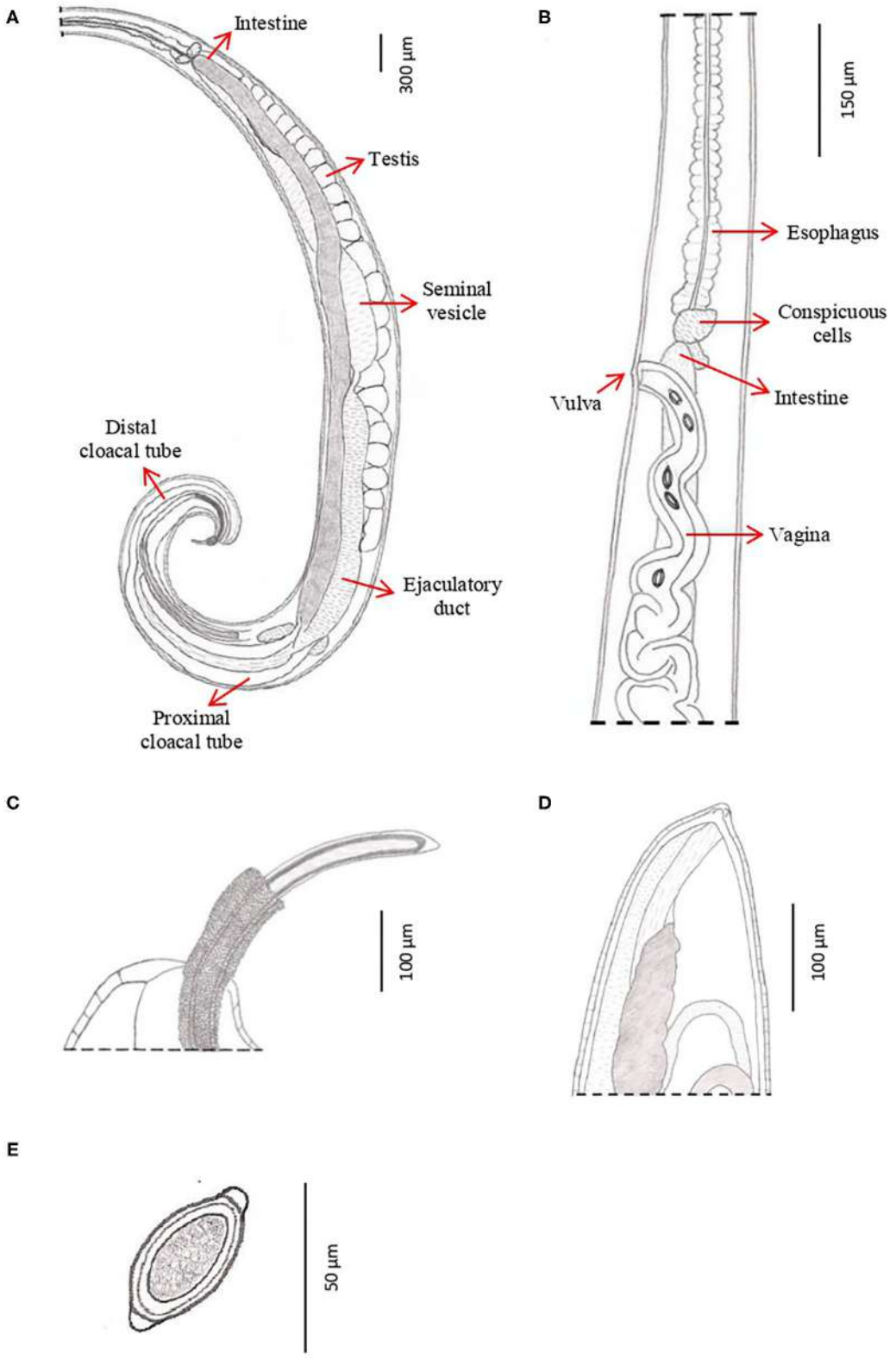

FIGURE 5 | Drawings of *T. trichiura* from *P. papio*. **(A)** Male, posterior end, spiny spicule sheath, spicule, spicule tube and proximal and distal cloacal tube, lateral view. **(B)** Female, esophagus–intestine junction, vulva and vagina, lateral view. **(C)** Male, detail of the posterior end, lateral view. **(D)** Female, posterior end, lateral view. **(E)** Eggs.

TABLE 3 | Biometrical data of males of *T. trichiura*.

	***T. trichiura* (Present study, updated)**	***T. trichiura* from *P. troglodytes* [Cutillas et al., (7)]**				***T. trichiura* from *Homo sapiens* [Nissen et al. (14)]**			***T. trichiura* from *M. sylvanus* [García-Sánchez et al. (21)]**				***T. trichiura* from *P. papio* (Present study)**		
	MIN-MAX	**MIN**	**MAX**	**X**	**δ**	**MIN**	**MAX**	**X**	**MIN**	**MAX**	**X**	**δ**	**MIN-MAX**	**X**	**δ**
M1	25.0–49.3	32.0	36.0	33.5	0.19	37.2	49.3	43.9	30.0	39.0	34.5	0.25	25.0	25.0	-
M2	18.0–28.9	18.0	23.0	20.5	0.21	20.9	28.9	25.6	19.0	28.0	21.9	0.27	18.0	18.0	-
LP	7.0–20.4	12.0	14.0	13.0	0.05	14.9	20.4	18.4	8.0	17.0	1.25	0.22	7.0	7.0	-
M3	0.09–0.31	0.09	0.31	0.15	0.02	0.10	0.20	0.15	0.12	0.18	0.14	0.02	0.12	0.12	-
M4	0.39–0.74	0.39	0.60	0.50	0.08	0.50	0.70	0.63	0.49	0.74	0.61	0.07	0.56	0.56	-
M5	0.15–0.49	0.15	0.24	0.2	0.04				0.25	0.49	0.37	0.06	0.35	0.35	-
M6	0.33–0.64	-	-	-	-				0.33	0.64	0.49	0.09	0.50	0.50	-
M7	1.10–1.62	-	-	-	-				1.10	1.57	1.33	0.14	1.62	1.62	-
M8	1.61–3.81	1.61	2.22	1.90	0.23	2.88	3.81	3.19	2.23	3.23	2.65	0.23	2.86	2.86	-
M9	0.22–1.23	0.22	0.22	0.22	-				0.53	1.23	0.93	0.20	0.63	0.63	-
M10	0.01–0.08	0.01	0.06	0.02	0.02				0.04	0.08	0.06	0.01	0.07	0.07	-
M11	0.04–0.09	0.05	0.09	0.07	0.02				0.04	0.08	0.06	0.01	0.05	0.05	-
M12	0.06–0.11	0.09	0.11	0.09	0.01				0.06	0.09	0.07	0.01	0.07	0.07	-
M13	1.79–5.19	1.79	1.95	1.88	0.07				2.90	5.19	4.06	0.58	3.31	3.31	-
M14	1.07–2.34	1.07	1.07	-	-				1.32	2.34	1.97	0.29	1.74	1.74	-
M15	1.10–2.75	1.10	1.10	-	-				1.55	2.75	2.20	0.36	1.35	1.35	-

M1, total body length of adult worm; M2, length of the esophageal region of the body; LP, length of the posterior region of the body; M3, width of the esophageal region of the body; M4, maximum width of the posterior region of the body (thickness); M5, body width in the place of the junction of the esophagus and the intestine; M6, Distance from the head end to beginning of bacillary stripes; M7, length of bacillary stripes; M8, length of spicule; M9, maximum length of spicule sheath; M10, width of the proximal end of the spicule; M11, width of the spicule sheath at the tail end of the body; M12, maximum width of the spicule sheath; M13, distance between the posterior part of the testis and tail end of body; M14, length of ejaculatory duct; M15, length of distal cloacal tube. **Ɛ**, *arithmetic mean.* σ, *standard deviation.*

(**Figures 4E**, **5A**). The spicule is surrounded by a peculiar spicule sheath (0.22–1.23 mm long), which may protrude externally together with the spicule (**Figure 4G**). The maximum width of the spicule sheath is 0.06–0.11 mm and is covered throughout its length by densely located chitinous spines from the proximal to distal portion and is cylindrical without a distal bulb (**Figures 4G**, **5C** and **Table 3**).

Female: The body is 20–48.6 mm long. The ratio between anterior and posterior body length is 2.1–2.2:1. The thin anterior part is 1: 0.65–0.67 of the entire length of the body. The length of the esophagus is 13–33 mm. The width of the body around the esophageal end is 0.13–0.48 mm, and the maximum width (in the posterior part of the body) is 0.38–0.90 mm (**Table 4**). The uterus is unpaired. The vulva is located at esophagus–intestine junction level (**Figures 5B**, **6A,B**). This part of body thereafter changes into a transversely striated body cuticle. The vulva is non-protrusive and has no ornamentation (**Figures 6A,B**). The vagina has strong walls and, when everted, shows small papillae (**Figure 6B**). This vagina is long and presents one zone straight near the vulva while presenting circumvolutions nearly the uterus (**Figures 5B**, **6A**). The ovary is long and continues with the oviduct in the back of the body (**Figure 6C**). The anus lies, subterminal, at the tip of the tail (**Figures 5D**, **6D**). The eggs are barrel-shaped with clear, mucoid-appeared polar plugs, in addition to a vitelline membrane, and have a triple shell, the outermost layer of which is brown (**Figure 5E**).

DISCUSSION

In the present paper, we address an updated morphological and biometric description of *T. trichiura*, based on the results provided in the present study as well as on previous studies by different authors who characterized this species combining the analysis of morphological, biometric, and molecular characteristics. Thus, for the emendation of the description of *T. trichiura*, we have considered the populations of *P. troglodytes* (7), *H. sapiens* (14), *M. sylvanus* (17, 21), and *P. papio* (present study). For many years, different authors have based on the description of *T. trichiura* isolated from humans and NHP, on morphological and biometric characteristics exclusively (6, 25, 39–43), which was not molecularly confirmed to have corresponded to *T. trichiura*.

Thus, we found that females of *T. trichiura* are characterized by a non-protrusive vulva without ornamentation. However, some authors have described the vulva with a surface covered with spines like those of the male's spicule sheath (6, 25). Likewise, based on the morphological characteristics of males, *T. trichiura* is characterized by the presence of a lanceolate spicule that tapers at the end (41). However, Tenora et al. (42) observed a spicule with a cylindrical end in isolated samples of *H. sapiens*. Furthermore, males of *T. trichiura* isolated from *P. papio* and *M. sylvanus* presented a spicule with two chitinized extreme zones and a lighter central part (17). This feature was not observed in

TABLE 4 | Biometrical data of females of *T. trichiura*.

	T. trichiura (Present study, updated)	*T. trichiura* from *P. troglodytes* [Cutillas et al. (7)]				*T. trichiura* from *H. sapiens* [Nissen et al. (14)]			*T. trichiura* from *M. sylvanus* [García-Sánchez et al. (21)]				*T. trichiura* from *P. papio* (Present study)			
	MIN-MAX	MIN	MAX	X	δ	MIN	MAX	X	MIN	MAX	X	δ	MIN	MAX	X	δ
F1	20–48.6	20.0	42.0	33.4	0.78	29.1	48.6	38.4	30.0	38.0	34.1	0.25	24.0	28.0	26	0.28
F2	13–33	13.0	33.0	25.3	0.68	16.2	32.1	25.6	18.0	26.0	21.9	0.23	17.0	20.0	18.5	0.21
LP	6–15.60	6.0	10.0	8.1	0.14	10.5	15.60	13.7	09.0	14.0	12.1	0.16	7	8	7.5	0.07
F3	0.09–0.20	0.09	0.19	0.14	0.04	0.11	0.20	0.17	0.13	0.18	0.15	0.01	0.14	0.15	0.14	0.00
F4	0.38–0.90	0.38	0.64	0.45	0.08	0.52	0.90	0.73	0.64	0.81	0.72	0.05	0.60	0.63	0.62	0.02
F5	0.13–0.48	0.13	0.23	0.17	0.03				0.36	0.48	0.42	0.03	0.31	0.41	0.36	0.07
F6	0.38–0.76	0.48	0.64	0.56	0.11				0.42	0.76	0.50	0.09	0.38	0.42	0.40	0.03
F7	0.36–1.71	0.36	0.63	0.50	0.19				0.90	1.71	1.44	0.21	1.08	1.35	1.22	0.19
F8	0.68–1.99	0.68	1.29	0.96	0.20				0.73	1.99	1.12	0.35	0.87	0.91	0.89	0.03
F9	0.02–0.09	0.03	0.05	0.04	0.01				0.02	0.09	0.05	0.02	0.05	0.05	0.05	0.00
F10	0.33–0.11	0.11	0.24	0.15	0.05				0.15	0.33	0.25	0.05	0.18	0.19	0.18	0.01
F11	0.13–0.84	0.13	0.24	0.21	0.04				0.40	0.84	0.61	0.14	0.35	0.37	0.36	0.01
F12	0.19–0.62	0.32	0.53	0.44	0.11				0.19	0.48	0.30	0.09	0.56	0.62	0.59	0.04
F13	0.05–0.81	0.66	0.67	0.67	0.01				0.05	0.14	0.11	0.04	0.73	0.81	0.77	0.06

F1, total body length of adult worm; F2, length of the esophageal region of the body; LP, length of the posterior region of body; F3, width of the esophageal region of the body; F4, maximum width of the posterior region of the body (thickness); F5, body width in the place of the junction of the esophagus and the intestine; F6, distance from the head end to beginning of bacillary stripes; F7, length of bacillary stripes; F8, length of vagina; F9, diameter of vulva turned over the surface of the body; F10, distance of vulva from the place of junction of the esophagus and the intestine; F11, distance of the posterior loop of the uterus from the tail end of the body; F12, distance of the tail end of the body and posterior fold of the seminal receptacle; F13, length of the muscular zone of the esophagus. Ƀ*, arithmetic mean.* ơ*, standard deviation.*

males of *T. trichiura* described by several authors who exclusively carried out morphological and biometric studies (6, 7, 25, 39–42).

On the other hand, the spicule sheath is cylindrical without a distal bulb with triangular spines; however, other authors described these spines with different shapes and sizes, some of them with blunt points in *T. trichiura* from humans and NHP (25, 42). Besides, we found that the males of *T. trichiura* are characterized by the presence of a pair of paracloacal papillae which are observed when the sheath is invaginated (17); however, a proximal group of small papillae is not observed, as described by Ooi et al. (6) in a morpho-biometric study carried out on samples of monkeys, baboons, and humans. These papillae cluster was described in *T. colobae* by Cutillas et al. (19) who characterized a new species of *Trichuris* present in *C. g. kikuyuensis* based on morphological, biometric, and molecular data. This fact could suggest that the descriptions carried out by Ooi et al. (6) of *Trichuris* populations from humans and NHP based exclusively on morphological and biometric characters could correspond to different species closed to *T. trichiura*.

Biometrically, there is a concordance between the measures of *T. trichiura* obtained by different authors and those provided in the present paper, since all the values overlapped within the range of defined measures. However, regarding males, Dinnik et al. (40) reported that the maximum total body length and the maximum length of the anterior part [total body length of adult worm (M1) and length of esophageal region of body (M2)] were slightly larger (52 and 34 mm, respectively). Furthermore, the maximum width of the posterior region of body (thickness) (M4), length of spicule (M8), and length of ejaculatory duct (M14) has also been reported as slightly higher by Skrjabin et al. (25) (M4 = 0.76 mm, M8 = 3.9 mm, and M14 = 3.9 mm). Regarding females, the maximum total body length of adult worm (M1) showed slightly higher values in the studies of Dinnik et al. (40), Skrjabin et al. (25), and Vigot-Fréres (39) (50, 52.7, and 50 mm, respectively). In addition, the maximum length of the esophageal region of body (M2) and the maximum body width in the place of junction of esophagus and the intestine (F5) were slightly higher (35.6 and 0.50 mm, respectively) in the data provided by Skrjabin et al. (25).

In addition, there are differences in the population of *Trichuris* sp. from chimpanzees showing a shorter size of the males and females collected from chimpanzees with respect to *T. trichiura* from *H. sapiens*, *M. sylvanus*, and *P. papio*. García-Sánchez et al. (21) who reported the differentiation of *Trichuris* species using a morphometric approach cited these results previously. The occurrence of different biometrical measurements in the same species may be explained by the phenotypic plasticity of these organisms themselves (8–11).

Morphological and biometric data for *T. trichiura* provided in the present study allow the differentiation of this species with respect to other species of *Trichuris* parasitizing NHP such as *T. colobae* (19) and *T. ursinus* (20). The typical *T. trichiura* spicule has a clear central part, which is not present in that of *T. colobae* and *T. ursinus*. Furthermore, the typical papilla group is only present in *T. colobae* (19). Females of *T. trichiura* and *T. ursinus* appear to have a non-protrusive vulva (20); nevertheless, females of *T. colobae* present a vulva like a crater with papillae (19). The vagina is very long and straight in *T. ursinus* (20) but appear with circumvolutions in *T. trichiura*.

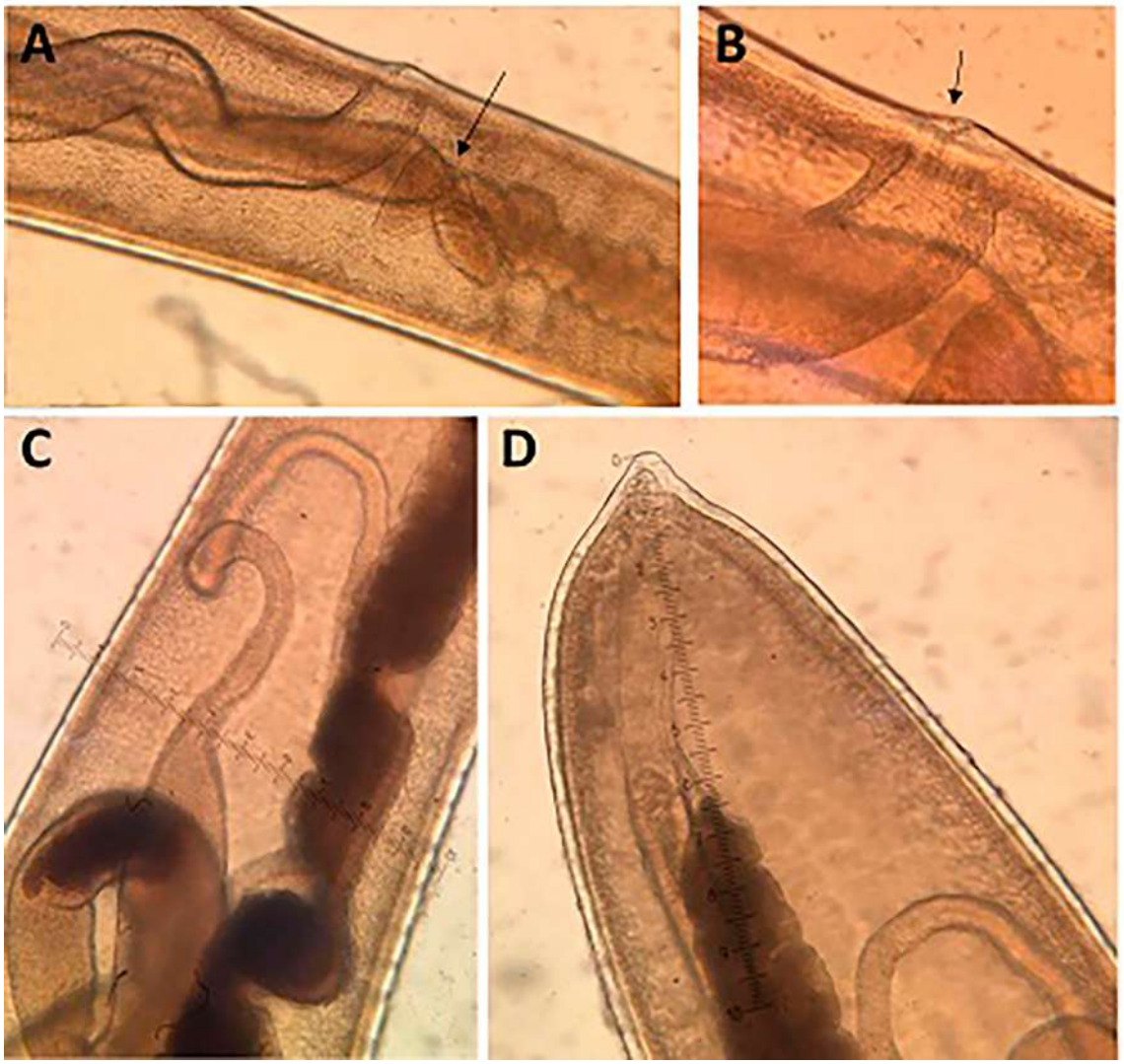

FIGURE 6 | Morphology of females of *T. trichiura*. **(A)** Esophagus–intestinal junction level (arrowed), vulva and vagina. **(B)** Vulva (arrowed). **(C)** Oviduct. **(D)** Posterior end.

With respect to the biometric characteristics, most of the measurements do not allow the specific differentiation between *T. trichiura* and other *Trichuris* spp. of NHP since these values overlapped for most of the measurements. However, males of *T. trichiura* have a smaller range of values in terms of distance from the end of the head to beginning of bacillary stripes and length of bacillary stripes (M6 and M7) compared to *T. colobae*, and values of length of minor bacillary stripes regarding *T. ursinus*. Regarding the specific differentiation of females based on biometric measurements, *T. trichiura* presents a lower range of values of length of bacillary stripes and distance of the tail end of the body and posterior fold of the seminal receptacle (F7 and F12) respect to *T. colobae*. In addition, the maximum and minimum values of distance of the vulva from the place of junction of the esophagus and the intestine were below than those observed in *T. ursinus* (19, 20).

From a molecular point of view, molecular markers which have been used by different authors to resolve species-level questions in *Trichuris* include the ITS1 and ITS2 nuclear regions (7, 44–53), 18S nuclear rRNA gene (38, 53, 54), mtDNA 16S rRNA gene (*rrn*L) (17, 50, 55), and protein-coding mitochondrial genes, including the 13 common genes obtained from the mitochondrial complete genome (12, 16, 56), *cox*1 mtDNA partial gene (17, 20, 38, 53, 55, 57), and *co*b mtDNA partial gene (17, 20, 55, 57, 58).

Different ITS rDNA types have been cited by different authors in some species of *Trichuris* (45–47). These sequence differences among ITS repeats in the rDNA array appeared to be a consequence of (intrachromosomal) mutational exchange during DNA replication (59). Thus, different reports suggest that different sequence types are most likely to be a result of base changes at certain positions in the sequence of a proportion of rDNA repeats because of mutational exchange during DNA replication, the extent of which appears to differ depending on the taxonomic group (60–63).

The results observed in ITS sequences revealed a percentage of similarity between 2a, 2b, 2c, and 2d subclades higher than those previously observed by other authors for species of the genus *Trichuris* (50, 57). Genetic analysis revealed that ITS regions were saturated and showed poor nucleotide diversity for clade 2 (*T. trichiura*). Thus, ITS sequences were not useful to infer the phylogenetic relationships between the different populations of *T. trichiura* (clade 2). On the other hand, some sequences from *T. trichiura* from Uganda and Cameroon (4) appeared within clade 1, suggesting that this population could be included on *T. ursinus* due to the high interpopulation similarity observed between both populations (**Supplementary Table 4** and **Figure 1**). Therefore, the different genetic lineages within *T. trichiura* were delimited exclusively based on analysis of the mitochondrial genes in agreement with Chan et al. (64), who evaluated the utility of mitochondrial and ribosomal genes for molecular systematics of parasitic nematodes. ITS regions accumulated substitutions substantially more slowly than mtDNA and showed nucleotide saturation.

Studies of interpopulation similarity between the different 2a, 2b, 2c, and 2d subclades, based on the mitochondrial markers (*cox*1 and *co*b), revealed a range of similarity lower than those observed by ITS sequences, and similar to those observed among other clearly defined species such as for example *T. suis* and *T. colobae*, *T. colobae*, and *T. ursinus* [cited by present authors, **Supplementary Tables 5, 6** and previously by Callejón et al. (20) and Rivero et al. (17)]. However, this similarity range showed higher values between *rrn*L sequences presenting a strong resolution of the different *T. trichiura* lineages (**Supplementary Table 7**). These results agree with those of Chan et al. (64), who evaluated the utility of mitochondrial ribosomal genes for molecular systematics of parasitic nematodes. These authors cited that 18S and 28S rRNA genes as well as 12S (*rrn*S) and 16S (*rrn*L) rRNA and *cox*1 genes showed a higher resolution for phylogenetic studies indicating that these five genes have potentially to be used as markers. Furthermore, they demonstrated that mitochondrial 12S and 16S genes present a resolution power at both lower and higher taxonomic levels for species and clade discrimination (64). For this reason and considering the similarity values observed in the different markers, we suggest that the populations of *T. trichiura* corresponding to the 2a, 2b, 2c, and 2d subclades correspond to different genetic lineages.

On the other hand, within clade 2 "*T. trichiura* lineage," results based on *rrn*L revealed an interpopulation similarity of 83.80–86.31% between *T. trichiura* populations and *Trichuris* sp. from *T. francoisi*. Hence, we suggest that this species has a close relationship with *T. trichiura*. Our results agree with Liu et al. (12) who considered that *Trichuris* populations from *T. francoisi* are a new species of *Trichuris*.

The phylogeny inferred from mitochondrial datasets revealed the same topology of those based on rDNA with respect to the two main clades (clade 1 and clade 2). We were able to identify several genetically distinct subgroups (subclades) of whipworms, which were present in the sampled primates. The subclades 2b and 2c showed a broad host range and were not restricted to NHP species. However, the subclades 2a and 2d showed a higher host specificity corresponding with the *T. trichiura* population from *M. sylvanus* and *H. sapiens* (subclade 2a) and *M. fuscata* (subclade 2d) exclusively. In agreement to this study, similar results were observed on *Trichuris* sp. from *M. fuscata* (44, 55). This population also showed two potentially distinct entities of *Trichuris* present in two different subclades: subclade 2d [analogous to subclade MF reported by Cavallero et al. (44, 55)] and subclade 2c. These authors suggested the possibility of two different sources of infection for Japanese macaques corresponding with two *Trichuris* taxa, one potentially able to also infect humans.

In our study, all phylogenetic trees (partitioned and concatenated) reported the existence of two main clades, which has been previously reported (3, 7, 12, 17, 20, 33, 44, 55, 58). A clear differentiation between *T. suis* (clade 1) and *T. trichiura* (clade 2) can be confirmed according to our results. Within clade 2 "*T. trichiura* lineage," *T. trichiura* can be also divided into 4 subclades, suggesting a complex of different genetic lineages. The analysis of the intraspecific similarity between the populations of *T. trichiura* from *P. papio*, *C. aethiops*, and *E. patas* from Spain revealed their highest value when compared with the populations of *T. trichiura* belonging to subclade 2c [previously described by Rivero et al. (17)] in all analyzed markers.

In addition to *T. trichiura*, a complex of *Trichuris* species showed infecting primates (*Trichuris* sp. from *T. francoisi* within clade 2 "*T. trichiura* lineage" and from *C. aethiops* from Italy and *C. sabaeus* from Czech Republic within clade 1 "*T. suis* lineage"). Different authors (17, 65) have previously reported the presence of several groups of *T. trichiura*. These results emphasize that the taxonomy and genetic variations of *Trichuris* are more complicated than previously acknowledged (65). Ravasi et al. (3) identified two distinct *Trichuris* genotypes that infect both humans and NHP. These authors identified "heterozygotes" confirming the identification of two distinct *Trichuris* genotypes in primates. On the other hand, Nissen et al. (14) identified "heterozygote" worms isolated from humans suggesting that *T. trichiura* might consist of several subspecies, some being found mainly in NHP. Ghai et al. (66) suggested that the *Trichuris* taxon should be considered a multi-host pathogen that is capable of infecting wild primates and humans. Finally, Betson et al. (5) reported that *Trichuris* infecting primates represents a complex of cryptic species with some species being able to infect both humans and NHP.

On the other hand, the existence of populations of *Trichuris* sp. associated with clade 1 "*T. suis* lineage" (based on all partitioned and concatenated markers) could indicate the possible origin of the change of host from NHP to pigs and, therefore, the origin of a new species, *T. suis*. This fact has been suggested by Hawash et al. (16) who found evidence of an African origin of *T. trichiura* that was later transmitted with human ancestors to Asia and then to South America. These authors suggested the possibility of a change of host to pigs in Asia where *T. suis* appears to have been transmitted globally by a combination of natural host dispersion and anthropogenic factors.

The present work examines the taxonomy, genetics, and phylogeny of *T. trichiura* parasitizing human and NHP and its

relationship to *Trichuris* spp. from other NHP host species. A similar analysis was carried out on whipworms from humans (5). These authors suggested the existence of zoonotic transmission, especially regarding *T. trichiura* infections in NHP and, possibly, also for *T. suis* from pigs and *Trichuris vulpis* from dogs. In consequence, *Trichuris* may represent different species with the potential differences in endemicity, which may have important implications for implementing effective control strategies (5).

Several studies have reported that NHP represent an important reservoir for several known zoonotic infectious diseases (67–69). In this context, *Trichuris* infections have been found in a range of NHP species living in natural habitats including colobus monkeys, macaques, baboons, and chimpanzees (70–78). Based on molecular studies described above, some *Trichuris* species seem to be specific to a particular NHP, while others likely have the potential to circulate between humans and NHP, as they are genetically identical. This is particularly important when humans and NHP live in close proximity, as it is becoming increasingly common with human encroachment on pristine habitats and NHP accessing gardens and farms in search of food, and it has significant implications for human health and wildlife conservation (5).

The taxonomic, genetic, and phylogenetic results obtained in the present study confirm that *T. trichiura* exists as a complex (four subclades) with different host affinities and cross-infection capabilities corresponding with four different genetic lineages. Specifically, two of the four subclades show little host specificity and can develop trichuriasis in a wider variety of NHP species (*C. sabaeus*, *C. aethiops*, *M. fuscata*, *M. sylvanus*, *E. patas*, *P. papio*, *P. hamadryas*, *Papio* sp., and *P. anubis*) shared with humans. For this reason, we suggest the existence of a possible reservoir in the previously mentioned NHP species for human trichuriasis, which constitutes a serious public health risk (17). However, previous studies showed that the majority of the population of *M. fuscata* was included in a specific subclade [2d, subclade MF cited by Cavallero et al. (44, 55)] including only specimens from macaques.

Therefore, considering the following arguments for the taxonomic and phylogenetic study of populations of *T. trichiura*: (i) ITS regions were saturated and accumulated substitutions substantially slowly than mtDNA; therefore, they are not good genetic markers to delimit different genetic lineages. This is in agreement with previous studies where intraspecific variation was not observed when using nuclear DNA (64) suggesting that is not a useful genetic marker for intraspecies discrimination. (ii) The mitochondrial genes *cox*1, *co*b, and *rrn*L were not saturated, indicating that these three genes could have potential to be used as markers. Nevertheless, although *cox*1 presents the advantage for the extensive availability of database sequences, allowing for thorough comparisons of unknown sequences, *cox*1 sequences showed a high intraspecific variability which hinders resolution between closely related species. (iii) Nucleotide diversity showed that the *rrn*L gene present a lower intraspecific variability than *cox*1 and *co*b genes. However, *rrn*L interspecific genetic distance values allowed the phylogenetic resolution of the different *T. trichiura* subclades. Whence, *rrn*L was used successfully for inter-lineage discrimination of closely related populations within the *T. trichiura* lineage.

We suggest the utility of *rrn*L rRNA gene as a useful genetic marker for *Trichuris* species discrimination. Different authors who evaluated the utility of different mitochondrial genes for phylogenetic analyses (64, 79–81) have cited similar results. Future studies should focus on developing sequence databases for rRNA genes, which can serve as alternative genetic.

In conclusion, the present work provides an extensive study of biometric, morphological and molecular data for the unification of criteria that allows an update description of *T. trichiura* as well as a complex taxonomic, genetic, and phylogenetic study of the cited species applying multiple genetic markers to whipworm populations collected from humans and NHP from sympatric areas and worldwide locations. This study provides useful results for future studies aimed at the identification of new subspecies and hybridization events between existing species and enables a much clearer and more detailed understanding of dispersal patterns. This will reveal parasite transmission routes between these primates and will allow the implementation of appropriate control and prevention measures.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JR, RC, and CC contribute conception, design of the study, and wrote the manuscript. All the authors contributed to the manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.626120/full#supplementary-material

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Characterization of trichuris species from porcupine (*Hystrix cristata*) at zoological garden of Spain

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ABSTRACT

Population of *Trichuris* sp. isolated from *Hystrix cristata* was analyzed based on morphological, biometrical characteristics and mitochondrial (*cox*1, *cob*, *rrn*L) and ribosomal (ITS1) (rDNA) region sequences. Morphological and biometrical results revealed that *Trichuris* sp. from *H. cristata* present a high similarity with *Trichuris landak* from *Hystrix javanica* and less similarity with other *Trichuris* species from porcupine species (*Trichuris hystricis, Trichuris lenkorani* and *Trichuris mettami*). The lack of molecular data corresponding to *Trichuris* species that parasitize the porcupine (genus *Hystrix* and *Atelerix*) has not allowed a comparative molecular or phylogenetic study. Molecular analyses revealed the existence of two different haplotypes that did not correspond to different morphospecies. Relationships among *Trichuris* sp. from *H. cristata* and other *Trichuris* spp. have been resolved by molecular sequence data in this study. Thus, the combined analysis of one ribosomal and three mitochondrial markers revealed a sister relationship between whipworms parasitizing porcupine and other *Trichuris* spp. from rodents and canids and separated from the rest of *Trichuris* spp. from other hosts species. It is necessary accurate information on the possible zoonotic behavior of different *Trichuris* species for health workers to improve existing control measures. Thus, it is necessary to increase the studies of integrative taxonomy on *Trichuris* spp. based on morphological, biometrical and molecular data, which will inevitably contribute to our knowledge on the etiology of trichuriasis.

1. Introduction

Tissue-dwelling intestinal nematodes are spread throughout the world. It is estimated that these parasites currently infect over two billion people, causing morbidity and debility in infected individuals, especially in developing countries, being a food safety and health problem worldwide (Patel et al., 2009). Among the nematodes of high interest with respect to the mechanisms of inter-action with the host tissue are the hookworms, which interact with their hosts by inserting cuticular teeth in the intestinal epithelium, and the whipworms, which during infection create an intra-tissue niche in the cells of the large intestine (Hall et al., 2008). Whipworms of the genus *Trichuris* Roederer, 1761 (Nematoda: Trichuridae) have a cosmopolitan distribution and are widely disseminated among mammals regardless of their dietary habits and habitat preference (Cafrune et al., 1999; Anderson, 2000). For many years, *Trichuris* species have been described with a range of morphologic and biometric characteristics (Schwartz, 1926; Chandler, 1930; Knight, 1984; Babero and Murua, 1990). The presence or absence of the spicular tube, the length of the spicule and the cloacal tube, the shape of the proximal and distal cloacal tube, and the vulvar morphology, along with the classic morphometric characteristics, have been used as features with high discriminatory value for differentiating species of *Trichuris* (Gomes et al., 1992; Spakulová, 1994; Suriano and Navone, 1994; Rossin and Malizia, 2005; Robles et al., 2006). The spicule length has been considered as the main criterion to differentiate *Trichuris* species (Cutillas et al., 1995). Knight (1971) and lately Rickard and Bishop (1991) proposed a key to species of *Trichuris* based on spicule length greater than 5000 μm (*Trichuris ovis* = 5690 μm (Oliveros et al., 2000; Oliveros and Cutillas, 2003); *Trichuris tenuis* = 7200 μm (Rickard and Bishop, 1991)); or spicule length less than 5000 μm (*Trichuris discolor* (Callejón et al., 2012), *Trichuris rhinopiptheroxella* (Wang et al., 2019), *Trichuris trichiura* and *Trichuris suis* (Cutillas et al., 2009)).

Nevertheless, several whipworms of similar hosts share a similar morphological pattern (Tiner, 1950; Babero et al., 1976; Feliú et al., 2000; Robles et al., 2006, 2011). Consequently, recent studies have used molecular techniques to differentiate them (Feliú et al., 2000; Cutillas et al., 2002, 2004, 2007, 2014; Callejón et al., 2017, Rivero et al., 2020a, 2020b, 2021). Studies of integrative taxonomy on *Trichuris* spp. have

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been carried out in the last ten years (Cutillas et al., 2009, 2014; Robles et al., 2014, 2018; Callejón et al., 2016). These surveys combine different methodologies (morphological and molecular) with the aim of clarifying taxonomic problems and contributing to the description of new species (Robles et al., 2014, 2018).

To date, few molecular studies were carried out on *Trichuris* parasites of rodents. There are reports from Muridae (*Trichuris muris* Schrank,1788; *Trichuris mastomysi* Verster, 1960; *Trichuris carlieri* Gedoelst, 1916; *Trichuris duplantieri* Ribas et al. 2017), Cricetidae (*Trichuris arvicolae* Feliú et al. 2000; *Trichuris pardinasi* Robles et al. 2006; *T. navonae* Robles, 2011; *Trichuris bainae* Robles et al. 2014; *Trichuris massoiai* Robles et al. 2018), Ctenomyidae (*Trichuris pampeana* Suriano and Navone (1994) and Myocastoridae (*Trichuris myocastoris*) (Callejón et al., 2010, 2016; Robles et al., 2014; Ribas et al., 2017), and unpublished data from the GenBank), but none from Hystricidae.

Up to now, 4 *Trichuris* species have been described from *Hystrix* spp., *Trichuris landak* Purwaningsih (2013) from *Hystrix javanica, Trichuris hystricis* Syn. *Trichocephalus hystricis* Kreis, 1938 from *Hystrix cristata* and *Hystrix indica, Trichuris infundibulus* Linstow, 1906 from *H. cristata* and *Trichuris* [the authors use *Trichocephalus*] *lenkorani* n. sp., from *Hystrix hirsutirostris*.

The first description of *T. hystricis* might be thought doubtful given it was based on material from captive porcupines from Basel Zoo (Switzerland) (Skrjabin et al., 1957), *Trichuris infundibulus* has no locality in the original description, and no information appears available on trichurids from wild African crested porcupines (Ribas et al., 2013). In addition, *Trichocephalus mettami* has been described in porcupines of the genus *Atelerix* or *Aethehinus*.

The crested porcupine (*H. cristata*) is a large semi-fossorial, mainly nocturnal and herbivorous rodent mammal species that is widespread in both wild wood and areas as well as highly anthropic woody. *H. cristata*, currently distributed in most of North Africa and in Sub-Saharan Africa from Senegal to Ethiopia, up to northern Tanzania (Cabrera, 1932; Corbet and Jones, 1965; Ranck, 1968; Smit and Wijngaarden, 1981; Niethammer, 1982). Its European distribution is limited to peninsular Italy, Sicily and the island of Elba (Lovari, 1993; Masseti, 2008, 2009). This last occurrence is the result of very recent introductions, probably dating from the first half of the 1980s (De Marinis et al., 1996). Italy is the only European country where the crested porcupine lives, coexisting and sharing settlements with two other borrowing mammals, the red fox (*Vulpes vulpes*) and the badger (*Meles meles*) (Pigozzi, 1986). Although the crested porcupine is protected by European (Bern Convention, 1979) and Italian legislation (National Lawn.503/1981), this species is still under-research. Only one study deal with ectoparasites of *H. cristata* (Mori et al., 2015) living in Italy, while no data is available on endoparasites (Coppola et al., 2020).

The main purpose of this paper is to characterize the population of *Trichuris* sp. isolated from *H. cristata* from Spain (Bioparc zoo, Fuengirola), based on morphological and biometrical characteristics and mitochondrial (*cox*1, *cob*, *rrn*L) and nuclear (ITS1) markers. Molecular data are also used to analyze and discuss the phylogenetic relationships among the *Trichuris* species from different hosts.

2. Material and methods

2.1. Ethics statement

This study does not require approval by an ethics committee. *H. cristata*, from which *Trichuris* specimens were collected from their caeca post-mortem, was handled, and housed in a zoo in strict accordance with good animal practices.

2.2. Collection of samples

Trichuris specimens were obtained from one crested porcupine (*H. cristata*) at Bioparc Fuengirola in Málaga, Spain. Adult whipworms were washed in a saline solution of 0.9% sodium chloride and stored individually at -20 °C until further studies.

2.3. Morphological and biometrical studies

Thirty adult specimens (fifteen males and fifteen females) were recognized according to previous studies (Cutillas et al., 2009, 2014; Callejón et al., 2017). Morphological studies were carried out as described by Oliveros et al. (1998) and Skrjabin et al. (1957). *Trichuris* worms were measured according to parameters reported by Spakulová and Lýsek (1981), Suriano and Navone (1994), and Robles et al. (2006). Descriptive univariate statistics based on mean values, standard deviation and range for all parameters were determined for male and female population (Callejón et al., 2017). To test the equality of means for each variable, was used the Student's t-test ($P < 0.001$). Statistical analysis was carried out using Microsoft Excel v16.0 (Feliú et al., 2000). Thus, a comparative study of biometric data was performed between different previously described of *Trichuris* sp. from *H. cristata* and the most significant parameters were tested for a morphometric study.

2.4. PCR and sequencing of specimens

Total genomic DNA was isolated separately using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Düsseldorf, Germany). Quality of extractions was assessed using 0.8% agarose gel electrophoresis infused with SYBR® Safe DNA gel stain (Thermo Fisher Scientific, Waltham, MA, USA).

Molecular markers used [*cox*1, *cob* and *rrn*L mitochondrial DNA (mtDNA) and ITS1 ribosomal DNA (rDNA)] were amplified by polymerase chain reaction (PCR) using a thermal cycler (Eppendorf AG; Hamburg, Germany). PCR reactions were successful except for some reactions for the *rrn*L marker, for which newly primers were designed based on the alignments of the previously amplified sequences with the previously described primers (Liu et al., 2012) (Table 1). For ITS1 marker, a new reverse primer was designed because the total ITS1–5.8-ITS2 sequence was wrong (Table 1). PCR mix, PCR primers and PCR conditions are summarized in Table S1.

The PCR products were checked on SYBR® Safe stained 2% Tris-Borate-EDTA (TBE) agarose gels and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The purified PCR products were concentrated and sequenced in both directions with the same primers used for PCR by Stab Vida (Portugal).

2.5. Phylogenetic analysis

Nucleotide sequence data reported in this study are available in the GenBank ® database (Accession number in Table 2). For the purpose of analyze and understand the relationships between the different *Trichuris*

Table 1
Sequences of primers for amplifying DNA regions.

	Primers	Sequence (5' to 3')	References
ITS1	NC5	GTAGGTGAACCTGCGGAAGGATCATT	Gasser et al., 1996
	5.8SR	GAGTGTCACGTCGTTCTTCA	Present study
*cox*1	HCO2198F	TGATTTTTTGGTCACCCTGAAGTTTA	Folmer et al., 1994
	CORA	ACYACATAGTAGGTRTCATG	Nagano et al., 1999
cob	D769	GAGTAATTTTTATAATRCGRGAAGT	Callejón et al., 2015b
	D770	AATTTTCAGGRTCTCTRCTTCAATA	Callejón et al., 2015b
*rrn*L	TTrrnLF	TAAATGGCCGTCGTAACGTGACTGT	Liu et al., 2012
	TTrrnLR	AAAGAGAATCCATTCTATCTCGCAACG	Liu et al., 2012
	PErrnLF	CGTAACGTGACTGTGCTAAGG	Present study
	PErrnLR	CCATTCTATCTCGCAACGGT	Present study

Table 2
Sequences of *Trichuris* spp. and outgroups species obtained from GenBank and used for phylogenetic analysis.

Species	Host species/ Geographical origin	Marker	Accession number
Trichuris arvicolae	*Myodes glareolus*/Spain	*cox*1	FR851284
Trichuris bainae	*Necromys temchuki*/ Argentina		LN899585
Trichuris colobae	*Colobus guereza kikuyuensis*/Spain		HE653116
Trichuris discolor	*Bos grunniens mutus*/China		NC_018596
Trichuris muris	-/United Kingdom		LC050561
Trichuris navonae	*Akodon montensis*/ Argentina		HG934464
Trichuris ovis	*Addax nasomaculatus*/ China		NC_018597
Trichuris pampeana	*Ctenomys talarum*/ Argentina		KC614698
Trichuris pardinasi	*Phyllotis xanthopygus*/ Argentina		HG934451
Trichuris rhinopiptheroxella	*Rhinopithecus roxellana*/ China		MG189593
Trichuris skrjabini	*Capra hircus*/Spain		HE653121
Trichuris sp.	*Akodon azarae*/Argentina		LT221883
	Holochilus chacarius/ Argentina		LT221884
	Hystrix cristata/Italy		MK779003 (TIS1)
	Hystrix cristata/Spain		OU596152 (THC1) OU596153 (THC2)
	Mastomys coucha/South Africa		MG386207
	Sooretamys angouya/ Argentina		HG934466
	Trachypithecus francoisi/ China		KC461179
Trichuris suis	*Sus scrofa*/China		GU070737
Trichuris trichiura	*Homo sapiens*/Uganda		KT449826
Trichuris ursinus	*Papio ursinus*/South Africa		LT627353
Trichuris vulpis	*Canis lupus familiaris*/Spain		HE653135
Trichuris arvicolae	*Myodes glareolus*/Spain	*cob*	LM994698
Trichuris bainae	*Sooretamys angouya*/ Argentina		LN899575
Trichuris colobae	*Colobus guereza kikuyuensis*/Spain		LM994704
Trichuris discolor	*Bos grunniens mutus*/China		NC_018596
Trichuris leporis	*Lepus europaeus*/Spain		LM994705
Trichuris muris	-/United Kingdom		LC050561
Trichuris navonae	*Akodon montensis*/ Argentina		LN899565
Trichuris ovis	*Addax nasomaculatus*/ China		NC_018597
Trichuris pardinasi	*Phyllotis bonariensis*/ Argentina		LN899577
Trichuris rhinopiptheroxella	*Rhinopithecus roxellana*/ China		MG189593
Trichuris skrjabini	*Capra hircus*/Spain		LM994700
Trichuris sp.	*Holochilus chacarius*/ Argentina		LT221888
	Hystrix cristata/Italy		MK779004 (TIS8)
	Hystrix cristata/Spain		OU596147 (THC1) OU596148 (THC2)
	Trachypithecus francoisi/ China		KC461179
Trichuris suis	*Sus scrofa*/China		GU070737
Trichuris trichiura	*Homo sapiens*/Uganda		KT449826
Trichuris ursinus	*Papio ursinus*/South Africa		LT627357
Trichuris vulpis	*Canis lupus familiaris*/Spain		LM994699
Trichuris colobae	*Colobus guereza kikuyuensis*/Spain	*rrn*L	MN088583
Trichuris discolor	*Bos grunniens mutus*/China		NC_018596
Trichuris muris	-/United Kingdom		LC050561
Trichuris ovis	*Addax nasomaculatus*/ China		NC_018597
Trichuris sp.	*Hystrix cristata*/Spain		OU596145 (THC1) OU596146 (THC2)
	Trachypithecus francoisi/ China		KC461179
Trichuris trichiura	*Homo sapiens*/Uganda		KT449826
Trichuris rhinopiptheroxella	*Rhinopithecus roxellana*/ China		MG189593
Trichuris suis	*Sus scrofa*/China		GU070737
Trichuris arvicolae	-/Spain	ITS1	FR849652
Trichuris carlieri	*Gerbilliscus vicinus*/ Tanzania		JX683524
Trichuris colobae	*Colobus guereza kikuyuensis*/Spain		FM991956
	Nomascus gabriellae/Spain		FM991955
Trichuris cutillasae	*Hydrochoeris hydrochaeris*/ Argentina		LS481186
Trichuris discolor	*Bos taurus*/Spain		HE608850
Trichuris duplantieri	*Gerbillus gerbillus*/ Mauritania		KX669087
Trichuris skrjabini	*Capra hircus*/Spain		AJ489248
Trichuris mastomysi	*Mastomys natalensis*/ Tanzania		JX683525
Trichuris muris	*Mus domesticus*/Spain		FN543196
Trichuris myocastoris	*Myocastor coypus*/Czech Republic		MF077371
Trichuris ovis	-/Spain		AJ310662
Trichuris sp.	*Hystrix cristata*/Spain		OU624085 (THC1) OU624086 (THC2)
	Rhinopithecus roxellana/ China		KT344825
	Trachypithecus francoisi/ China		KT186234
Trichuris trichiura	*Homo sapiens*/Cameroon		GQ301555
Trichuris suis	*Sus scrofa*/China		AM992999
Trichuris vulpis	*Canis lupus familiaris*/Spain		AM234616
Trichinella spiralis	USA		KC006415
Outgroup mitochondrial markers			
Trichinella spiralis	USA		AF293969
Trichinella pseudospiralis	Australia		KM357411
Outgroup ribosomal marker			
Trichinella spiralis	USA		KC006415

species and *Trichuris* sp. from *H. cristata*, additional sequences from the National Centre for Biotechnology Information (NCBI) GenBank ® database were incorporated into alignments (Table 2).

To evaluate similarity between all *Trichuris* sp. marker sequences obtained with other *Trichuris* species, the number of nucleotide differences per sequence was analyzed using Compute Pairwise Distances as a function of the number of differences method of MEGA X v10.1.8 (Kumar et al., 2018).

The nucleotide sequence alignment files were obtained using MUSCLE alignment method in MEGA X (Kumar et al., 2018).

Phylogenetic trees were deduced based on nucleotide data and produced by two methods: Maximum Likelihood (ML) and Bayesian Inference (BI). To generate ML trees was used PHYML package (Guindon and Gascuel, 2003), and to generate BI, MrBayes v.3.2.6 (Ronquist and Huelsenbeck, 2003). jModelTest (Posada, 2008) was used to determinate the best-fit substitution model for the parasite data. Models of evolution were chosen for subsequent analysis according to the Akaike information criterion (Huelsenbeck and Rannala, 1997; Posada and Buckley, 2004). To investigate the dataset containing the concatenation of three mitochondrial markers (*cox*1, *cob* and *rrn*L), analyses based on BI were partitioned by gene and models for individual genes

within partitions were those selected by jModeltest. For ML inference, best-fit nucleotide substitution models included a general time-reversible model with gamma-distributed rate variation and a proportion of invariable sites GTR + G + I (ITS1, *cox*1, *cob* and *rrn*L). Support for the topology was examined using bootstrapping (heuristic option) Felsenstein (1985) over 1000 replications to assess the relative reliability of clades. The commands used in MrBayes, version 3.2.6 for BI were nst = 6 with invgamma rates (ITS1, *cox*1, *cob* and *rrn*L). For BI, the standard deviation of split frequencies was used to determine whether the number of generations completed was sufficient; the chain was sampled every 500 generations and each dataset was run for 10 million generations. Trees from the first million generations were discarded based on an assessment of convergence. Burn-in was determined empirically by examination of the log likelihood values of the chains. The Bayesian posterior probabilities (BPP) comprise the percentage converted.

3. Results

3.1. Morphological and biometrical results

General: Cuticle with fine transversal striation, body divides into two parts, characteristic of the *Trichuris* genus: anterior part of body long, narrow, tapered, where bacillary band is located, whip-like; posterior part of body broad, handle-like (Fig. 1A). The transition of the thin to thick portion of the body occurs at esophagus-intestinal junction (Fig. 1A, Fig. 2A, arrowed). There is a stichosome with one row of stichocytes, observed internally and externally a bacillary band. In addition, it showed cervical alae (like cuticular inflammations) in a thinner portion on the ventrolateral face (Fig. 1B, Fig. 2B and C).

Male: Body length was 35.6 (22.0–41.0) mm, esophagus length: 21.6 (13.0–26.0) mm, ratio anterior to posterior part was (1.4–1.7):1 (Table 3). The maximum width of the body in the esophageal region was 0.16 (0.11–0.20) mm and the maximum width in the posterior part of the body was 0.67 (0.30–0.81) mm (Table 3). Cervical alae length was 0.017 (0.014–0.021) mm, and the width was 0.008 (0.006–0.011) mm (Fig. 1B, Fig. 2B). Distance from the head end to beginning of bacillary stripes: 0.28 (0.21–0.38) mm (Table 3, Fig. 2C). Length of bacillary stripes: 3.13 (2.64–3.46) mm. Posterior end of the body ventrally incurved (Figs. 1A, C, Fig. 2D). The genital apparatus of the male is a long tube whose sections differ from each other in structure bearing different functions (Fig. 1C, Fig. 2D). The first section of the genital apparatus is the testis, which is very long and strongly convoluted, beginning in the posterior part of the male body, directed anteriorly, and lying along the long axis of the body terminating at a short distance from the transition of the esophagus into the intestine (Fig. 1A). The testis ends near the union of the ejaculator conduct and intestine. The testis is followed by the vas deferens. The seminal vesicle runs parallel to the intestine but does not describe sharp convolutions and via a narrow tube with thick muscular walls joins the ejaculatory duct, which was 2.52 (2.08–3.14) mm in length (Fig. 1A, Fig. 2D). The ejaculatory duct joins the intestine to form the cloaca, which opens at the posterior end of the male body. The cloaca presents anus subterminal. No paracloacal papilla was observed (Figs. 1A, Fig. 2D). The proximal cloacal tube was wide and continued with the distal cloacal tube measuring 2.76 (1.70–3.38) mm, that contains the spicule which projects into the anterior portion of the body in a spicule tube (Fig.1A, Fig. 2D). There is single spicule, which is elongated, slender, attenuated at proximal end forming a knob, slightly narrowed distally, funnel shaped, straight tip. (Fig. 1C, Fig. 2D, 2E). The length of spicule was 3.4 (2.99–4.00) mm. The width of proximal end of spicule was 0.05 (0.02–0.11) mm. Spicule sheath is tubular, the surface covered by regular spine, its shorter and less dense at proximal part (Fig. 1C, 2F), length of spicular sheath was 0.33 (0.16–044) mm and the width was 0.11 (0.10–0.12) mm.

Female: Body length was 55.2 (50.0–61.0) mm, esophagus length was 35.8 (30.0–40.0) mm. The ratio between the esophageal part and the posterior part was (1.5–1.9):1 (Table 4). The width of the esophageal region was 0.20 (0.17–0.25) mm, and the maximum width (in the posterior part of the body) 0.84 (0.60–0.91) mm. Cervical alae length was 0.023 (0.020–0.027) mm, and the width was 0.008 (0.006–0.011) mm (Fig. 1B, Fig. 2B). The distance from the head end to beginning of bacillary stripes was 0.23 (0.20–0.27) mm. The length of bacillary stripes was 3.64 (2.80–4.51) mm (Table 4). The vulva is non-protrusive and has rugose cuticle (Figs. 1D, Figure 2G, 2H). Diameter of vulva turned over the surface of body was 0.08 (0.06–0.11) mm (Table 4, Fig. 2G, 2H). The vagina has strong walls, and, when everted, does not protrude appreciably (Fig. 1D, 2G). This vagina is long, measuring 1.76 (1.29–2.87) mm in length, near vulva, the vagina becomes straight and gradually expands forming a vast egg reservoir, and presents circumvolutions nearly the uterus (Figs. 1D, 2G). The ovary is long and continues with the oviduct in the back of the body. Posterior end bluntly rounded, anus subterminal (Fig. 1E, 2I).

3.2. Molecular study

3.2.1. Annotation and features of mitochondrial and ribosomal genomes

The *cox*1, *cob* and *rrn*L mtDNA partial genes and ITS1 rDNA partial region were amplified and sequenced from 30 individuals of *Trichuris* sp. from *H. cristata* and two different haplotypes were found (*Trichuris* sp. from *H. cristata* haplotype 1 (THC1) and *Trichuris* sp. from *H. cristata* haplotype 2 (THC2)). *Cox*1 sequences were 370 bp in length and their G + C content was 34.32–34.86%. *Cob* sequences were 520 bp in length and their G + C content was 25–27.12%. *Rrn*L sequences were 360 bp to THC1 and 373 bp to THC2 in length and their G + C content was 23.06–23.86%. ITS1 rDNA partial sequences were 550 and 552 bp in length from THC1 and THC2, respectively. Their G + C content was 61.23–61.45%. Nucleotide sequence data reported in this study were deposited at the GenBank, EMBL, and DDBJ databases, and the accession numbers are available in Table 2.

3.2.2. Phylogenetic analysis

Sequences of mitochondrial and ribosomal datasets revealed the existence of two haplotypes (THC1 and THC2) presents in *Trichuris* population of *H. cristata* from Spain (Figs. 3 and 4 and Supplementary Figures S1-S2).

The multiple alignment of 25 *cox*1 nucleotide sequences (including outgroups) yielded a dataset of 291 characters. The similarity between the two *Trichuris* haplotypes sequences from *H. cristata* from Spain found in this paper was 86.25%. When we compared both populations of porcupine from Spain with *Trichuris* sp. from *H. cristata* from Italy (TIS1), THC1 sequence shared the maximum inter-specific similarity (99.66%), while haplotype THC2 was 86.60% (Table 5). The lower inter-specific similarity of *Trichuris* species based on cox1 mtDNA sequences was 69.42% (*Trichuris* sp. from *H. cristata* (Italy) (TIS1) and THC1, with *Trichuris* sp. from *Trachypithecus francoisi*) (Table 5). Comparing between *Trichuris* spp. that differ in host, *Trichuris* sp. from *H. chacarius* and *A. azarae* and *T. bainae*, have the maximum similarity with THC1 (79.73%), and *Trichuris vulpis* Froelich, 1789 and *T. pardinasi* with THC2 (79.38%) (Table 5).

The multiple alignment of 22 *cob* nucleotide sequences (including outgroups) yielded a dataset of 432 characters. The similarity between the two *Trichuris* haplotypes sequences from *H. cristata* from Spain was 90.05%. THC2 and THC1 sequences shared the maximum inter-specific similarity with *Trichuris* sp. from *H. cristata* sequence (TIS8) from Italy (91.20% and 89.12%, respectively) (Table 6).

The multiple alignment of 12 *rrn*L nucleotide sequences (including outgroups) yielded a dataset of 397 characters. The inter-population similarity between the two *Trichuris* haplotypes sequences from *H. cristata* was 91.18% (Table 7). THC1 and THC2 sequences shared the maximum inter-specific similarity with that of *T. discolor* (82.87%) and *T. suis* (83.12%), respectively (Table 7).

The multiple alignment of 20 ITS1 nucleotide sequences (including

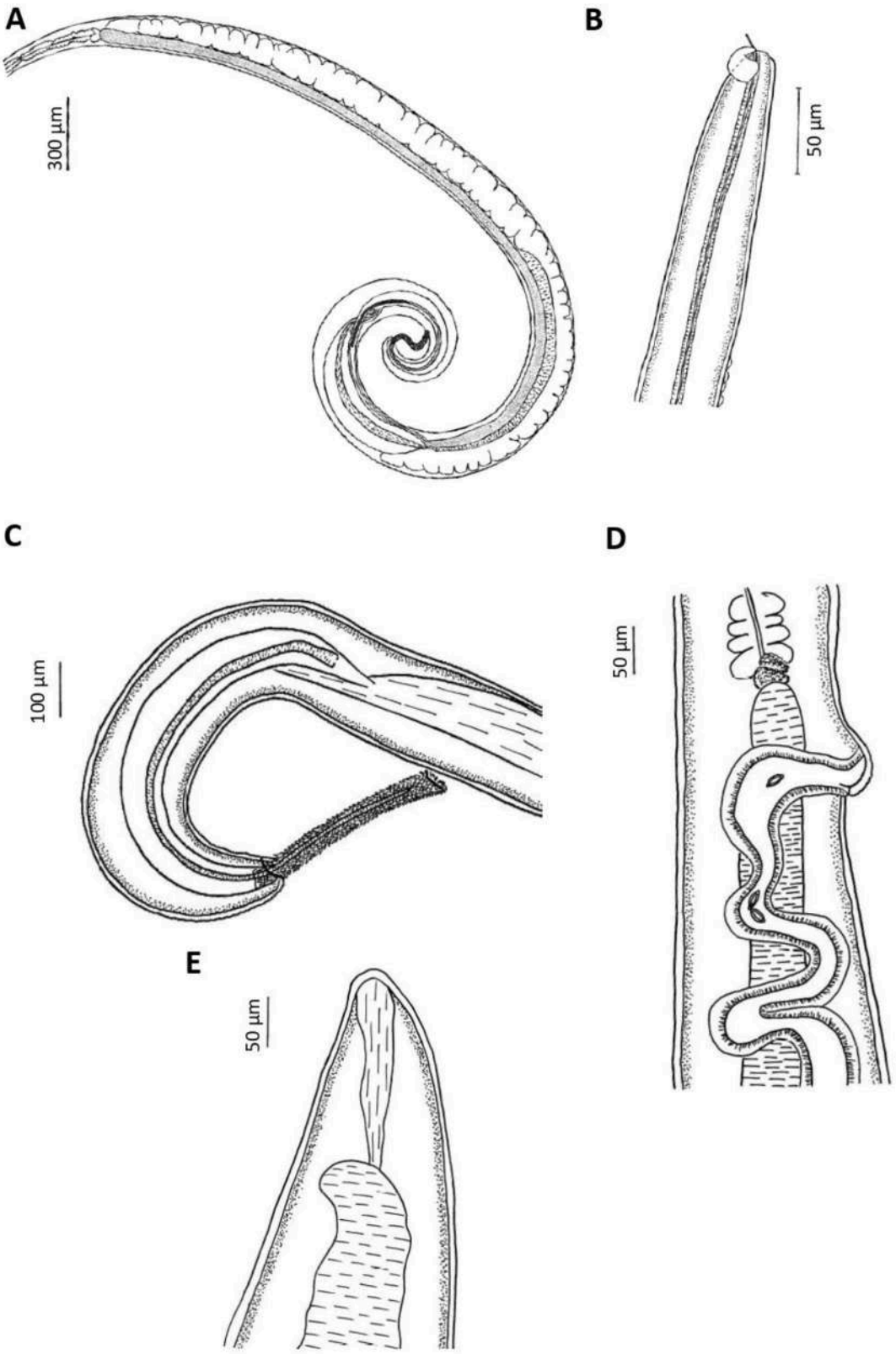

Fig. 1. Drawings of *Trichuris* sp. from *Hystrix cristata*. A. Male, posterior end, spiny spicule sheath, spicule, spicule tube and proximal and distal cloacal tube, lateral view. B. General, anterior part with cervical alae, lateral view. C. Male, detail of the posterior end, lateral view. D. Female, esophagus-intestine junction, vulva and vagina, lateral view. E. Female, posterior end, lateral view.

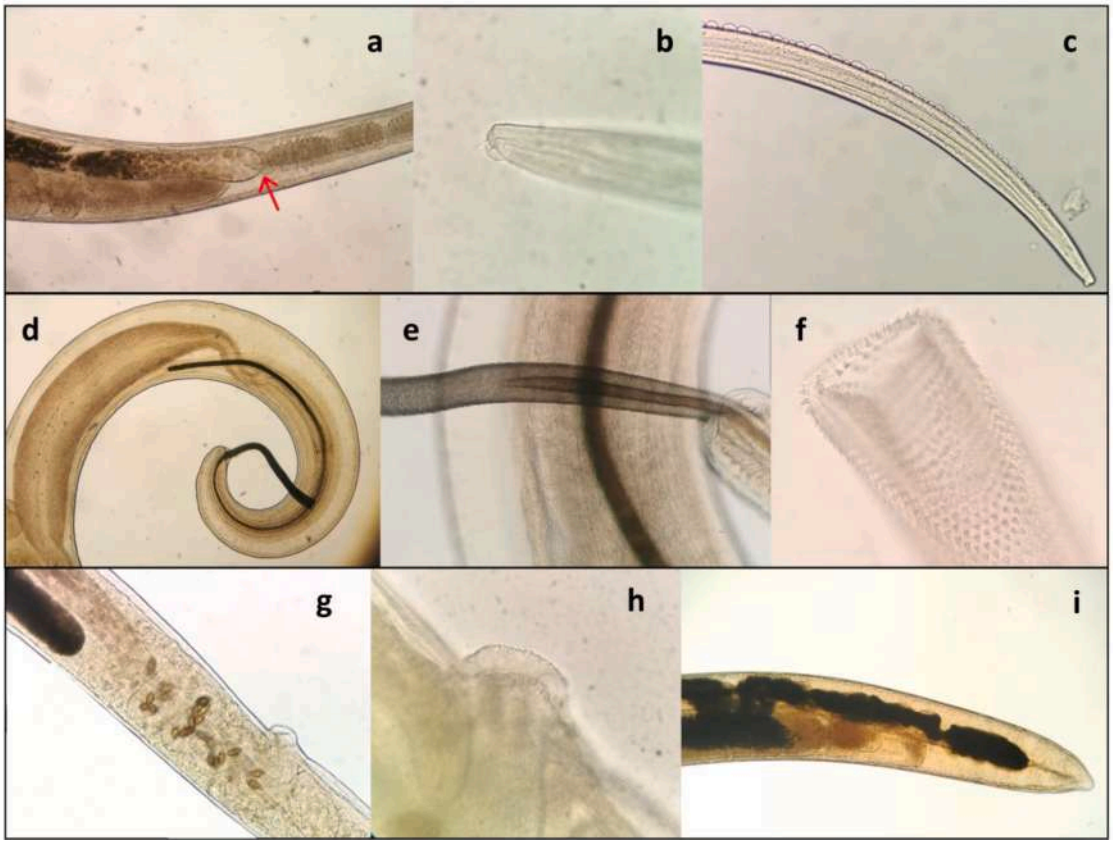

Fig. 2. Morphology of *Trichuris* sp. from *Hystrix cristata*. A. Male, esophagus-intestine junction. B. Cervical alae. C. Bacillary band showing typical cuticular inflations. D. Male, posterior end, spiny spicule sheath, spicule, spicule tube and proximal and distal cloacal tube. E. Male, spicule and spicule sheath. F. Male, spicule sheath. G. Female, vagina and vulva. H. Female, vulva. I. Female, posterior end.

outgroups) yielded a dataset of 1110 characters. The inter-population similarity between the two Trichuris haplotypes sequences from H. cristata was 99.91% (Table 8). The maximum similarity between Trichuris sp. from H. cristata (present study) and the different Trichuris species was 87.03% (both haplotypes) with T. vulpis, and the minimum was 80.63–80.90% for THC1 and THC2 with T. myocastoris, respectively (Table 8).

The best-fit model for all mtDNA datasets (*cox*1, *co*b and *rrn*L) determined by jModelTest was GTR + G + I that was used for ML and for BI. Phylogenetic trees based on mitochondrial (cox1, cob and rrnL) markers (partitioned and concatenated) confirmed the existence of two main groups within genus *Trichuris*: group 1, including *Trichuris* species parasitizing herbivorous, while group 2 clustered Trichuris species parasitizing omnivores (suids, primates (including human) and rodents). Within these two groups, according to interspecific similarity, there were 5 clades with similar percentages of similarity; clade 1 including *Trichuris* species from *H. cristata* (Rodentia: Hystricidae); clade 2, including *Trichuris* species from rodents and canids (Rodentia: Ctenomyidae, Sigmodontinae, Muridae; Carnivora: Canidae); clade 3, including including *Trichuris* species from *Homo sapiens* and non-human primates (NHP); clade 4, *Trichuris* species from suids and NHP (*T. suis, Trichuris ursinus* and *Trichuris colobae*); clade 5, including *Trichuris* species from herbivorous (*T. ovis, T. discolor, Trichuris skrjabini* and *Trichuris leporis*) (Figs. 3 and 4 and Supplementary Figures S1-S2).

Since the trees obtained by separate analyses of both mitochondrial genes do not show enough resolution supporting the relationships between *Trichuris* spp. from *H. cristata* (clade 1) with other *Trichuris* species, thus, we present the analysis of mitochondrial concatenated sequences (*cox*1 (Fig. 3A, *co*b (Fig. 3B) and *rrn*L (Supplementary Figure S1)) that included 1112 aligned sites and 12 taxa, including outgroups.

The phylogeny of *Trichuris* inferred using the BI and ML methods based on concatenated mitochondrial genes revealed similar results (Fig.4A). The comparison of BI and ML test revealed no significant differences between their topologies. The general structure of *Trichuris* tree shows a monophyletic lineage for the genus (Fig. 4A) separated of *Trichinella* spp. (outgroups). The five main clades are recovered within genus *Trichuris* strongly supported. The *Trichuris* sequences from *H. cristata* were clustered within clade 1 [sequences from Spain (present study)] and separately from the rest of the sequences with absolute support. A sister relationship between *Trichuris* spp. from Rodentia (clade 1 and clade 2) are strongly supported (Fig. 4A). Furthermore, both clades were clustered with clades 3 and 4 including *Trichuris* spp. from human, suids and NHP, respectively and separated of clade 5 including *Trichuris* species from herbivorous (see Fig. 4A).

The best-fit model for ITS1 rDNA datasets determined by jModelTest was GTR + G + I that was used for ML and BI. This phylogenetic tree revealed two clades highly support. Clade 1 clustered *Trichuris* from *H. cristata*, and clade 2 clustered all the *Trichuris* sequences from different hosts. ML inferences based on ribosomal rDNA revealed a basal position within the genus (99%). Nevertheless, resolution based on BPP methods is very poor (Supplementary material Figure S2). ITS1 dataset is not able to resolve questions about relationships of the different groups within *Trichuris* genus.

The concatenated dataset of mtDNA sequences (*cox*1, *co*b and *rrn*L) and rDNA sequences (ITS1) included 2230 aligned sites and 11 taxa, including outgroup. As in the phylogenetic analysis of mtDNA concatenated, the different clades were strongly supported by ML method (>99 % BV), but on the other hand, the BI method did not resolve. The sequences of *Trichuris* from *H. cristata* (present study) clustered with T.

Table 3
Comparative biometrical measurements between different adult males of *Trichuris* spp. from *Hystrix* spp.

	Trichuris hystricis from *Hystrix cristata* (Kreis, 1938)			*Trichuris hystricis* from *Hystrix indica* (Yousefi et al., 2010)			*Trichuris landak* from *Hystrix javanica* (Purwaningsih, 2013)			*Trichuris lenkorani* (male holotype) from *Hystrix hirsutrirostris* (Petrov and Sadikhov, 1961)			*Trichuris mettami* from *Atelerix* or *Aethechinus* sp. (Baylis, 1935)			*Trichuris* sp. from *Hystrix cristata* (Present authors, 2021)		
	MAX	MIN	X	MAX	MIN	X	MAX	MIN	X	MAX	MIN	X	MAX	MIN	X	MAX	MIN	X	σ
M1	45.70	39.40	-	27.4	23.8	-	42.6	36.0	39.3	17.0	14.0	15.5	38	27	32.5	41.0	22.0	35.6	0.56
M2	-	-	-	-	-	-	23.6	24.1	22.4	-	-		22	16	19	26.0	13.0	21.6	0.60
LP	-	-	-	-	-	-	19.0	11.9	15.45	-	-					17.0	11.0	14.0	0.20
M3	0.405	0.342	0.374	-	-	-	-	-	-	-	-					0.20	0.11	0.16	0.02
M4	0.683	0.595	0.639	-	-	-	0.77	0.71	0.74	-	-		0.7	0.6	0.65	0.81	0.30	0.67	0.12
M5	0.405	0.342	0.374	-	-	-	0.29	0.28	0.29	-	-		0.35	0.30	0.32	0.35	0.29	0.32	0.17
M6	-	-	-	-	-	-	-	-	-	-	-					0.38	0.21	0.28	0.05
M7	-	-	-	0.09	0.06	-	-	-	-	-	-					3.46	2.64	3.13	0.26
M8	1.212	0.896	1.054	1.98	1.87	-	4.7	3.3	4.0	-	-		3.8	3.5	3.65	4.00	2.99	3.39	0.30
M9	-	-	-	-	-	0.76	0.47	0.33	0.40	-	-		0.26	0.2	0.23	0.44	0.16	0.33	0.03
M10	0.030	0.017	0.024	-	-	-	0.05	0.04	0.04	-	-					0.11	0.02	0.05	0.02
M11	0.244	0.171	0.208	-	-	-	-	-	-	-	-					0.07	0.05	0.06	0.01
M12	-	-	0.085	-	-	-	0.05	0.04	0.04	-	-					0.12	0.10	0.11	0.02
M13	-	-	-	-	-	-	-	-	-	-	-	-	4.9	4.2	4.55	3.60	1.85	2.93	0.45
M14	-	-	-	-	-	-	-	-	-	-	-	-	4.0	3.3	3.65	3.14	2.08	2.52	0.34
M15	-	-	-	-	-	-	-	-	-	-	-	-				3.38	1.70	2.76	0.53
M16	2.3	1.7	2	-	-	-	1.4	1.3	1.35	-	-	-				1.70	1.40	1.55	0.03
M17	-	-	-	-	-	-	0.023	0.019	0.021	-	-					0.021	0.014	0.017	0.043
M18	-	-	-	-	-	-	0.0094	0.0078	0.0086	-	-					0.011	0.0064	0.0084	0.02

muris sequence (Fig. 4B), while *Trichuris* sequences from primates and NHP were clustered together, *T. suis* and *T. colobae*, and *T. discolor* and *T. ovis* as well.

4. Discussion

In the present paper, we address a morphologic, biometric and molecular description of *Trichuris* sp. from *H. cristata*. In addition, based on morphological and biometric criteria, a comparative study was carried out with the results obtained in previous studies, performing a description of different *Trichuris* species isolated from close species of porcupine genus *Hystrix* (Yousefi et al., 2010; Torres et al., 2011). One of the objectives of the present study was differentiated the populations of *Trichuris* sp. from *H. cristata* from the other *Trichuris* spp. from porcupine by comparing the morphologic features and morphometric measurements of the adult worms. Similar approach was reported by Purwaningsih, (2013) in the discovery of *T. landak* n. sp. from *H. javanica* based on morphological and biometrical characters and Robles et al. (2011) in the discovery of *Trichuris navonae* n. sp. from forest-dwelling mice in Argentina.

Thus, for the description of *Trichuris* sp. from *H. cristata*, we have compared with the *Trichuris* spp. described previously by different authors for porcupine: *T. landak* from *H. javanica, T. hystricis* from *H. cristata* and *Hystrix indica, T. thrichomysi* from *Thrichomysi apereoides, T. infundibulum* from *H. cristata, Trichuris metami* Baylis, 1935 from *Atelerix* or *Aethehinus, Trichuris lenkorani* from *Hystrix hirsutrirostris* (Petrov and Sadikhov, 1961). In addition, others *Trichuris* spp. have been included for the comparative study.

Trichuris sp. from *H. cristata* showed a short lateral alae and stylet, and similar characters were found in *T. landak* (Purwaningsih, 2013). The presence of a short lateral alae and/or stylet was cited in other *Trichuris* species such as *T. muris, T. ovis* and *Trichuris parvispicularis* Skrjabin (1957). Nevertheless, both morphological characters have not showed a diagnostic value to differentiate whipworm species.

The male reproductive system of *Trichuris* sp. from *H. cristata* showed a general morphological pattern. In fact, identification of closely related species is very difficult. Thus, males showed a spicule sheath and spicule similar to *T. landak*, except because *T. landak* is characterized by a spicule with a conical tip, and some specimens have sheath without bulb and others have tubular sheath with bulb and the males analyzed in the present study showed a sheath without bulb Purwaningsih (2013)

The comparative study between other populations of *Trichuris* from porcupine of the genus *Hystrix* (*H. cristata, H. indica* and *H. javanica*) and *Atelerix* revealed males of *Trichuris* sp. and *T. hystricis* from *H. cristata* have in common the appearance of the tip of the spicule with a rounded shape. Nevertheless, *T. mettami* have a conical tip of the spicule. Females of *Trichuris* sp. from porcupine showed a general morphological pattern. In fact, the overlapped of some morphological characteristics between the individual species proved to be high that they were not useful for interspecific differentiation. This is partly due to the phenotypic plasticity of the organisms themselves, host-induced variation, the paucity of morphological features, and the extensive overlap in morphometric characteristics that occur among species (Spakulová, 1994; Knight, 1984; Cutillas et al., 1995; Robles et al., 2006). For biometrical report, only previously reported porcupine species of *Trichuris* were analyzed: *T. hystricis, T. landak, T. lenkorani* and *T. mettami* (Tables 3 and 4). Our population revealed a high concordance with *T. landak*, since most of the values overlap within the range of defined measurements except the total body length of females that was showed slightly shorter in *T. landak* (Purwaningsih, 2013). The lack of many biometric characters studied for others *Trichuris* species for porcupine limited the comparative study to few characters. Thus, based on those characters, *T. hystricis* from *H. indica, T. lenkorani* and *T. mettami* differ of *Trichuris* sp. from *H. cristata* (present study) in a maximum and minimum total body length that were considerably shorter (Table 3 and 4).

Furthermore, the analysis of additional biometrical characters

Table 4
Comparative biometrical measurements between different adult females of *Trichuris* spp. from *Hystrix* spp.

	Trichuris hystricis from *Hystrix cristata* (Kreis, 1938)			*Trichuris hystricis* from *Hystrix indica* (Youseffi el al., 2010)			*Trichuris landak* from *Hystrix javanica* (Purwaningsih, 2013)			*Trichuris lenkorani* (female holotype) from *Thystrix hirsutrirostris* (Petrov and Sadikhov, 1961)			*Trichuris mettami* from *Atelerix* or *Aethechinus* sp. (Baylis, 1935)			*Trichuris* sp. from *Hystrix cristata* (Present authors, 2021)			
	MAX	MIN	X	MAX	MIN	X	MAX	MIN	X	MAX	MIN	X	MAX	MIN	X	MAX	MIN	X	σ
F1	49.5	47.2	-	35.9	32.9	-	41.9	36.0	36.4	28.0	20.0	24.0	47.0	36.0		61.0	50.0	55.2	0.34
F2	-	-	-	15.3	12.3	-	23.8	22.2	23.1	-	-		29	24		40.0	30.0	35.8	0.35
LP	-	-	-	-	-	-	-	-	-	-	-					23.0	13.0	19.4	0.26
F3	0.49	0.32	-	0.50	0.45	-	-	-	-	-	-		0.8	0.76		0.25	0.17	0.20	0.02
F4	0.94	0.79	-	-	-	-	0.810	0.800	0.805	-	-		0.44	0.39		0.91	0.60	0.84	0.09
F5	-	-	-	-	-	-	0.310	0.300	0.305	-	-					0.44	0.36	0.39	0.03
F6	-	-	-	-	-	-	-	-	-	-	-	-				0.27	0.20	0.23	0.02
F7	-	-	-	-	-	-	-	-	-	-	-	-				4.51	2.80	3.64	0.61
F8	-	-	-	-	-	-	-	-	-	-	-	-				2.87	1.29	1.76	0.48
F9	0.51	0.36	-	-	-	-	-	-	-	-	-	-			0.15	0.11	0.06	0.08	0.02
F10	-	-	-	-	-	-	-	-	-	-	-	-				0.53	0.32	0.43	0.07
F11	-	-	-	-	-	-	-	-	-	-	-	-				0.67	0.35	0.46	0.09
F12	-	-	-	-	-	-	-	-	-	-	-	-				2.70	1.14	1.55	0.54
F13	-	-	-	-	-	-	-	-	-	-	-	-				1.35	0.99	1.12	0.12
F14	2	1.3	-	-	-	-	1.7	1.5	-	-	-	-				1.9	1.5	-	-
F15	-	-	-	-	-	-	0.023	0.023	0.023	-	-					0.027	0.020	0.023	0.03
F16	-	-	-	-	-	-	-	-	-	-	-					0.011	0.006	0.008	0.02

comparing males of *Trichuris* sp. and *T. hystricis* from *H. cristata*, revealed that width of the esophageal region of the body, body width in the place of the junction of the esophagus and the intestine, width of the spicule sheath at the tail end of the body, ratio between the length of the posterior part of body and that of the anterior, were higher for *T. hystricis*. Nevertheless, length of spicule was considerably shorter (0.89 mm-1.21 mm) (Table 3). On the other hand, the females showed a width of the esophageal region of the body and the diameter of vulva when turned over the surface of the body, hardly higher (Table 4).

Spakulová (1994) used a discriminant analysis as a method for the numerical evaluation of taxonomic characters in male whipworms. The length and width of the spicule and body length proved the best diagnostic characters for the trichurid males studied. Knight (1971) found that spicule length is the most dependable character in species differentiation and, the others morphological characters are more variable. Therefore, considering the greater morphological and biometrical similarity between *T. landak* and the populations of *Trichuris* sp. analyzed in the present study with a similar size of the spicule, clearly larger than those of other species of whipworms from porcupine, we suggest our population correspond with *T. landak*. From a molecular point of view, we amplified molecular markers which have been used previously by different authors to resolve species-level questions in *Trichuris* including the ITS1 nuclear regions (Cutillas et al., 2002, 2004, 2007; Oliveros et al., 2000; Callejón et al., 2012; Salaba et al., 2013; Cavallero et al., 2015; Robles et al., 2014), *rrn*L mtDNA partial gene (Callejón et al., 2012; Cavallero et al., 2019; Rivero et al., 2020b), and protein-coding mitochondrial genes, including *cox*1 mtDNA partial gene (Callejón et al., 2012, 2013, 2017; Doležalová et al., 2015; Cavallero et al., 2019; Rivero et al., 2020a, 2020b) and *cob* mtDNA partial gene (Callejón et al., 2015a, 2015b, 2016, 2017; Cavallero et al., 2015; Rivero et al., 2020a, 2020b). The lack of molecular data corresponding to *Trichuris* species that parasitize the porcupine (genus *Hystrix* and *Atelerix*) has not allowed a comparative molecular study or a phylogenetic study of different species. Only a partial sequence of the cox1 gene corresponding to the individual named TIS1 and *cob* gene corresponding to the individual named TIS8 isolated from *Trichuris* sp. of *H. cristata* from Italy, has been possible to add (Genbank Nulceotide sequences, Cavallero et al., 2019 (unpublished)).

Thus, the value observed between *Trichuris* sp. from *H. cristata* from Spain and Italy are within the range of inter-specific variation reported for the same genus suggesting that both populations could correspond with the same species and, hence, based on morphological and biometrical characters could be correspond with *T. landak*.

On the other hand, taking into account the inter-specific similarity observed in the genus *Trichuris* based on *cox*1, *cob* and *rrn*L mtDNA partial genes and ITS1 rDNA, the values observed between *Trichuris* sp. from *H. cristata* from Spain and *Trichuris* spp. were within the range of inter-specific variation reported for the genus suggesting that *Trichuris* sp. from *H. cristata* must be considered a different new species of *Trichuris* (Tables 5–8) (Callejón et al., 2013, 2016; Rivero et al., 2020a, 2020b). *Cox*1 and *cob* mtDNA partial gene sequences revealed the existence of two haplotypes (THC1 and THC2) present in the *Trichuris* population of *H. cristata* from Spain that did not correlate with two different morphospecies (Tables 5–6). Considering the percentages of intra-population similarity of *Trichuris* sp. of *H. cristata* from Spain and Italy, it could suggest the possibility of the existence of different haplotypes in the Italian population since the individual TIS1 showed a high percentage of similarity with the THC1 haplotype of the Spanish population and, based on the *cob* gene, the individual TIS8 from Italy showed a high percentage of similarity with the THC2 haplotype from Spain. However, for the confirmation of the existence of different haplotypes in *Trichuris* sp. of *H. cristata* from Italy, the number of individuals and additional molecular markers should be increased. This fact would agree with Rivero et al. (2020a) who found the presence of two different haplotypes on *T. trichiura* from *Macaca sylvanus* corresponding with two different genotypes. Furthermore, Ghai et al. (2014), who found that the host range of *Trichuris* sp. varies by taxonomic group, with some groups showing host specificity and others were showing host generality. For this reason, these authors observed that one group was specific to humans, another had an intermediate host, and an additional group could infect all the primates sampled, including humans. In addition, Ravasi et al. (2012) found two different genotypes of *Trichuris* sp. of *P. ursinus* from two different geographical locations, but they did not carry out a morphological study to characterize different morphospecies.

In previous phylogenetic studies of *Trichuris* based on ribosomal or mitochondrial genes, certain relationships have been consistently supported. Thus, the phylogenetic inferences for genus *Trichuris* revealed the existence of several clades clustering species according to the nutritional requirements of their hosts (Callejón et al., 2010, 2013, 2015a, 2017; Robles et al., 2014; Ravasi et al., 2012; Nissen et al., 2012, Cutillas et al., 2004, 2007; Rivero et al., 2020a, 2020b).

In the present study, phylogenetic analyses based on partitioned and combined mitochondrial genes supported all these afore mentioned

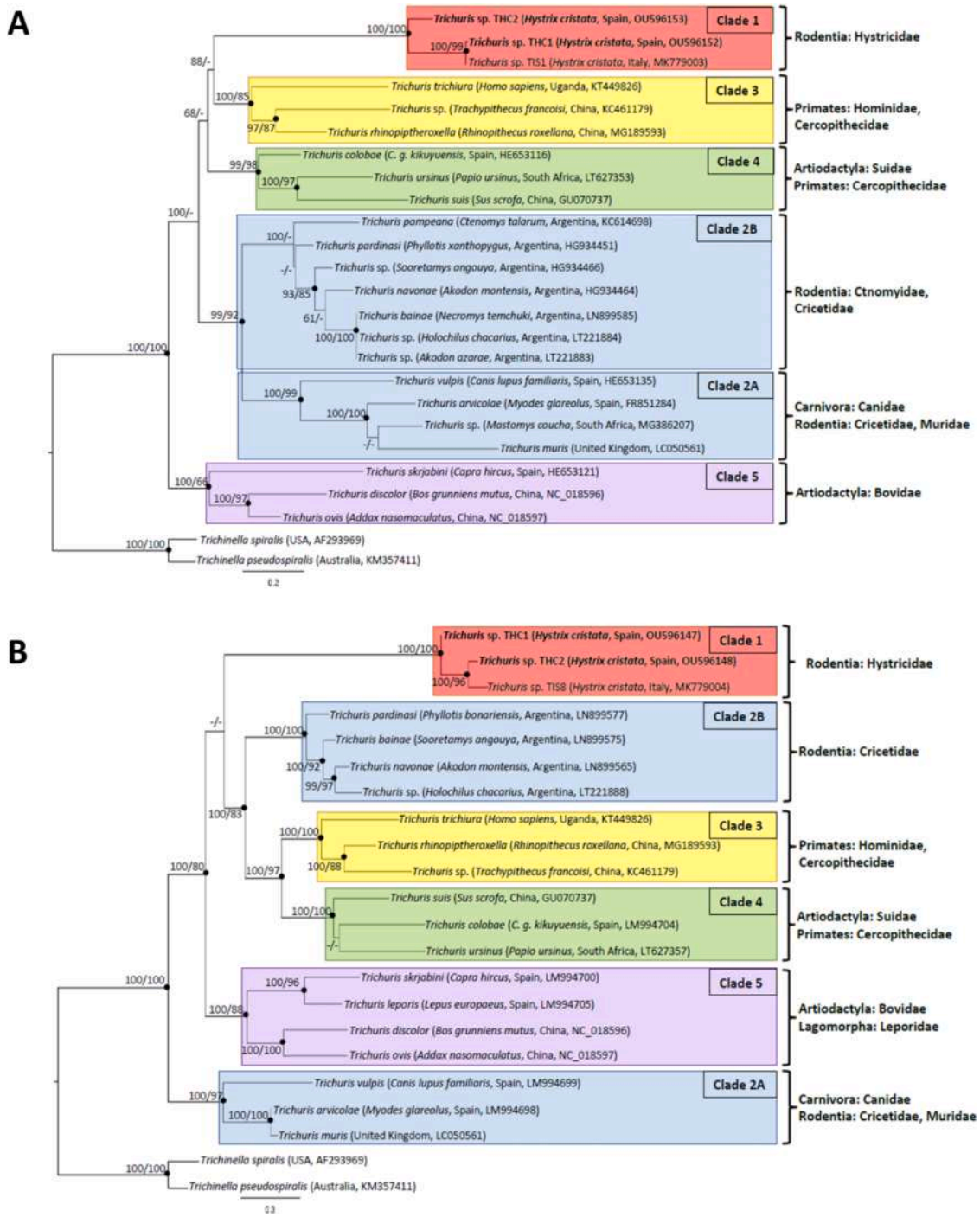

Fig. 3. A. Phylogenetic tree of *Trichuris* species based on *cox*1 mtDNA sequences inferred using Maximum Likelihood. **B.** Phylogenetic tree of *Trichuris* species based on *cob* mtDNA sequences inferred using Maximum Likelihood. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

clades (clade 2–5) (Fig. 3A-B, and Supplementary Figure S1). Thus, *Trichuris* sp. from *H. cristata* formed a separated clade (clade1) related to clade 2 including *Trichuris* species from rodents and canids. Nevertheless, ITS1 inferences showed a poor resolution between clades. This result could be since the ITS regions were saturated and showed poor nucleotide diversity. Therefore, to infer the relationships between different genetic lineages within genus *Trichuris* were delimited exclusively based on analysis of the mitochondrial genes in agreement with Rivero et al. (2020b) and Chan et al. (2020), who evaluated the utility of mitochondrial and ribosomal genes for molecular systematics of *Trichuris* and parasitic nematodes respectively. These authors reported ITS regions accumulated substitutions substantially more slowly than mtDNA and showed nucleotide saturation.

From the point of view of medical implication as consequence of the advance in the taxonomy of *Trichuris* species, the study of parasitic species of trichuriasis in zoonotic hosts is important in tropical parasitology. Even though there seems to be a pattern of infection with different *Trichuris* species infecting particular host species, there are several issues that need to be further explored (Betson et al., 2015). Are the different *Trichuris* species specific to host species? It has been suggested that humans also occasionally can be infected by *T. vulpis* and *T. suis*, and therefore these are considered zoonotic species (Areekul

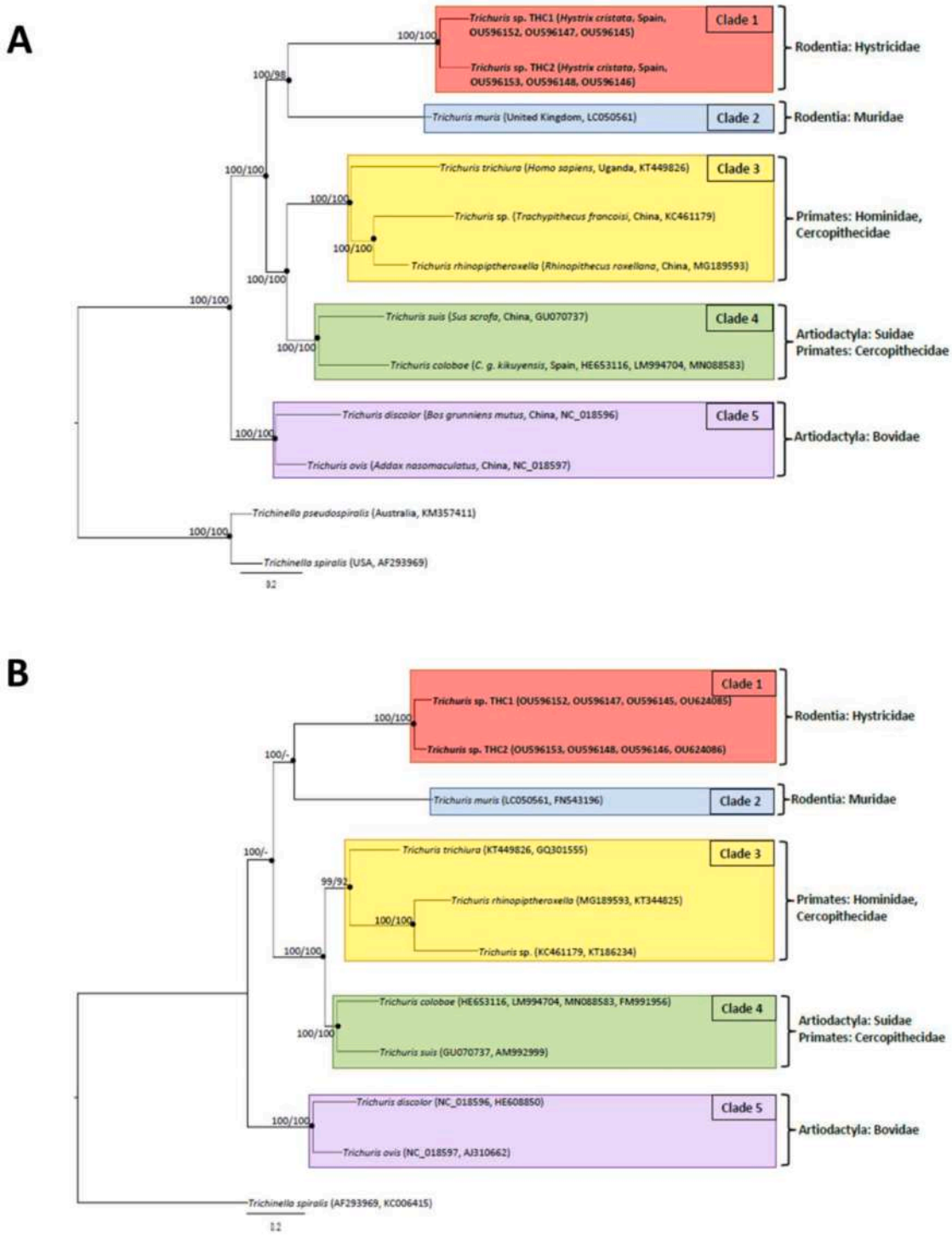

Fig. 4. **A**. Phylogenetic tree of *Trichuris* species based on combined analysis of mtDNA (*cox*1, *cob* and *rrn*L) inferred using Maximum Likelihood. **B.** Phylogenetic tree of *Trichuris* species based on combined analysis of mtDNA (*cox*1, *cob* and *rrn*L) and nuclear rDNA (ITS1) inferred using Maximum Likelihood. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

et al., 2010; Nissen et al., 2012; Ravasi et al., 2012).

Cantlay et al. (2017) carried out a review of zoonotic infection risks associated with the wild meat trade in Malaysia. These authors indicated that two wildlife taxa appear to harbor very few zoonotic pathogens, Manidae (zero) and Hystricidae (one), related to the deficiency of published studies on these taxa, which may lead to an underestimate of their zoonotic infection potential. This lack of data could be attributed to the difficulty of observing these animals in their environment due to their size and reserved behavior. Further research is required to determine whether Hystricidae species (Order: Rodentia) harbor more zoonoses, since surveys of other rodents have shown they can harbor several viruses and bacteria from human and other mammals (Easterbrook et al., 2007; Firth et al., 2014). Hence, the potential threat of other *Trichuris* species such as *Trichuris* spp. infecting porcupine to human population is a health concern especially among rural folks whose environment is surrounded by zoonotic hosts.

Table 5
Intra-specific and inter-specific similarity observed in *cox*1 partial sequences in *Trichuris* species isolated from different host species.

*Cox*1	*THC1*	*THC2*	*Trichurissp.TIS1 (H.cristata)*	*T.muris*	*T.suis*	*T.trichiura*	*T.colobae*	*Trichurissp.(T.francoisi)*	*T.rhinopiptheroxella*	*T.ovis*	*T.discolor*	*T.skrjabini*	*T.vulpis*	*T.arvicolae*	*T.pardinasi*	*Trihurissp.(S.angouya)*	*Trichurissp.(H.chacarius)*	*T.ursinus*	*T.navonae*	*T.pampeana*	*Trichurissp.(A.azarae)*	*Trichurissp.(M.coucha)*
THC2	86.25																					
Trichuris sp. TIS1 (H. cristata)	99.66	86.60																				
T. muris	75.95	74.91																				
T. suis	74.57	76.98	74.57	74.23																		
T. trichiura	76.63	76.63	76.63	70.45	77.66																	
T. colobae	73.88	74.91	73.88	72.51	80.41	76.29																
Trichuris sp. (T. francoisi)	69.42	73.20	69.42	71.48	75.95	77.32	78.35															
T. rhinopiptheroxella	76.29	79.04	76.29	78.00	78.35	79.73	81.10	79.38														
T. ovis	70.79	75.26	70.79	74.91	77.66	75.60	79.38	78.01	79.04													
T. discolor	73.20	73.54	73.20	72.51	75.26	75.26	76.98	75.60	77.32	85.22												
T. skrjabini	72.51	71.82	72.51	76.63	73.88	74.23	79.38	75.26	74.91	80.41	76.63											
T. vulpis	76.63	79.38	76.98	78.00	78.11	76.63	75.26	71.82	79.38	78.01	75.95	75.26										
T. arvicolae	76.98	75.26	76.98	81.79	78.35	75.60	74.91	71.13	78.69	75.60	72.16	76.98	82.13									
T. pardinasi	74.91	79.38	74.91	76.98	79.73	79.38	78.69	76.98	79.73	78.35	76.29	76.98	77.66	79.38								
Trichuris sp. (S. angouya)	74.91	75.60	74.91	74.91	77.66	79.04	78.35	77.32	78.00	79.04	76.29	75.26	78.35	78.69	89.35							
Trichuris sp. (H. chacarius)	79.73	77.32	79.73	76.29	75.60	78.01	77.32	74.23	78.35	78.01	75.60	75.95	79.73	78.35	85.57	89.69						
T. ursinus	76.63	76.98	76.63	72.16	82.13	75.94	82.47	76.63	76.98	78.01	76.98	74.91	77.32	75.95	81.44	79.04	77.66					
T. navonae	76.29	76.29	76.29	74.91	78.35	79.04	78.35	78.69	79.38	79.04	76.63	75.26	79.04	80.07	87.97	89.69	90.38	79.38				
T. pampeana	77.32	78.35	77.32	75.60	76.98	78.35	79.04	75.60	76.98	78.69	74.91	76.98	78.35	79.38	86.97	84.88	84.19	79.04	83.85			
Trichuris sp. (A. azarae)	79.73	77.32	79.73	76.29	75.60	78.01	77.32	74.23	78.35	78.01	75.60	75.95	79.73	78.35	85.57	89.69	100	77.66	90.38	84.19		
Trichuris sp. (M. coucha)	78.69	78.35	78.69	80.76	76.29	76.98	74.57	71.48	79.04	74.57	73.20	73.88	81.10	86.94	79.04	76.63	78.35	75.94	79.73	80.41	78.35	
Trichuris bainae	79.73	77.32	79.73	76.29	75.60	78.01	77.32	74.23	78.35	78.01	75.60	75.95	79.73	78.35	85.57	89.69	100	77.66	90.38	84.19	100	78.35

Table 6
Intra-specific and inter-specific similarity observed in *cob* partial sequences in *Trichuris* species isolated from different host species.

Cob	THC1	THC2	Trichuris sp. TIS8 (H. cristata)	T. muris	T. suis	T. trichiura	T. colobae	Trichuris sp. (T. francoisi)	T. rhinopp theroxella	T. ovis	T. discolor	T. skrjabini	T. vulpis	T. arvicolae	T. leporis	T. pardinasi	T. bainae	Trichuris sp. (H. chacarius)	T. ursinus
THC2	90.05																		
Trichuris sp. TIS8 (H. cristata)	89.12	91.20																	
T. muris	72.22	72.00	73.15																
T. suis	72.69	71.99	74.31	71.76															
T. trichiura	71.76	70.60	72.22	72.69	74.07														
T. colobae	71.30	69.44	70.60	71.06	77.78	72.22													
Trichuris sp. (T. francoisi)	68.52	67.59	67.82	68.06	74.54	75.46	70.83												
T. rhinopiptheroxella	70.83	68.98	70.14	74.22	78.70	78.94	75.00	80.09											
T. ovis	72.92	71.53	72.69	74.07	75.00	76.85	72.92	70.14	74.77										
T. discolor	74.77	72.45	72.45	74.07	71.76	71.76	70.37	69.44	73.84	79.86									
T. skrjabini	70.83	70.60	69.68	74.31	74.54	71.06	70.37	68.75	75.23	74.54	75.93								
T. vulpis	73.61	72.45	72.92	79.40	72.69	72.69	71.53	65.97	71.53	73.38	71.06	70.37							
T. arvicolae	72.92	72.22	72.92	96.99	71.30	73.61	71.06	68.98	71.76	74.54	74.31	73.84	80.56						
T. leporis	71.53	70.14	71.30	74.07	74.31	71.06	73.84	67.36	74.54	75.23	79.17	83.10	73.38	73.84					
T. pardinasi	72.22	69.44	70.60	75.69	75.23	74.54	72.45	72.69	74.31	72.69	73.15	75.00	73.15	75.23	74.54				
T. bainae	73.15	72.22	72.92	77.55	75.23	74.54	74.07	73.61	75.23	74.77	74.07	73.61	75.00	77.31	74.77	85.19			
Trichuris sp. (H. chacarius)	73.84	70.60	72.45	75.69	73.61	73.84	72.22	72.92	71.99	75.69	74.04	72.69	72.45	74.77	73.15	84.03	86.57		
T. ursinus	72.92	70.37	71.53	72.22	77.78	72.45	78.70	72.22	75.00	71.76	71.30	73.15	72.92	71.30	73.61	74.07	73.38	71.30	
T. navorae	73.61	71.76	73.84	77.31	75.96	75.23	73.61	71.76	75.93	75.00	75.46	76.62	73.38	76.62	75.93	84.95	89.35	88.43	72.69

Table 7
Intra-specific and inter-specific similarity observed in *rrn*L partial sequences in *Trichuris* species isolated from different host species.

RrnL	THC1	THC2	T. muris	T. suis	T. trichiura	T. colobae	Trichuris sp. (T. francoisi)	T. rhinopiptheroxella	T. ovis
THC2	91.18								
T. muris	79.60	80.86							
T. suis	82.12	83.12	84.38						
T. trichiura	77.83	77.08	81.36	81.11					
T. colobae	80.60	79.34	82.37	85.14	80.10				
Trichuris sp. (T. francoisi)	76.83	76.32	78.09	79.85	83.63	77.33			
T. rhinopiptheroxella	78.59	79.60	80.60	81.61	84.13	78.84	86.90		
T. ovis	80.60	81.11	81.11	81.86	79.09	83.88	76.07	76.83	
T. discolor	82.87	82.62	81.11	81.11	80.10	81.11	74.56	77.58	89.92

Table 8
Intra-specific and inter-specific similarity observed in ITS1 sequences in *Trichuris* species isolated from different host species.

ITS1	THC1	THC2	T. muris	T. suis	T. trichiura	T. colobae (N. gabriellae)	T. colobae (C. g. kikuyensis)	Trichuris sp. (T. francoisi)	T. rhinopitheroxella	T. ovis	T. discolor	T. skrjabini	T. vulpis	T. arvicolae	T. carlieri	T. mastomysi	T. cutillasae	T. myocastoris
THC2	99.91																	
T. muris	86.04	85.95																
T. suis	84.95	84.95	85.68															
T. trichiura	84.50	84.50	85.95	89.19														
T. colobae (N. gabriellae)	84.95	84.95	86.04	91.53	90.09													
T. colobae (C. g. kikuyensis)	84.95	84.95	86.04	91.71	90.18	99.91												
T. sp (francoisi)	85.77	85.68	85.77	85.95	85.59	85.77	85.86											
T. rhinopitheroxella	86.13	86.03	84.95	84.95	86.04	85.22	85.32	90.81										
T. ovis	86.31	86.04	84.95	81.53	80.72	81.89	81.98	82.79	83.33									
T. discolor	85.14	84.77	84.14	78.83	78.38	80.99	81.08	82.25	81.71	90.45								
T. skrjabini	85.41	85.14	84.95	82.70	82.88	82.52	82.43	82.52	83.51	83.51	83.06							
T. vulpis	87.03	87.03	90.54	88.02	87.48	87.93	87.84	88.11	86.31	88.38	87.57	88.02						
T. arvicolae	85.86	85.59	93.42	85.86	85.95	86.31	86.22	86.31	85.41	84.50	83.78	84.68	90.27					
T. carlieri	85.86	85.59	91.71	86.40	87.03	87.39	87.30	87.03	85.68	83.78	83.33	84.59	89.01	90.54				
T. mastomysi	85.77	85.59	91.17	88.38	87.66	87.03	86.94	86.22	86.22	83.96	83.51	84.14	90.63	89.37	89.19			
T. cutillasae	86.49	86.13	88.20	83.24	82.52	83.33	83.42	84.05	86.85	82.16	81.89	83.87	88.92	86.76	87.57	87.39		
T. myocastoris	80.90	80.63	82.25	74.95	75.86	76.67	76.76	78.74	78.29	75.86	74.23	78.74	85.41	82.34	82.43	83.42	79.82	
T. duplanteri	86.67	86.49	87.66	87.18	87.39	87.39	87.48	87.66	85.59	88.20	87.18	87.48	87.48	86.49	86.58	88.47	89.73	83.15

Purwaningsih (2013) carried out an important discovery of *T. landak* n. sp. from *H. javanica*, a new species of whipworm parasitizing porcupine in Indonesia. Nevertheless, the author separated the new species from the other *Trichuris* species exclusively by comparing morphologic features and morphometric measurements of adult worms. Due to the inherently limited number of unique external morphological features in the adult worms, molecular analysis tools should be included in future studies to improve the speciation of *Trichuris* spp. The morphological and biometrical results obtained by the present study suggest that the population of *Trichuris* sp. from *H. cristata* could be correspond with *T. landak*. Yet, further investigations to confirm our suggestions include: a) experimental trail to perform parasitic life cycle maintenance in *T. landak* hosts; and b) DNA analysis for more comparative and phylogenetics studies. Accurate information on the possible zoonotic behavior of different *Trichuris* species is pertinent for health workers to improve on the existing control measures. Thus, it is necessary to increase the molecular and phylogenetic studies on *Trichuris* spp., which will inevitably contribute to our knowledge on the etiology of human trichuriasis.

In fact, whether there is no good sanitation system in place to maintain clean contact with the porcupine and its flesh, the local people like health workers might get an infection. Furthermore, Fayer et al. (2010) reported the porcupine is recorded as the host for many new parasites, such as *Cryptosporidium ubiquitum* n. sp and the risk for transmission to human has been confirmed. Consequently, it could become the new public health concern to prevent the possibility of a new zoonosis by this parasite.

Thus, it is necessary to increase the studies of integrative taxonomy on *Trichuris* spp. based on morphological, biometrical and molecular data, which will inevitably contribute to our knowledge on the etiology of trichuriasis.

5. Conclusions

In conclusion, morphological and biometrical data revealed a high similarity between *Trichuris* parasitizing *H. cristata* with *T. landak*, suggesting that both populations correspond with the same *Trichuris* species. We report the first molecular characterization of *Trichuris* sp. from porcupine. Molecular analyses revealed the existence of two different haplotypes that did not correspond to different morphospecies. Relationships among *Trichuris* sp. from *H. cristata* and other *Trichuris* species have been resolved by molecular sequence data in this study. Thus, the combined analysis of four markers (*cox*1, *cob*, *rrn*L and ITS1) revealed a sister relationship between *Trichuris* parasitizing porcupine and other *Trichuris* species from rodents and canids and separated from the rest of *Trichuris* spp. from other hosts species. Supplementary data to this article can be found online.

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Availability of data and materials

The datasets generated and analyzed during the current study are available in the GenBank™, EMBL and DDBJ repository, [Accession numbers: OU596152. OU596153, OU596145–48 (Table 2)].

Ethics approval and consent to participate

Not applicable. This study does not require approval by an ethics committee. H. cristata, from which Trichuris specimens were collected from their caeca post-mortem, died of natural death. The specimen was handled and housed in a zoo in strict accordance with good animal

practices.

Consent for publication

Not applicable.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi

CRediT authorship contribution statement

Julia Rivero: Methodology, Validation, Investigation, Formal analysis, Software, Data curation, Writing – original draft, Visualization. **Ángela María García-Sánchez:** Conceptualization, Funding acquisition, Resources, Project administration, Supervision. **Rocío Callejón:** Methodology, Formal analysis, Software, Data curation, Writing – original draft, Visualization, Writing – review & editing, Supervision. **Cristina Cutillas:** Formal analysis, Software.

Declaration of Competing Interest

The authors declare no competing interests.

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New genetic lineage of whipworm present in Bactrian camel (*Camelus bactrianus*)

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ABSTRACT

With a global population of around 35 million in 47 countries, camels play a crucial role in the economy of many marginal and desert areas of the world where they survive in harsh conditions. Nonetheless, there is insufficient knowledge regarding camels' parasite fauna which can reduce their milk and meat production. A molecular study for the *Trichuris* population of *Camelus bactrianus* from Spain is presented based on sequences of mitochondrial (*cox*1, *cob*, *rrn*L) and ribosomal (ITS1 and ITS2) DNA regions. Bayesian Inference and Maximum Likelihood methods were used to infer phylogenies for (i) each gene separately, (ii) the combined mitochondrial data, and (iii) the combined mitochondrial and ribosomal dataset. Molecular analyses revealed the existence of two different genetic lineages in the *Trichuris* parasites populations of *C. bactrianus*. Future studies should focus on whether there is a coevolution process corresponding to the wild or domestic character of *C. bactrianus* and *Camelus dromedarius*. Furthermore, it is necessary to increase integrative taxonomic studies on *Trichuris* spp. based on morphological, biometric, and molecular data, which will inevitably contribute to our knowledge of the etiology of trichuriasis.

1. Introduction

Camels are an important livestock species that have adapted to hot and arid environments. The global camel population is estimated to be around 35 million animals in 47 countries. Approximately 85% of the population is found in Africa and the rest in the Indian subcontinent and Middle East countries (Demelash et al., 2014). The genus *Camelus* has two species, the one-humped camel (*Camelus dromedarius*) and the two-humped camel (*Camelus bactrianus*). Camels have been an important component of the desert ecosystem since time immemorial; humans depend on this animal not only for meat, milk and hide, but also as one of the most important means of transport in the desert and is recognized as the "Ship of the desert". Camel production is practiced by herding communities under diverse constraints in dry and marginal areas. It is known that parasitic diseases of the gastrointestinal tract are the primary health problem of camels worldwide (Kinne et al., 2002; Radfar and Aminzadeh, 2013). Parasitic infections in camels may reduce milk and meat production, affect fertility and decrease calving rates (Sazmand and Joachim, 2017). They can also decrease work efficiency or even cause death and, consequently, significant economic loss. Camels play an important role in the epidemiology of parasitic diseases under the three aspects of zoonoses, animal health and transmission to other livestock.

Whipworms are widespread soil-transmitted helminths that can be found in a wide range of hosts, including humans (*Trichuris trichiura*), non-human primates (NHP) (*Trichuris* spp.), pigs (*Trichuris suis*), dogs (*Trichuris vulpis*), goats, sheep, bovines and camelids (*Trichuris ovis*, *Trichuris discolor* and *Trichuris skrjabini*), among others (Cutillas et al., 2007, 2009; Liu et al., 2012; Callejón et al., 2017; Ahmad et al., 2019). These parasites are transmitted by the direct fecal-oral route and are among the most common intestinal parasites of humans and animals, causing significant disease and economic loss globally (Jex et al., 2014; Roepstorff et al., 2011).

For many years, the systematics of the genus *Trichuris* Roeder, 1761 at the species level has been considered controversial. Morphological and biometric studies were performed initially to identify the different *Trichuris* species. However, these approaches are not always satisfactory since *Trichuris* species overlap in most morpho-biometric features, especially in the closest species where differentiation is challenging (Cutillas et al., 2009; Robles, 2011). rDNA markers have provided

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genetic information that allows taxonomically characterizing different species of the genus and even establishing relationships between sister species (Gasser et al., 2001; Rivero et al., 2020). Subsequently, mitochondrial markers have been used due to their rapid rate of evolutionary change, less recombination, and moderately conserved genomic structures for population genetics, systematics and phylogenetic relationships, such as *cytochrome oxidase* 1 (*cox*1), *cytochrome* b (*cob*) or 16 S gene (Cavallero et al., 2015, 2019; Callejón et al., 2017; Rivero et al., 2020, 2021a). In addition, some studies on the complete analysis of the mitochondrial genome have been determined to obtain the greatest amount of information for the taxonomic and phylogenetic studies (Liu et al., 2012; Hawash et al., 2015; Wang et al., 2019; Rivero et al., 2021b). Some authors evaluated the utility of mitochondrial and ribosomal genes for the molecular systematics of *Trichuris* and parasitic nematodes and concluded that combined molecular analysis based on nuclear and mitochondrial genes allows for a more complete understanding of the taxonomy and evolution of *Trichuris* species (Chan et al., 2020; Rivero et al., 2020). In recent years, the designation of parasitic species within the *Trichuris* genus has been determined using an integrative approach based on the combination of morphological and molecular methodologies (Rivero et al., 2020, 2021a, 2022). Within the genus *Trichuris*, various authors have referenced synonymies (Oliveros et al., 2000), cryptic species (Callejón et al., 2012), different lineages (Rivero et al., 2020), and new species (Liu et al., 2013; Cutillas et al., 2014; Robles et al., 2014; Callejón et al., 2017; Wang et al., 2019).

From ruminants, more than 23 *Trichuris* species have been previously described (Knight, 1974). Several studies have reported that whipworms from domestic ruminants (*T. ovis* Abildgaard, 1795, *T. globulosa* Von Linstow, 1901, *Trichuris discolor* Von Linstow, 1906 and *T. skrjabini* Baskakov, 1924) may parasitize different hosts since nematodes isolated from sheep or cattle have been found in camels. Hence, in addition to the species of *Trichuris* specific to camelids (*Trichuris tenuis*) (Rickard and Bishop, 1991; Cafrune et al., 1999), these can host more *Trichuris* species (e.g., *T. ovis*, *T. globulosa*, *T. skrjabini* and *Trichuris* spp.) (Tait et al., 2002; Callejón et al., 2015a; Montalbano Di Filippo et al., 2020). Therefore, a complex of *Trichuris* species can be found in camels.

Detailed epidemiological studies on camel parasites strongly demand molecular diagnostic tools to correctly classify species and genotypes in order to improve existing diagnostic tools and provide more detailed insight into the epidemiology, transmission and risk factors of these parasites. These results will advance an economically important integrated control program against camel parasites and include parasites in camel health surveillance. For this reason, we carried out a molecular and phylogenetic study of *Trichuris* population from *C. bactrianus* obtained from a zoological garden. We analyzed the relationships among the different species of *Trichuris* that parasitize camels and other closed *Trichuris* species.

2. Materials and methods

2.1. Ethics statement

This study does not require approval by an ethics committee. *Trichuris* specimens were collected from stool samples from *C. bactrianus* that were housed in a zoological garden in Spain and handled in strict accordance with good animal practices.

2.2. Collection samples

Whipworms of *Trichuris* spp. were collected from the fecal samples after anthelmintic treatment of *C. bactrianus.* These camels were housed in "Parque de la Naturaleza de Cabárceno" in Cantabria province, Spain. Four adult worms were recollected and washed extensively in 0.9% saline solution and then, stored at − 20º C until further studies.

Adults of *Trichuris* sp. from *C. bactrianus* were identified at the genus level exclusively based on the external characteristics of the adults (Cutillas et al., 1995; Callejón et al., 2015a) due to adult worms were deteriorated due to the treatment received to obtain the fecal samples from the camels, and morphological studies could not be completed.

2.3. Molecular and phylogenetic analysis

The total genomic DNA from whipworms were extracted according to the manufacturer's protocol using DNeasy Blood and Tissue Kit (Qiagen). 0.8% agarose gel electrophoresis with SYBR® Safe DNA gel stain was assessed for the quality of extractions.

The molecular markers sequenced (*cox*1, *cob* and *rrn*L mtDNA, and ITS1 and ITS2 rDNA) were amplified by the polymerase chain reaction (PCR) using a thermal cycler (Eppendorf AG; Hamburg, Germany). PCR primers, PCR conditions and PCR mix were summarized in Supplementary Table S1. The PCR products were verified on SYBR® Safe stained 2% Tris-Borate-EDTA (TBE) agarose gels. PCR products were purified using QWizard SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, U.S.A.). Then, the purified products were concentrated and sequenced by Stab Vida (Lisbon, Portugal).

For all markers analyzed, the identity of the of *Trichuris* sequences from this study were compared to other *Trichuris* spp. sequences obtained from the National Centre for Biotechnology Information (NCBI) GenBank database. The number of nucleotide differences per sequence was determined using Compute Pairwise Distances based on the number of differences method of MEGA X v10.1.8 (Kumar et al., 2018).

To understand and analyze the relationships among the *Trichuris* species and *Trichuris* sp. from *C. bactrianus* (obtained in this study), additional sequences from the NCBI GenBank™ database were incorporated into alignments (Table 1). All phylogenetic trees were rooted by including *Trichinella* spp. as an outgroup. In addition, nucleotide sequence data obtained in this work are available in the GenBank™ database with the accession number reported in Table 1 and Supplementary Table S2. MUSCLE alignment method in MEGA X was used to obtain the nucleotide sequence alignment files (Kumar et al., 2018). Two methods based on nucleotide data were inferred to produce the phylogenetic trees, based on either Maximum Likelihood (ML) and Bayesian Inference (BI). ML was generated using PhyML 3.0 (Guindon et al., 2010), and MrBayes v3.2.6 to produce BI (Ronquist and Huelsenbeck, 2003). To determine the best-fit substitution model for the parasite data, jModelTest was utilized (Posada, 2008). According to Akaike information criterion, evolution models were chosen for subsequent analysis (Huelsenbeck and Rannala, 1997; Posada and Buckley, 2004). To

Table 1
List of *Trichuris* samples obtained in the present work based on the different markers (*cox*1, *cob*, *rrn*L, ITS1 and ITS2) including GenBank accession numbers, G + C content and length of sequences.

ID sample	Accession number	Marker	Length (bp)	G + C content (%)
TCH1	OM920890	***cox*1**	369	39.60
TCH2	OM920891		369	39.60
TCH5	OM920892		369	39.60
TCM1	OM920893		369	39.60
TCH1	OM912468	***cob***	519	31.60
TCH2	OM912469		519	31.98
TCH5	OM912470		519	31.98
TCM1	OM912471		519	31.60
TCH1	OM920886	***rrn*L**	362	27.60
TCH2	OM920887		362	27.60
TCH5	OM920888		362	27.90
TCM1	OM920889		362	27.60
TCH1	OM920875	**ITS1**	521	59.90
TCH2	OM920876		521	59.30
TCH5	OM920877		521	59.50
TCM1	OM920878		521	59.30
TCH1	OM920881	**ITS2**	522	58.60
TCH2	OM920882		522	58.30
TCH5	OM920883		522	58.50
TCM1	OM920884		522	58.30

investigate the phylogenetic relationships, the partitioned datasets were analyzed by gene and models for individual genes (two rDNA (ITS1 and ITS2) and three mtDNA (*cox*1, *cob* and *rrn*L)), and the concatenation of two mitochondrial markers (*cox*1 and *cob*), two ribosomal markers (ITS1 and ITS2), and the concatenation of two mitochondrial and two ribosomal markers (*cox*1, *cob*, ITS1 and ITS2). The *rrn*L marker was not used for concatenated trees due to the lack of sequences. For ML inference, the best-fit nucleotide substitution model included a transition model with gamma-distributed rate variation and a proportion of invariable sites, TIM2 + I + G for the *cox*1 marker and TIM1 + I + G for *rrn*L marker; a transversion model with gamma-distributed rate variation and a proportion of invariable sites, TVM + I + G for *cob* marker; a general time reversible with gamma-distributed rate variation and a proportion of invariable sites, GTR + I+ G for ITS1; and a general time reversible with gamma-distributed rate variation among sites for ITS2. Bootstrapping was utilized to examine the support for the topology (heuristic option) (Felsenstein, 1985) using over 1000 replications to evaluate the relative reliability of clades. For MrBayes, the command used was nst= 6 with gamma rates (ITS2) and nst= 6 with invgamma rates (ITS1, *cox*1, *cob* and *rrn*L). For BI, the standard deviation of split frequencies was used to determine whether the number of generations completed was sufficient. The chain was sampled every 500 generations and each dataset was run for 10 million generations. Based on an assessment of convergence, trees from the first million generations were discarded. Burn-in was resolved empirically by examination of the log-likelihood values of chains. The Bayesian Posterior Probabilities (BPP) include the significance scores for the nodes.

3. Results

3.1. Molecular Results

Four individuals of *Trichuris* sp. from *C. bactrianus* were extracted and all the markers (ITS1, ITS2, *cox*1, *cob* and *rrn*L) were amplified and sequenced of each specimen. The length of the sequences, the G+C content and the GenBank accession numbers is summarized in Table 1.

The multiple alignment of 45 *cox*1 nucleotide sequences, including outgroups, yielded a dataset of 291 characters. For *cob*, the multiple alignment was among 44 nucleotide sequences and a dataset of 384 characters. For *rrn*L, the multiple alignment of 17 nucleotide sequences yielded a dataset of 392 characters. Finally, for ITS1 and ITS2, the multiple alignment of 35 nucleotide sequences yielded a dataset of 1114 characters and the multiple alignment of 40 nucleotide sequences yielded a dataset of 910 characters, respectively.

For *cox*1, *cob*, *rrn*L, ITS1 and ITS2 sequences the percentages of interspecific identity between our sequences and the other species of *Trichuris* from *Camelus* spp. are shown in Supplementary Tables S3, S4, S5, S6 and S7, respectively.

Phylogenetic trees inferred by ML and BI methods of each marker showed similar results among *Trichuris* sequences assessed in this study.

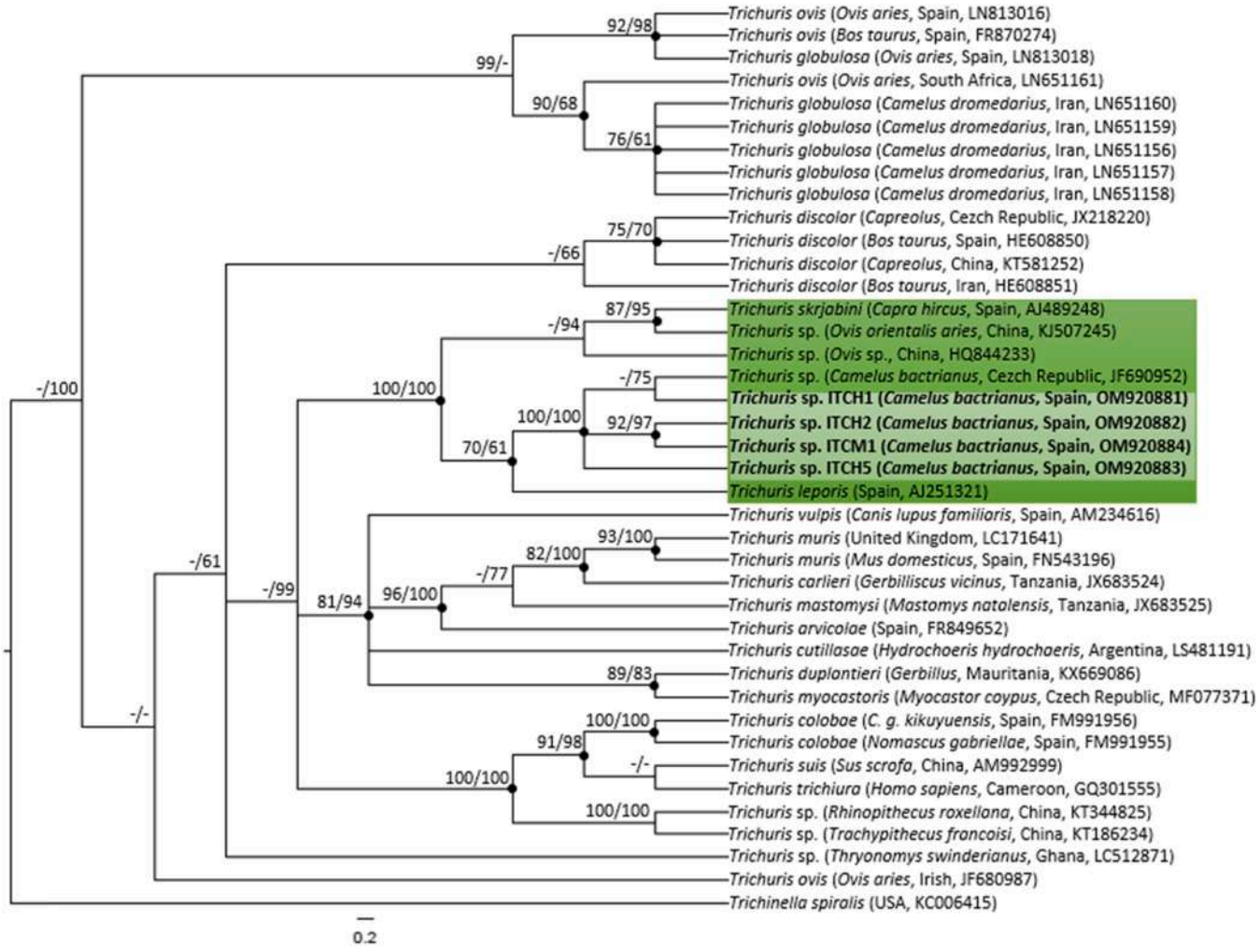

Fig. 1. Phylogenetic tree of *Trichuris* species based on analysis of ITS2 ribosomal DNA inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

In all trees, the four sequences from *Trichuris* sp. from *C. bactrianus* from Spain were clustered together in the same clade with high bootstrap values and BPP (Figs. 1–5). Due to the lack of *Trichuris* sequences in some markers, not all the trees could be compared with the same sequences and non-representative trees were obtained for each marker separately. Hence, the *rrn*L marker could not be concatenated with other markers and instead, the ITS2 dataset was analyzed independently due to many sequences of *Trichuris* sequenced for this marker.

The phylogenetic analysis of the ITS2 dataset yielded a tree with no well-supported values to infer the phylogenetic relationship between *Trichuris* species (Fig. 1). However, it revealed a moderated support to delimit species. Thus, *Trichuris* sp. from *C. bactrianus* from Spain and the Czech Republic were grouped with strongly supported nodes related to *T. leporis*, but not associated with the *T. globulosa* sequences from *C. dromedarius*.

The concatenated dataset of partial ITS1 and ITS2 rDNA sequences included 2024 aligned sites and 30 taxa (including the outgroup). The phylogeny revealed the existence of four different clades: clade 1 including *Trichuris* spp. from humans, NHP and suid; clade 2, including *Trichuris* spp. from canids and rodents; clade 3, including *Trichuris* spp. from camel, sheep, goat, and hare; and clade 4 including cows, goats, sheep, and the greater cane rat. Clades were well supported based on ML and BI methods except that clade 4 with a bootstrap value of less than 65% based on ML methods. Phylogenetic inferences of *Trichuris* sp. From *C. bactrianus* with respect to other *Trichuris* spp. revealed a sister group that includes *T. skrjabini*, *Trichuris* sp. from *Ovis* spp. and *T. leporis* with high support values (Fig. 2).

The partial *rrn*L mtDNA dataset included 392 aligned sites and 17 taxa (including outgroups). Phylogenetic inferences for the genus based on *rrn*L were not resolved because some were in polytomy (Fig. 3). Even so, *Trichuris* sp. from *C. bactrianus* formed a solid group related to *T. skrjabini* as a sister group with high bootstrap values and BPP.

The concatenated dataset of partial *cox*1 and *cob* mtDNA sequences included 675 aligned sites and 35 taxa (including outgroups). In congruence with concatenated dataset rDNA (ITS1 and ITS2), the four main clades cited were revealed with high branches support. An additional clade (clade 5), including *Trichuris* sp. from *Hystrix cristata*, was considered for mitochondrial datasets. *Trichuris* spp. from camels were separated into different clades. In this way, the population of *Trichuris* sp. from *C. bactrianus* from Spain was related to *T. skrjabini* as a sister group with high bootstrap values and BPP within clade 3, and on the other way, *Trichuris* sp. from *C. bactrianus* from Italy (rDNA sequences not available) appeared within clade 4 clustered with *T. ovis* and more related to *Trichuris* sp. from *Addax nasomaculatus* from Italy and *T. globulosa* from *C. dromedarius* from Iran. In any case, of all the clades in the genus *Trichuris*, both clades (which included *Trichuris* spp. parasitizing herbivorous host species) were the most phylogenetically related, being more separated from clades 1, 4 and 5 (Fig. 4).

Finally, the concatenated dataset of partial ribosomal (ITS1 and ITS2 rDNA) and mitochondrial (*cox*1 and *cob* mtDNA) sequences included 2728 aligned sites and 15 taxa (including outgroup). Due to missing sequences in most markers, many *Trichuris* species sequences could not be added to the analysis. The four main clades were obtained in congruence with phylogenetic inferences based on mitochondrial and ribosomal datasets. *Trichuris* sequences obtained in this study clustered together, and this population appeared as a sister group to *T. skrjabini* (clade 3) with high BPP and bootstrap values separated from all other *Trichuris* clades (Fig. 5).

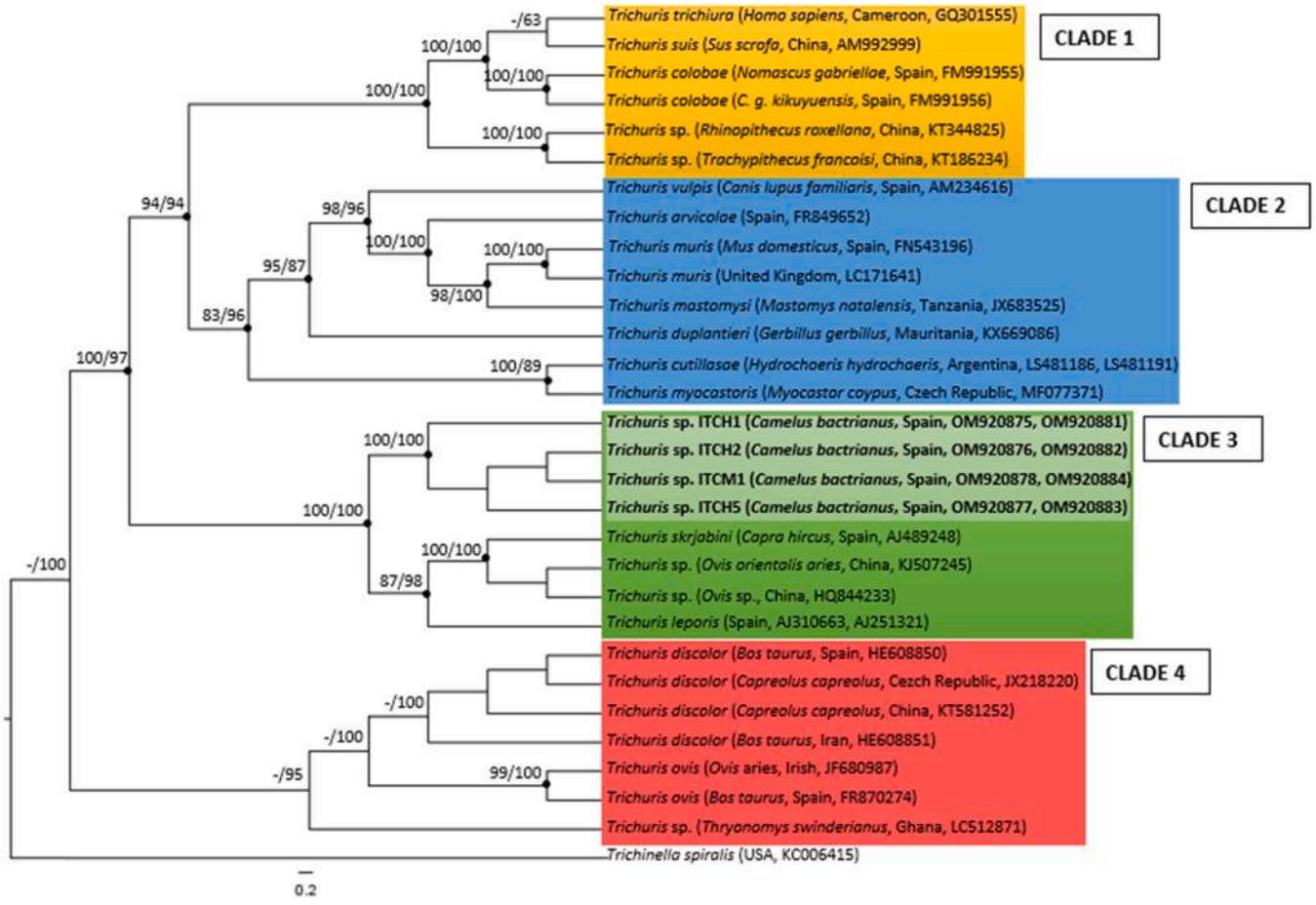

Fig. 2. Phylogenetic tree of *Trichuris* species based on combined analysis of ribosomal DNA (ITS1 and ITS2) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

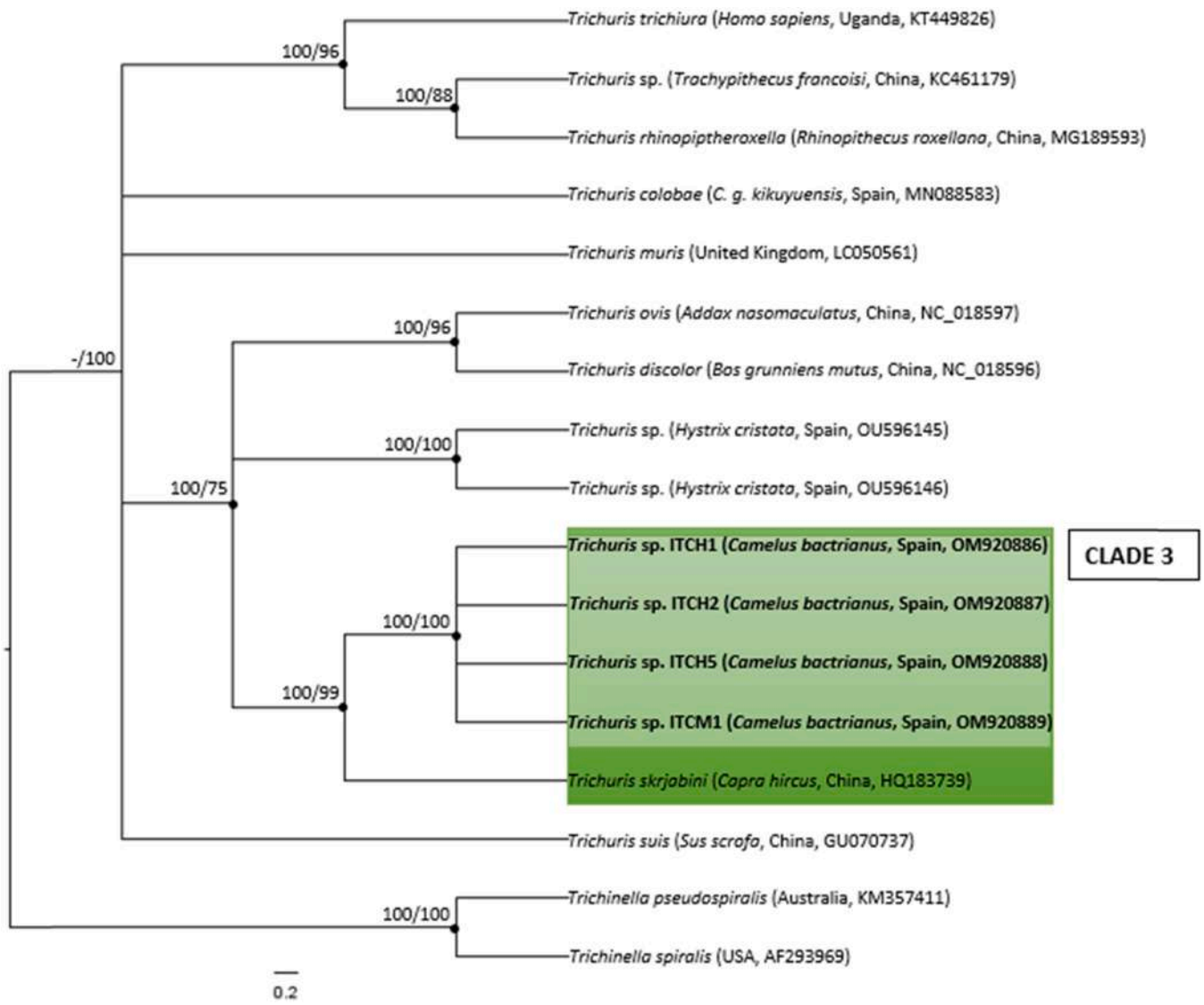

Fig. 3. Phylogenetic tree of *Trichuris* species based on analysis of *rrn*L mitochondrial DNA inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

4. Discussion

The present paper reflects the current knowledge of the phylogenetic analysis of the *Trichuris* lineages of camels from *C. bactrianus* from Spain and other camel populations from different geographical origins. Molecular techniques have been carried out to clarify the *Trichuris* systematics in camelids. No biometrical and morphological data were obtained for *Trichuris* from *C. bactrianus* in this work and, therefore, comparative morpho-biometrical studies could not be performed. To assess the utility of all the different markers (*cox*1, *cob* and *rrn*L mtDNA, and ITS1 and ITS2 rDNA) used in this study, intra and inter-specific identity and phylogenetic tree studies have been performed.

In all markers analyzed, the identity percentage obtained among the *Trichuris* sequences obtained in this study from *C. bactrianus* showed values corresponding to intra-specific identity according to the range cited by several authors in different studies for the genus *Trichuris* (Blouin et al., 2002; Callejón et al., 2015b; Chan et al., 2020).

Comparative analysis of *Trichuris* sequences obtained in this study with other *Trichuris* sequences from camels revealed that only two sequences of *Trichuris* from *C. bactrianus* from the Czech Republic, one sequenced for the *cox*1 mtDNA and one for ITS2 rDNA markers, showed the same percentage of identity (98–100%) with our sequences, corresponding with the same species. Furthermore, *Trichuris* sp. sequences from *C. bactrianus* from Italy and *T. globulosa* showed values within the range of inter-specific identity (Blouin et al., 2002). Thus, the identity percentage observed between *T. globulosa* and *Trichuris* sp. sequences from *C. bactrianus* from Italy were higher than the values of *T. globulosa* with *Trichuris* sp. from *C. bactrianus* from Spain. Hence, the species compared above, having a lower inter-specific identity, also appeared to be in the range of different species. Moreover, the greatest inter-specific identity among our sequences and other *Trichuris* species was with *T. skrjabini*. These values were higher in rDNA markers (approximately 95%) than in mtDNA markers. In addition, the inter-specific identity was usually higher in rDNA markers (ITS1 and ITS2) than in mtDNA markers (*cox*1, *cob*). However, the *rrn*L mitochondrial marker presented intermediate values. This fact agrees with several authors (Callejón et al., 2015b; Chan et al., 2020; Rivero et al., 2021a), who support the hypothesis that ITS regions show a higher nucleotide saturation than mitochondrial markers due to this region accumulating substitutions more slowly. For this reason, ITS markers were not sufficient to infer phylogenetic relationships among the different related populations of *Trichuris*, but they were effective in differentiating among other species that were more evolutionarily separated. With respect to mitochondrial markers, the *rrn*L partial gene has been suggested as the

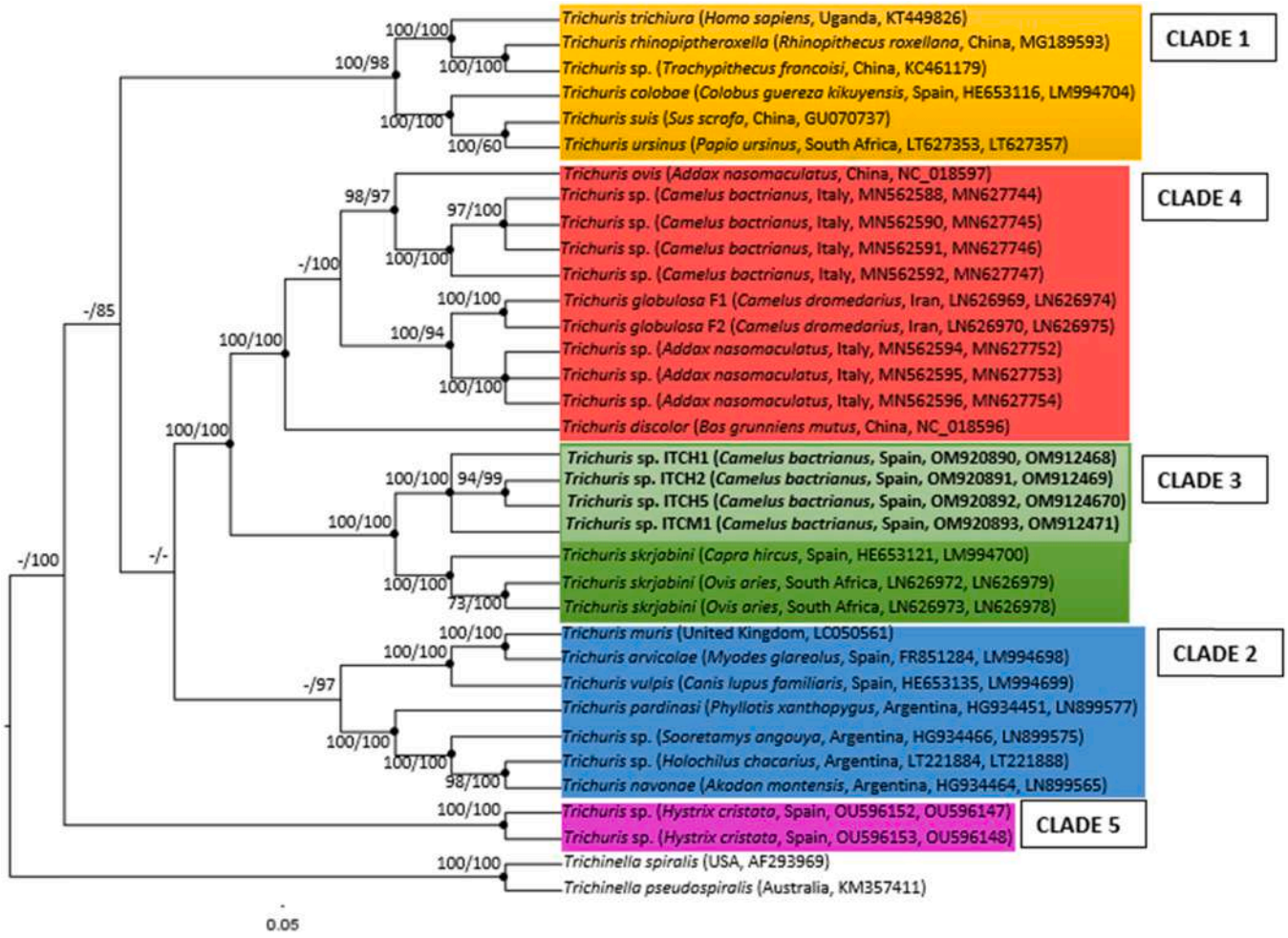

Fig. 4. Phylogenetic tree of *Trichuris* species based on combined analysis of mitochondrial DNA (*cox*1, *cob*) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

best marker to differentiate among close genetic lineages since nucleotide saturation was lower than ITS markers but higher than *cox*1 and *cob* genes. This means that the *rrn*L gene provided a higher resolution for phylogenetic studies than the other mitochondrial markers due to the *rrn*L gene presenting a lower intraspecific variability (Rivero et al., 2021a). Nevertheless, because of the lack of sequences for the *rrn*L partial gene in *Trichuris* species, only a few sequences have been able to be studied. For this reason, a concatenated dataset with *rrn*L could not be done. Consequently, a concatenated dataset of mitochondrial (*cox*1 and *cob* genes) and ribosomal (ITS1 and ITS2) markers supplied higher resolution for phylogenetic studies (Rivero et al., 2020, 2021a), providing in this study strongly supported nodes, differentiating among the different clades. Attending to the identity percentage observed for the other markers, we suggest that the population of *Trichuris* sp. from *C. bactrianus* from Spain corresponds to a different species from those cited for *C. bactrianus* and *C. dromedarius* and other herbivores. Therefore further studies based on parallel morpho-biometric and molecular data are necessary for its description.

The results obtained in the identity study corresponded with the phylogenetic results. The sequences obtained in this study were grouped in the same clade as the *Trichuris* sequence of *C. bactrianus* from the Czech Republic (clade 3). However, other populations of *Trichuris* parasitizing camels (*C. bactrianus* from Italy and *C. dromedarius* from Iran) were shown separated into a different clade (clade 4) (Fig. 4). In all phylogenetic trees, both in the individual marker data sets and in the concatenated data sets, our sequences showed a close relationship with *T. skrjabini* as a sister group with both populations forming a well-supported clade separate from all other clades. The identity results were consistent with the phylogenetic results. Hence, our sequences obtained the highest identity values compared to *T. skrjabini* and lower compared to *T. globulosa* and *Trichuris* sp. from *C. bactrianus* from Italy, appearing these last populations within clade 4, suggesting that these populations correspond to different species according to interspecific and phylogenetic results but closely related genetically, being separated from the population of *Trichuris* sp. from *C. bactrianus* from Spain.

This result may be due to the geographical difference among them, the type of food, and the inter-specific transmission route among related host species, or it may be related to the passive transport of eggs through zookeepers or freely circulating animals (Kvapil et al., 2017; Montalbano Di Filippo et al., 2020). On the other hand, the evolutionary relationship between the domestic and wild Bactrian camel (two-humped camels) and the actual origin of the domestic Bactrian is not clarified. Ji et al. (2009) revealed that the extant wild two-humped camel might not share a common ancestor with the domestic Bactrian camel and they are not the same subspecies except in their maternal origins. Complete mitochondrial genome sequences indicated that the wild camel is a separate lineage but not the direct progenitor of the domestic Bactrian camel. Later, phylogenetic analyses suggested that the wild Bactrian camel appeared monophyletic in evolutionary origin and that the domestic Bactrian camel could come from a single wild population.

Our results showed two different genetic lineages in the populations of *Trichuris* parasites of *C. bactrianus*, one of them (from Spain and the Czech Republic) genetically related to *T. skrjabini* and another

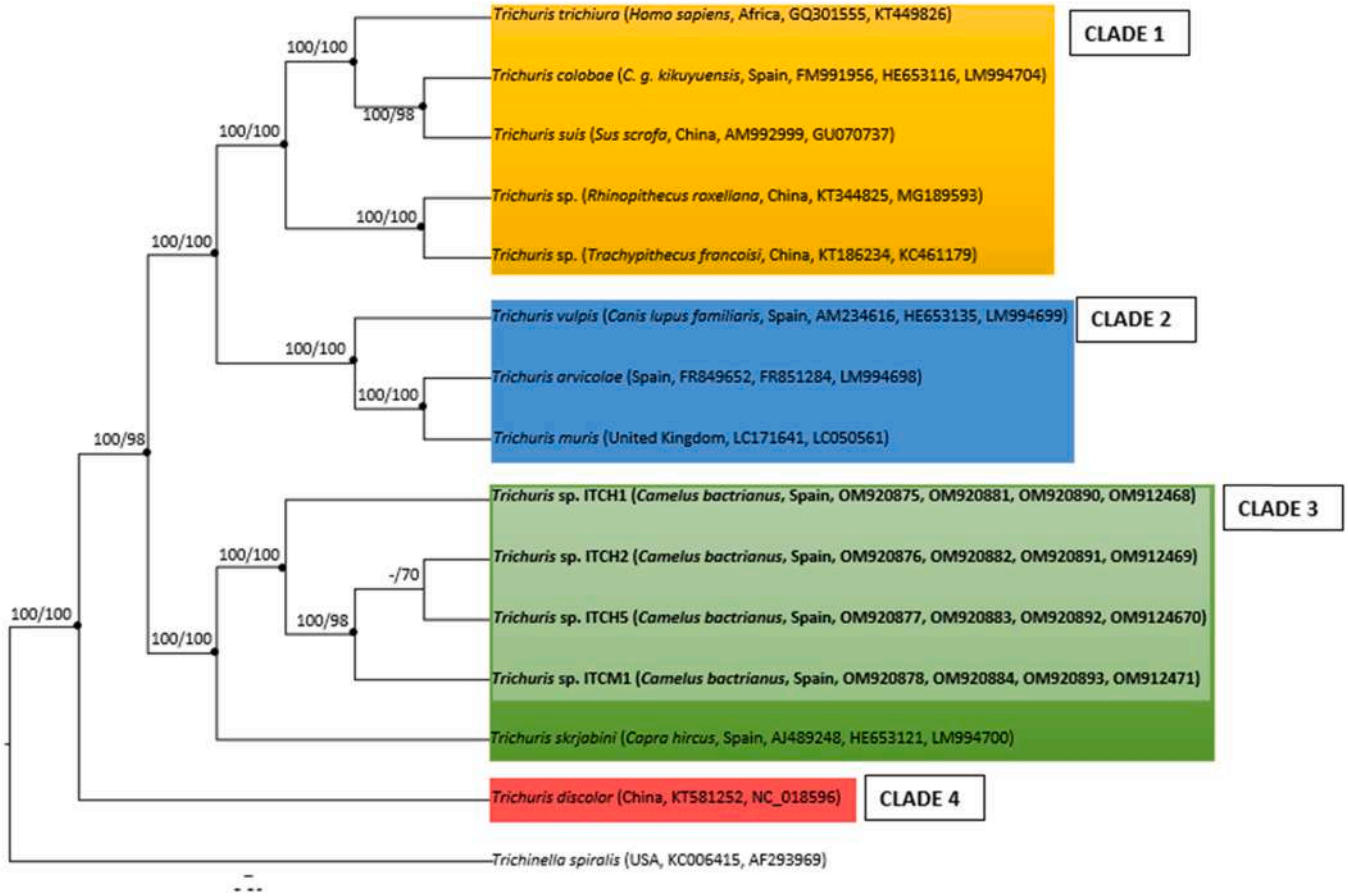

Fig. 5. Phylogenetic tree of *Trichuris* species based on combined analysis of mitochondrial DNA (*cox*1, *cob*) and ribosomal DNA (ITS1 and ITS2) inferred using Bayesian Inference. Maximum Likelihood bootstrap values of clades are listed first, followed by Bayesian Posterior Probabilities, respectively, for clade frequencies exceeding 60%.

population (from Italy) associated with *T. globulosa* of *C. dromedarius*. Future studies should focus on whether a coevolution process corresponds to the wild or domestic character of *C. bactrianus* and *C. dromedarius*.

5. Conclusions

Camels play a crucial role in the economy of many marginal and desert areas of the world, where they survive under harsh conditions. Nevertheless, there is insufficient knowledge regarding camel's parasite fauna which can reduce their milk and meat production. This work provides data on *Trichuris* species parasitizing *C. bactrianus* based on ribosomal and mitochondrial markers. The percentage of identity observed for the different markers revealed that the population of *Trichuris* sp. from *C. bactrianus* from Spain corresponds to a different species from those cited for camels and other herbivores showing a sister relationship with respect to *T. skrjabini*. These results could allow the scientific community and organizations responsible for formulating camel health policies to clarify the role of these animals in the epidemiology of zoonotic parasitic diseases and view a possible increase in their prevalence. Hence, further studies based on parallel morpho-biometric and molecular data are necessary to clarify the knowledge of *Trichuris* species that may be responsible for trichuriasis in camels.

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CRediT authorship contribution statement

Julia Rivero: Methodology, Validation, Investigation, Formal analysis, Software, Data curation, Writing – original draft, Visualization. **Cristina Cutillas:** Conceptualization, Formal analysis, Writing – review & editing, Funding acquisition, Resources, Project administration, Supervision. **Rocío Callejón:** Methodology, Formal analysis, Software, Data curation, Writing – original draft, Visualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare no competing interests.

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Ethics approval and consent to participate

Not applicable. This study does not require approval by an ethics committee. *C. bactrianus*, from which *Trichuris* specimens were collected from their feces post-treatment with anthelminthic. The specimen was handled and housed in a zoo in strictly following good animal practices.

Consent for publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2023.109886.

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The Use of MALDI-TOF MS as a Diagnostic Tool for Adult *Trichuris* Species

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Trichuriasis is considered a neglected tropical disease, being the second most common helminthiasis in humans. Detection of *Trichuris* in routine diagnosis is usually done by microscopic detection of eggs in fecal samples. Other molecular analyses are more reliable and could be used, but these analyses are not routinely available in clinical microbiology laboratories. The use of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) is increasing since the last decades due to its recent evidence as a potential role for reliable identification of microorganisms and a few nematodes. But, for parasites detection, normalized protocols and the acquisition and introduction of new species to the database are required. We carried out a preliminary study confirming the usefulness of MALDI-TOF MS for the rapid and reliable identification of *Trichuris suis* used as control and the creation of an internal database. To create main spectra profiles (MSPs), the different parts of five whipworms (esophagus and intestine) were used, developing different tests to verify the repeatability and reproducibility of the spectra. Thus, to validate the new internal database, 20 whipworms, separating the esophagus and intestine, were used, of which 100% were accurately identified as *T. suis*, but could not distinguish between both parts of the worm. Log score values ranged between 1.84 and 2.36, meaning a high-quality identification. The results confirmed that MALDI-TOF MS was able to identify *Trichuris* species. Additionally, a MALDI-TOF MS profile of *T. suis* proteome was carried out to develop the first internal database of spectra for the diagnosis of trichuriasis and other *Trichuris* spp.

Keywords: *Trichuris*, MALDI-TOF MS, diagnosis, nematode, internal database

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INTRODUCTION

Helminths constitute one of the most common, ecologically diverse, and speciose animal groups in the world. Several species of nematodes are of huge economic importance or medical interest. The most important species of soil-transmitted helminth (STH) infections, infecting humans are *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm) and *Necator americanus* and *Ancylostoma duodenale* (hookworms). Nearly 24% of the world's population is infected with soil-borne helminths (1). The prevalence is higher in marginalized populations in the tropics and subtropics, where there is a lack of basic sanitation services (2).

T. trichiura is a nematode, which is the etiological agent of the parasitic disease known as "trichuriasis." Trichuriasis is considered as a neglected tropical disease and has a worldwide geographical distribution. *T. trichiura* is the second most common helminth in humans. Moreover,

whipworms are among the most common intestinal parasites of humans and animals, causing significant diseases and economic losses globally (3, 4). Whipworms can be found in a large range of hosts, in addition to humans (*T. trichiura*), in suids (*T. suis*), sheep, goats, and bovines (*T. ovis* and *Trichuris discolor*), dogs (*Trichuris vulpis*), non-human primates (NHP) (*Trichuris* spp.) and several putative new *Trichuris* species (5–10).

Traditionally, the identification of *Trichuris* species was carried out according to the host where the whipworm was found, and later, by studies based on morphological and biometrical features of adults. But due to the phenotypic plasticity of these parasites (host-induced variation, lack of morphological characteristics, and overlap of morphological characteristics and biometrical data between species), it is highly difficult to distinguish between closely related *Trichuris* species (11–14). Hence, molecular studies, such as polymerase chain reaction (PCR) and sequencing, are used as a tool to differentiate species (5–7, 9, 10, 15–20). While the morphological identification is a rapid and less costly procedure, the related *Trichuris* species are hardly morphologically distinguishable. Moreover, the diagnosis of *Trichuris* is made by observing eggs in fecal samples and requires qualified personnel. Thus, the improvement of an accurate, fast, less expensive, and more accessible diagnostic technique for the identification of parasites would be desirable.

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been commonly introduced as a diagnostic method in laboratories, that analyzed complex molecules, such as proteins, by producing protein fingerprint signatures (spectra) from proteins extracts of organisms (21). The creation of reference spectra database by the acquisition of spectra has been used to identify species of parasites (22–25). MALDI-TOF MS has been suggested as a rapid and reliable identification technique of bacteria (26, 27). Lately, authors have demonstrated that MALDI Biotyper software can be used, in addition to bacteria (28–31), mycobacteria (28, 32), fungi (31), and most recently, in viruses (33), protozoans, arthropods (22, 34, 35), and a few nematodes (24, 36–40).

MALDI-TOF MS has revealed many advantages compared with other diagnostic tools (such as PCR assays). When the mass spectrometer and the corresponding databases are available in a laboratory, the identification is inexpensive, and the sample preparation procedure does neither require highly skilled technicians or complex additional laboratory infrastructure (41–43). Other advantages are that MALDI-TOF MS is significantly less susceptible to contamination, since the samples do not require special collected and preserved conditions, and the results are available within a few minutes. Nevertheless, the constant power supply is a limitation for the suitability of the technique in resource-limited environments. However, MALDI-TOF MS is more and more available in reference laboratories around the world including in developing countries (41–45). Furthermore, recent studies suggested that MALDI-TOF MS technique is of great importance due to its applicability in the discovery of antibiotic resistance in microorganisms, disinfectants, and the production of toxins from pathogens (29, 46–48).

Recently, a review about the use of MALDI-TOF in human and veterinary helminthology confirms that this technique is reliable and reproducible for nematode parasites. They suggested that it is necessary for many studies for more different species of nematodes, especially in common nematode parasites in the world, and to create a single database with all nematode species. And also, add the necessary spectra to the internal o commercial database to be able to identify larvae and eggs to open the possibility to analyze fecal samples by MALDI-TOF MS and obtain a fast and reliable identification and diagnosis (49).

Based on this background, the aim of this study was to evaluate the usefulness of MALDI-TOF MS as an effective diagnostic tool for the specific identification of *Trichuris* species. For this purpose (i) a standard protocol for the extraction of proteins was developed using *Trichuris suis* as control, (ii) a preliminary specific reference spectra database was created characterizing this species, *T. suis*, (iii) the standardized protocol of *T. suis* was validated for more species of *Trichuris* using MALDI-TOF MS analysis: *Trichuris* sp. from *Hystrix cristata*, *T. trichiura* from *Macaca sylvanus*, *T. vulpis* from *Canis lupus familiaris* and *T. ovis* from *Capra hircus* collected on Spain, (iv) an MS reference spectra in-house database for *Trichuris* species-specific identification was created based on molecular *Trichuris* identification comparatively with the new species of *Trichuris* analyzed, and (v) an analysis of the results obtained by MALDI-TOF MS was performed to verify the usefulness of MALDI-TOF MS in the phylogenetic and taxonomic study of *Trichuris* species.

MATERIALS AND METHODS

Ethics Statement

This study does not require approval by the ethics committee. Adult *Trichuris* samples were obtained from their caecum postmortem. The specimens of *T. suis* and *T. ovis* were obtained through a slaughterhouse; in a zoo, for adults of *T. trichiura* from *M. sylvanus* and *Trichuris* sp. from *H. cristata*, and in groups of dogs, for adults of *T. vulpis*, in strict accordance with good animal practices.

Sample Collection

For this study, whipworms belonging to the species *T. suis* from swine (*Sus scrofa domestica*) and *T. ovis* from goats (*Capra hircus*) were collected in slaughterhouses in the provinces of Seville and Huelva, and Seville (Spain), respectively. Specimens identified as *Trichuris* sp. from *H. cristata* and *T. trichiura* from *M. sylvanus* were collected from Bioparc Fuengirola (Spain), and Zoo Castellar (Spain), respectively. Finally, *T. vulpis* adults were collected from dogs (*Canis lupus familiaris*) in the provinces of Seville and Huelva. Separately, whipworm samples were washed several times in saline solution (0.9% w/v), and then frozen at −20°C until posterior analysis.

Sample Preparation and Morphological Identification

Adult worms were defrosted and dried at room temperature. The morphological identification of different *Trichuris* species was carried out according to previous studies (15, 18, 20, 50–52). Nonetheless, morphologically the different species of

Trichuris are difficult to differentiate. Thus, a molecular study was carried out.

Then, two different samples of the *T. suis* adults (esophagus and intestine) were used separately for MALDI-TOF MS analysis to check which part of the worm gave greater reliability and easier reproducibility.

For this reason, the preliminary study with only adults of *T. suis* was performed using 31 *T. suis* worms, using the esophagus and the intestine of adults as samples. Further, these two samples were assayed in the rest of the species of *Trichuris* for proteomic and molecular analysis, respectively.

Molecular Analysis

DNA Extraction, PCR, and Sequencing

To verify the accuracy of the species identification of the samples collected for MALDI-TOF MS, the morphological studies were corroborated with molecular analysis. The total genomic DNA from intestine worms was extracted using DNeasy Blood and Tissue Kit (Qiagen; Hilden, Germany) according to the manufacturer's instructions. The quality of extractions was assessed using 0.8% agarose gel electrophoresis infused with SYBR® Safe DNA gel stain (Thermo Fisher Scientific, MA, USA).

The *cytochrome* b (*cyt*b) mitochondrial DNA (mtDNA) gene of two to five intestines DNA samples of each *Trichuris* species (*T. suis*, *Trichuris* sp. from *H. cristata*, *T. trichiura*, *T. vulpis,* and *T. ovis*), were amplified by standard PCR by a thermal cycler (Eppendorf AG; Hamburg, Germany), while the esophagus was reserved for MALDI-TOF MS. The forward primer utilized was D769 (5′-GAGTAATTTTTATAATRCGRGAAGT-3′) (53) and the reverse primer utilized was D770 (5′-AATTTTCAGGRTCTCTRCTTCAATA-3′) (53), and the following PCR mix: 5 μl each primer (10 μM), 25 μl GoTaq G2 Green Master Mix, 5 μl template DNA, and nuclease-free water to 50 μl. The following conditions were applied: 94°C at 5 min (denaturing); 36 cycles at 94°C at 30 s (denaturing), 50°C at 30 s (annealing), and 72°C at 30 s (primer extension); followed by 7 min at 72°C (final extension). The PCR products were visualized on SYBR Safe stained with 2% w/v Tris–Borate–EDTA (TBE) agarose gels. Then, bands were eluted and purified using the Wizard SV Gel and PCR Clean-Up System Kit (Promega, WI, USA). Once purified and concentrated, the PCR products were sequenced in both directions by Stab Vida (Lisbon, Portugal).

Sequence Analysis Species Identification

The forward and reverse sequences obtained were analyzed using Multiple Sequence Alignment by CLUSTALW, to generate a consensus sequence for each specimen. The sequences were compared with sequences available in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLASTn) algorithm for identification (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences obtained were submitted to the NCBI GenBank database.

MALDI-TOF Analysis

Preliminary MALDI-TOF Analysis

Protein Extraction

For the preliminary study with *T. suis* worms, each worm sample, esophagus, and intestine, separately was placed in a 1.5 ml sterile Eppendorf. Afterward, each sample was pooled with 10 mg zirconia/silica beads (0.5 mm) along with 20–30 μl of a mix of 70% (v/v) formic acid and 50% (v/v) acetonitrile. Then, the samples were homogenized using the TissueLyser II system (Qiagen GmbH) in three cycles of 30 s at a frequency of 30 Hz. The homogenized samples were centrifuged at 10,000 g for 30 s.

Target Plate Preparation and Measurements

From each sample, 1 μl of the supernatant was carefully dropped on to the MALDI-TOF target in eight different spots for the creation of the main spectrum profiles (MSPs) (25), and four times for the validation test (54). Air dried and each spot was then recovered with 1 μl of CHCA matrix solution composed of saturated α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich, Co., MO, USA), 50% acetonitrile (v/v), 2.5% trifluoroacetic acid (v/v) (Thermo Scientific, Rockford, IL, USA), and 47.5% (v/v) high-performance liquid chromatography (HPLC) grade water (34). Bacterial test standard (BTS) (Bruker Daltonics, Bremen, Germany) was used to calibrate the machine (an *Escherichia coli* extract), which is spiked with two high molecular weight proteins. The matrix solution was loaded in quadruplicate to control the matrix quality (54). Then, at room temperature, having dried for several minutes, the plate was placed into the Microflex LT Mass Spectrometer (Bruker Daltonics) for MALDI-TOF MS.

MALDI-TOF Parameters

The MALDI-TOF MS measurements were carried out on a range of 2,000–20,000 Da, *m*/*z* (mass to charge) and with detection in the linear positive in mode with a laser frequency of 50 Hz, following the calibration with BTS.

For each spot, 240 laser shots were performed in four regions, and the measurements were automatically acquired using the AutoXecute method of the flexControl v3.4 software (Bruker Daltonics; Bremen, Germany). The spectrum profiles (protein mass profiles) were generated and visualized by Flex Analysis v3.3 software and were exported to ClinProTools v2.2 and MALDI-Biotyper v3.1.66 (Bruker Daltonics; Bremen, Germany) for data processing (smoothing, baseline subtraction, and peak picking). The acceleration voltage was 20 kV, and the extraction delay time was 200 ns (54). Concisely, the maximum mass error of each individual spectrum was 2,000 Da, the desired peak frequency minimum was 25% and the desired mass error for the MSP was 200 Da.

Spectral Analysis and Preliminary Database Creation

For the creation of species-specific MSPs, according to Diarra et al. (55), 2–6 specimens of each species is enough. Hence, protein extracts of five esophagus from *T. suis* worms and three intestines from *T. suis* worms were spotted on the MALDI-TOF target plate eight times per sample. Then, each spot was measured four times. For each sample worm, this procedure was carried out

on two replicates on different days to demonstrate repeatability and the reproducibility analysis. The combination of the results of the spectra from each specimen was used to create MSP by the automated function of the MALDI-Biotyper using the default parameter set of the "Bio Typer MSP Creation Standard Method" (54). The quality of each raw spectra was assessed with Flex Analysis software version 3.4. (Bruker Daltonics; Bremen, Germany). This program was also used to remove all flatlines and outlier peaks and smooth intensities and edit peak changes within spectra whenever they exceed 500 ppm.

Validation Test

The recently developed internal database underwent two different validation procedures. Starting with an internal validation, where all the spectra of each group of samples obtained through the MSP creation process were analyzed. And finally, a blind test analysis, in which the samples were measured by MALDI-TOF to evaluate the ability of the database to reliably identify these samples. For the blind test analysis, 20 specimens were analyzed. Protein extract from each sample was spotted on the MALDI-TOF target in quadruplicate. Hence, each sample was associated with four spectra. A total of 72 high-quality spectra from *T. suis*'s esophagus (for two samples the protein extract process failed) and 80 from *T. suis*'s intestine, were selected to query the database. The results obtained against the reference library were shown as log score values (LSVs) for each spectrum. LSV range from 0 to 3 reflects the results of a comprehensive comparison of peak position and intensity between two spectra (LSV from 0 to 1.699: no reliable identification; 1.7 to 1.999: probable genus identification; 2.0 to 2.299: secure genus identification and probable species identification, and 2.300 to 3.000: highly probable species identification) (25, 40).

Validation of MALDI-TOF Analysis

The analysis was carried out according to the preliminary study to validate and verify the procedures and the parameters used.

Spectral Analysis and Database Creation

For each *Trichuris* species (*Trichuris* sp. from *H. cristata*, *T. trichiura*, *T. vulpis,* and *T. ovis*), MSPs were created as previously described by the preliminary protocol. To actualize the internal database, five specimens for *Trichuris* sp. from *H. cristata* and for *T. trichiura* from *M. sylvanus*, two specimens for *T. vulpis*, and four specimens for *T. ovis* were used by running the MALDI Biotyper software automatically.

Validation Test

To assess the ability of the database to reliably identify these samples, a blind test was performed. A total of 63 good quality spectra from the four different *Trichuris* species analyzed were selected to examine the updated database. For each specimen, the protein extracted was spotted on the MALDI-TOF target four times, generating four spectra associated with the same sample. The results obtained for the query of the internal database are shown as log score values (LSVs) for each spectrum.

To determine the distances and similarity among MSPs, a hierarchical clustering of the mass spectra was performed, using the spectra utilized for the MSP creation, and the dendrogram function within MALDI Biotyper software.

Phylogenetic Analysis

To evaluate the similarity among all *cyt*b *Trichuris* sequences, the number of nucleotide and amino acids differences per sequence was calculated using Compute Pairwise Distances based on the number of differences method of MEGA X v10.1.8 (56).

For phylogenetic analysis, two methods—maximum likelihood (ML) and Bayesian inferences (BI)—were used. PhyML 3.0 was used to generate the ML tree (57), and for BI was used MrBayes v.3.2.6 (58). jModelTest to resolve the best-fit substitution model for the nucleotide data set was employed (59). According to Akaike Information Criterion, models of evolution were determined (57, 60). GTR + I + G model, with rate variation along the length of the alignment (+ G) and allowing for a proportion of invariant sites (+ I), was selected for the nucleotide data set. Topology support was examined using bootstrapping (heuristic option) (61) over 1,000 replications to assess the relative reliability of clades. The Bayesian posterior probabilities (BPPs) comprise the percentage converted. To determine if the number of generations completed was sufficient, was used the standard deviation of split frequencies; every 500 generations the chain was sampled, and each dataset was run for 10 million generations. Based on an assessment of convergence, trees from the first million generations were discarded. Examination of the log-likelihood values of the chains is determined empirically during the burn.

RESULTS

Preliminary MALDI-TOF Analysis

Comparative Analysis of *T. suis* Samples Used for MSP Database Creation (MALDI-TOF Analysis)

As mentioned before, for the creation of different reference spectra, firstly, two distinct body parts (esophagus and intestine) of *T. suis* worms were used. We included five specimens of which five esophagus and three intestines were used. The *cox*1 mtDNA partial sequence valid the specific analysis in the reference samples against previously deposited sequences by BLASTn, revealing approximately a 100% identity between *T. suis* (**Supplementary Table S1**) and the *T. suis* sequences from this study (reference accession number: OU756954). From the three *T. suis* worm sequences obtained, only 1 haplotype was generated.

The homogenization and sample preparation protocol described above provided high-quality spectra (**Figure 1**). Both, results based on esophagus and intestines samples showed similar spectra, with profiles of high intensity and strongest peaks placed in the same range, however, peaks intensity in the esophagus samples was slightly higher. The reference MSPs obtained for each type of sample by MALDI-TOF with high-intensity peaks in the 2–20 kDa range are presented in **Figure 1**. The highest density peaks were in the region comprised between 2 and 9.5 kDa, with clusters of signals in ranges corresponding to 2.1–2.3, 3–3.3, 5.2–5.6, and 6.2–6.6 kDa (**Figure 2**). There were no

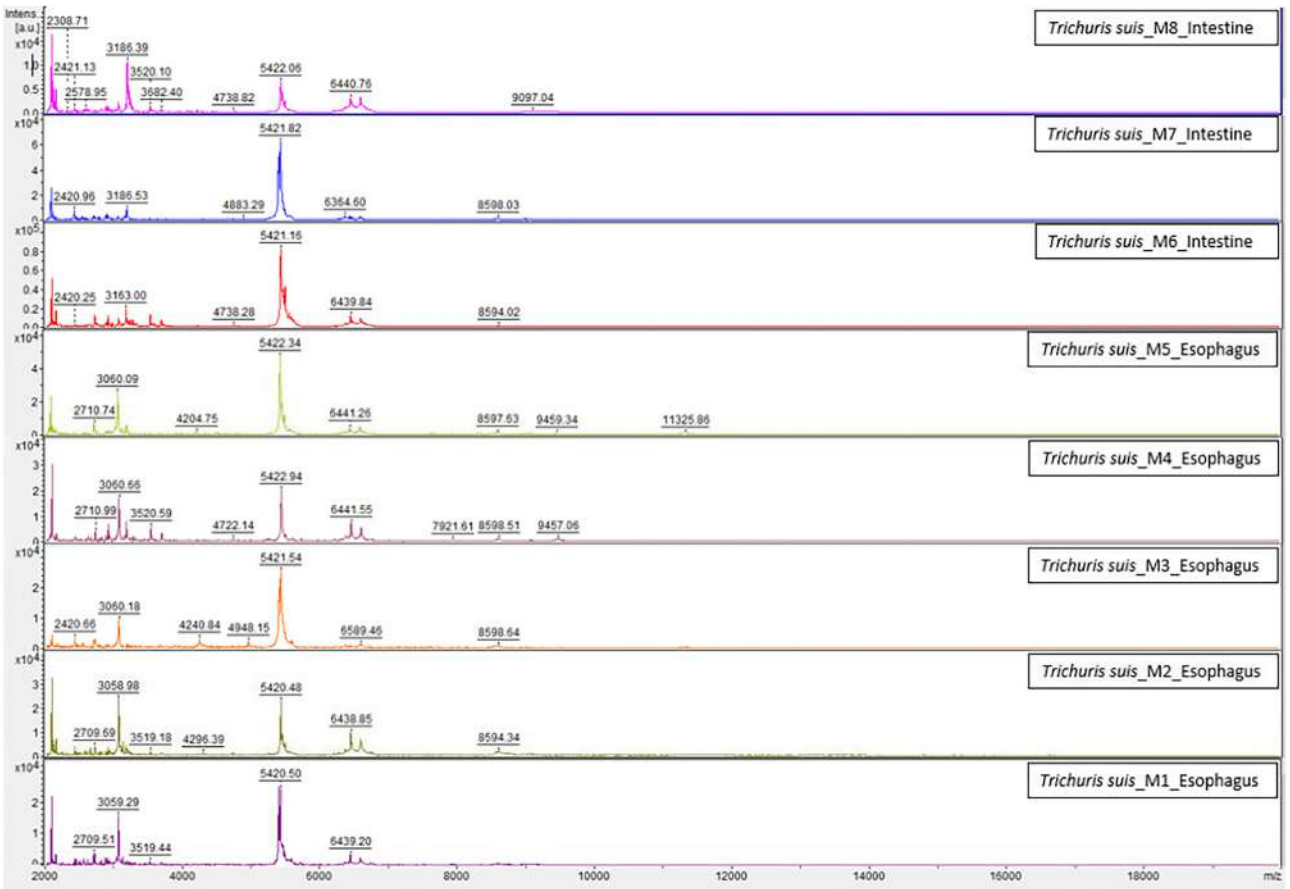

FIGURE 1 | Comparison of the spectra created for the internal database between esophagus and intestine samples of *T. suis* worms by MALDI-TOF MS. a.u., arbitrary units; *m/z*, mass-to-charge ratio (Da).

significant differences between esophagus and intestine samples of *T. suis*.

Internal Database Creation

All MSPs obtained were analyzed with the commercially common database using FlexAnalysis software for bacteria and fungi identification and reliable identification was not achieved with all LSVs < 1.7. The posterior analysis of the raw spectra obtained during MSP creation, which used a combination of the commercial and internal database, revealed the identification of *T. suis* in all the samples, but it was not possible to differentiate between esophagus and intestines of the same *T. suis* species. LSVs of *T. suis* esophagus sample ranging from 2.43 to 2.82, identified as *T. suis* esophagus, and from 2.10 to 2.27 identified as *T. suis* intestine. LSVs of *T. suis* intestine samples ranged from 2.67 to 2.78 with *T. suis* intestine, and from 2.24 to 2.36 with *T. suis* esophagus. The LSVs observed for each sample appeared higher when they were compared with their corresponding body part (esophagus or intestine), but with both could be correctly identified.

Analysis of Samples for External Database Validation

During the study, a total of 20 *Trichuris* worms were utilized for blind testing 20 esophagus and 20 intestines for MALDI-TOF analysis. Of the 40 samples, two esophagus samples were not successfully extracted.

By using the newly developed internal database and considering a threshold of 1.70, MALDI-TOF MS correctly identified 38/38 (100%) of the samples at the probable genus level. When an LSV threshold of ≥2.0 was obtained, the identification rate was 9/18 (50%) for esophagus *T. suis* samples while for the intestine samples it was 16/20 (80%) for a probable species identification. Lastly, LSV ≥ 2.3 was 1/18 (5.55%) for esophagus samples and 1/20 (5%) for intestine samples with a highly probable species identification (see **Table 1**).

Validation of MALDI-TOF Analysis

To provide a molecular identification, 19 *cyt*b mtDNA partial sequences were assessed, of which the intestines of the whipworms were used, and were tested against previously deposited sequences by BLASTn, demonstrating high values of identity (98.81–100%) (see **Supplementary Table S1**).

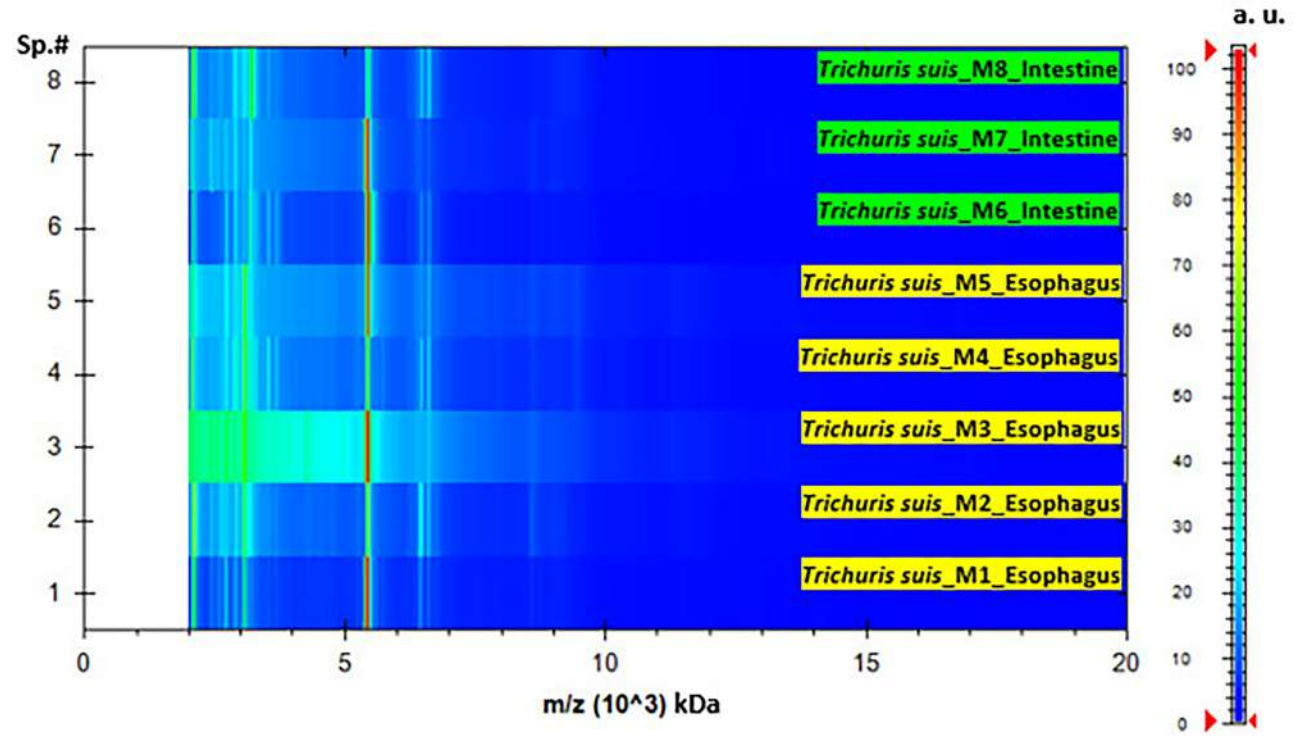

FIGURE 2 | Pseudo-gel representing the protein profile obtained after MALDI-TOF MS analysis of *T. suis* specimens' representative of esophagus and intestine samples. On the *x*-axis, the mass-to-charge values (*m/z*, kDa) are reported, at the same time, on the right *y*-axis, the scale bar reveal the relationship between the color intensity and the peak intensity, expressed by arbitrary units (a.u.). The spectra samples are represented on the left *y*-axis (Sp.#).

TABLE 1 | Identification of 38 *T. suis* samples by MALDI-TOF MS, using a preliminary developed internal database.

Species/body part (sample type)	Number of samples correctly extracted	Identification			LSV range
		LSV ≥ 1.70	LSV ≥ 2.00	LSV ≥ 2.30	
Trichuris suis/Esophagus	18	18 (100%)	9 (50%)	1 (5.55%)	1.84–2.36
Trichuris suis/Intestine	20	20 (100%)	16 (80 %)	1 (5%)	1.90–2.34

LSV, log-score value.

The preliminary protocol used for *Trichuris* samples preparation provided high-quality spectra with elevated reproducibility and intensity of MS spectra. To observe the characteristic high-intensity peaks, a representative protein spectral profile for each *Trichuris* species (*T. suis*, *Trichuris* sp. from *H. cristata*, *T. trichiura*, *T. vulpis,* and *T. ovis*) is shown in **Figure 3**. Hence, for each *Trichuris* species, specific and reproducible MALDI-TOF MS spectra profiles were obtained. Moreover, the internal database was updated with four new *Trichuris* species, and 16 new reference spectra: *Trichuris* sp. from *H. cristata* ($n = 5$), *T. vulpis* ($n = 2$), *T. ovis* ($n = 4$), and *T. trichiura* ($n = 5$).

When submitting the new MSPs of *Trichuris* species to the in-house database (combined with those data commercially available), all the 79 specimens used in the blind test were successfully queried in the extended database. The blind test results yielded 100% correct identification for the specimens evaluated since all samples were LSVs greater than 1.70 (**Table 2**). For the blind test, the percentage of samples with LSVs ≥ 2.00 was for *T. trichiura* from *M. sylvanus*: 88.89%; for *Trichuris* sp. from *H. cristata*: 72.22%; for *T. ovis*: 100%; and for *T. vulpis*: 87.5% (**Table 2**). Finally, the blind test confirmed a complete agreement with the molecular and MALDI-TOF MS identification methods.

Furthermore, to confirm the accurate analysis, a dendrogram based on MSPs was added to the internal database with the five *Trichuris* species (including *T. suis*), and all species provided highly specific spectra with separated clades for each species (**Figure 4**).

Molecular Study

The accession number of all nucleotide sequence data obtained in this work were deposited at the GenBank™, EMBL, and DDBJ databases, and are available in **Table 3**.

To compare the intraspecific and interspecific similarities obtained among *Trichuris* species, pairwise nucleotide and amino acid distances for the *cyt*b partial gene sequences were performed (**Table 4**). The intraspecific similarity between all the samples of the same species studied was similar with values close to

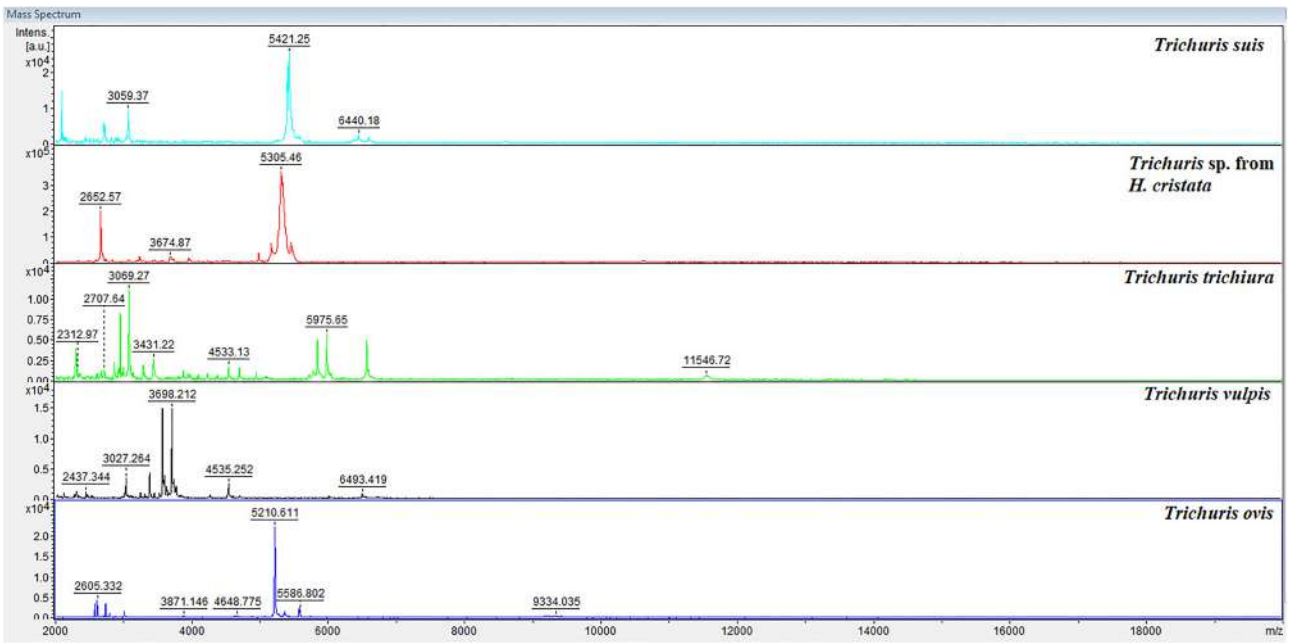

FIGURE 3 | Representative spectral profiles of the esophagus of *Trichuris* species acquired for the creation of the internal database. a.u., arbitrary units; *m/z*, mass-to-charge ratio.

TABLE 2 | Identification of *Trichuris* species esophagus samples by MALDI-TOF MS, using an internal database.

Species	Number of samples correctly extracted	Identification							LSV range
		LSV <1.7	LSV 1.7–1.799	LSV 1.8–1.999	LSV 2–2.299	LSV ≥ 2.3	LSV ≥ 1.70	LSV ≥ 2.00	
Trichuris trichiura from *M. sylvanus*	18	0	0	2 (11.11%)	13 (72.22%)	3 (16.67%)	18 (100%)	16 (88.89%)	1.89–2.38
Trichuris sp. from *H. cristata*	18	0	1 (5.55%)	4 (22.22%)	12 (66.67%)	1 (5.55%)	18 (100%)	13 (72.22%)	1.72–2.42
Trichuris ovis	19	0	0	0	1 (5.26%)	18 (94.74%)	19 (100%)	19 (100%)	2.185–2.577
Trichuris vulpis	8	0	0	1 (12.5%)	5 (62.5%)	2 (25%)	8 (100%)	7 (87.5%)	1.95–2.52

LSV, log-score value.

100% (**Table 4**), except for the intraspecific similarity among *Trichuris* sp. from *H. cristata*, which showed two different groups. The first group consisted of THCM1, THCM2, and THCF3 samples, and the second group of THCF1 and THCF2 samples, with intraspecific nucleotide similarity values of 100 and 99.8%, respectively, and 100% for amino acid distances. The interspecific nucleotide similarity between both groups was 88.2–88.4%, and amino acid similarity was 90.8%. The minimum interspecific similarity among all *Trichuris* species obtained was 68% (between *T. trichiura* from *M. sylvanus* and *Trichuris* sp. from *H. cristata*) in nucleotide distances, and the maximum was 73.6% (between *T. ovis* and *Trichuris* sp. from *H. cristata*) and the minimum in amino acid distances was 65.3% (between *T. trichiura* from *M. sylvanus* and *Trichuris* sp. from *H. cristata*) and the maximum was 76.3% (between *T. trichiura* from *M. sylvanus* and *T. suis*) (**Table 4**).

Phylogenetic tree based on *cyt*b sequences, was rooted including outgroup *Trichinella spiralis* and *Trichinella pseudospiralis*. The alignment of 20 sequences of *Trichuris* species (including outgroups), yielded a dataset of 505 characters. The phylogenetic tree revealed five main clades, corresponding each clade with one different species of *Trichuris*. All clades separately were highly supported (100% ML and 100% BPP). In addition, the clades *T. suis* and *T. trichiura* are more related to each other, but this relation is only supported by BPP, since there is a bootstrap value of <60% between the clades in ML. Both clades are, at the same time, more related to *T. vulpis*. And all the above is more related to *Trichuris* sp. from *H. cristata*, with the clade of *T. ovis* separated from all of them, although within the group of *Trichuris*. In addition, two different lineages, highly supported, among *Trichuris* sp. from *H. cristata* were revealed (**Figure 5**).

DISCUSSION

The genus *Trichuris* has been the subject of a wide-ranging controversy due to its difficult specific differentiation. Many authors have evidenced synonymies (62), cryptic species (18, 63),

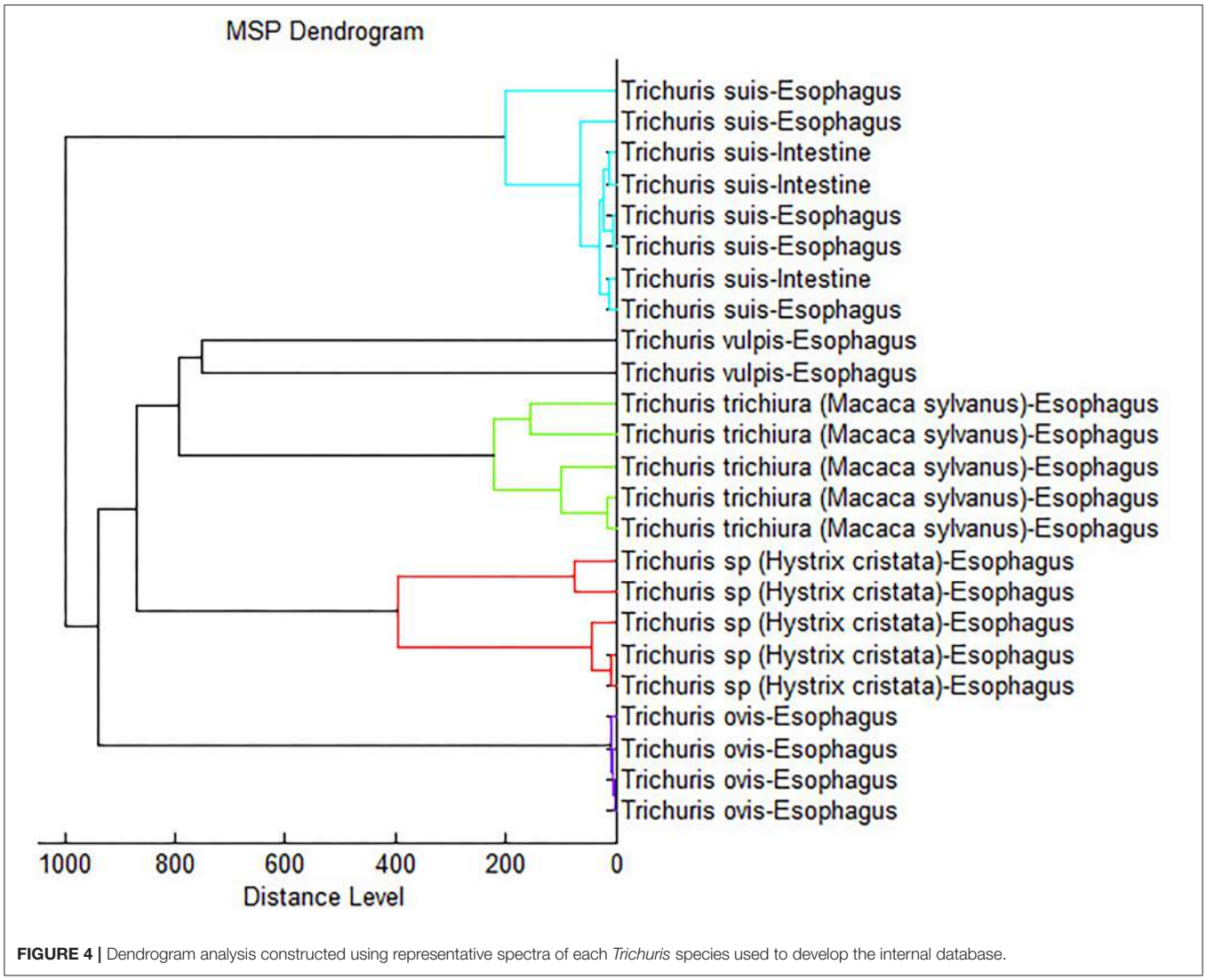

FIGURE 4 | Dendrogram analysis constructed using representative spectra of each *Trichuris* species used to develop the internal database.

and new species (6, 7, 10, 64, 65). Since Dujardin (66) reviewed the genus of *Trichuris* for the first time, many studies have been based on morphometric and molecular analyses. The difficulty of relating the species described with exclusively morphological studies and with species described with only molecular studies makes the study of taxonomy even more difficult to solve. In support of this, additional complementary methods might be necessary to elucidate the different species.

The aim of this study was to confirm whether MALDI-TOF MS analysis could be used as a diagnostic tool for the identification of adult *Trichuris* species. Our results showed high-quality spectra and a high similarity among the samples, regardless of the type of sample analyzed, so we confirm the use of both parts of the body of the nematode for its diagnosis using this technique. To achieve this goal, an internal database has been developed with samples of both, the esophagus and intestine of *T. suis*, providing similar spectra between them, and, therefore, being adequate to identify this species. All specimens (38/38) were accurately identified. Moreover, the preliminary protocol was validated for more species of *Trichuris*, including four more species of *Trichuris* in the internal database. These *Trichuris* species were also 100% correctly identified. As the values revealed in the preliminary study of samples, both esophagus and intestines, showed similar results, we suggest the preference of using the worm's esophagus through MALDI-TOF MS technique since it is easier to manage the esophagus and saving the part of the worm's intestine for molecular studies.

MALDI-TOF MS has been used previously as an effective diagnostically tool in microbiology clinical to identify pathogenic microorganisms. Even now, a systematic review of about MALDI-TOF MS in human and veterinary helminthology was carried out, concluding that more studies are needed since there is evidence for the reliable and rapid identification of nematodes using MALDI-TOF MS, and the identification of these nematodes, whether larvae, adults, or eggs in fecal samples (49). Hence, the internal database for helminth identification is being generated with species-specific MSPs. It is necessary the advancement of one standardized approach for protein

TABLE 3 | Summary of the *Trichuris* sequences obtained in this work and available in GenBank database.

Species	Host	Sample ID	Accession number	Sequence length	Content of G + C%
Trichuris sp.	*Hystrix cristata*	THCM1	OM457227	519	27.2
Trichuris sp.	*Hystrix cristata*	THCM2	OM457228	519	27.2
Trichuris sp.	*Hystrix cristata*	THCF1	OM457229	519	25.1
Trichuris sp.	*Hystrix cristata*	THCF2	OM457230	519	24.9
Trichuris sp.	*Hystrix cristata*	THCF3	OM457231	519	27.2
Trichuris vulpis	*Canis lupus familiaris*	TVM1	OM457232	519	28.5
Trichuris vulpis	*Canis lupus familiaris*	TVF1	OM457233	519	28.3
Trichuris ovis	*Capra hircus*	TOF1	OM457234	519	31.4
Trichuris ovis	*Capra hircus*	TOF2	OM457235	519	31.6
Trichuris ovis	*Capra hircus*	TOM1	OM457236	519	31.4
Trichuris ovis	*Capra hircus*	TOM2	OM457237	519	31.4
Trichuris trichiura	*Macaca sylvanus*	TMSF1	OM457238	519	30.7
Trichuris trichiura	*Macaca sylvanus*	TMSF2	OM457239	519	30.7
Trichuris trichiura	*Macaca sylvanus*	TMSF3	OM457240	519	31.1
Trichuris trichiura	*Macaca sylvanus*	TMSM1	OM457241	519	31.1
Trichuris trichiura	*Macaca sylvanus*	TMSM2	OM457242	519	30.7
Trichuris suis	*Sus scrofa domestica*	TSM1	OM457243	519	28.7
Trichuris suis	*Sus scrofa domestica*	TSF1	OM457244	519	28.9
Trichuris suis	*Sus scrofa domestica*	TSF2	OM457245	519	28.9

TABLE 4 | Pairwise nucleotide and amino acid distances for the *cytochrome* b partial gene sequences for *Trichuris* species studied in this work.

	THCM1	THCM2	THCF1	THCF2	THCF3	TVM1	TVF1	TOF1	TOF2	TOM1	TOM2	TMSF1	TMSF2	TMSF3	TMSM1	TMSM2	TSM1	TSF1	TSF2
THCM1		100	90.8	90.8	100.0	68.2	68.2	68.8	68.8	68.8	68.8	65.3	65.3	65.9	65.3	65.3	69.4	69.4	69.4
THCM2	100.0		90.8	90.8	100.0	68.2	68.2	68.8	68.8	68.8	68.8	65.3	65.3	65.9	65.3	65.3	69.4	69.4	69.4
THCF1	88.4	88.4		100.0	90.8	68.8	68.8	69.4	69.4	69.4	68.8	65.9	65.9	66.5	65.9	65.9	69.9	69.9	69.9
THCF2	88.2	88.2	99.8		90.8	68.8	68.8	69.4	69.4	69.4	68.8	65.9	65.9	66.5	65.9	65.9	69.9	69.9	69.9
THCF3	100.0	100.0	88.4	88.2		68.2	68.2	68.8	68.8	68.8	68.8	65.3	65.3	65.9	65.3	65.3	69.4	69.4	69.4
TVM1	70.7	70.7	73.2	73.2	70.7		99.4	71.7	71.7	71.7	71.7	72.8	72.8	71.7	72.8	72.8	69.9	69.9	69.9
TVF1	70.7	70.7	73.2	73.2	70.7	99.8		71.7	71.7	71.7	71.7	72.8	72.8	71.7	72.8	72.8	69.9	69.9	69.9
TOF1	71.7	71.7	73.6	73.6	71.7	69.9	69.9		100.0	100.0	99.4	72.3	72.3	71.1	72.3	72.3	73.4	73.4	73.4
TOF2	71.5	71.5	73.4	73.4	71.5	69.7	69.7	99.8		100.0	99.4	72.3	72.3	71.1	72.3	72.3	73.4	73.4	73.4
TOM1	71.5	71.5	73.4	73.4	71.5	69.7	69.7	99.2	99.4		99.4	72.3	72.3	71.1	72.3	72.3	73.4	73.4	73.4
TOM2	71.5	71.5	73.2	73.2	71.5	69.7	69.7	99.2	99.4	98.8		72.8	72.8	71.7	72.8	72.8	73.4	73.4	73.4
TMSF1	68.2	68.2	69.4	69.6	68.2	70.3	70.5	70.1	69.9	69.7	70.1		100.0	98.8	100.0	100.0	76.3	76.3	76.3
TMSF2	68.2	68.2	69.4	69.6	68.2	70.3	70.5	70.1	69.9	69.7	70.1	100.0		98.8	100.0	100.0	76.3	76.3	76.3
TMSF3	68.2	68.2	69.4	69.6	68.2	69.9	70.1	69.7	69.6	69.4	69.7	99.6	99.6		98.8	98.8	76.3	76.3	76.3
TMSM1	68.0	68.0	69.2	69.4	68.0	70.1	70.3	70.1	69.9	69.7	70.1	99.6	99.6	99.2		100.0	76.3	76.3	76.3
TMSM2	68.2	68.2	69.4	69.6	68.2	70.3	70.5	70.1	69.9	69.7	70.1	100.0	100.0	99.6	99.6		76.3	76.3	76.3
TSM1	71.1	71.1	72.1	72.1	71.1	72.1	72.1	72.1	71.9	72.1	71.9	71.1	71.1	71.1	70.9	71.1		100.0	100.0
TSF1	70.9	70.9	71.9	71.9	70.9	71.9	71.9	72.1	71.9	72.1	71.9	70.9	70.9	70.9	70.7	70.9	99.8		100.0
TSF2	70.9	70.9	71.9	71.9	70.9	71.9	71.9	72.1	71.9	72.1	71.9	70.9	70.9	70.9	70.7	70.9	99.8	100.0	

Nucleotide genetics distances are given below the diagonal and amino acid genetic distance above the diagonal.

extraction and MALDI-TOF MS spectra to create accurate databases and specific and reproducible results in different laboratories for further research (25). Nonetheless, these internal databases are created in each reference laboratory, and for that reason, we find ourselves with the added difficulty of not being able to compare the different species recorded in the different databases created, being of huge importance to the creation of a single common database or being able to share the spectra obtained from the analyzes of each research laboratory.

Nagorny et al. (67), in their research for the identification of different nematodes (*Dirofilaria* and *Ascaris*) by MALDI-TOF MS using tissues from adult worms, suggested that in the range from 8 to 20 kDa, the spectra allowed differentiating between different species of nematodes, and in the range of 2 to 6 kDa, the

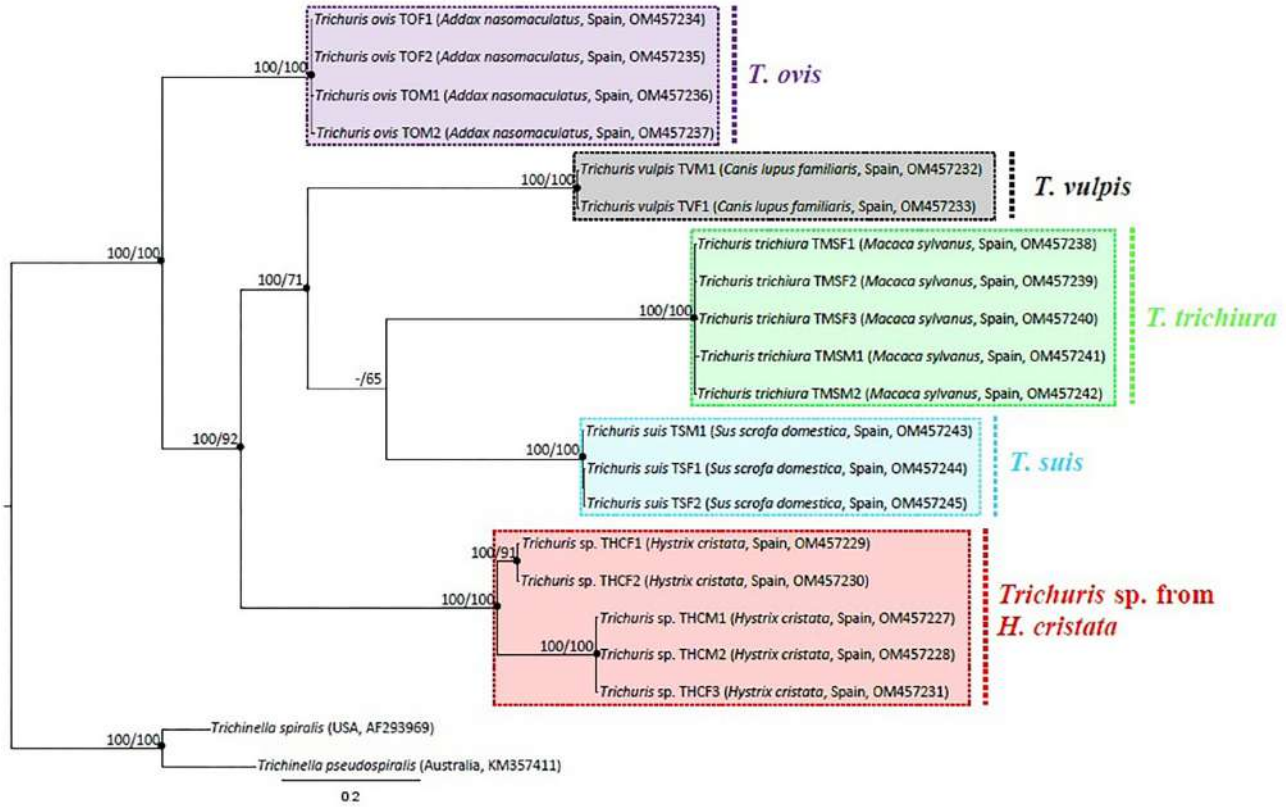

FIGURE 5 | Phylogenetic tree based on analysis of mtDNA *cyt*b partial gene using Bayesian Inference. Maximum likelihood bootstrap values are listed first, followed by Bayesian Posterior Probabilities of clades, for clade frequencies exceeding 60%.

entire genus of nematodes could probably be characterized. In our spectra obtained for the MSP, the most frequent peaks were observed in the range from 2 to 7 kDa but extended up to 10 kDa. Consequently, the necessity for the identification of more species of *Trichuris* to know whether really exist a differential range for genus and species of nematodes in the spectra obtained by MALDI-TOF MS in further research.

Furthermore, MALDI-TOF MS technique has been advanced in the last few years as a significant tool for taxonomic identification and for the phylogenetic classification of microorganisms (68). Subsequently, Zurita et al. (22) supported the evidence with flea vector species, obtaining agreement with the data obtained in the dendrogram and the phylogenetic studies carried out. Nevertheless, those authors in agree with Yssouf et al. (69), argued that due to the lack of specimens, MALDI Biotyper software cannot yet determine reliablility for the phylogenetic study of arthropods. Likewise, studies of parasitic nematodes suggest the usefulness of MALDI-TOF as an efficient taxonomic tool in parasitological studies (67). In this study, the obtained dendrogram separated each *Trichuris* species into a different clade. Moreover, it related more to the clades of *T. vulpis* and *T. trichiura*, and on the other hand, the clades of *T. suis* and *Trichuris* sp. from *H. cristata*, resulting in the clade of *T. ovis* more separated from all other clades. This last argument is the same as the one found in the phylogenetic tree based on *cyt*b mtDNA partial gene, where *T. ovis* clade is always separated from the other clades; however, the phylogenetic relationships among the other clades are different and were highly supported. Thus, according to previously described authors, we suggest that MALDI-TOF MS should not be used to establish phylogenetic relationships in nematode species, but further studies are needed with more different species and genera of nematode parasites.

Soil-transmitted helminth (STH) infections are among the most common infections worldwide, affecting the poorest and most disadvantaged communities. The global strategy for controlling the morbidity of STH infections is preventive chemotherapy with periodic medicinal treatment (deworming) without a previous individual diagnosis for all at-risk people living in endemic areas (preschool and school-age children and women of reproductive age and adults in certain high-risk occupations). The medicines recommended by WHO are albendazole or mebendazole, which are effective, inexpensive, and easy to administer by non-medical personnel (1). Both drugs show low efficacy against *T. trichiura* using single, oral doses. Frightening, according to a recent network meta-analysis looking at interactions over time, the efficacy of both drugs is declining over time, which could be associated with resistance to anthelmintic drugs (70). Moreover, MALDI-TOF MS analysis is used as a tool to discover the antibiotic resistance in microorganisms by detection of precise biomarkers within the

protein spectra produced (46, 47, 71), and could be used to find different targets and to develop treatments to combat the resistance. A preliminary study about a new diagnostic tool for *Anisakis* spp. by MALDI-TOF MS, has managed to identify a set of signs as potential consensus "biomarkers" peak list (24). In this study, we started by characterizing a genus and a species that has never been determined by MALDI-TOF analysis and developing the first internal database with *Trichuris* nematode parasites. But more *Trichuris* species would be needed to differentiate between their protein spectra profile and draw conclusions.

CONCLUSION

This study validates the usefulness of MALDI-TOF MS technique as a reliable, fast, and economical identification tool for the diagnosis of *Trichuris* species. The creation of the internal database should be expanded with more samples of different species of the genus *Trichuris* and other nematodes species. The results obtained by MALDI-TOF MS showed a dendrogram that is not reliable to phylogenetic studies in *Trichuris* species. In addition, the necessity to discover and analyze potential biomarkers and targets to focus future studies on developing new anthelmintic drugs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JR, AZ, and RC: conceptualization and design of the study, investigation, validation, and methodology. JR: data curation and writing–original draft. JR and AZ: formal analysis. CC: funding acquisition. RC and CC: project administration and supervision. All authors contributed to the manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.867919/full#supplementary-material

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CAPÍTULO IV. DISCUSIÓN

I. DISCUSIÓN

IV.1. ESTUDIOS MORFOLÓGICOS, BIOMÉTRICOS, DE CARACTERIZACIÓN MOLECULAR Y FILOGENÉTICOS DE DIFERENTES ESPECIES DEL GÉNERO *TRICHURIS*

En las últimas décadas, diversos autores han analizado la sistemática del género *Trichuris* debido a su controversia a nivel de especies. La diferenciación específica de especies estrechamente relacionadas ha sido un problema complejo durante muchos años. Así, los estudios más recientes han evidenciado sinonimias (Oliveros y col., 2000), especies crípticas (Callejón y col., 2012), diferentes linajes genéticos (Cavallero y col., 2019; Rivero y col., 2020, 2022a) y nuevas especies (Liu y col., 2013; Cutillas y col., 2014; Callejón y col., 2017; Wang y col., 2019) dentro de este género.

Tradicionalmente a la especie del género *Trichuris* aislada de humanos y de PNH se le denominaba *T. trichiura*. En la última década, algunas publicaciones abordaron la cuestión de si las especies de *Trichuris* son compartidas entre humanos y PNH o, si existen especies diferentes (Liu y col., 2012a, 2013; Hawash y col., 2015; Callejón y col., 2017). Además, la relación de *Trichuris* procedentes de humanos y PNH, en términos de genética y de aspectos evolutivos, ha sido poco conocida hasta la fecha. Durante los últimos años se ha revelado la existencia de más de un taxón capaz de infectar a humanos y otros PNH, incluidos los individuos que permanecían en cautiverio. Por consiguiente, varios autores sugirieron que *T. trichiura* debía considerarse como un complejo de especies que incluía diferentes unidades crípticas (Cutillas y col., 2009; Hawash y col., 2015). Asimismo, basándose en parámetros morfo-biométricos y moleculares se han descrito nuevas especies de *Trichuris* pertenecientes a hospedadores primates, como *T. rhinopitheroxella* procedente de *R. roxellana* (Wang y col., 2019), *T. colobae* procedente de *C. g. kikuyensis* (Cutillas y col.,

2014) y *T. ursinus* procedente de *P. ursinus* (Callejón y col., 2017). Estos estudios confirmaban que *T. trichiura* no es el único tricocéfalo que se podía encontrar en hospedadores primates.

En vista de estos antecedentes, se ha requerido de métodos de identificación adicionales para dilucidar entre las diferentes especies de *Trichuris* procedentes de primates.

En la presente Tesis Doctoral hemos realizado un análisis morfo-biométrico de diferentes especies del género *Trichuris* procedentes de diversos hospedadores primates (*M. sylvanus*, *P. troglodytes*, *C. g. kikuyensis*, *P. ursinus*) y de cerdo (*S. s. domestica*), resultando que el análisis basado en tres medidas representativas de los machos de las especies de *Trichuris* (la máxima anchura de la región posterior del cuerpo, la longitud de la espícula y la longitud máxima de la vaina espicular), mostraba las diferencias globalizadas de la población de *Trichuris* sp. de *M. sylvanus* (García-Sánchez y col., 2019), mostrando valores mayores en los machos aislados de los macacos con respecto a los machos de *T. trichiura* procedentes de chimpancés (Cutillas y col., 2009). Por otro lado, se ha llevado a cabo, en las especies citadas anteriormente, el primer estudio morfométrico utilizando la técnica de morfometría geométrica (García-Sánchez y col., 2019). En cuanto a los resultados obtenidos, se ha logrado la diferenciación de todas las poblaciones estudiadas (*T. trichiura*, *T. colobae*, *Trichuris* sp. de *M. sylvanus*, *T. ursinus* y *T. suis*), obteniendo áreas diferenciadas y bien definidas para cada población de los machos de *Trichuris*. Estos resultados están en concordancia con el análisis molecular aplicado a las muestras de los machos que permitían la identificación de las diferentes especies de *Trichuris* (Cutillas y col., 2009, 2014; Callejón y col., 2017). Los resultados confirmaron que cada población de machos de *Trichuris* analizada, poseía su propia identidad morfológica (García-Sánchez y col., 2019).

En cambio, el tamaño de las poblaciones de las hembras de *Trichuris* no seguía un patrón dependiente del hospedador, probablemente debido a la ausencia de medidas representativas. Únicamente, las hembras procedentes de suidos presentaron un tamaño mayor que las hembras procedentes de primates (García-Sánchez y col., 2019). Estos resultados estaban de acuerdo con los reportados previamente, en los cuales no se había logrado la diferenciación de las hembras de *Trichuris* (Cutillas y col., 2009, 2014; Callejón y col., 2017). Además, diversos autores han citado que las hembras son más difíciles de diferenciar que los machos del género (Gibbons, 1986; Cutillas y col., 2009).

Por otro lado, hemos realizado el análisis biométrico resultando que el análisis basado en tres medidas representativas de los machos (la máxima anchura de la región posterior del cuerpo, la longitud de la espícula y la longitud máxima de la vaina espicular), mostraba las diferencias globalizadas de la población de *Trichuris* sp. de *M. sylvanus*, mostrando valores mayores en los machos aislados de los macacos con respecto a los machos de *T. trichiura* procedentes de chimpancés (Cutillas y col., 2009; García-Sánchez y col., 2019; Rivero y col., 2020). Varios autores han explicado esta ocurrencia de diferentes medidas biométricas en una misma especie como adaptaciones fenotípicas (Nissen y col., 2012; Cutillas y col., 2009, 2014).

Durante muchos años, diferentes autores se han basado únicamente en características morfológicas y biométricas para realizar una descripción de *T. trichiura* aislados de primates. Por esa razón, hemos llevado a cabo un estudio morfo-biométrico y molecular, además de una actualización en la descripción de *T. trichiura*, basada en los resultados proporcionados de *Trichuris* procedentes de diferentes primates de la presente Tesis Doctoral junto con otras descritas previamente, procedentes de diferentes hospedadores humanos y PNH (*H. sapiens* (Nissen y col., 2012), *P.*

troglodytes (Cutillas y col., 2009), *M. sylvanus* (Rivero y col., 2020) y *P. papio* (Rivero y col., 2021b)).

Por otra parte, los adultos obtenidos de *M. sylvanus*, atendiendo a las características morfológicas observadas, correspondían con *T. trichiura*. Igualmente, de acuerdo con varios autores, los machos de esta población mostraban un par de papilas paracloacales típicas de la especie *T. trichiura* (Zaman, 1984; Tenora y col., 1988; Cutillas y col., 2009). De acuerdo con los resultados obtenidos, Ooi y col. (1993) reportaron que la existencia de un par de papilas paracloacales no estaba presente únicamente en los machos de *T. trichiura* de humanos sino también en los machos de *T. trichiura* de otros PNH como *M. fuscata* o *P. papio*. En cambio, en las hembras, observaron que mostraban la vagina evertida y cubierta con espinas puntiagudas. Este tipo de vagina no se observó en las hembras de *Trichuris* de *M. sylvanus* (Rivero y col., 2020). Además, los adultos procedentes de *C. g. kikuyensis* y *P. ursinus* (Cutillas y col., 2014; Callejón y col., 2017), revelaron claras diferencias con respecto a los adultos encontrados en *M. sylvanus*. De ese modo, se observaron que los tricocéfalos procedentes de *M. sylvanus* podían diferenciarse de *T. colobae* por la presencia de una papila paracloacal subterminal típica, estando ausente un racimo de pequeñas papilas, características de la especie *T. colobae* (Cutillas y col., 2014) y, además, presentaba una espícula diferente a la de *T. colobae* y *T. ursinus*. En cambio, las hembras presentan una vulva no evertida y no ornamentada. Asimismo, como Steinmann y col. (2015) citaron previamente, se observó la presencia de dos tipos de huevos diferentes de *Trichuris* en el mismo hospedador.

En consecuencia, en la redescripción de *T. trichiura* realizada en la presente Tesis Doctoral, se mostró que las hembras se caracterizaban por presentar una vulva no protrusiva sin ornamentación (Rivero y col., 2020, 2021b). En cambio, algunos autores la han descrito con una superficie cubierta de espinas, similares a las espinas presentes en la vaina espicular

del macho (Skrjabin y col., 1957; Ooi y col., 1993). Asimismo, los machos de *T. trichiura* se caracterizaron por la presencia de una espícula lanceolada que se estrecha en el extremo (Zaman, 1984). Sin embargo, Tenora y col. (1988) observaron, en muestras procedentes de *H. sapiens*, que las espículas acababan con un extremo cilíndrico. Además, los machos de *T. trichiura* procedentes de *P. papio* y *M. sylvanus* presentaban una espícula con dos zonas extremas quitinizadas y una parte central más clara (Rivero y col., 2020, 2021b). Esta característica no fue observada en otros machos de *T. trichiura* descritos previamente. Por otro lado, la vaina espicular era cilíndrica sin bulbo distal con espinas, mientras que otros autores han descrito éstas con espinas de diferentes formas y tamaños (Skrjabin y col., 1957; Tenora y col., 1988). Igualmente, observamos que los machos de *T. trichiura* se caracterizaban por la presencia de un par de papilas paracloacales, que se observaban cuando la vaina estaba invaginada (Rivero y col., 2020, 2021b); sin embargo, otros autores no lo apreciaron (Ooi y col., 1993). Este hecho podría sugerir que las descripciones realizadas por Ooi y col. (1993) de las poblaciones de *Trichuris* aisladas de humanos y PNH basadas exclusivamente en caracteres morfológicos y biométricos, podrían corresponder a diferentes especies cercanas a *T. trichiura*.

Biométricamente, existe una concordancia entre las medidas de *T. trichiura* obtenidas por diferentes autores y las aportadas en la presente Tesis Doctoral, ya que todos los valores medidos se superponían dentro del rango de medidas definidas para esta especie (Rivero y col., 2021b). En cambio, respecto a los machos, la longitud total máxima del cuerpo y la longitud máxima de la parte posterior, fueron ligeramente más grandes en todas las especies analizadas en la presente Tesis Doctoral (Dinnik y col., 1938). Además, el ancho máximo de la región posterior del cuerpo, la longitud de la espícula y la longitud del conducto eyaculador, presentaban valores ligeramente superiores (Skrjabin y col., 1957).

En las hembras, varios autores citaron valores ligeramente superiores acerca de la longitud total máxima del cuerpo del adulto (Frères y Neveu-Lemaire, 1936; Dinnik y col.,1938; Skrjabin y col., 1957). Además, la longitud máxima de la región esofágica, el ancho máximo del cuerpo en el lugar de unión del esófago y el intestino fueron ligeramente superiores en los datos proporcionados por Skrjabin y col., (1957). Asimismo, existen diferencias entre la población de *Trichuris* sp. procedente de chimpancés (Cutillas y col., 2009), que muestran un tamaño más corto de los machos y hembras con respecto a *T. trichiura* procedentes de *H. sapiens*, *M. sylvanus* y *P. papio*, pudiéndose explicar por la plasticidad fenotípica de estos organismos (Knight, 1984; Spakulová, 1994; Robles y Navone, 2006; Robles, 2011).

Los datos aportados en el estudio permiten la diferenciación de esta especie con respecto a otras que parasitan PNH como *T. colobae* (Cutillas y col., 2014) y *T. ursinus* (Callejón y col., 2017). La espícula de *T. trichiura* presenta una parte central más clara, que no está presente en la de *T. colobae* y *T. ursinus*. Además, el grupo de papilas en la zona posterior del cuerpo solo está presente en *T. colobae*. Las hembras de *T. trichiura* y *T. ursinus* presentan una vulva no protrusiva, mientras que las hembras de *T. colobae* presentan una vulva protusiva con papilas. Asimismo, la vagina es larga y recta en *T. ursinus*, pero aparece con circunvoluciones en *T. trichiura.*

Por otro lado, la combinación de ciertos marcadores nucleares y mitocondriales se han considerado una herramienta útil para inferir relaciones filogenéticas dentro del género *Trichuris*. Así, los árboles filogenéticos inferidos de los conjuntos de datos ribosómicos y mitocondriales, de los hospedadores analizados, revelaron la existencia de dos clados principales, el clado 1 o "Linaje *T. suis*" y el clado 2 o "Linaje *T. trichiura*" (Rivero y col., 2020, 2021b), citados previamente por diversos autores (Ravasi y col., 2012; Callejón y col., 2015, 2017;

Cavallero y col., 2019). Sin embargo, los árboles filogenéticos inferidos únicamente con los conjuntos de datos ribosómicos no resolvían correctamente la filogenia del clado 2. Asimismo, en el clado 2 se incluían todas las especies de *Trichuris* procedentes de humanos y de PNH a excepción de algunas secuencias de *T. trichiura* de Uganda y Camerún (Ghai y col., 2014) que aparecieron dentro del clado 1 junto a *T. ursinus* (Rivero y col., 2021b).

Paralelamente, la similitud entre los dos clados principales obtenidos para los marcadores ribosómicos mostraron valores claramente bajos. Además, se mostró un porcentaje de similitud entre los subclados del clado 2 (*T. trichiura*) superiores a los observados previamente por otros autores en el género *Trichuris* (Callejón y col., 2012, 2016). Este hecho pudo justificarse en cuanto a que los análisis genéticos revelaron que las regiones de ITS estaban saturadas y, por lo tanto, no eran útiles para inferir relaciones filogenéticas entre las poblaciones del clado 2.

Por otro lado, en las secuencias ribosómicas obtenidas se han observado microsatélites. Este hecho ha sido observado previamente por varios autores (Oliveros y col., 2000; Cutillas y col., 2002, 2004) y parecía ser debido a una consecuencia del intercambio mutacional durante el proceso de replicación del ADN (Schlötterer y Tautz, 1994; Wesson y col., 1992; Campbell y col., 1995; Hoste y col., 1995; Gasser y col., 1996).

Basándonos en los marcadores mitocondriales *cox*1 y *co*b, los estudios de similitud interpoblacional entre los diferentes subclados del clado 2, revelaron un rango de similitud inferior a los observados por las secuencias de ITS, pero similares a las observadas entre las especies del clado 1 (*T. ursinus*, *T. suis* y *T. colobae*). En cambio, estos valores eran superiores para el marcador molecular *rrn*L, presentando una fuerte resolución entre los diferentes linajes de *T. trichiura*. Estos resultados concuerdan con Chan y col. (2020) donde evaluaron la utilidad de los marcadores ribosómicos y mitocondriales y concluyeron que los

marcadores mitocondriales mostraban una mayor resolución para estudios filogenéticos. Además, demostraron que los genes del ARNr (*rrn*L y *rrn*S) presentaban mayor poder resolutivo a niveles taxonómicos tanto inferiores como superiores para la discriminación entre especies. Además, puesto que estos marcadores mitocondriales, al ser material genético que se hereda por vía materna, mutan a un ritmo rápido en comparación con los genes nucleares, pueden ser útiles para los análisis evolutivos y filogenéticos (Hu y col., 2002). Por esta razón y considerando los valores de similitud observados, sugerimos que las poblaciones de *T. trichiura* del clado 2 corresponden a diferentes linajes genéticos. Nuestros resultados concuerdan con Liu y col. (2013) quienes consideraron, basándose en el marcador molecular *rrn*L, y gracias a los valores de similitud, que la población de *Trichuris* de *T. francoisi* era una nueva especie de *Trichuris*. En los árboles filogenéticos inferidos de los conjuntos de datos mitocondriales pudimos identificar cuatro subclados genéticamente distintos que estaban presentes en los primates muestreados. Los subclados 2b y 2c mostraron un amplio rango de hospedadores, y no estaban restringidos a las especies de PNH. Sin embargo, los subclados 2a y 2d mostraron una mayor especificidad de hospedador correspondiente a *Trichuris* sp. de *M. sylvanus* y *H. sapiens* (subclado 2a), y *M. fuscata* (subclado 2d) exclusivamente. De acuerdo con este estudio se observaron resultados similares en *Trichuris* sp. de *M. fuscata* (Cavallero y col., 2015a, 2019). Además, se observó que la población de *Trichuris* de *M. sylvanus* se dividía en dos subclados diferentes, un clado formado únicamente por el haplotipo minoritario y otro clado formado por el haplotipo mayoritario y secuencias de *Trichuris* procedentes de diferentes hospedadores primates. Además, basándonos en las secuencias del gen parcial *cox*1, estos dos subclados aparecían distribuidos según el origen, africano o europeo, de *T. trichiura* de *H. sapiens*. Este patrón filogenético de distribución podría sugerir que diferentes poblaciones están circulando,

aunque las muestras de *Trichuris* de *M. sylvanus* se tomaron del mismo hospedador (Rivero y col., 2020). Además, el hecho de que los tricocéfalos procedentes de *M. sylvanus* estén agrupados y separados de los tricocéfalos presentes en los otros hospedadores del género, puede ser debido a que *M. sylvanus* es el único representante de origen africano existente del primate macaco, y todas las demás especies proceden de países asiáticos, sugiriendo un proceso coevolutivo de los vermes junto con el hospedador (Modolo y col., 2005).

Del mismo modo, otros autores no encontraron diferencias entre *T. trichiura* de humanos y de PNH procedentes de Uganda, e indicaron un origen africano específico del parásito, que posteriormente se habría transmitido a Asia y América del Sur, lo que sugiere que *Trichuris* en humanos representa un parásito que ha ido evolucionando al igual que sus ancestros (Hawash y col., 2015). Además, se observa que la mayoría de las secuencias de *Trichuris* sp. de *M. sylvanus* (haplotipo mayoritario) se agrupaba con *T. trichiura* de *H. sapiens* de Uganda (África) y, el haplotipo minoritario, con *Trichuris* sp. de *H. sapiens* de República Checa (Europa). Dado que únicamente se utiliza una secuencia de referencia africana, se deberían llevar a cabo más estudios moleculares para aclarar si existe un origen específico africano del parásito y una transmisión posterior a Europa y Asia.

De acuerdo con este estudio (Rivero y col., 2020), se observaron resultados similares en *Trichuris* sp. procedente de *M. fuscata* (Cavallero y col., 2019). Esta población mostró dos genotipos diferentes de *Trichuris* dispuestos en dos subclados distintos. Estos autores sugirieron la posibilidad de dos fuentes diferentes de infección para los macacos japoneses correspondiendo a dos taxones de *Trichuris* diferentes. Dentro del clado 2, los subclados TT2a, TT2b y TT2c correspondían con especies taxonómicas capaces de infectar a humanos y primates sin una estricta especificidad de hospedador. Estos resultados están de acuerdo con

Doležalová y col. (2015) revelando la existencia de especies de *Trichuris* que son compartidas por humanos y PNH (mandriles y macacos). Asimismo, Ghai y col. (2014) observaron que el rango de hospedadores de las especies de *Trichuris* varía según el grupo taxonómico, así, con algunos grupos muestran especificidad de hospedador, con otros una especificidad intermedia, y otros muestran generalidad en los hospedadores que parasitan. De esa manera, un grupo era específico para parasitar a los humanos, otro presentaba un rango de hospedadores intermediario y un grupo adicional podría infectar a todos los primates muestreados, incluido los humanos.

Asimismo, los análisis moleculares basados en el ADNmt (*cox*1 y *co*b) revelaron la existencia de dos genotipos diferentes en la población de *Trichuris* sp. de *M. sylvanus*, que correspondían con dos linajes genéticos diferentes dentro del "Linaje *T. trichiura*". A su vez, estos dos linajes no se correlacionaron con dos morfoespecies distintas. Sin embargo, debemos ser cautelosos debido a que el número de individuos de una de las poblaciones fue más bajo que de la otra (Rivero y col., 2020). En concordancia con este hecho, también se han encontrado dos genotipos diferentes de *Trichuris* sp. procedentes de *P. ursinus* de dos localizaciones geográficas distintas. Sin embargo, no se realizaron los estudios morfológicos para caracterizar las diferentes morfoespecies (Ravasi y col., 2012). Posteriormente, Callejón y col. (2017) realizaron el estudio y la descripción morfológica en una de estas poblaciones presentes en *P. ursinus* y describieron una nueva especie de *Trichuris* denominada *T. ursinus*, que forma parte del "Linaje *T. suis*". Asimismo, el análisis de la similitud intraespecífica entre las poblaciones de *T. trichiura* procedentes de *P. papio, C. aethiops, E. patas* y el haplotipo mayoritario de *Trichuris* sp. de *M. sylvanus* de España, reveló su valor más alto en comparación con las demás poblaciones de *T. trichiura* perteneciente al subclado 2c, en todos los marcadores analizados.

Por otra parte, la existencia de poblaciones de *Trichuris* sp. asociadas con el clado 1 o "Linaje *T. suis*" podría indicar el posible origen del cambio de hospedador de PNH a cerdos y, por lo tanto, el origen de una nueva especie, *T. suis*. Este hecho ha sido sugerido por varios autores previamente, ya que Hawash y col. (2015) encontraron evidencias de un origen africano de *T. trichiura* que luego se transmitió con los ancestros humanos a Asia y luego a América, y también sugieren un cambio de hospedador a los cerdos en Asia, donde *T. suis* parece haberse transmitido globalmente por una combinación de dispersión natural del hospedador y factores antropogénicos.

Un estudio similar sobre tricocéfalos procedentes de humanos sugería la existencia de transmisión zoonótica especialmente con *T. trichiura* procedente de PNH, y posiblemente, también con *T. suis* de cerdos y *T. vulpis* de perros. Así, recientemente, se caracterizaron molecularmente poblaciones de *Trichuris* aisladas de humanos y perros de una comunidad rural en Malasia Peninsular, basándose en el marcador del ARNr *rrn*S, y las relaciones filogenéticas y el análisis molecular demostró que *T. trichiura* y *T. vulpis* se encontraban, ambas especies, en ambos hospedadores (tanto en humanos como en perros), y eran genéticamente diferentes, sugiriendo una transmisión cruzada entre ambos hospedadores (Mohd-Shaharuddin y col., 2019). Asimismo, varios estudios han demostrado que los PNH representan un importante reservorio para enfermedades infecciosas zoonóticas (Geerts y Gryseels, 2001; WHO, 2011; GBDS, 2015). En este contexto, las infecciones por *Trichuris* han sido encontradas en una amplia variedad de PNH que viven en diferentes hábitats que incluyen los colobos, macacos, babuinos y chimpancés (Gillespie y col., 2010; Arizono y col., 2012; Kooriyama y col., 2012; Levecke y col., 2015; Zanzani y col., 2016; Li y col., 2015; Yao y col., 2018), entre otros. Basándonos en los estudios moleculares descritos previamente, algunas especies de *Trichuris* parecen ser específicas de un

PNH, mientras que otras probablemente tengan el potencial de circular entre humanos y PNH, ya que son genéticamente similares. Este hecho es particularmente importante cuando los humanos y PNH viven en estrecha proximidad y tiene implicaciones significativas para la salud y para implementar estrategias de control efectivas (Betson y col., 2015).

A pesar de que parece existir un patrón de infección con diferentes especies de *Trichuris* que infectan especies particulares de hospedadores, la existencia de más especies de *Trichuris* en primates abre la posibilidad de estudiar el potencial zoonótico de diversos hospedadores que albergan *T. trichiura* y otras supuestas nuevas especies de tricocéfalos (Ghai y col., 2014).

En consecuencia, sería necesario realizar más estudios morfológicos y moleculares de distintas poblaciones de *Trichuris* que parasitan humanos y PNH de diferentes orígenes geográficos para clarificar la taxonomía y las diferentes especies de *Trichuris* en primates y, realmente, conocer en profundidad la diversidad de las especies de *Trichuris* que parasitan PNH, y si se debe a un proceso específico del hospedador, o si estas especies comparten diferentes hospedadores primates, así como, para evaluar la posibilidad de estos primates como reservorio de la tricuriasis humana.

Con el fin de dilucidar los diferentes linajes genéticos encontrados en la población de *Trichuris* procedente de *M. sylvanus*, se llevó a cabo un estudio del genoma mitocondrial completo de los dos haplotipos, el haplotipo mayoritario (TMF31) y el haplotipo minoritario (TMM5), y el genoma mitocondrial completo de *Trichuris* aislado de *P. papio* (TPM1) (Rivero y col., 2021a).

El tamaño de los genomas mitocondriales completos obtenidos estuvo dentro del rango citado para la familia Trichuridae (Liu y col., 2012a, 2012b, 2013; Hawash y col., 2015; Ahmad y col., 2019). El genoma mitocondrial completo es una molécula circular que codifica 37 genes: 13 PCG (*atp*6, *atp*8, *cox*1–3, *co*b, *nad*1–6 y *nad*4L), dos ARNr (*rrn*S y *rrn*L),

y 22 ARNt. Los codones de inicio (ATG, ATA y ATT) coincidieron con los reportados en *Trichuris* procedentes de cerdo y humano (Hawash y col., 2015). Sin embargo, las especies *T. ovis* y *T. discolor* emplearon también el codón TTG (Liu y col., 2012b), mientras que *T. skrjabini* utilizó únicamente dos codones de inicio (ATG y ATA) (Ahmad y col., 2019). En cuanto a los codones de terminación, se utilizaron TAA y TAG en todas las especies de *Trichuris* analizadas hasta la fecha, exceptuando *T. skrjabini*, que presentaba el codón TGA (Liu y col., 2012a, 2012b; Hawash y col., 2015; Ahmad y col., 2019). Las secuencias analizadas en este estudio presentaban una mayor concordancia con las especies de *Trichuris* procedentes de primates (Liu y col., 2012a; Hawash y col., 2015).

El haplotipo mayoritario de *Trichuris* procedente del macaco (TMF31) y el procedente del babuino (TPM1), eran similares genéticamente, con una diferencia de 7 pb en su longitud de secuencia (14091 pb en TMF31 y 14098 pb en TPM1). La diferencia nucleotídica y aminoacídica entre ellas fue del 0,25% y el 0,41%, respectivamente. Además, ambos genomas mitocondriales eran similares a los genomas de *T. trichiura* procedente de humano de Uganda, y a *Trichuris* sp. procedente de *P. hamadryas* (Hawash y col., 2015), con una variación del 0,28 – 0,47%. El genoma mitocondrial completo del haplotipo minoritario de macaco analizado, TMM5, contenía 14047 pb, similar a *T. trichiura* procedente de humano de China (14046 pb). En cambio, la diferencia nucleotídica entre estas secuencias con *Trichuris* sp. procedente de *P. anubis* de EE. UU fue del 15,9%, y del 15,8%, respectivamente. La diferencia nucleotídica y aminoacídica entre las secuencias TMF31 y TMM5 fue del 18,7% y el 14,5%, respectivamente. Además, la variación entre las secuencias de los 13 PCG fue del 20,3% en la secuencia de nucleótidos, y 34,4% en las secuencias de aminoácidos. Este porcentaje de diferencia entre las secuencias analizadas correspondían con rangos citados previamente de

especies diferentes de nematodos. Por esa razón, Hawash y col. (2015) sugirieron la presencia de dos especies diferentes de *Trichuris* en humanos, una procedente de Uganda, y otra procedente de China, con porcentajes de diferencia nucleotídica y aminoacídica de 18,8% y 14,6%, respectivamente. De acuerdo con esto, en estudios previos, *T. suis* y *T. trichiura* presentaron una diferencia aminoacídica del 33,3 – 39,2% (Hawash y col., 2015), *T. trichiura* procedente de humano y *Trichuris* sp. procedente de langur (*T. francoisi*) del 29,4% (Liu y col., 2013), *T. ovis* y *T. discolor* del 11% al 33,9% (Liu y col., 2012b). En cambio, dentro de la misma familia, el género *Trichinella* presentaba entre sus especies una diferencia aminoacídica del 4 al 18% (Mohandas y col., 2014). Blouin (2002) indicó que la diferencia entre las secuencias de los genomas mitocondriales entre especies más cercanas era generalmente del 10-20%. En consecuencia, en el presente trabajo, los datos moleculares obtenidos soportaban la hipótesis de que los diferentes haplotipos obtenidos de *Trichuris* sp. de *M. sylvanus* mostraban diferencias en las secuencias aminoacídicas y nucleotídicas que estaban en el rango que otros autores han considerado como especies diferentes (Rivero y col., 2021a).

Por otra parte, se evaluaron los genomas mitocondriales completos de las diferentes especies de *Trichuris* procedentes de diversos hospedadores para clarificar las relaciones evolutivas.

En consecuencia, se obtuvieron dos árboles filogenéticos; el primero fue inferido utilizando un conjunto de datos de secuencias aminoacídicas de 12 PCG de nematodos de la clase Enoplia incluyendo, además, las secuencias de *Trichuris* obtenidas en el presente trabajo y todas las secuencias de *Trichuris* disponibles en la base de datos de GenBank. Dentro del *phylum* Nematoda, el gen *atp*8 únicamente está presente en la subclase Trichocephalia (Lavrov y Brown, 2001; Mohandas y col., 2014; Kern y col., 2020), de ese modo, no se pudo incluir en el análisis. En segundo lugar, el árbol filogenético se infirió utilizando un conjunto de

datos de secuencias nucleotídicas de las especies de *Trichuris* obtenidas en el presente trabajo, junto con todas las disponibles en GenBank, conteniendo las 13 PCG y los dos ARNr (*rrn*L y *rrn*S). Ambos árboles filogenéticos estaban altamente soportados y mostraron congruencia con la diferenciación entre las especies de *Trichuris* y los diferentes clados de las poblaciones de *Trichuris* que parasitan a humanos y PNH. Además, apoyaban fuertemente la monofilia del género y agrupaban las especies de *Trichuris* con Trichinellida excluyendo los miembros de Dorylaimida y Mermithida (Rivero y col., 2021a). Estos resultados concordaban con los citados por otros autores que caracterizaron genomas mitocondriales completos de varios tricocéfalos y los compararon con otros nematodos enoplidos (Liu y col., 2012a, 2012b).

El análisis filogenético apoyó la evolución de las diferentes especies de *Trichuris*, mostrando tres clados dentro del "Linaje *T. trichiura*". El clado 1 estaba formado por *T. trichiura* de Asia y EE. UU., quedando más relacionado con el clado 2, que estaba formado a su vez por *Trichuris* sp. de *M. sylvanus* (haplotipo TMM5). Además, ambos clados (1 y 2), estaban relacionados y a su vez separados del clado 3, que estaba formado por *T. trichiura* de *H. sapiens* de África y *Trichuris* sp. de PNH de Europa. Por tanto, el clado formado por *Trichuris* sp. de *T. francoisi* se mostraba aislado del resto (Rivero y col., 2021a).

Previamente, varios autores han informado que las poblaciones de *Trichuris* que parasitan humanos y PNH muestran un complejo de especies (Ravasi y col., 2012; Hawash y col., 2015; Cavallero y col., 2015, 2019). Además, la alta diferencia aminoacídica y nucleotídica entre las secuencias de *T. trichiura* (clado 3) y el haplotipo TMM5 de *Trichuris* sp. de *M. sylvanus* (clado 2) y *Trichuris* sp. procedente de Asia y EE. UU. (clado 1), se confirmaron en los árboles filogenéticos inferidos, presentando los diferentes clados con altos valores de soporte de rama.

Por consiguiente, basándose en los resultados obtenidos, se sugiere la existencia de dos especies crípticas que parasitan a *M. sylvanus* (Rivero y col., 2021a). En concordancia con lo citado anteriormente, Nadler y De León (2011) sugirieron que el género *Trichuris* es un gran candidato para contener especies crípticas debido a su distribución geográfica y que infecta a muchas especies diferentes de hospedadores. Asimismo, otros estudios revelaron que hay más de un taxón capaz de infectar a humanos y PNH, incluyendo individuos en cautividad, sugiriendo que *T. trichiura* debe considerarse un complejo de especies que incluye diferentes unidades crípticas (Hawash y col., 2015).

Este hecho tiene dos grandes repercusiones epidemiológicas. En primer lugar, el potencial zoonótico de las especies de *Trichuris* de PNH en los humanos. Este hecho cobra una relevante importancia cuando los humanos y los PNH viven en proximidad, y cada vez se está volviendo más común la ocupación humana en hábitats donde los PNH acceden a jardines y granjas en busca de alimentos y tiene implicaciones tanto para la salud humana como para la conservación silvestre (Betson y col., 2015). Esto implica que en comunidades donde el acceso de los PNH es común, se deben implementar medidas de salud pública. En segundo lugar, la presencia de diferentes especies crípticas también debe ser muy importante para la implementación de estrategias adecuadas de control, como se ha realizado para otros parásitos (Saijuntha y col., 2007).

Para profundizar en la taxonomía del género *Trichuris* de diferentes hospedadores vertebrados, se ha realizado un estudio morfológico, biométrico y molecular de *Trichuris* sp. aislado de puercoespín (*H. cristata*) procedente de un parque zoológico de España (Rivero y col., 2022a). Además, en base a criterios morfo-biométricos, se ha realizado un estudio comparativo de diferentes especies de *Trichuris* aisladas de especies de puercoespines (cercanos al género *Hystrix*) descritas

previamente (Youssefi y col., 2010; Torres y col., 2011; Purwaningsih, 2013).

Así, en el análisis morfológico, *Trichuris* sp. procedente de *H. cristata* presentó un ala lateral corto y un estilete en el extremo anterior, similares a los encontrados en *T. landak* (Purwaningsih, 2013). Estas características fueron citadas en otras especies de *Trichuris* como *T. muris, T. ovis* y *Trichuris parvispicularis* (Skrjabin, 1957). Sin embargo, estos caracteres no mostraron valores diagnósticos para diferenciar entre las especies de tricocéfalos.

Los adultos mostraron patrones morfológicos generales. Así, los machos mostraron la espícula y la vaina espicular similares a *T. landak*, que se caracterizaba por tener una espícula con punta cónica, y la vaina espicular con o sin bulbo (Purwaningsih, 2013) y los analizados, mostraron una vaina espicular sin bulbo. El estudio comparativo junto con otras poblaciones de *Trichuris* de puercoespines del género *Hystrix* (*H. cristata* (Kreis, 1938), *H. indica* (Youssefi y col., 2010) e *H. javanica* (Purwanungsih, 2013)) y *Atelerix* (Baylis, 1935) reveló que los machos de *Trichuris* sp. y *T. hystricis* procedentes de *H. cristata* tenían en común la forma de la punta de la espícula, redondeada. Sin embargo *T. mettami* tenía la punta cónica. En cambio, las hembras de *Trichuris* sp. procedentes de *H. cristata* mostraron la superposición de la mayoría de las características morfológicas analizadas con respecto a las demás especies analizadas, lo que ha demostrado que no son útiles para la diferenciación interespecífica.

El estudio biométrico comparativo de nuestros resultados y los publicados previamente por otros autores (*T. hystricis* (Kreis, 1938; Youssefi y col., 2010), *T. landak* (Purwaningsih, 2013), *T. lenkorani* (Petrov y Sadikhov, 1961) y *T. mettami* (Baylis, 1935)), reveló una alta concordancia con las características descritas para *T. landak*, ya que la mayoría de los valores se superponían dentro del rango de medidas definidas, exceptuando la

longitud total corporal de las hembras, que mostró ser ligeramente más corta en *T. landak* (Purwaningsih, 2013). Además, mostraron el ancho de la región esofágica del cuerpo y el diámetro de la vulva, ligeramente mayor (Kreis, 1938). La falta de muchos caracteres estudiados en otras especies limitó el estudio comparativo. Así, en base a esos caracteres, *T. hystricis*, *T. landak*, *T. lenkorani* y *T. mettami* diferían de *Trichuris* sp. procedente de *H. cristata* en la longitud total corporal máxima y mínima, ya que eran considerablemente más cortos. Asimismo, el análisis de caracteres biométricos adicionales entre machos de *Trichuris* sp. y *T. hystricis* de *H. cristata* (Kreis, 1938) reveló que el ancho de la región esofágica del cuerpo, el ancho en el lugar de unión del esófago y el intestino, el ancho de la vaina espicular en el extremo posterior del cuerpo y la relación entre la longitud de la parte posterior y la parte anterior del cuerpo, eran mayores para *T. hystricis*. Sin embargo, la longitud de la espícula fue considerablemente menor.

Spakulová (1994) sugirió como las mejores características diagnósticas en los machos tricúridos las medidas de la longitud y el ancho de la espícula y la longitud del cuerpo. Knight (1971), también encontró que la longitud de la espícula era el carácter más fiable en la diferenciación de especies y los demás caracteres morfológicos eran más variables. Por lo tanto, considerando la mayor similitud morfológica y biométrica entre *T. landak* y la población de *Trichuris* sp. analizada en el presente estudio, con el tamaño de la espícula claramente mayor que las otras especies de tricocéfalos de puercoespines, sugerimos que nuestra población se correspondía con *T. landak.*

Desde un punto de vista molecular, hemos utilizado marcadores moleculares que han sido utilizados previamente por diferentes autores para resolver cuestiones a nivel de especies del género *Trichuris*, incluyendo la región ITS1 del ADNr (Cutillas y col., 2002, 2004, 2007; Oliveros y col., 2000; Callejón y col., 2012; Salaba y col., 2013; Robles y

col., 2014; Cavallero y col., 2015), el fragmento del gen parcial *rrn*L del ARNr (Callejón y col., 2012; Cavallero y col., 2019; Rivero y col., 2021b) y los genes parciales *cox*1 y *co*b del ADNmt (Callejón y col., 2012, 2013, 2017; Dolezalová y col., 2015; Cavallero y col., 2019; Rivero y col., 2020, 2021b). La falta de datos moleculares correspondientes a las especies de *Trichuris* que parasitan puercoespines (género *Hystrix* y *Atelerix*) no ha permitido realizar un amplio estudio molecular y filogenético comparativo. Únicamente una secuencia parcial del gen *cox*1 (TIS1) y una secuencia parcial del gen *co*b (TIS8) se han podido añadir (secuencias procedentes de GenBank, Cavallero y col. (2019) de Italia (no publicado)).

Las secuencias parciales de los genes mitocondriales y del ARNr, revelaron la existencia de dos haplotipos que no se correlacionaron con diferentes morfoespecies (Rivero y col., 2022a).

Por otra parte, teniendo en cuenta la similitud interespecífica observada en el género *Trichuris* basándonos en todos los marcadores moleculares utilizados, los valores observados entre *Trichuris* sp. de *H. cristata* procedente de España y otras especies de *Trichuris*, estaban dentro del rango de variación interespecífica reportada para el género (Callejón y col., 2013, 2016; Rivero y col., 2020, 2021a, 2021b), lo que sugiere que *Trichuris* sp. de *H. cristata* debe considerarse una nueva especie de *Trichuris*. Asimismo, analizando las poblaciones de *Trichuris* sp. de *H. cristata* de España e Italia, sugerimos la existencia de dos haplotipos diferentes en la población italiana ya que el individuo TIS1 mostró un alto porcentaje de similitud con el haplotipo THC1 en base al gen *cox*1, mientras que el individuo TIS8 mostró un alto porcentaje de similitud con el haplotipo THC2 en base al gen *co*b. Sin embargo, es necesario un mayor número de individuos para obtener una confirmación. Este hecho estaría de acuerdo con Rivero y col. (2020), quienes encontraron la presencia de diferentes haplotipos en una población de *Trichuris* sp. procedente de *M.*

sylvanus, correspondientes con dos genotipos diferentes, con Ravasi y col. (2012) los cuales encontraron dos genotipos diferentes de *Trichuris* sp. procedentes de *P. ursinus* de dos ubicaciones geográficas distintas, pero no llevaron a cabo el estudio morfológico en paralelo para caracterizar las morfoespecies y con Cavallero y col. (2019), los cuales encontraron dos poblaciones diferentes de *Trichuris* parasitando *M. fuscata*, ambas poblaciones dentro del clado de "*T. trichiura*", una población se encontraba dentro de clado principal, con más hospedadores humanos y PNH, y la otra población separada en un subclado diferente.

Al igual que en estudios previos, los árboles filogenéticos inferidos para el género *Trichuris* revelaron la existencia de varios clados que agrupaban las especies según los requerimientos nutricionales de los hospedadores (Callejón y col., 2010, 2013, 2015a, 2017; Robles y col., 2014; Nissen y col., 2012; Ravasi y col., 2012; Cutillas y col., 2004, 2007). En la presente Tesis Doctoral, los resultados de los análisis filogenéticos, basándonos en todos los marcadores moleculares, individuales y de forma combinada, apoyaban lo anteriormente mencionado. Así, *Trichuris* sp. procedente de *H. cristata* formó un clado separado, el clado 1, relacionado con el clado 2, que incluía especies de *Trichuris* de roedores y cánidos. Sin embargo, el árbol filogenético del marcador ITS1, mostró poca resolución entre los diferentes clados. Este resultado puede deberse a lo citado anteriormente, que las regiones de ITS estaban saturadas y mostraban una pobre diversidad de nucleótidos. Por lo tanto, para inferir las relaciones entre los diferentes linajes genéticos nos basamos exclusivamente en el análisis de los marcadores mitocondriales de acuerdo con Chan y col. (2020) y con Rivero y col. (2020, 2021b).

Debido a que la especie *T. landak*, procedente de *H. javanica*, fue descrita exclusivamente mediante la comparación de características morfológicas y medidas morfométricas de los adultos, son necesarias herramientas

moleculares para confirmar si la población de *Trichuris* sp. procedente de *H. cristata* de España podría corresponderse con *T. landak*.

Finalmente, llevamos a cabo el estudio molecular y filogenético de una población de *Trichuris* procedente de *C. bactrianus* de España y se comparó con otras poblaciones de *Trichuris* procedentes de camellos de diferentes orígenes geográficos disponibles en la base de datos de GenBank (Rivero y col., 2023).

En todos los marcadores analizados (ITS1 e ITS2 del ADNr, *cox*1 y *co*b del ADNmt y *rrn*L del ARNr), los porcentajes de similitud intrapoblacional obtenidos entre las secuencias de *Trichuris* analizadas procedentes de *C. bactrianus* de España, mostraron valores dentro del rango correspondiente al género *Trichuris* (Blouin, 2002; Callejón y col., 2015b; Chan y col., 2020).

Las secuencias de *Trichuris* de camellos analizadas revelaron que solo dos secuencias de *Trichuris* de *C. bactrianus* de la República Checa, una secuencia perteneciente al gen parcial *cox*1 del ADNmt y una secuencia al fragmento ITS2 del ADNr, mostraron el mismo porcentaje de identidad con las secuencias obtenidas en el presente trabajo, correspondientes con la misma especie. Por otra parte, las secuencias de *Trichuris* de *C. bactrianus* de Italia y *T. globulosa*, mostraron valores dentro del rango de similitud interespecífica (Blouin, 2002). Además, el porcentaje de similitud observado entre estas especies fueron superiores a los valores de *T. globulosa* con *Trichuris* sp. de *C. bactrianus* de España, las cuales parecen estar también en el rango de especies diferentes. Asimismo, la mayor similitud interespecífica entre las secuencias obtenidas en el presente trabajo y otras especies de *Trichuris* fue con *T. skrjabini*. Estos valores fueron más elevados en los marcadores ribosómicos que en los marcadores mitocondriales. Además, la similitud interespecífica generalmente es mayor en los marcadores ribosómicos que en los mitocondriales. Sin embargo, el marcador *rrn*L del ARNr presentó valores

intermedios. Este hecho concuerda con varios autores citados previamente (Callejón y col., 2015b; Chan y col., 2020; Rivero y col., 2021b, 2022a). Atendiendo al porcentaje de similitud observado para los otros marcadores, sugerimos que la población de *Trichuris* de *C. bactrianus* de España corresponde a una especie diferente a las citadas para *C. bactrianus* y *C. dromedarius* y otros herbívoros de orígenes geográficos diferentes.

En cambio, debido a la falta de secuencias para el marcador molecular *rrn*L, no se ha podido concatenar con los datos mitocondriales y ribosómicos. Consecuentemente, se ha realizado un conjunto de datos concatenados de los marcadores mitocondriales (*cox*1 y *co*b) y ribosómicos (ITS1 e ITS2) proporcionando una mayor resolución para los estudios filogenéticos (Cavallero y col., 2019; Rivero y col., 2020, 2021b, 2022a) y proporcionando nodos fuertemente respaldados, diferenciando entre los diferentes clados. Los resultados obtenidos en el estudio de similitud concuerdan con los resultados filogenéticos.

Las secuencias obtenidas en este estudio se agruparon en el clado con las secuencias de *Trichuris* de *C. bactrianus* de República Checa. En cambio, el resto de las poblaciones de *Trichuris* de hospedadores camélidos estaban en un clado separado. En todos los árboles filogenéticos obtenidos, tanto individuales como concatenados, nuestras secuencias tenían una relación hermana con *T. skrjabini*, con ambas poblaciones formando un clado bien soportado y separado de los demás clados. Por lo tanto, teniendo en cuenta que nuestras secuencias mostraron los valores de similitud más altos respecto a *T. skrjabini* y los más bajos con *T. globulosa* y *Trichuris* de *C. bactrianus* de Italia, apareciendo estas últimas poblaciones en otro clado, se sugirió que estas poblaciones correspondían a especies diferentes según los resultados de similitud y filogenéticos, pero estrechamente relacionadas genéticamente, estando separadas de la población de *Trichuris* de *C. bactrianus* de España. Estos resultados

podían ser debidos a las diferencias geográficas, el tipo de alimentación, la ruta de transmisión interespecífica entre las especies hospedadoras, o con el transporte pasivo de huevos a través de cuidadores de los zoológicos o animales en libre circulación (Kvapil y col., 2017; Montalbano Di Filippo y col., 2020).

Por otro lado, actualmente no está clara la relación evolutiva entre el camello bactriano doméstico (*C. bactrianus*) y el salvaje (*C. bactrianus ferus*) y el origen real del bactriano doméstico. Ji y col. (2009) revelaron que el camello bactriano salvaje podría no compartir un ancestro común con el camello bactriano doméstico y no son la misma subespecie exceptuando en sus orígenes maternos. Así, las secuencias del genoma mitocondrial completo indicaron que el camello salvaje es un linaje separado, pero no corresponde con el progenitor del camello bactriano doméstico. Más tarde, los análisis filogenéticos han sugerido que el camello bactriano salvaje es monofilético en su origen evolutivo y que el doméstico podría provenir de una sola población salvaje.

IV.2. ESTUDIO DE LA TÉCNICA DE DIAGNÓSTICO MALDI-TOF MS PARA LA IDENTIFICACIÓN DE ESPECIES DE *TRICHURIS*

Durante muchos años, el género *Trichuris* ha sido objeto de una amplia controversia debido a su difícil diferenciación específica entre especies estrechamente relacionadas. La dificultad de relacionar las especies descritas con criterios exclusivamente morfológicos y con especies descritas únicamente con estudios moleculares, hace que el estudio de la taxonomía sea aún más difícil de resolver. Por esta razón, son necesarios métodos complementarios para dilucidar entre las diferentes especies.

El objetivo de este estudio ha sido confirmar si el análisis a través de la técnica de MALDI-TOF MS podría usarse como una herramienta de

diagnóstico para la identificación de especies adultas de *Trichuris* (Rivero y col., 2022b).

Para lograr este objetivo se ha desarrollado, en primer lugar, una base de datos interna con muestras de la parte anterior (nombrado como "esófago") y de la parte posterior (nombrado como "intestino") de *T. suis*, proporcionando espectros similares entre ellos, siendo ambas partes adecuadas para identificar esta especie. Todos los espectros fueron identificados correctamente, independientemente del tipo de muestra analizada, por lo que confirmamos el uso de ambas partes del cuerpo del nematodo para su correcta identificación utilizando esta técnica. Como consecuencia de los resultados obtenidos, sugerimos utilizar preferentemente la parte anterior del verme o el esófago para el diagnóstico a través de la técnica de MALDI-TOF MS por su fácil manejo y, reservar la parte posterior para estudios posteriores o moleculares.

En segundo lugar, el protocolo preliminar fue validado para utilizarlo con otras especies de *Trichuris*, incluyendo cuatro especies más en la base de datos interna. Las cuatro especies añadidas, utilizando únicamente la parte anterior del verme, fueron correctamente identificadas. Los resultados obtenidos mostraron espectros de alta calidad y similitud entre las muestras permitiendo la identificación específica y, por lo tanto, mostrando la utilidad de dicha técnica para la diferenciación específica del género *Trichuris* como una posible herramienta para el diagnóstico de la tricuriasis (Rivero y col., 2022b).

La técnica de MALDI-TOF MS ha sido utilizada en microbiología clínica como una herramienta diagnóstica eficaz para identificar microorganismos patógenos (Sjöholm y col., 2008; Angeletti, 2017; Luethy y Johnson, 2019). Incluso se ha llevado a cabo una revisión sistemática del uso del MALDI-TOF MS en helmintología humana y veterinaria, concluyendo que son necesarios más estudios ya que existe

evidencia para la correcta y rápida identificación de nematodos, ya sean larvas, adultos o huevos en muestras fecales (Feucherolles y col., 2019).

Por consiguiente, es necesario avanzar en el desarrollo de la base de datos interna para la identificación de helmintos generando MSP específicos de las especies y estandarizar la extracción de proteínas, para que los resultados sean precisos y reproducibles en los diferentes laboratorios para futuras investigaciones (Sy y col., 2020). No obstante, las bases de datos son privadas y se crean en cada laboratorio, por lo que nos encontramos con la dificultad añadida de no poder comparar las diferentes especies registradas en las diferentes bases de datos, siendo de gran importancia la creación de una única base de datos de referencia o poder compartir los espectros obtenidos de cada laboratorio.

En la investigación para la identificación de diferentes nematodos (*Dirofilaria* y *Ascaris*) usando la técnica MALDI-TOF MS, utilizando tejidos de gusanos adulos, sugirieron que en el rango de 8 a 20 kDa, los espectros permitieron identificar entre diferentes especies de nematodos y, además, en el rango de 2 a 6 kDa, probablemente podría caracterizarse a nivel de género (Nagorny y col., 2019). En el presente trabajo, los espectros obtenidos se observaron en el rango de 2 a 7 kDa, extendiéndose hasta 10 kDa (Rivero y col., 2022b). Consecuentemente, es necesario identificar más especies de nematodos para profundizar en si existe un rango diferencial entre especie y género en los espectros obtenidos de los nematodos mediante la técnica de MALDI-TOF MS.

Por otro lado, la técnica MALDI-TOF MS ha avanzado en los últimos años en la identificación taxonómica y la clasificación filogenética de microorganismos (Freiwald y Sauer, 2009). Este hecho fue apoyado por Zurita y col. (2019) utilizando especies de pulgas vectoras, obteniendo concordancia con los datos resultantes en el dendrograma y los estudios filogenéticos realizados. Sin embargo, Yssouf y col. (2013) argumentaron

que, debido a la falta de especímenes, el software MALDI Biotyper no puede determinar con fiabilidad el estudio filogenético de los artrópodos. Asimismo, otros estudios sugieren la utilidad del MALDI-TOF MS como una herramienta eficiente en estudios taxonómicos parasitológicos (Nagorny y col., 2019).

En el presente estudio, el dendrograma obtenido separó cada especie de *Trichuris* en un clado diferente. Además, relacionó más los clados de *T. vulpis* y *T. trichiura*, y por otro lado los clados de *T. suis* y *Trichuris* sp. de *H. cristata*, resultando el clado de *T. ovis* el más distanciado de todos los clados. Este último argumento está de acuerdo con el árbol filogenético obtenido basado en el gen parcial *co*b del ADNmt, donde el clado de *T. ovis* quedaba separado de los demás clados. Sin embargo, las relaciones filogenéticas entre los otros clados difieren del dendrograma, y estas relaciones, además, estaban fuertemente apoyados por altos valores de rama (Rivero y col., 2022b). Por esta razón, de acuerdo con los autores citados previamente, sugerimos que la técnica de MALDI-TOF MS no debe usarse para establecer relaciones filogenéticas en especies de nematodos, ya que se necesitan más estudios con más especies y géneros de diferentes nematodos parásitos.

Por otra parte, de forma alarmante, los fármacos administrados para el tratamiento de la infección por *Trichuris* muestran una baja eficacia clínica. Además, según un reciente estudio observando las interacciones a lo largo del tiempo, la eficacia de los fármacos va disminuyendo con el tiempo, lo que podría estar asociado con la resistencia a los fármacos antihelmínticos (Moser y col., 2017). La técnica de MALDI-TOF MS se ha utilizado como una herramienta para descubrir la resistencia a los antibióticos en microorganismos mediante la detección de biomarcadores dentro de los espectros de proteínas obtenidos (Burckhardt y Zimmermann, 2011; Hrabák y col., 2013; Feucherolles y col., 2019). Así, un estudio preliminar sobre el diagnóstico de especies de *Anisakis*

utilizando la técnica MALDI-TOF MS ha logrado identificar un conjunto de signos como una lista de potenciales “biomarcadores” (Marzano y col., 2020). Este hecho abre puertas esperanzadoras sobre la aplicación de dicha técnica para descubrir nuevas dianas y desarrollar tratamientos para combatir la resistencia antihelmíntica.

IV.3. BIBLIOGRAFÍA

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CAPÍTULO V. CONCLUSIONES

II. CONCLUSIONES

V.1. CONCLUSIONES GENERALES

1. Los estudios morfológicos y biométricos llevados a cabo en las distintas especies de *Trichuris* analizadas nos permiten concluir que dichos análisis no son muy resolutivos para la diferenciación de estas especies. Del mismo modo, no permiten la diferenciación de los diferentes linajes genéticos presentes.
2. Los resultados obtenidos llevando a cabo el estudio de diferentes especies de *Trichuris* utilizando la técnica de morfometría geométrica, revelaron un fuerte apoyo frente a esta técnica como una herramienta útil para diferenciar poblaciones de machos de *Trichuris*. Por lo tanto, la morfometría geométrica en combinación con otras técnicas, como los análisis moleculares, deben aplicarse para promover la diferenciación de las poblaciones masculinas. Sin embargo, aplicada a las hembras de las especies de *Trichuris* no parece aportar más información, ya que todas las combinaciones de medidas obtenidas siempre mostraron resultados similares.
3. Tras la amplificación y secuenciación de los fragmentos parciales de ITS1 e ITS2 del ADNr en *Trichuris* spp. podemos concluir que dichos marcadores son apropiados para la resolución de problemas taxonómicos de nematodos parásitos pertenecientes al género *Trichuris*. En cambio, los genes parciales *cox*1 y *co*b del ADNmt y el gen parcial *rrn*L del ARNr son más adecuados para establecer relaciones filogenéticas entre las diferentes especies del género *Trichuris*, e incluso a nivel poblacional o entre los diferentes linajes genéticos, siendo el marcador *rrn*L el de mayor poder resolutivo.
4. Combinar los análisis morfométricos y moleculares es el factor clave para permitir la diferenciación de las especies de *Trichuris*.

5. Tras los análisis moleculares del clado "Linaje *T. trichiura*" podemos concluir que existe un complejo de especies relacionadas genéticamente parasitando a hospedadores humanos y PNH.
6. La base de datos interna obtenida mediante la obtención de los espectros por la técnica MALDI-TOF MS para las especies *T. suis*, *T. trichiura*. de *M. sylvanus*, *Trichuris* sp. de *H. cristata*, *T. vulpis* y *T. ovis*, nos permiten concluir la utilidad de esta técnica para la diferenciación específica de las diferentes especies de adultos de *Trichuris*.

V.2. CONCLUSIONES ESPECÍFICAS

7. Los resultados del estudio morfológico y biométrico de los tricocéfalos procedentes de *M. sylvanus* mostraron que eran *T. trichiura*.
8. Los análisis moleculares realizados de la población de *Trichuris* sp. de *M. sylvanus* revelaron la existencia de dos linajes genéticos distintos dentro del "Linaje *T. trichiura*", que no se correspondían con morfoespecies diferentes.
9. Los resultados moleculares obtenidos en el estudio del genoma mitocondrial completo de diferentes vermes de *Trichuris*, sugieren la existencia de dos especies diferentes en los tricocéfalos aislados de *M. sylvanus*. Los adultos procedentes de *P. papio* y el haplotipo mayoritario de *M. sylvanus*, molecularmente, eran *T. trichiura*.
10. Los resultados obtenidos del estudio de datos biométricos y morfológicos de *Trichuris* de diferentes hospedadores PNH y humanos, nos han permitido una descripción actualizada de *T. trichiura*.

11. El estudio taxonómico y filogenético de las especies de *Trichuris* aisladas de humanos y PNH, ha confirmado la existencia de un complejo en *T. trichiura*, formado por cuatro subclados, con diferentes afinidades de hospedador y capacidades de infección cruzada correspondientes a cuatro linajes genéticos diferentes. En consecuencia, ha proporcionado resultados útiles para la identificación de nuevas subespecies y eventos de hibridación entre especies existentes y permite una comprensión más clara y detallada de los patrones de dispersión.
12. Los resultados del análisis morfológico y biométrico de la población de *Trichuris* sp. procedente de *H. cristata* de España, revelaron una alta similitud con *T. landak*, sugiriendo que ambas poblaciones se podrían corresponder con la misma especie.
13. Los estudios moleculares de la población de *Trichuris* sp. procedente de *H. cristata* de España revelaron la existencia de dos haplotipos diferentes que no correspondían con dos morfoespecies diferentes.
14. Las relaciones filogenéticas manifestaron una relación hermana entre *Trichuris* parasitando puercoespín y otras especies de *Trichuris* de hospedadores roedores y cánidos, separados del resto de especies de *Trichuris* de otras especies hospedadoras.
15. Los resultados obtenidos del estudio molecular de una población de *Trichuris* sp. procedente de *C. bactrianus* de España nos permite concluir que corresponde a una especie diferente a las citadas previamente por otros autores para camellos y otros herbívoros, mostrando una relación hermana con respecto a *T. skrjabini*.
16. El estudio de la técnica MALDI-TOF MS validó la utilidad de la técnica como una herramienta de identificación fiable, rápida y económica para la caracterización de especies del género *Trichuris*. Sin embargo, la base de datos interna creada debe ampliarse con más

muestras de diferentes especies del género *Trichuris* y otras especies de nematodos.

17. Tras los resultados obtenidos del uso de la técnica MALDI-TOF MS concluimos que no es resolutiva para los estudios filogenéticos en las especies de *Trichuris* estudiadas.

CAPÍTULO VI. CONCLUSIONS

III. CONCLUSIONS

VI.1. GENERAL CONCLUSIONS

1. Morphological and biometrical studies carried out on the different *Trichuris* species analyzed, allow us to conclude that these analyzes are not resolute for the differentiation of these species. Similarly, they do not allow the differentiation of the different genetic lineages studied.
2. The results obtained using the technique of geometric morphometry for different *Trichuris* species revealed strong support for this technique as a useful tool to differentiate males *Trichuris* populations. Hence, morphometrics should be applied in combination with other techniques, such as molecular analyses, to promote the differentiation of male populations. However, when applied to females *Trichuris* species, it did not seem to provide new information, since all the combinations of measurements obtained always showed similar results.
3. The amplification and sequencing of the partial fragments of ITS1 and ITS2 of the rDNA in *Trichuris* spp. showed that these molecular markers are appropriate for resolve taxonomic issues of parasitic nematodes of the genus *Trichuris*. In contrast, the partial genes *cox*1 and *co*b of the mtDNA and the partial gene *rrn*L of the rRNA are more useful for establishing phylogenetic relationships among the different *Trichuris* species, and even at the population level or among the different genetic lineages, being the *rrn*L the molecular marker with greater discrimination power.
4. Combining morphometric and molecular analyzes is the key to allow the differentiation among *Trichuris* species.

5. The molecular analyzes of the clade "*T. trichiura* lineage" showed that there is a complex of genetically related species that parasitize human and non-human primate (NHP) hosts.
6. The internal database created by obtaining the spectra profiles by the MALDI-TOF MS technique for the *T. suis*, *T. trichiura* from *M. sylvanus*, *Trichuris* sp. from *H. cristata*, *T. vulpis* and *T. ovis* species, allow us to conclude the usefulness of this technique for the specific differentiation among the different adults of *Trichuris* species.

VI.2. SPECIFIC CONCLUSIONS

7. The morphological and biometric results obtained from whipworms from *M. sylvanus* showed that they were *T. trichiura*.
8. The molecular analyzes obtained from *Trichuris* sp. from *M. sylvanus* population revealed the existence of two different genetic lineages within "*T. trichiura* lineage" that did not correspond to different morphospecies.
9. The molecular results obtained of complete mitochondrial genome analyzes of different specimens of *Trichuris* suggested the existence of two different *Trichuris* species from *M. sylvanus*. The *Trichuris* specimens from *P. papio* and the majority haplotype from *M. sylvanus*, molecularly, were *T. trichiura*.
10. The results obtained from the study of biometric and morphological data of *Trichuris* from different NHP and human hosts, allowed us to update the description of *T. trichiura*.
11. The taxonomic and phylogenetic study of *Trichuris* species isolated from human and NHP has confirmed the existence of a species complex in *T. trichiura*, constituted by four subclades with different host affinities and cross-infection capacities corresponding to four different genetic lineages. Consequently, it has provided useful results for the identification of new subspecies and hybridization events

among existing species and allows a clearer and more detailed understanding of dispersal patterns.

12. The results obtained from the morphological and biometric analyzes of a population of *Trichuris* sp. from *H. cristata* from Spain, revealed a strong similarity to *T. landak*, suggesting that both populations could be the same species.
13. The molecular studies of *Trichuris* sp. from *H. cristata* from Spain revealed the existence of two different haplotypes that did not correspond to different morphospecies.
14. The phylogenetic relationships showed a sister relationship between *Trichuris* parasitizing porcupine and other *Trichuris* species from rodent and canid hosts, separated from the rest of *Trichuris* species from other host species.
15. The molecular study of a population of *Trichuris* sp. from *C. bactrianus* from Spain, allowed us to conclude that it corresponds to a different species from those previously cited by other authors for camels and other herbivores, showing a sister relationship with respect to *T. skrjabini*.
16. The study of the MALDI-TOF MS technique validated the usefulness of the technique as a reliable, rapid, and inexpensive identification tool for the characterization of species of the *Trichuris* genus. However, the internal database created must be expanded with more samples from different species of the *Trichuris* genus and other nematode species.
17. The results obtained from the use of the MALDI-TOF MS technique showed that this technique does not have resolving capacity for phylogenetic studies in the *Trichuris* species studied.

ANEXO I. MATERIAL Y MÉTODOS

A.I. MATERIAL Y MÉTODOS

A.I.1. MATERIAL

El material objeto de estudio han sido adultos y huevos de distintas especies de *Trichuris* procedentes de diferentes hospedadores vertebrados y zonas geográficas de España (Tabla 1).

Tabla 1. Distribución de las muestras de *Trichuris* por tipo de especie, muestra, especie hospedadora, lugar de recogida de muestra y zona geográfica.

Especie	Tipo de muestra	Especie hospedadora	Lugar de recogida	Zona geográfica
Trichuris sp.	Adultos	*Macaca sylvanus*	Zoo de Castellar	Cádiz, España
Trichuris trichiura	Adultos y huevos	*Papio papio*	Parque de la Naturaleza de Cabárceno	Cantabria (Cabárceno), España
Trichuris trichiura	Huevos	*Chlorocebus aethiops*	Selwo Aventura	Málaga (Estepona), España
Trichuris trichiura	Huevos	*Erythrocebus patas*	Zoo Barcelona	Barcelona, España
Trichuris colobae	Adultos	*Colobus guereza kikuyensis*	Bioparc Fuengirola	Málaga (Fuengirola), España
Trichuris sp.	Adultos	*Hystrix cristata*	Bioparc Fuengirola	Málaga (Fuengirola), España
Trichuris suis	Adultos	*Sus scrofa domestica*	Matadero	Sevilla y Huelva, España
Trichuris ovis	Adultos	*Capra hircus*	Matadero	Sevilla, España
Trichuris vulpis	Adultos	*Canis lupus familiaris*	Cacería	Sevilla y Huelva, España
Trichuris sp.	Adultos	*Camelus bactrianus*	Parque de la Naturaleza de Cabárceno	Cantabria (Cabárceno), España

A.I.1.1. Recogida del material

A.I.1.1.1. Adultos

Los adultos aislados de diferentes especies de *Trichuris* fueron recogidos a través de dos procedimientos distintos:

- Mediante la obtención de los ciegos procedentes de los diferentes hospedadores tras el fallecimiento y la posterior necropsia. Los distintos ciegos fueron transportados en bolsas herméticamente cerradas hasta el laboratorio. Posteriormente, con ayuda de pinceles y pinzas, los adultos fueron extraídos del ciego y depositados en placas de Petri con solución salina fisiológica al 0,9% (p/v). Los tricocéfalos una vez lavados y desprovistos de los restos procedentes del hospedador, fueron clasificados por sexo y depositados individualmente en tubos Eppendorf para ser almacenados a -20º C hasta ser utilizados para posteriores estudios.
- Mediante el procesamiento de muestras de heces procedentes de los diferentes hospedadores analizados tras tratamiento antihelmíntico. Las heces resultantes fueron sometidas a varios lavados mediante solución salina fisiológica al 0,9% (p/v) hasta aislar los vermes con ayuda de pinceles y se siguió el procedimiento descrito previamente para la recogida de adultos provenientes de los ciegos.

A.I.1.1.2. Huevos

Las heces procedentes de los distintos hospedadores fueron analizadas y las muestras positivas, que presentaban huevos de *Trichuris*, fueron sometidas a técnicas de concentración basadas en la flotación de Sheather modificada, fundamentada en la flotación de huevos de parásitos en una solución de azúcar saturada (1,59 g/cm^3) que posee una mayor densidad que ellos (Gupta y Singla, 2013).

Tras la técnica de concentración se procedió a embrionar los huevos concentrados mediante la incubación a 32º C durante 3 o 4 semanas con una solución de dicromato potásico al 0,2% para proporcionar humedad al medio y prevenir el crecimiento de hongos y bacterias (Horii y Usui, 1985).

Protocolo de concentración por flotación de Sheather modificada

1. Homogeneizar de todo el volumen de materia fecal en 500 ml de agua destilada. Mantener en agitación durante una hora.
2. Colocar un embudo de vidrio con una gasa doblada en la abertura del tubo de ensayo y filtrar el material homogeneizado. Colocar tantos tubos de ensayo como solución tengamos.
3. Centrifugar los tubos con el material homogeneizado a 1.500 r.p.m. durante 10 minutos.
4. Eliminar el sobrenadante, y agregar la solución de azúcar saturada hasta 1 cm del borde del tubo. Agitar hasta disolver el sedimento y dejar reposar 1 hora.
5. Recoger la superficie del tubo y centrifugar a 1.500 r.p.m. durante 10 minutos.
6. Eliminar el sobrenadante y almacenar la muestra a -20º C para su posterior utilización.

Parte del material ha sido facilitado por los siguientes colaboradores:

- *Trichuris* sp. procedente de *M. sylvanus* ha sido facilitado por Dña. Andrea Morales del Zoo de Castellar.
- *T. trichiura* procedente de *P. papio* y *Trichuris* sp. aisaldo de *C. bactrianus* de Parque de la Naturaleza de Cabárceno han sido facilitados por D. Santiago Borragán.

- *T. colobae* procedente de *C. g. kikuyensis* y *Trichuris* sp. de *H. cristata* de Bioparc Fuengirola han sido facilitados por D. Jesús Recuero.
- *T. trichiura* procedente de *C. aethiops* de Selwo Aventura ha sido facilitado por D. Alfredo Gargallo.
- *T. trichiura* procedente de *E. patas* del Zoo de Barcelona ha sido facilitado por D. Hugo Fernández.

A.I.2. MÉTODOS

A.I.2.1. Estudios morfológicos y biométricos

A.I.2.1.1. Estudios morfológicos y biométricos clásicos

Todas las muestras de huevos y de adultos de *Trichuris* spp. analizadas en la presente Tesis Doctoral fueron estudiadas morfológicamente en nuestro laboratorio. Los estudios morfológicos y biométricos se realizaron de acuerdo con los parámetros citados por Skrjabin y col. (1957), Oliveros y col. (1998), Spakulová y Lysek (1981), Suriano y Navone (1994) y Robles y col. (2006). Las muestras fueron montadas entre porta y cubreobjetos con solución salina fisiológica al 0,9% (p/v) para ser posteriormente examinadas y fotografiadas con el microscopio óptico Olympus BX51 equipado con una cámara fotográfica Nikon D90.

Asimismo, se calcularon los parámetros estadísticos básicos (media y desviación estándar) para las diferentes especies estudiadas y se utilizó la prueba t de Student ($P < 0.001$) para probar la igualdad de medidas de cada variable. El análisis estadístico se realizó utilizando Microsoft Excel (Feliú y col., 2000).

Para el análisis biométrico llevado a cabo en algunas de las poblaciones de *Trichuris* estudiadas se utilizaron entre 15 y 13 parámetros diferentes según el sexo. Esta parte del estudio se realizó utilizando el equipo microscópico Zeiss con un sistema de cámara clara y de fotografía. Las

medidas obtenidas fueron comparadas entre poblaciones de diferentes hospedadores y sexos.

A.I.2.1.2. Análisis basados en morfometría geométrica

Se utilizó la técnica de morfometría geométrica para cuantificar la variación morfológica de las especies de *Trichuris* analizadas (Rohlf y Marcus, 1993). Es una técnica que ofrece una estimación de tamaño por el cual diferentes ejes de crecimiento se integran en uno solo (Bookstein, 1989). La estimación del tamaño está contenida en una sola variable que refleja la variación en muchas direcciones, tantas como puntos de referencia en estudio haya, y la forma se define como sus posiciones relativas después de la corrección por tamaño, posición y orientación. Con estos datos informativos y el software correspondiente (disponible gratuitamente) para realizar los análisis complejos, las características biológicas y epidemiológicas significativas pueden cuantificarse con mayor precisión (Dujardin, 2008).

Asimismo, se aplicaron análisis multivariante para calcular las variaciones fenotípicas entre las especies de *Trichuris*, utilizando un análisis discriminante canónico libre de tamaño en la covarianza de las medidas transformadas logarítmicamente (Dos Reis y col., 1990). El análisis de componentes principales (ACP) se utilizó para resumir la mayoría de las variaciones en un conjunto de datos multivariante en unas pocas dimensiones (Dujardin y Le Pont, 2004). El resultado se sometió a un análisis de variación canónica (AVC), y se obtuvieron las distancias de Mahalanobis (Mahalanobis, 1936). En el presente análisis se evaluó el grado de similitud entre poblaciones de *Trichuris* a través de distancias de Mahalanobis por pares. Además, se realizó un análisis fenotípico de los adultos de *Trichuris* usando varios módulos del software CLIC v97 (Dujardin y Slice, 2007; Dujardin y col., 2010), (disponible en http://momeclic.com), y el software BAC v2 (Dujardin, 2002; Valero y

col., 2009). Además, las distancias de Mahalanobis se calcularon utilizando el software CLIC y se probaron mediante pruebas de permutación no paramétricas con 1000 iteraciones cada una. Los resultados fueron estadísticamente significativos cuando $P < 0,05$.

A.I.2.2. Estudios moleculares y filogenéticos

A.I.2.2.1. Selección de marcadores moleculares

En la presente Tesis Doctoral se han utilizado los marcadores moleculares más utilizados en la identificación, estudios taxonómicos y filogenéticos del género *Trichuris* hasta la fecha. La correcta elección de un tipo u otro de marcadores se justifica por las características diferenciales de cada tipo y el tipo de información que aportan. Destacan los marcadores del ADNr nuclear y los marcadores del ADNmt. Así, se han seleccionado los marcadores de ADNr ITS1 e ITS2, los marcadores del ADNmt *cox*1 y *co*b, y los marcadores del ARNr *rrn*L o 16S, y se ha estudiado cada marcador individualmente, así como en combinación con las secuencias concatenadas.

A.I.2.2.2. Extracción de ADN

La extracción del ADN genómico se ha llevado a cabo siguiendo el protocolo del kit de extracción de ADN, DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Düsseldorf, Alemania) (Figura 26), que se realiza utilizando columnas DNeasy Mini spin.

El proceso de extracción de ADN consiste en el empleo de una matriz que retiene los productos de la lisis celular y permite la obtención de un extracto de ADN de un modo sencillo y rápido.

Como material de partida hemos utilizado individualmente cada adulto completo, la parte posterior del tricocéfalo (conteniendo el intestino y el aparato reproductor), o por lote de huevos de *Trichuris* de un mismo

hospedador previamente sometidos a la técnica de concentración por flotación y posteriormente embrionados.

Protocolo de extracción de ADN

1. Disponer cada individuo, libre de restos del hospedador, en un tubo Eppendorf de 1,5 ml. Presionar con un pistilo estéril para triturarlo en su totalidad. Añadir 180 µl de tampón de lisis (ATL).
2. Añadir 20 µl de proteinasa K. Mezclar bien en un vórtex e incubar a 56ºC hasta que el tejido esté completamente lisado, aproximadamente de 1 a 3 horas. Mezclar ocasionalmente durante la incubación.
3. Añadir 4 µl de ARNasa (4 mg/ml), mezclar e incubar durante 2 min a temperatura ambiente.
4. Agitar en un vórtex durante 15 s. Añadir 200 µl de tampón AL y mezclar. A continuación, añadir 200 µl de etanol (96 – 100%), y agitar vigorosamente mediante un vórtex.
5. Pipetear la mezcla del paso 4 en la columna de centrifugación Dneasy Mini spin colocada en un tubo de recolección de 2 ml. Centrifugar a 8.000 r.p.m. durante 1 min. Desechar el filtrado y el tubo de recolección. En este paso el ADN de la muestra queda atrapado en la membrana de la columna de QIAGEN.
6. Colocar la columna de centrifugación DNeasy Mini spin en un tubo de recogida nuevo de 2 ml. Añadir 500 µl de tampón AW1 y centrifugar durante 1 min a 8.000 r.p.m. para hacerlo pasar a través de la membrana de la columna. Desechar el filtrado y el tubo de recolección.
7. Colocar la columna de centrifugación DNeasy Mini spin en un tubo de recogida nuevo de 2 ml. Añadir 500 µl de tampón AW2 y centrifugar durante 3 min a 14.000 r.p.m. para secar la membrana DNeasy. Desechar el filtrado y el tubo de recolección.

8. Colocar la columna de centrifugación DNeasy Mini spin en un tubo Eppendorf de 1,5 ml y pipetear 100 µl de tampón AE directamente en la membrana DNeasy. Incubar a temperatura ambiente durante 1 min y luego centrifugar durante 1 min a 8.000 r.p.m. para eluir el ADN.
9. Repetir el paso 8 una segunda vez utilizando un tubo Eppendorf de 1,5 ml nuevo, de modo que se obtenga un volumen de ADN más concentrado y otro volumen de ADN más diluido.
10. Almacenar el ADN obtenido a -20º C hasta su posterior uso.

Figura 26. Esquema del procedimiento de extracción de ADN de acuerdo con el protocolo DNeasy® Blood & Tissue Kit de QIAGEN.

A.I.2.2.3. Reacción en cadena de la polimerasa (PCR)

En la presente Tesis Doctoral se ha utilizado la técnica de PCR estándar para realizar la amplificación de diversos marcadores moleculares de diferentes especies del género *Trichuris* (Tabla 2). Todas las reacciones de PCR se realizaron utilizando los termocicladores Mastercycler® ep (EPPENDORF) y T100^{TM} Thermal Cycler (Bio-Rad).

La mezcla de reactivos, el nombre de los cebadores y las condiciones de PCR utilizadas para cada marcador molecular individualmente están resumidos en la Tabla 3. Del mismo modo, para los marcadores moleculares utilizados para el genoma mitocondrial completo, están dispuestos en la Tabla 4.

A.I.2.2.4. Electroforesis de ADN en gel de agarosa

La electroforesis en gel de agarosa se realiza con el propósito de, en primer lugar, verificar la correcta extracción del ADN, y, en segundo lugar, verificar la amplificación de los fragmentos tras la reacción de PCR, y poder identificar distintos fragmentos de ADN en base a su tamaño.

El ADN es una molécula que posee carga negativa. Por esta razón, cuando el ADN se coloca en un gel de agarosa, y es sometido a un campo eléctrico, migra hacia el ánodo o polo positivo. La migración de las moléculas a través de la matriz del gel de agarosa es inversamente proporcional al logaritmo de su peso, por lo tanto, los fragmentos pequeños de ADN migrarán más rápido, situándose más cercanos al ánodo, mientras que los fragmentos más grandes se desplazarán más lentamente y quedarán más próximos al cátodo o polo negativo.

El éxito de la extracción y de la amplificación se corrobora por la adición de tinte de gel de ADN, SABR^{TM} safe (ThermoFisher Scientific), que proporciona una tinción de alta sensibilidad para la visualización de ADN en geles de agarosa y es más seguro que el bromuro de etidio. Para la visualización es necesario la exposición del gel a la luz ultravioleta entre

302 y 312 nm mediante el uso de un transiluminador. Se utilizaron dos patrones de pesos moleculares, el primero fue DNA Molecular Weight Marker IX (Roche), el cual contiene fragmentos de ADN de diez longitudes distintas, desde 72 pb hasta 1353 pb. El segundo fue 1Kb DNA gTP-Ladder, el cual contenía 13 fragmentos de ADN, desde 250 pb hasta 10.000 pb.

Para llevar a cabo la electroforesis se prepararon geles de agarosa a dos concentraciones; al 0,8% (p/v), para la visualización del ADN extraído y los fragmentos amplificados para la obtención del ADNmt completo; y al 2% (p/v) para la visualización de los fragmentos parciales de ADN amplificados mitocondriales y ribosómicos.

Protocolo de preparación de geles de agarosa

1. Pesar 0,32 g de agarosa para los geles al 0,8% (p/v), o 0,80 g para los geles al 2% (p/v).
2. Añadir 40 ml de tampón de electroforesis TBE (Tris-Borato-EDTA) 0,5X y calentar la mezcla en el microondas hasta su total disolución.
3. Templar la solución y añadir 1 µl de SABR^{TM} safe para la tinción del ADN. Posteriormente, volcar la solución sobre el molde de la cubeta de electroforesis (Bio-Rad), a la que previamente se le tienen que colocar los peines que formarán los pocillos sobre los que se carga la muestra.
4. Tras la solidificación, retirar los peines y colocar el gel en la cubeta de electroforesis, sumergido en el tampón TBE 0,5X, teniendo precaución de recubrir totalmente la superficie del gel.
5. Añadir 5 µl de cada producto de PCR (conteniendo previamente el tampón de carga) en los pocillos formados en el gel de agarosa y aplicar voltaje a 90 V. La electroforesis se realiza a voltaje constante, lo que permite separar los fragmentos de ADN gracias a su tamaño.

6. Finalmente, retirar el gel de agarosa de la cubeta de electroforesis y fotografiar el gel sobre un transiluminador de luz ultravioleta a una longitud de onda de 360 nm a través de una cámara digital Kodak (Gel Logic 100 Imaging System) asociada al programa Kodak 1D 3.6.

Tabla 2. Secuencias y números de acceso en GenBank obtenidos de los diferentes marcadores moleculares de *Trichuris* spp. analizados en la presente Tesis Doctoral.

Especie	Hospedador	Origen geográfico	ITS1	ITS2	*cox*1	*cob*	*rrn*L	Genoma mitocondrial
Trichuris sp.	*Macaca sylvanus*	Cádiz, España	LR898004 LR898005 LR898006 LR898007 LR898008 LR898009	LR535742 LR535746 LR535747 LR535748 LR535749 LR535750 LR535751 LR898025	LR130781 LR130782 LR130783 LR130784	LR132031 LR132032 LR132033 LR132034 LR757999 OM457238 OM457239 OM457240 OM457241 OM457242	LR898063 LR898064 LR898065 LR898066 LR898067	MW448470 MW448471
Trichuris trichiura	*Papio papio*	Cantabria (Cabárceno), España	LR898010 LR898011	LR898032 LR898033 LR898034 LR898035 LR898036	LR898050	LR898053 LR898054 LR898055 LR898056	LR898057	MW448472
Trichuris trichiura	*Chlorocebus aethiops*	Málaga (Estepona), España	LR898012 LR898013	LR898026 LR898027 LR898028 LR898029 LR898030	LR898048	LR898051		
Trichuris trichiura	*Erythrocebus patas*	Barcelona, España	LR898014	LR898031	LR898049	LR898052		

Trichuris colobae	*Colobus guereza kikuyensis*	Málaga (Fuengirola), España					LR898058 LR898059 LR898060 LR898061 LR898062
Trichuris sp.	*Hystrix cristata*	Málaga (Fuengirola), España	OU624085 OU624086		OU596152 OU596153	OU596147 OU596148 OM457227 OM457228 OM457229 OM457230 OM457231	OU596145 OU596146
Trichuris suis	*Sus scrofa domestica*	Sevilla y Huelva, España				OM457243 OM457244 OM457245	
Trichuris ovis	*Capra hircus*	Sevilla, España				OM457234 OM457235 OM457236 OM457237	
Trichuris vulpis	*Canis lupus familiaris*	Sevilla y Huelva, España				OM457232 OM457233	
Trichuris sp.	*Camelus bactrianus*	Cantabria (Cabárceno), España	OM920875 OM920876 OM920877 OM920878	OM920881 OM920882 OM920883 OM920884	OM920890 OM920891 OM920892 OM920893	OM912468 OM912469 OM912470 OM912471	OM920886 OM920887 OM920888 OM920889

Tabla 3. Mezcla de reactivos, cebadores y condiciones de PCR utilizadas para cada marcador molecular analizado en la presente Tesis Doctoral.

	ITS1	ITS2	*cox*1	*cob*	*rrn*L
Mezcla de PCR					
Cebador directo (10 µM)	5 µl	5 µl	5 µl	5 µl	5 µl
Cebador inverso (10 µM)	5 µl	5 µl	5 µl	5 µl	5 µl
GoTaq G2 Green Master Mix	25 µl	25 µl	25 µl	25 µl	25 µl
ADN	5 µl	5 µl	5 µl	5 µl	5 µl
Agua libre de nucleasas, c.s.p.	50 µl	50 µl	50 µl	50 µl	50 µl
Cebadores					
Cebador directo	NC5 (Gasser y col., 1996)	5,8SF (Robles y col., 2014)	HC02198F (Folmer y col., 1994)	D769 (Callejón y col., 2015)	TTrrnLF (Liu y col., 2012)
Cebador inverso	5,8SR (Rivero y col., 2022)	NC2 (Gasser y col., 1996)	CORA (Nagano y col., 1999)	D770 (Callejón y col., 2015)	TTrrnLR (Liu y col., 2012)
Condiciones de PCR					
Desnaturalización inicial	94° C/10 min	94° C/3 min	94° C/5 min	94° C/5 min	95° C/15 min
Número de ciclos	35	35	40	36	35
Desnaturalización	94° C/1 min	94° C/1 min	94° C/1 min	94° C/30 s	95° C/30 s
Hibridación	55° C/1 min	50° C/1 min	48° C/1 min	50° C/30 s	55° C/30 s
Extensión de los cebadores	72° C/1 min	72° C/1 min	72° C/1 min	72° C/30 s	72° C/1 min
Extensión final	72° C/10 min	72° C/10 min	72° C/7 min	72° C/5 min	72° C/10 min

Tabla 4. Mezcla de reactivos, cebadores y condiciones de PCR utilizadas para amplificar y secuenciar el genoma mitocondrial completo del género *Trichuris* en la presente Tesis Doctoral.

Genoma mitocondrial completo		
Mezcla de PCR		
Cebador directo (10 µM)	2,5 µl	
Cebador inverso (10 µM)	2,5 µl	
Q5® High-Fidelity 2X Master Mix	25 µl	
ADN	5 µl	
Agua libre de nucleasas, c.s.p.	50 µl	
Cebadores		
	1er fragmento	2º fragmento
Cebador directo	MS1F (Rivero y col., 2021)	TTB1rrnLF (Hawash y col., 2015)
Cebador inverso	MS1R (Rivero y col., 2021)	TTB1nad1R (Hawash y col., 2015)
Condiciones de PCR		
	1er fragmento	2º fragmento
Desnaturalización inicial	92° C/4 min	92° C/4 min
Número de ciclos	35	35
Desnaturalización	92° C/20 s	92° C/20 s
Hibridación	59° C/30 s	50° C/30 s
Extensión de los cebadores	67° C/7 min	67° C/7 min
Extensión final	67° C/10 min	67° C/10 min

A.I.2.2.5. Purificación y cuantificación del ADN amplificado

La purificación del ADN amplificado se ha llevado a cabo utilizando el kit QWizard®SV Gel and PCR Clean-Up System (Promega), que permite extraer y purificar el ADN a partir del gel de agarosa o del producto de PCR amplificado directamente. La purificación del ADN amplificado se realiza para eliminar los componentes de la PCR que puedan interferir en procesos posteriores.

Una vez purificado es necesario una cuantificación aproximada. Para ello, generalmente comparamos la intensidad de la banda del ADN purificado con la del marcador de pesos moleculares, de tamaño y concentración conocidos. Asimismo, en algunos casos, para llevar a cabo una cuantificación más precisa, utilizamos el aparato NanoDrop 2000 (Thermo Scientific), el cual permite realizar medidas de espectrofotometría y cuantificar ADN.

Protocolo de purificación del ADN amplificado

1. En el caso de purificar el producto de PCR directamente, añadir un volumen igual de tampón Membrane Binding Solution al tubo Eppendorf y pasar directamente al paso 6. Si, por el contrario, se purifica a través del aislamiento de la banda del gel, pasamos al paso 2.
2. Cargar el producto de PCR completo en un gel de agarosa al 0,2 o al 0,8% (p/v) (dependiendo del tamaño de la banda que queramos obtener), y realizamos una electroforesis.
3. Cortar la banda que interesa y colocarlo en un tubo Eppendorf de 1,5 ml previamente pesado. Pesar la cantidad de gel que se ha cortado.
4. Añadir tampón Membrane Binding Solution en proporción de 10 µl por cada 10 mg de gel de agarosa obtenido.

5. Mezclar mediante vórtex e incubar a 50 – 65ºC durante 10 min o hasta que el gel esté completamente disuelto. Aplicar vórtex cada pocos minutos para facilitar la disolución.
6. Colocar una columna de purificación SV Minicolumn en un tubo de recolección por cada muestra. Posteriormente, transferir la mezcla, del gel disuelto o el producto de PCR, con el tampón a la columna de purificación e incubar durante 1 min a temperatura ambiente. Centrifugar durante 1 min a 13.000 r.p.m. y desechar el filtrado del tubo de recolección.
7. Colocar la columna de purificación en el tubo de recolección y añadir 700 µl de Membrane Wash Solution previamente diluido con etanol de 95º. Centrifugar durante 1 min a 13.000 r.p.m. y desechar el filtrado del tubo de recolección.
8. Repetir el paso 7 con 500 µl de Membrane Wash Solution y durante 5 min a 13.000 r.p.m.
9. Vaciar el filtrado del tubo de recolección y volver a centrifugar durante 1 min a 13.000 r.p.m. para evaporar completamente los residuos del etanol.
10. Cuidadosamente transferir la columna de purificación a un tubo Eppendorf de 1,5 ml.
11. Añadir 50 µl de Nuclease-Free Water a la columna e incubar durante 1 min a temperatura ambiente. Centrifugar durante 1 min a 13.000 r.p.m.
12. Desechar la Minicolumn y almacenar el ADN a 4ºC o a -20ºC hasta análisis posteriores.

A.I.2.2.6. Secuenciación

Una vez purificado el producto de PCR, se cuantifica la concentración obtenida utilizando NanoDrop 2000. Posteriormente, las muestras son enviadas a la empresa Stab Vida (Portugal) para que se proceda a la secuenciación de las

mismas, mediante la secuenciación Sanger. Todas las secuencias, junto con los números de acceso (GenBank) obtenidas en la presente Tesis Doctoral están disponibles en la Tabla 2.

A.I.2.2.7. Análisis de las secuencias

Los análisis de las secuencias parciales obtenidas de los marcadores moleculares ribosómicos y mitocondriales se llevaron a cabo, en primer lugar, utilizando el programa Chromas 2.6.6 (Technelysium) y ClustalW (Multiple Sequence Alignment, GenomeNet), con el fin de obtener la secuencia consenso para cada muestra analizada. Posteriormente, utilizando Nucleotide BLAST (Basic Local Aligment Search Tool) (Sayers y col., 2021) para identificar y confirmar la correcta secuenciación de las especies requeridas comparándolas con otras secuencias disponibles en la base de datos de GenBank.

Para el alineamiento de las secuencias se utilizó el método MUSCLE en MEGA7 (v7.0), MEGAX (v10.1.8) y MEGA11 (v11.0.11) (Molecular Evolutionary Genetics Analysis) (Kumar y col., 2016, 2018; Tamura y col., 2021). Esta aplicación permitió la comparación de las secuencias obtenidas entre ellas junto con las secuencias extraídas de la base de datos de GenBank, además de, el cálculo de los parámetros de longitud, la composición de las bases nucleotídicas (mutaciones y deleciones) y el porcentaje de variabilidad. Además, en algunos estudios se llevaron a cabo los análisis de saturación de sustitución de nucleótidos, un indicador de si el marcador genético utilizado es útil, utilizando el programa DAMBE v5.3.32 (Xia y col., 2003, 2009), y la diversidad haplotípica y nucleotídica, utilizando DnaSP v6.12.03 (Rozas y col., 2017).

El ensamblaje y la anotación de las secuencias obtenidas del genoma mitocondrial completo se llevaron a cabo utilizando el programa MacVector v17.5.4 (Oxford Molecular Group, Waterbeach, Cambridge, UK). Se utilizó el programa BLASTn para conocer la identidad de las secuencias comparándolas con otras disponibles en la base de datos de GenBank. La anotación del genoma se perfeccionó utilizando el servidor web MITOS (Bernt y col., 2013). La mayoría de los genes de ARNt se identificaron utilizando la herramienta ARWEN (Laslett y Canbäck, 2008) y tRNAScan-SE (Lowe y Chan, 2016). Sin embargo, no todos los genes fueron posibles de identificar a través de estas herramientas y, el resto, se reconocieron manualmente por comparación.

Los genomas mitocondriales completos fueron comparados con distintas secuencias obtenidas de la base de datos de GenBank. Los alineamientos y la estimación de las distancias genéticas se realizaron utilizando MEGAX (Kumar y col., 2018). Las regiones ambiguas de la alineación se excluyeron usando Gblocks Server (v0.0.91b), con la configuración predeterminada que se utiliza para seleccionar la opción de conservación menos estricta de las posiciones flanqueantes (Castresana, 2000; Talavera y Castresana, 2007). Asimismo, se calculó la diversidad nucleotídica utilizando DnaSP v5 (Librado y Rozas, 2009).

Los diferentes análisis de los alineamientos se han realizado con el fin de obtener:

- La similitud intraespecífica: basada en conocer el porcentaje de similitud entre las secuencias de un mismo marcador molecular y de una misma especie y población. Además, se estudió la variabilidad intrapoblacional, entre las muestras de una misma población (de la misma especie y zona geográfica), y la variabilidad interpoblacional, entre muestras de una misma especie, pero de diferentes poblaciones.

- La similitud interespecífica: basada en conocer el porcentaje de similitud entre secuencias de un mismo marcador molecular entre individuos de diferentes especies y poblaciones.

A.I.2.2.8. Análisis filogenéticos

De acuerdo con los alineamientos obtenidos de las diferentes matrices de datos de los marcadores moleculares ribosómicos y mitocondriales, se seleccionaron los modelos evolutivos más adecuados utilizando el programa jModelTest v0.1.1 (Posada, 2008) mediante la selección de un modelo utilizando el Criterio de Información de Akaike (AIC) (Posada y Buckley, 2004). Los árboles filogenéticos se construyeron a partir de estos modelos seleccionados.

Por otra parte, todos los árboles filogenéticos necesitan ser enraizados con grupos externos ("outgroups"), una o más secuencias que están relacionadas de forma distante con las secuencias objeto de estudio para el análisis. Por ello, en la presente Tesis Doctoral, en los análisis filogenéticos realizados dentro del género *Trichruis,* se eligieron como grupos externos, distintas especies del género *Trichinella*, y en los análisis de la clase Enoplea, se utilizaron distintas especies del orden Rhabditida (Tabla 5).

Tabla 5. Especies, números de acceso, genes o regiones amplificadas, orden y hospedadores y origen geográfico de los taxones utilizados en los análisis moleculares de la presente Tesis Doctoral.

Especie	Orden	Hospedador	Origen geográfico	Gen o región	Número de acceso
Trichuris arvicolae	Trichinellida	*Myodes glareolus*	España (Europa)	*cox*1	FR851284
Trichuris bainae	Trichinellida	*Necromys temchuki*	Argentina (América del Sur)		LN899585
Trichuris colobae	Trichinellida	*Colobus guereza kikuyensis*	España (Europa)		HE653116
	Trichinellida				HE653118
					JF690968
			Italia (Europa)		MK762948
Trichuris discolor	Trichinellida	*Bos grunniens mutus*	China (Asia)		NC_018596
Trichuris globulosa	Trichinellida	*Camelus dromedarius*	Irán (Asia)		LN626969
					LN626970
					LN626971
Trichuris muris	Trichinellida	-	Reino Unido (Europa)		LC050561
Trichuris navonae	Trichinellida	*Akodon montensis*	Argentina (América del Sur)		HG934464
Trichuris ovis	Trichinellida	*Addax nasomaculatus*	China (Asia)		NC_018597
Trichuris pampeana	Trichinellida	*Ctenomys talarum*	Argentina (América del Sur)		KC614698
Trichuris pardinasi	Trichinellida	*Phyllotis xanthopygus*	Argentina (América del Sur)		HG934451
Trichuris rhinopiptheroxella	Trichinellida	*Rhinopithecus roxellana*	China (Asia)		MG189593
Trichuris skrjabini	Trichinellida	*Capra hircus*	España (Europa)		HE653121
		Capra hircus	China (Asia)		HQ183745
		Ovis aries	Sudáfrica (África)		LN626972
					LN626973
***Trichuris* sp.**	Trichinellida	*Addax nasomaculatus*	Italia (Europa)		MN562593
					MN562594
					MN562595
					MN562596
		Akodon azarae	Argentina (América del Sur)		LT221883
		Camelus bactrianus	Italia (Europa)		MN562588
					MN562589
					MN562590

				MN562591
				MN562592
		Chlorocebus aethiops	Italia (Europa)	MK762929
				MK762931
		Chlorocebus sabaeus	República Checa (Europa)	MK762923
				MK762924
				MK762925
				MK762927
		Colobus guereza kikuyensis	República Checa (Europa)	JF690968
		Holochilus chacarius	Argentina (América del Sur)	LT221884
		Hystrix cristata	Italia (Europa)	MK779003
		Homo sapiens	República Checa (Europa)	JF690962
		Macaca fuscata	Italia (Europa)	MK762905
				MK762906
				MK762908
				MK762909
				MK762915
				MK762919
				MK762920
				MK762921
		Mastomys coucha	Sudáfrica (África)	MG386207
		Papio anubis	EE. UU. (Norte América)	KT449825
		Papio hamadryas	República Checa (Europa)	JF690963
		Papio hamadryas	Dinamarca (Europa)	KT449824
		Papio hamadryas	Europa	MK762943
				MK762945
		Sooretamys angouya	Argentina (América del Sur)	HG934466
		Trachypithecus francoisi	China (Asia)	KC461179
Trichuris trichiura	Trichinellida	*Homo sapiens*	China (Asia)	GU385218
				NC_017750
			Japón (Asia)	AP017704
			Uganda (África)	KT449826
		Papio sp.	España (Europa)	HG003692
Trichuris suis	Trichinellida	*Sus scrofa*	China (Asia)	NC_017747
		Sus scrofa domestica	China (Asia)	GU070737
				HQ204208

					HQ204209 HQ204210
			Dinamarca (Europa)		KT449822
			España (Europa)		HE653124
			Uganda (África)		KT449823
		Sus scrofa scrofa	España (Europa)		HE653127
Trichuris ursinus	Trichinellida	*Papio ursinus*	Sudáfrica (África)		LT627353
Trichuris vulpis	Trichinellida	*Canis lupus familiaris*	España (Europa)		HE653135
Trichuris arvicolae	Trichinellida	*Myodes glareolus*	España (Europa)	*cob*	LM994698
Trichuris bainae	Trichinellida	*Sooretamys angouya*	Argentina (América del Sur)		LN899575
Trichuris colobae	Trichinellida	*Colobus guereza kikuyensis*	España (Europa)		LM994704 MK914578
Trichuris discolor	Trichinellida	*Bos grunniens mutus*	China (Asia)		NC_018596
Trichuris globulosa	Trichinellida	*Camelus dromedarius*	Irán (Asia)		LN626974 LN626975
Trichuris leporis	Trichinellida	*Lepus europaeus*	España (Europa)		LM994705
Trichuris muris	Trichinellida	-	Reino Unido (Europa)		LC050561
Trichuris navonae	Trichinellida	*Akodon montensis*	Argentina (América del Sur)		LN899565
Trichuris ovis	Trichinellida	*Addax nasomaculatus*	China (Asia)		NC_018597
		Ovis aries	Sudáfrica (África)		LN626976 LN626977
Trichuris pardinasi	Trichinellida	*Phyllotis bonariensis*	Argentina (América del Sur)		LN899577
Trichuris rhinopiptheroxella	Trichinellida	*Rhinopithecus roxellana*	China (Asia)		MG189593
Trichuris skrjabini	Trichinellida	*Capra hircus*	España (Europa)		LM994700
		Ovis aries	Sudáfrica (África)		LN626978 LN626979
		Pseudois nayaur	China (Asia)		KY971755
***Trichuris* sp.**	Trichinellida	*Addax nasomaculatus*	Italia (Europa)		MN627752 MN627753 MN627754
		Camelus bactrianus	Italia (Europa)		MN627744 MN627745 MN627746 MN627747

				MN627748
				MN627749
				MN627750
				MN627751
		Chlorocebus aethiops	Italia (Europa)	MK914564
				MK914570
				MK914571
		Chlorocebus sabaeus	República Checa (Europa)	MK914562
				MK914563
		Holochilus chacarius	Argentina (América del Sur)	LT221888
		Hystrix cristata	Italia (Europa)	MK779004
		Macaca fuscata	Italia (Europa)	MK914550
				MK914551
				MK914554
				MK914555
				MK914556
				MK914557
				MK914560
		Papio anubis	EE. UU. (Norte América)	KT449825
		Papio hamadryas	Dinamarca (Europa)	KT449824
			República Checa (Europa)	MK914573
				MK914574
				MK914575
				MK914576
				MK914577
		Papio sp.	España (Europa)	LM994703
		Trachypithecus francoisi	China (Asia)	KC461179
Trichuris trichiura	Trichinellida	*Homo sapiens*	China (Asia)	GU385218
				NC_017750
			Uganda (África)	KT449826
Trichuris suis	Trichinellida	*Sus scrofa*	China (Asia)	NC_017747
		Sus scrofa domestica	China (Asia)	GU070737
			Dinamarca (Europa)	KT449822
			Uganda (África)	KT449823
			España (Europa)	LM994696
Trichuris ursinus	Trichinellida	*Papio ursinus*	Sudáfrica (África)	LT627357
				LT627358
				LT627359
				LT627360

Trichuris vulpis	Trichinellida	*Canis lupus familiaris*	España (Europa)		LM994699
Trichuris colobae	Trichinellida	*Colobus guereza kikuyensis*	España (Europa)	*rrn*L	MN088583 MN088584 MN088585
Trichuris discolor	Trichinellida	*Bos grunniens mutus*	China (Asia)		NC_018596
Trichuris muris	Trichinellida		Reino Unido (Europa)		LC050561
Trichuris ovis	Trichinellida	*Addax nasomaculatus*	China (Asia)		NC_018597
Trichuris rhinopiptheroxella	Trichinellida	*Rhinopithecus roxellana*	China (Asia)		MG189593
Trichuris skrjabini	Trichinellida	*Capra hircus*	China (Asia)		HQ183739
***Trichuris* sp.**	Trichinellida	*Chlorocebus aethiop*	Italia (Europa)		MN088565
		Chlorocebus sabaeus	República Checa (Europa)		MN088559 MN088560 MN088561
		Chlorocebus sabaeus	San Cristóbal y Nieves (Norte América)		KU524595 KU524599 KU524600 KU524601 KU524602
		Macaca fuscata	Italia (Europa)		MN088542 MN088543 MN088544 MN088546 MN088551 MN088553 MN08855 MN088557
		Papio anubis	EE. UU. (Norte América)		KT449825
		Papio hamadryas	República Checa (Europa)		MN088578 MN088580
			Dinamarca (Europa)		KT449824
		Papio sp.	Dinamarca (Europa)		KU524558 KU524559 KU524564 KU524573 KU524574 KU524575 KU524576 KU524581 KU524584
		Trachypithecus francoisi	China (Asia)		KC481232 KC481233 KC481234

					KC481235
					KC461179
Trichuris trichiura	Trichinellida	*Homo sapiens*	China (Asia)		GU385218
					NC_017750
					AM993017
					AM993018
					AM993019
					AM993020
			Ecuador (América del sur)		KP781898
					KP781899
					KP781900
					KP781901
					KP781906
			Uganda (África)		KT449826
					KU524541
					KU524542
					KU524544
					KU524545
					KU524548
					KU524557
Trichuris suis	Trichinellida	*Sus scrofa*	China (Asia)		NC_017747
		Sus scrofa domestica	China (Asia)		GU070737
			Dinamarca (Europa)		KT449822
			Ecuador (América del sur)		KP781894
			Uganda (África)		KT449823
					KU524537
					KU524540
Trichuris arvicolae	Trichinellida	-	España (Europa)	ITS1	FR849652
Trichuris carlieri	Trichinellida	*Gerbilliscus vicinus*	Tanzania (África)		JX683524
Trichuris colobae	Trichinellida	*Colobus guereza kikuyensis*	España (Europa)		FM991956
		Nomascus gabriellae	España (Europa)		FM991955
Trichuris cutillasae	Trichinellida	*Hydrochoerus hydrochaeris*	Argentina (América del Sur)		LS481186
Trichuris discolor	Trichinellida	*Bos taurus*	España (Europa)		HE608850
			Irán (Asia)		HE608851
		Capreolus capreolus	China (Asia)		KT581252
			República Checa (Europa)		JX218220
Trichuris duplanteri	Trichinellida	*Gerbillus gerbillus*	Mauritania (África)		KX669087
Trichuris leporis	Trichinellida	-	España (Europa)		AJ310663

Trichuris mastomysi	Trichinellida	*Mastomys natalensis*	Tanzania (África)	JX683525
Trichuris muris	Trichinellida	*Mus domesticus*	España (Europa)	FN543196
		-	Reino Unido (Europa)	LC171641
Trichuris myocastoris	Trichinellida	*Myocastor coypus*	República Checa (Europa)	MF077371
Trichuris ovis	Trichinellida	*Bos taurus*	España (Europa)	FR870274
		Ovis aries	Irlanda (Europa)	JF680987
			España (Europa)	AJ310662
Trichuris skrjabini	Trichinellida	*Capra hircus*	España (Europa)	AJ489248
***Trichuris* sp.**	Trichinellida	*Capricomis sumatraensis*	China (Asia)	OK142776
		Chlorocebus aethiops	China (Asia)	KT344827
		Macaca leonina	China (Asia)	KT344828
		Macaca mulatta	China (Asia)	KT344829 MH390369
		Nomascus leucogenys	China (Asia)	KT344830
		Ovis aries	China (Asia)	HQ844233
		Ovis orientalis aries	China (Asia)	KJ507245
		Papio anubis	China (Asia)	KT344826
		Papio hamadryas	China (Asia)	KT344831
		Papio ursinus	Sudáfrica (África)	GQ301551
		Pseudois nayaur	China (Asia)	MG573307
		Rhinopithecus roxellana	China (Asia)	KT344825
		Trachypithecus francoisi	China (Asia)	KT186231 KT186232 KT186233 KT186234
		Thryonomys swinderianus	Gana (África)	LC512871
Trichuris trichiura	Trichinellida	*Cercopithecus ascanius*	Uganda (África)	KJ588097
		Cercopithecus lhoesti	Uganda (África)	KJ588102
		Cercopithecus mitis	Uganda (África)	KJ588104
		Colobus guereza	Uganda (África)	KJ588076
		Homo sapiens	Camerún (África)	GQ301555
			China (Asia)	AM992987

			Uganda (África)		KJ588075
		Lophocebus albigena	Uganda (África)		KJ588096
		Pan troglodytes	Uganda (África)		KJ588094
		Papio anubis	Uganda (África)		KJ588099
		Papio hamadryas	Turquía (Europa, Asia)		KC877992
		Procolobus rufomitratus	Uganda (África)		KJ588122
Trichuris suis	Trichinellida	*Sus scrofa*	China (Asia)		AM992999
		Sus scrofa domestica	España (Europa)		AJ781762
Trichuris ursinus	Trichinellida	*Papio ursinus*	Sudáfrica (África)		GQ301554
Trichuris vulpis	Trichinellida	*Canis lupus familiaris*	España (Europa)		AM234616
Trichuris arvicolae	Trichinellida	-	España (Europa)	ITS2	FR849652
Trichuris carlieri	Trichinellida	*Gervilliscus vicinus*	Tanzania (África)		JX683524
Trichuris colobae	Trichinellida	*Colobus guereza kikuyensis*	España (Europa)		FM991956
		Nomascus gabriellae			FM991955
Trichuris cutillasae	Trichinellida	*Hydrochoeris hydrochaeris*	Argentina (América del Sur)		LS481191
Trichuris discolor	Trichinellida	*Bos taurus*	Irán (Asia)		HE608851
		Bos taurus	España (Europa)		HE608850
		Capreolus capreolus	China (Asia)		KT581252
		Capreolus capreolus	República Checa (Europa)		JX218220
Trichuris duplanteri	Trichinellida	*Gerbillus tarabuli*	Mauritania (África)		KX669086
Trichuris globulosa	Trichinellida	*Camelus dromedarius*	Irán (Asia)		LN651156 LN651157 LN651158 LN651159 LN651160
		Ovis aries	España (Europa)		LN813018
Trichuris leporis	Trichinellida	-	España (Europa)		AJ251321
Trichuris mastomysi	Trichinellida	*Mastomys natalensis*	Tanzania (África)		JX683525

Trichuris muris	Trichinellida	*Mus domesticus*	España (Europa)	FN543196
		-	Reino Unido (Europa)	LC171641
Trichuris myocastoris	Trichinellida	*Myocastor coypus*	República Checa (Europa)	MF077371
Trichuris ovis	Trichinellida	*Bos taurus*	España (Europa)	FR870274
		Ovis aries	Irlanda (Europa)	JF680987
			España (Europa)	LN813016
			Sudáfrica (África)	LN651161
Trichuris skrjabini	Trichinellida	*Capra hircus*	España (Europa)	AJ489248
***Trichuris* sp.**	Trichinellida	*Camelus bactrianus*	República Checa (Europa)	JF690952
		Chlorocebus aethiops	Tanzania (África)	JF690949
				JF690950
		Homo sapiens	República Checa (Europa)	JF690940
		Macaca leonina	China (Asia)	MH390365
				KT344828
		Macaca mulatta	China (Asia)	MH390367
				MH390369
		Macaca silenus	República Checa (Europa)	JF690945
		Nomascus leucogenys	China (Asia)	KT344830
		Ovis aries	China (Asia)	HQ844233
		Ovis orientalis aries	China (Asia)	KJ507245
		Papio anubis	República Checa (Europa)	JF690942
		Papio hamadryas	República Checa (Europa)	JF690941
		Papio hamadryas ursinus	Sudáfrica (África)	GQ301551
				GQ301552
				GQ301553
		Rhinopithecus roxellana	China (Asia)	KT344825
		Trachypithecus francoisi	China (Asia)	KT186231
				KT186232
				KT186233
				KT186234

		Thryonomys swinderianus	Gana (África)		
Trichuris trichiura	Trichinellida	*Cercopithecus ascanius*	Uganda (África)		KJ588135
		Cercopithecus lhoesti	Uganda (África)		KJ588163
		Cercopithecus mitis	Uganda (África)		KJ588156
		Colobus guereza	Uganda (África)		KJ588167
		Homo sapiens	Camerún (África)		GQ301555
			China (Asia)		AM992987
			Uganda (África)		JN181859
		Lophocebus albigena	Uganda (África)		KJ588147
		Macaca fuscata	China (Asia)		AM992987
			Japón (Asia)		AB586133
		Pan troglodytes	Uganda (África)		KJ588160
		Papio anubis	Uganda (África)		KJ588152
		Papio hamadryas	Turquía (Europa, Asia)		KC877992
		Procolobus rufomitratus	Uganda (África)		KJ588162
Trichuris suis	Trichinellida	*Sus scrofa*	China (Asia)		AM992999
			Eslovaquia (Europa)		JF690951
			Tanzania (África)		JN181811
		Sus scrofa domestica	España (Europa)		AJ249966
Trichuris ursinus	Trichinellida	*Papio ursinus*	Sudáfrica (África)		GQ301554
Trichuris vulpis	Trichinellida	*Canis lupus familiaris*	España (Europa)		AM234616
Trichuris trichiura	Trichinellida	*Homo sapiens*	-	Mit. completo	AP017704
			China (Asia)		NC_017750
			China (Asia)		GU385218
			Uganda (África)		KT449826
***Trichuris* sp.**	Trichinellida	*Papo anubis*	EE. UU. (Norte América)		KT449825
		Papio hamadryas	Dinamarca (Europa)		KT449824
		Trachypithecus francoisi	China (Asia)		KC461179

Trichuris rhinopiptheroxella	Trichinellida	*Rhinopithecus roxellana*	China (Asia)		MG189593
Trichuris ovis	Trichinellida	*Addax nasomaculatus*	China (Asia)		NC_018597
Trichuris discolor	Trichinellida	*Bos grunniens mutus*	China (Asia)		NC_018596
Trichuris muris	Trichinellida		Reino Unido (Europa)		LC050561
Trichuris suis	Trichinellida	*Sus scrofa*	Uganda (África)		KT449823
			China (Asia)		GU070737
Trichinella pseudospiralis	Trichinellida	*Coragypus atratus*	EE. UU. (Norte América)		KM357411
Xiphinema americanum	Dorylaimida	-	-		NC_005928
Hexamermis agrotis	Mermithida	-	-		NC_008828
Agamermis sp.	Mermithida	-	-		NC_008231
Romanomermis culicivorax	Mermithida	-	-		NC_008640
Romanomermis iyengari	Mermithida	-	-		NC_008693
Romanomermis nielseni	Mermithida	-	-		NC_008692
Strelkovimermis spiculatus	Mermithida	-	-		NC_008047
Thaumamermis cosgrovei	Mermithida	-	-		NC_008046
Outgroups					
Trichinella spiralis	Trichinellida			*cox*1	AF293969
Trichinella pseudospiralis	Trichinellida			*cox*1	KM357411
Trichinella spiralis	Trichinellida			*co*b	NC_002681
Trichinella pseudospiralis	Trichinellida			*co*b	KM357411
Trichinella nativa	Trichinellida			ITS	KP307966
Trichinella spiralis	Trichinellida			ITS	KC006415
Trichinella pseudospiralis	Trichinellida			Mit. completo	KM357411
Brugia malayi	Rhabditida			Mit. completo	NC_004298
Ascaris suum	Rhabditida			Mit. completo	HQ704901

Los métodos de reconstrucción filogenética pueden dividirse en dos categorías en base al tipo de datos que se emplean, ya sean a analizando los caracteres discretos o analizando las distancias. Los métodos basados en caracteres discretos usan directamente las columnas individuales de nucleótidos o aminoácidos alineados, mientras que los métodos basados en distancias usan medidas de las diferencias generales entre todos los pares de secuencias del alineamiento (representado como una matriz de distancias genéticas por pares). Asimismo, para realizar dichos análisis, se utilizaron las secuencias obtenidas en la presente Tesis Doctoral (Tabla 2), junto con un número variable de las secuencias depositadas por otros autores en la base de datos de GenBank (Tabla 5).

Los métodos basados en la distancia se basan en el cálculo de distancias genéticas entre cada par de secuencias en un conjunto de datos con árboles filogenéticos que se construyen a partir de la matriz de distancia resultante utilizando un modelo evolutivo seleccionado. Dada una matriz de distancias evolutivas, se puede obtener un árbol usando la agrupación de pares no ponderados con media aritmética (UPGMA) (Michener y Sokal, 1956), mínimos cuadrados (LS, least squares) (Fitch y Margoliash, 1967), unión de vecinos (NJ, "Neighbor-Joining") (Saitou y Nei, 1987), o métodos BIONJ (Gascuel, 1997). La principal ventaja de estos métodos es su velocidad de cálculo (De Bruyn y col., 2014).

En cambio, los métodos basados en caracteres aprovechan toda la información disponible de las secuencias en cada sitio homólogo, considerando cada sitio del alineamiento directamente. Los métodos más utilizados son los métodos de MP (MP = "Maximum Parsimony") (Sober, 1988), MV (ML = "Maximum Likelihood") (Felsenstein, 1981) e IB (Huelsenbeck y col., 2001). Mientras que los métodos de MP generalmente asumen implícitamente un modelo de

evolución muy simple (normalmente uno donde todas las posibles sustituciones de nucleótidos son igualmente probables), el principal atractivo de los métodos de ML es que son probabilísticos y permiten la aplicación de una amplia variedad de modelos evolutivos explícitos (De Bruyn y col., 2014). Asimismo, los métodos bayesianos incorporan modelos estadísticos complejos en el proceso de reconstrucción y maximizan la probabilidad posterior (Holder y Lewis, 2003).

En la presente Tesis Doctoral se han utilizado los métodos basados en caracteres para la reconstrucción filogenética:

- Método de Máxima Parsimonia

El método de MP establece que cuando se proponen varias hipótesis con diferentes grados de complejidad para explicar el mismo fenómeno, se debe elegir la hipótesis más simple, proponiendo que el árbol filogenético más creíble o parsimonioso será el árbol que invoque el menor número de cambios evolutivos durante la divergencia de las secuencias que representa (Kluge y Farris, 1969; Farris, 1970; Fitch, 1971).

Este método utiliza únicamente posiciones filogenéticamente informativas para inferir los árboles. Así, un sitio es informativo cuando hay dos o más clases de nucleótidos en una posición determinada en las secuencias analizadas (Li y Graur, 1991). Asimismo, permite utilizar como quinto sitio informativo, las inserciones o supresiones.

Un problema importante que se encuentra al inferir el árbol más parsimonioso para un conjunto de secuencias de nucleótidos dado es que con frecuencia habrá múltiples árboles igualmente parsimoniosos (De Bruyn y col., 2014).

Para llevar a cabo este análisis se utilizó el programa MEGA7, MEGAX y MEGA11 (Kumar y col., 2016, 2018; Tamura y col., 2021).

- Método de Máxima Verosimilitud

El método de MV o ML presenta un concepto estadístico. Este concepto básico de probabilidad corresponde con una matriz de secuencias de nucleótidos o aminoácidos, un modelo de evolución y la probabilidad de un conjunto de parámetros (topología de árbol, longitudes de rama de árbol, parámetros del modelo de sustitución) (Huelsenbeck y Crandall, 1997). Las estimaciones de MV de los valores de los parámetros corresponden al conjunto de valores que maximizan esta probabilidad. Consecuentemente, el método busca el árbol con la mayor probabilidad o verosimilitud. Se basa en el uso de una cantidad conocida como *log-likelihood*, en base a la cual se evalúan las diferentes topologías y encontrar aquella que maximice este valor. Así, la filogenia inferida bajo este método trata de encontrar estimaciones de los valores de cada parámetro y luego comparar las verosimilitudes de los distintos modelos, eligiendo la mejor topología en función de su verosimilitud (Guindon y Gascuel, 2003). Para llevar a cabo este análisis se utilizó el programa PhyML (Phylogenetic estimation using Maximum Likelihood) package (Guindon y col., 2010).

- Método de Inferencia Bayesiana

El método de IB se fundamenta en la relación cuantitativa existente entre la función de verosimilitud y las distribuciones anteriores y posteriores de probabilidad. Esta relación tiene su origen en el Teorema de Bayes (generalizado por Laplace en el año 1763), por el cual se permite calcular la probabilidad posterior partiendo de la verosimilitud y la probabilidad anterior de los datos. Se basa en la definición de probabilidades conjuntas. Para llevar a cabo este análisis se utilizó el programa MrBayes 3.1.2 (Ronquist y Huelsenbeck, 2003).

Para la obtención de los soportes estadísticos de los árboles filogenéticos inferidos, se realizó el *bootstrap test*, una técnica utilizada para evaluar la fiabilidad de un árbol filogenético. Este método está basado en el remuestreo de las propias muestras partiendo de la idea de que la distribución del parámetro verdadero puede ser estimada por la generación de repeticiones y análisis de los datos artificiales. En la presente Tesis Doctoral se realizó un muestreo repetitivo entre 100 y 1000 réplicas (*bootstrap*), creando numerosos conjuntos de datos. En tal caso, el soporte estadístico de los árboles obtenidos mediante IB fue expresado en forma de probabilidades bayesianas transformadas en porcentajes.

A.I.2.3. Análisis basados en la técnica MALDI-TOF MS

A.I.2.3.1. Extracción de proteínas

En los estudios realizados en la presente Tesis Doctoral utilizando la técnica MALDI-TOF MS, se utilizaron adultos de diferentes especies del género *Trichuris*. Para ello, los adultos, previamente identificados y congelados a – 20ºC, fueron divididos en dos partes (parte anterior, conteniendo el esófago principalmente, y la parte posterior, conteniendo el intestino y el aparato reproductor). Posteriormente, ambas partes fueron depositadas en Eppendorf de 1,5 ml. No todas las muestras obtenidas fueron analizadas, en primer lugar, para el estudio preliminar se utilizó la especie de *T. suis* y se analizaron ambas partes de los adultos, y posteriormente, en el resto de las especies analizadas, se analizaron las partes anteriores. Seguidamente, a cada Eppendorf se le añadió 10 mg de unas perlas de zirconio/sílice (0,5 mm) junto con 20 – 30 µl de una mezcla al 50% (v/v) de ácido fórmico al 70% (v/v) y acetonitrilo al 50% (v/v). A continuación, cada muestra fue homogeneizada utilizando el aparato TyssueLyser II (Qiagen GmbH) en 3 ciclos de 30 s cada uno, a una frecuencia de 30 Hz y, posteriormente, centrifugada a 10.000 r.p.m. durante 30 s.

A.I.2.3.2. Preparación y medidas de la placa de análisis

Para la creación de los espectros de referencia, de cada muestra, se depositaron 1 μl de sobrenadante en la placa de MALDI-TOF en ocho pocillos diferentes (Sy y col., 2020). En cambio, para realizar la prueba de validación se utilizaron 4 pocillos diferentes por muestra (Nebbak y col., 2017). Cada muestra fue secada a temperatura ambiente y, seguidamente, recubierta con 1 μl de matriz CHCA (solución saturada de ácido α-cyano-4-hidroxicinámico, acetonitrilo al 50% (v/v), ácido trifluoroacético al 2,5% (v/v) y agua para cromatografía HPLC) (Yssouf y col., 2016). Además, se añadieron muestras del "estándar de prueba bacteriano" (BTS = "bacterial test standard", un extracto de *Escherichia coli* enriquecido con dos proteínas de alto peso molecular) para calibrar la máquina (Nebbak y col., 2017). La placa de MALDI-TOF se dejó secar a temperatura ambiente para luego ser introducida en el espectrómetro Microflex LT (Bruker Daltonics) para MALDI-TOF MS.

A.I.2.3.3. Parámetros utilizados en MALDI-TOF MS

Las mediciones utilizadas en la técnica MALDI-TOF MS se llevaron a cabo en un rango de 2.000 – 20.000 Da m/z (masa de carga) y con la detección en modo positivo lineal con una frecuencia de láser de 50 Hz, tras la calibración con BTS. En cada pocillo se realizaron 240 disparos de láser en cuatro regiones, y las medidas fueron automáticamente adquiridas utilizando el método AutoXecute del software flexControl v3.4 (Bruker Daltonics). Los espectros se generaron y visualizaron mediante el software Flex Analysis v3.3 y se exportaron a MALDIBiotyper v3.1.66 (Bruker Daltonics) para el procesamiento de los datos. El voltaje de aceleración fue de 20 kV y el tiempo de retardo de extracción de 200 ns (Nebbak y col., 2017). De forma precisa, el error de masa máximo para cada espectro individual fue de 2.000 Da, la frecuencia de pico mínima deseada

fue del 25%, y el error de masa deseado para los espectros de referencia fue de 200 Da.

A.I.2.3.4. Análisis de los espectros y base de datos interna

De 2 a 6 muestras de cada especie son suficientes para la creación de los espectros de referencia (Diarra y col., 2017), por ello, se utilizaron de 2 a 5 muestras de adultos de las diferentes especies de *Trichuris* analizadas. En la placa de análisis de MALDI-TOF se depositaron 8 veces cada muestra y cada pocillo se midió 4 veces. La combinación de los resultados de los espectros de cada muestra se utilizó para crear los espectros de referencia mediante la función MALDI-Biotyper, utilizando el conjunto de parámetros predeterminados del "Bio Typer MSP Creation Standard Method" (Nebbak y col., 2017). La calidad de cada espectro sin procesar se evaluó utilizando el software Flex Analysis v3.4 (Bruker Daltonics). Este programa también se utilizó para eliminar las líneas planas y picos atípicos e intensidades suaves y editar los cambios de pico dentro de que los espectros siempre superen los 500 ppm.

A.I.2.3.5. Prueba de validación

Con el fin de confirmar la reproducibilidad de los espectros añadidos a la base de datos, se realizaron, en primer lugar, para la especie *T. suis*, utilizada en el estudio preliminar, dos pruebas de validación, una validación interna y una validación externa o test ciego, y posteriormente, para el resto de las especies de *Trichuris*, el test ciego. En la validación interna todos los espectros obtenidos para formar parte de los espectros de referencia de cada grupo de muestra fueron analizados. Asimismo, en el test ciego, se midieron las muestras sometidas a la técnica MALDI-TOF MS para evaluar la capacidad de la base de datos para identificar de forma fiable las muestras.

Los resultados obtenidos de las pruebas de validación fueron representados en LSV que oscilaban entre 0 y 3 en función del grado de similitud entre el espectro analizado y el espectro de referencia depositado previamente en la base de datos interna. La identificación del test ciego fue considerada óptima cuando se obtuvo una identificación correcta a nivel de género con valores logarítmicos superiores a 1,7, y con valores superiores a 2 a nivel de especie.

Del mismo modo, para determinar las distancias y la similitud entre las especies analizadas, se realizó un dendrograma, mediante el software MALDI-Biotyper, utilizando los espectros obtenidos en la creación de los espectros de referencia de cada especie.

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ANEXO II. LISTADO DE ABREVIATURAS

A.II. LISTADO DE ABREVIATURAS

ACP: Análisis de componentes principales

ADNmt: ADN mitocondrial

ADNr: ADN ribosómico

AIC: "Akaike information criterion" o criterio de información de Akaike

ARNr: ARN ribosómico

AVC: Análisis de variación canónica

BTS: "Bacterial test standard"

***cob*:** "*cytochrome* b" o *citocromo* b

***cox*1:** "*cytochrome c-oxidase* subunit 1" o *citocromo c-oxidasa* 1

ddPCR: "Droplet Digital™ polymerase chain reaction"

EPG: "Eggs per gram of faeces" o número de huevos por gramo de heces

FDA: "Food and Drug Administration"

FECM: "Formol-Eter concentration method" o técnica de concentración de formol-éter

IB: Inferencia bayesiana

ITS: "Internal transcribed spacer" o espaciadores transcritos internos

ITS1: "Internal transcribed spacer 1" o espaciadores transcritos internos 1

ITS2: "Internal transcribed spacer 2" o espaciadores transcritos internos 2

KFM: "Kubic FLOTAC microscope"

KK: Método Kato-Katz

LE: Longitud del esófago

LOD: "Lab-on-a-disk" o laboratorio en un disco

LP: Longitud de la parte posterior del cuerpo

LSV: "Log-score values" o valores logarítmicos

MALDI-TOF MS: "Matrix-assisted laser desorption/ionization time of flight mass spectrometry" o "espectrometría de masas por ionización-desorción asistida por matriz con tiempo de vuelo"

MDA: "Mass drug administration" o distribución de medicamentos antihelmínticos a gran escala

MP: Máxima parsimonia

MV: Máxima verosimilitud

OMS: Organización Mundial de la Salud

PC1: Primer componente principal

PCG: "Protein coding genes" o proteínas que contienen genes

PCR: "Polymerase chain reaction" o reacción en cadena de la polimerasa

PNH: Primates no humanos

PPB: Probabilidades posteriores bayesianas

***rrn*L:** "Large subunit ribosomal RNA" o subunidad larga del ARN ribosómico

RT-PCR: "Real-time polymerase chain reaction" o PCR a tiempo real

STH: "Soil-transmitted helminths", geohelmintos o helmintos que transmiten infecciones por el suelo

TBE: Tris-Borato-EDTA