Origin, evolution, phylogeny and taxonomy of *Pulex irritans* (Siphonaptera:Pulicidae).

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Abstract

Within Pulicidae family, the so-called human flea, *Pulex irritans* Linnaeus, 1758 has been the most studied species together with *C. felis* Bouché, 1835, since they have a cosmopolitan distribution together with the fact that these species are closely related with humans. The main aim of this work was to carry out a comparative morphometric and molecular study of two different populations of *P. irritans* (Spain and Argentina). For this purpose, the ITS1, ITS2 of the rDNA and partial cytochrome-c oxidase (*cox1*) and cytochrome b (*cytb*) mtDNA gene of these taxa were sequenced. Furthermore, we assessed the taxonomy, origin, evolution and phylogeny of *P. irritans*. In our study, morphometric data did not show significant differences between *P. irritans* specimens from Spain and Argentina, even when these two populations were collected from different hosts; however, we found a considerable degree of molecular divergence between both populations based on nuclear and mitochondrial markers. Thus, we could suggest that *P. irritans*, in contrast to other generalist fleas, maintain a certain degree of morphological similarity, at least between Western Palearctic and Neotropical areas. Furthermore, we provided the existence of two well defined geographical genetic lineages within *P. irritans* species suggesting the existence of two cryptic species which could be discriminated by PCR-linked RFLP.

Introduction

Pulicidae (Order Siphonaptera) has remained as the most studied family of fleas over the world since most fleas of medical or veterinary importance, such as *Ctenocephalides felis*, *Ctenocephalides canis*, *Pulex irritans* or *Xenopsylla cheopis*, are members of this family. Currently, Pulicidae consists of four tribes, 21 genera, and 167 species (Whiting *et al.*, 2008). Some authors (Lewis, 1998) considered Pulicidae as including Tungidae; however, Whiting *et al.* (2008) placed this family as a monophyletic group and phylogenetically distant from Tungidae. Recently, Krasnov *et al*. (2015) placed Pulicidae family on the basis of the flea phylogeny together with Leptopsyllidae. These authors suggested that the mainly Palearctic distribution and origin of Pulicidae and Leptopsyllidae and the mainly Nearctic distribution of the most derived family Ceratophyllidae, indicates an eastward earlier (pre-glaciation) migrations from Palearctic to Nearctic zones through the Bering Land Bridge. On the other hand, Pulicidae exhibits an interesting diversity of host specificity patterns and ecological habits (Whiting *et al.*, 2008). Within this family, the so-called human flea, *P. irritans* Linnaeus, 1758 (the earliest flea species described) has been the most studied species together with *C. felis* Bouché, 1835, since they have a cosmopolitan distribution together with the fact that these species are closely related with humans. *P. irritans* parasitizes a wide variety of hosts, including rodents or birds (Graham *et al.*, 2016); however, it generally parasitizes large wild and domestic mammals, particularly carnivores, livestock and humans (Gratz, 1999). Furthermore, specific host associations vary between geographic regions (Hopla, 1980; Lewis, 1998). Thus, in the last years several authors have assessed the role of this species as a vector of several diseases carrying out epidemiological studies based on the detection and prevalence of certain pathogens such as *Yersinia pestis*, *Rickettsia felis* or *Bartonella* sp. in *P. irritans*

collected from different hosts and different geographical areas (Belthoff *et al.*, 2015; Fontalvo *et al.*, 2017; Palomar *et al.*, 2017). According to Yssouf *et al.* (2014) the rapid and reliable identification of fleas at species level is an essential component of the fight against flea borne diseases in order to establish epidemiological relationship between flea species and their borne zoonotic agents. Thus, before to assess the prevalence of certain pathogens in fleas it is needed to know accurately which species we are studying in order to establish appropriate prevention and control strategies.

During the last fifteen years, molecular approaches have contributed to significant data about the diagnostic determination of genus and species of fleas (Dittmar & Whiting, 2003; Vobis *et al.*, 2004; Gamerschlag *et al.*, 2008; Whiting *et al.*, 2008; Marrugal *et al.*, 2013; Zurita *et al.*, 2016). Nevertheless, the specific differentiation of fleas is generally based on a variety of morphological criteria such as the shape and structure of their complex genitalia or the distribution of setae, spines and ctenidia (Linardi & Santos, 2012; López Berrizbeitia *et al.*, 2016; Hastriter *et al.*, 2017). The combination of molecular and morphometric data has allowed to find synonymies or cryptic species on fleas (Zurita *et al.*, 2015; Zurita *et al.*, 2018). These studies revealed the existence of certain genetic plasticity in the Order Siphonaptera which should be taken into account in order to carry out further taxonomic studies.

The main aim of this work was to carry out a comparative morphometric and molecular study of two different populations of *P. irritans* (Spain and Argentina). For this purpose, the ITS1, ITS2 of the rDNA and partial cytochrome-c oxidase (*cox1*) and cytochrome b (*cytb*) mtDNA gene of these taxa were sequenced. The taxonomy, origin, evolution and phylogeny of *P. irritans* are discussed.

Materials and methods

Collection of samples

Fifty-five fleas from Seville (southwestern of Spain) were collected off-host from a neglected horse stable and near to a children school. On the other hand, thirty-three fleas isolated from one Andean fox (*Lycalopex culpaeus*) and three South American gray foxes (*Lycalopex griseus*) from Santa Cruz (south of Argentina) were obtained through the assistance of colleagues (see Acknowledgements). In addition, two individuals of *C. felis* collected from dogs (*Canis lupus familiaris*) from Argentina were molecularly studied only for comparative purposes.

Fleas obtained were kept in Eppendorf tubes with 70 % ethanol until required for subsequent identification and sequencing (Table 1).

Morphological identification

Flea specimens collected by us (Spain) were classified by ourselves whereas those fleas provided by our colleagues (Argentina) were classified firstly by them (see Acknowledgements) and then morphologically compared with our specimens in our laboratory. First of all for morphological analysis, whole specimens were examined under optical microscope, whereas, flea legs were cut off in order to carry out the posterior DNA extraction. Secondly, rest of body preserved in vials with 70 % ethanol were cleared with KOH, dehydrated in a growing series of alcohol, diaphanized in eugenol and mounted on permanent slides in Canada balsam or EUKITT for their detailed examination under optic microscope. Photographs were taken by using a Microscope Olympus BX51 equipped with Photographic Camera Olympus DP71. Diagnostic morphological characters of *P. irritans* were studied by comparing with figures, keys and descriptions given in Hopkins & Rothschild (1953), Barrera (1955),

Smit (1958) and Beaucournu & Launay (1990). After morphological identification, ten specimens from Argentina (seven females and three males) and fourteen specimens from Spain (six males and eigth females) were measured according to fifteen different parameters (Table 2). Descriptive univariate statistics (arithmetic means, standard deviations, and coefficient of variation) for all parameters were determined for two populations (Spain and Argentina) using IBM® SPSS® Statistics program version 24.0.0.0 (Pardo & Ruiz, 2002). Furthermore, morphometric data was explored using multivariate analysis in five measurements (HL, HW, PROL, MESL, and METL) (Table 2) by the principal component analysis (PCA), a technique for summarizing most of the variation in a multivariate dataset in few dimensions (Rohlf & Marcus, 1993; Klingenberg, 1996; Dujardin & Le Pont, 2004). The analyses were carried out using the BAC v.2 software (Dujardin, 2002; Valero *et al*., 2009).

Molecular study

Total DNA was extracted from fleas using flea legs by the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. Then, genomic DNA was checked using an electrophoresis in 0.8 % agarose gel electrophoresis infused with SYBR Safe.

All molecular markers sequenced in this study (ITS1 and ITS2 rDNA, *cox1* and *cytb* mtDNA) were amplified by polymerase chain reaction (PCR) using a thermal cycler (Eppendorf AG). PCR mix, PCR conditions and PCR primers are summarized in Table S1. The ITS1, ITS2, *cox1* and *cytb* partial gene sequences obtained from *P. irritans* from the two geographical areas were deposited in GenBank database (Table 1). In order to compare with other Pulicidae species, it were sequenced and analysed ITS1 and

ITS2 rDNA and *cox1* and *cytb* mtDNA partil genes of *C. felis* isolated from dogs (*C. l. familiaris*) from La Plata (Argentina).

The PCR products were checked on SYBR Safe stained 2 % Tris–Borate–EDTA (TBE) agarose gels. Bands were eluted and purified from the agarose gel by using the QWizard SV Gel and PCR Clean-Up System Kit (Promega). Once purified, the products were sequenced by Stab Vida (Portugal). To obtain a nucleotide sequence alignment file, we used MUSCLE alignment method (Edgar, 2004) by the MEGA program version 5.2 (Tamura *et al.*, 2011). The ITS1 rDNA intraindividual variation was determined by sequencing four to seven clones of two specimens from Spain and one specimen from Argentina. The PCR products were eluted from the agarose gel using the WIZARD® SV Gel and PCR Clean-Up System (Promega) and transformation was carried out as cited by Cutillas *et al.* (2009). Plasmids were purified using a Wizard Plus SV (Promega) and sequenced by Stab Vida (Portugal) with an universal primer (M13).

A restriction map of the *cox1* sequences of *P. irritans* from Spain and Argentina was constructed using The Sequence Manipulation Suite (Stothard, 2000; available at http://www.bioinformatics.org/sms2/rest_map.html) in order to identifiy certain endonucleases which could discriminate between both geographical origins by PCR-linked random-fragment-length polymorphism (RFLP).

In order to assess the similarity among all marker sequences of *P. irritans* obtained in this study and other Pulicidae species, we analyzed the number of base differences per sequence from between sequences studied using no. of differences method of MEGA 5 program version 5.2 (Tamura *et al.*, 2011). The program DOTMATCHER from the European Molecular Biology Open Software Suite package (Rice *et al.*, 2000) was used to find repeats within the ITS1 sequences.

Phylogenetic trees were inferred using nucleotide data and performed using two methods: Maximum Likelihood (ML) and Bayesian inferences (B). Maximum Likelihood trees were generated using the PHYML package from Guindon & Gascuel (2003), whereas Bayesian inferences were generated using Mr Bayes-3.2.6 (Ronquist & Huelsenbeck 2003). JMODELTEST (Posada 2008) program was used to determinate the best-fit substitution model for the parasite data (ITS2, *cox1* and *cytb*). Models of evolution were chosen for subsequent analyses according to the Akaike Information Criterion (Huelsenbeck & Rannala 1997; Posada & Buckley, 2004). For the study of the dataset containing the concatenation of three markers (ITS2, *cox1* and *cytb*), 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 general time-reversible model with gamma-distributed rate variation GTR+G (ITS2) and general time-reversible model with gamma-distributed rate variation and a proportion of invariable sites, GTR+I+G (*cox1* and *cytb*). 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-3.2.6 for BI were nst=6 with gamma rates (ITS2) and nst=6 with invgamma rates (*cox1* and *cytb*). For BI, the standard deviation of split frequencies was used to assess if the number of generations completed was sufficient; the chain was sampled every 500 generations and each dataset was run for 10 million generations. Adequacy of sampling and run convergence were assessed using the effective sample size diagnostic in TRACER program version 1.6 (Rambaut & Drummond, 2007). 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) are percentage converted.

The phylogenetic analyses, based on ITS2, *cox1* and *cytb* mtDNA sequences were carried out using our sequences and those obtained from GenBank database (appendix 1). Phylogenetic trees based on ITS2, *cox1*, *cytb* mtDNA and concatenated (ITS2, *cox1* and *cytb*) sequences were rooted including outgroup species representing members of the Order Mecoptera: *Panorpa meridionalis*. This choice was based on the the combination of morphological and molecular data obtained in former studies which provided compelling evidences for a sister group relationship between Mecoptera and Siphonaptera (Whiting, 2002; Whiting *et al.*, 2008). ITS1 sequence of *P. meridionalis* or other species of Mecoptera was not available neither by amplification of different individuals nor in any public database. Thus, no phylogenetic tree with other Siphonaptera species based on ITS1 sequences was constructed, as well as this molecular marker was discarded for the concatenated dataset. The selection of flea taxa for the concatenated phylogenetic tree was limited to flea species whose ITS2, *cox1* and *cytb* sequences were available on GenBank database.

NETWORK (v5.0.0.1) was used to create inter-population median-joining networks (Bandelt *et al.*, 1999; available at http://www.fluxus-engineering.com), to visualize the evolutionary relationships between *cox1* and *cytb* haplotypes. This approach has been shown to yield the best-resolved genealogies relative to other rooting and network procedures (Cassens *et al.*, 2003).

Results

Morphometric results

Specific morphological identification carried out in our lab was in agreement with those made by our colleagues. Thus, all specimens showed specific morphological characteristics of *P. irritans* (Fig. 1):

- Anterior margin of head smoothly rounded and without a tubercle (Fig. 1a).
- Vestigial genal comb which may or may not be present. Pronotal comb always absent (Fig. 1a).
- Club of antenna asymmetrical (Fig. 1b).
- Presence of three strong setae on the head (Genal, pre-ocular and post-antennal) (Fig 1c).
- Sternite VII of females with a sinus and with 4/5 setae on each side. Spermatheca with bulga subglobular, rather small and hilla longer than bulga (Fig 1d, 1e)
- Row of small spines inside of hind coxa near apex, consisting of 8-12 spines in females and 7-10 spines in males forming a patch (Fig. 1f, 1g)
- Male specimens show a clasper with process 1 very large and completely covering processes 2 and 3. The crochet appeared expanded apically and the dorsal aedeagal sclerite relatively long and slender (Fig. 1h, 1i)

Morphometric data showed salight differences between specimens from both geographical origins, thus, Spanish specimens showed ~~with~~ slight higher measurements (total length and width of the head, total length of prothorax, mesothorax and metathorax), than those from Argentina (Table 2). Furthermore, these results were compared with those obtained by PCA, consisting of regressing each character

separately on the within group first principal component (PC1), which is a multivariate estimate of size. Thus, the PCA carried out in adults variables from Spain and South America significantly correlated with PC1, contributing 71 % to the overall variation. The resulting maps (Fig. 2) clearly illustrated global size differences in the Spanish population analyzed, including a bigger size in adults from *Pulex irritan*s from Spain respect the South American population.

Molecular results

Internal Transcribed Spacer 1 and 2 (ITS1 and ITS2) analysis

The length of the ITS1 sequences of *P. irritans* from Spain ranged from 876 to 962 bp whereas ITS1 sequences of specimens from Argentina were 796 bp in length (Table 1). This difference resulted in the presence of repetitive sequences within the ITS1 sequences of population from Spain. The repeat unit was about 86 bp in length and arranged tandemly within the ITS1 spacer of Spain specimens (Fig. 3), this unit appeared twice and once within the ITS1 sequences of *P. irritans* from Spain (specimens with an ITS1 sequence length of 962 bp and 876 bp, respectively) whereas, this repeat unit was not observed in ITS1 sequences of *P. irritans* collected from Argentina (796 bp). Furthermore, this repeat unit showed three sites with nucleotide polymorphism (Fig. 3). The analysis of different clones from one individual showed sequences of 881-882 bp and 968 bp, from two individuals, respectively, from Spain and 796 bp in one individual from Argentina (Table 1). Specimens obtained from Argentina showed the same ITS1 sequence (Intrapopulation similarity = 100 %), whereas this value ranged from 99.5 % to 100 % for individuals collected from Spain (Table 3, asterisc). Furthermore, the similarity observed in the ITS1 sequences of *P. irritans* from different geographical origins ranged from 93.9 % (Iran-United States) to

99.9 % (Iran-Spain) (Table 3). Specimens collected from Spain, Cameroon and Iran showed high values of intraspecific similarity among them whereas these values were quite lower when we compared these specimens with those collected from United States and Argentina (Table 3).

ITS2 sequence length ranged from 322 bp (*P. irritans* from Spain) to 324 bp (*P. irritans* from Argentina) (Table 1). Likewise ITS1 sequences, the intrapopulation similarity of ITS2 sequences of *P. irritans* from Argentina was 100 %, however, this value ranged from 99.7 % to 100 % for *P.irritans* obtained from Seville, Spain. On the other hand, the intraspecific similarity observed between both populations ranged from 98.2 % to 98.5 % (Table not shown).

Phylogenetic tree inferred from ITS2 sequences of *P. irritans* and other ITS2 sequences retrieved from GenBank (see appendix 1) showed Pulicidae species forming a monophyletic group clustered together with high bootstrap and BPP values (100/100). Within Pulicidae clade, *P. irritans* specimens comprised a well-supported group (100/100) where individuals collected from Spain clustered together (Bootstrap and BPP values: 100/99) and in polytomy with *P. irritans* from Argentina (Fig. S1). *C. felis* from Argentina clustered together with *C. felis* from Spain, Iran and South Africa (Fig. S1).

Partial *Cox1* mtDNA gene analysis

The partial *cox1* mtDNA gene sequences of *P. irritans* from the two geographical areas were 658 bp in length (Table 1). When *cox1* sequences of *P. irritans* collected from Argentina were analyzed, three different haplotypes were obtained (H1, H2, H3) (Intra-population similarity = 99.2 % to 99.8 %). On the other hand, three different haplotypes were observed when *cox1* sequences of *P. irritans* from Spain were assessed (H4, H5,

H6) (Intrapopulation similarity = 99.6 % to 99.8 %). Intraspecific similarity observed between individuals from both geographical origins ranged from 91.4 % to 92.0 % (Table 4). When the *cox1* sequences of *P. irritans* from different geographical origins were compared, the highest values of intraspecific similarity were observed among specimens from Palearctic and Australian region (China, Spain, Hungary, Croatia and New Zealand) (96.0 % to 100 %). Lowest values of intraspecific similarity were observed when these specimens and those isolated from Neotropical region (Argentina) were compared (91.2 % to 93.4 %). In contrast to that, *C. felis* showed a 100 % intraspecific similarity.

Phylogenetic tree topology revealed a highly supported clade (Bootstrap and BPP values: 100/94) clustering all Pulicidae species (Fig. 4). Within this clade, *P. irritans* specimens clustered with high bootstrap and BBP support (100/99) but separated in two geographical zones (Neotropical: Argentina (100/96) and Palearctic and Australian: New Zealand, China, Croatia, Hungary and Spain (100/93) as different genetic lineages (Fig. 4). *C. felis* from Argentina clustered together with individuals of this species from different geographical origin (Fig. 4)

Based on *cox1* sequences, restriction mapping identified many endonucleases that could be used to delineate the different geographical haplotypes found in this study. Thus, *Afa*I, *Afl*II, *Bfr*I and *Hpa*II sites were present in the sequences of *P. irritans* from Argentina but not in *P. irritans* from Spain. Nevertheless, *Bgl*II, *Dra*I and *Hinc*II presented one restriction site in *cox1* haplotypes from Spain but not in those from Argentina.

Partial *Cytb* mtDNA gene analysis

298 The length of the partial *cytb* mtDNA gene sequences of *P. irritans* from Argentina and
299 Spain was 374 bp (Table 1). Seven haplotypes of *P. irritans* from Argentina were
300 obtained (H1-H7) (Intrapopulation similarity = 97.8 % to 99.7 %), while this value
301 ranged from 99.7 % to 100 % for specimens collected from Spain showing only two
302 haplotypes (H8 and H9) (Table 5). Intraspecific similarity between both populations
303 ranged from 90.9 % to 92.2 % (Table 5). The phylogenetic tree inferred from partial
304 *cytb* gene sequences revealed a monophyletic origin of Pulicidae with *P. irritans*
305 specimens appearing clustered with high support (Bootstrap and BPP values: 100/99).
306 Furthermore, *P. irritans* clade showed two highly supported subclades corresponding
307 with both geographical origins (Fig. 5). Likewise *cox1*, *C. felis* from Argentina
308 clustered together with individuals of this species from different geographical origin
309 (Fig. 5).

310 The network of the *cytb* sequences of *P. irritans* populations showed a general
311 congruence with the phylogenetic reconstruction. Thus, the minimum spanning network
312 showed the two divergent groups clearly separated based on their geographical location
313 (Argentina and Spain). The genetic divergence corresponded with 30-37 mutational
314 steps between both populations (Fig. 6). The general topology of the network showed an
315 radial structure for the haplotypes from Argentina, while population from Spain
316 presented a main haplotype (H8) including all individuals except one corresponding
317 with a different haplotype (H9) (Fig. 6). Furthermore, H2 represented the most common
318 ancestral haplotype from *P. irritans*.

319 The concatenated dataset of ITS2, partial *cytb* and *cox1* gene sequences included 1,406
320 aligned sites and 41 taxa, including outgroups. Phylogenetic analyses of the
321 concatenated dataset yielded a tree with branches strongly supported (Fig. 7). The
322 analysis based on the concatenated dataset is concordant with all trees constructed on

the basis of the single markers. Thus, all species belonging to Pulicidae family presented a monophyletic origin. Furthermore, within *P. irritans* clade we noticed two different genetic lineages (highly supported) corresponding to two geographical origins (Spain and Argentina).

Discussion

The present work represents the first study that provides morphometric, molecular, phylogeographic and phylogenetic comparative data of *P. irritans* isolated from South America and Europe in order to assess taxonomic and phylogenetic relationships between both populations and to shed light on the systematics and origins of *P. irritans*.

Some flea species from different families have a cosmopolitan or, at least, a very broad distribution. The most famous and important cosmopolitan fleas from a medical and veterinary point of view are several pulicids (*P. irritans*, *X. cheopis*, *C. felis*, *C. canis*), ceratophyllids (*Nosopsyllus consimilis*, *Nosopsyllus fasciatus*) and one leptopsyllid (*Leptopsylla segnis*) (Krasnov, 2008). Ubiquitous distribution of these species is related to dispersal via humans, their livestock, pets and commensals (mice and rats). Nevertheless, the origin of any of these fleas is not uniform. Instead of that, they are distributed in patches which are characterized by the host and environmental conditions that are favourable for each given species (Beaucournu & Pascal, 1998). For example, although it is accepted that the Pulicidae family originated in Palearctic zone (Krasnov *et al.*, 2015), several authors placed the origin of *P. irritans* in South America (Traub, 1980; Buckland & Sadler, 1989; Beaucournu *et al.*, 1993; Zhu *et al.*, 2015) with Vikings introducing this species to the Old World (Rothschild, 1973; Buckland & Sadler, 1989) and/or through ancient cultural contacts between Japan and Ecuador (Traub, 1980).

Because of morphological specializations, highly promiscuous fleas species such as *P. irritans* or *C. felis* which occurs on a wide variety of Carnivora, could show high levels of genetic variability especially when we assess populations which parasitize different hosts or they are settled in different geographical areas. Van der Mescht *et al.* (2015) suggested that the host specificity might influence the level of intraspecific genetic divergence since more generalist parasite species will show a higher level of intraspecific genetic variation enabling them to infest a broader host range. This fact has been recently demonstrated by Hornok *et al.* (2018) who found high mitochondrial sequence divergence in some synanthropic flea species such as *C. felis* or *P. irritans*.

In the present study, morphometric data showed slight differences between *P. irritans* specimens from Spain and Argentina. This result was corroborated by PCA appearing the Spanish adults of *P. irritans* slightly bigger. This fact could be explained according to the different geographical origins and/or different hosts. This is in agreement with Medvedev (1998) who characterized the human flea as a monotypic taxon, being inefficient traditional and classic morphological methods in separating its population groups. In our study, morphometric results did not correspond with molecular and phylogenetic ones since these showed a high degree of nucleotide divergence between individuals from both geographical origins. Our results are in agreement with Hornok *et al.* (2018) who did not observe morphological differences among *P. irritans* specimens isolated from humans and wild carnivores (badger, jackal and fox) from Hungary and Croatia; however, these authors found a considerable degree of molecular divergence between both populations based on mitochondrial markers. These results disagree with Krasnov *et al.* (2015) who supported the idea that the process of host selection by fleas is determined by reciprocal relationships between host traits and flea traits. Thus, flea species with similar traits, independently of their phylogenetic affinities, were found on

the same host species more often than expected by chance from the entire pool of flea species. In this sense, future morphometric studies of *P. irritans* from different hosts and continents would be necessary.

The Internal Transcribed Spacer 1 and 2 ribosomal DNA (ITS1 and ITS2) have been shown to be two of the best molecular markers to analyze genetic relationships at the species level in arthropods (Monje *et al.*, 2013; Zagoskin *et al.*, 2014).

At the present work, we observed that ITS2 sequences of *P. irritans* were markedly shorter than ITS1 sequences, which has already been noticed in other flea species such as *C. felis*, *Stenoponia tripectinata tripectinata*, *C. canis* and *N. fasciatus* (Vobis *et al.*, 2004, Zurita *et al.*, 2015; 2016 and 2018). Furthermore, the ITS1 rDNA regions revealed a considerable length variation between both geographical population caused by a long repetitive region of 86 bp length that appeared twice and once in specimens collected from Spain. Internal repeats in the ITS spacers are usual and have been frequently described. This fact have already noticed in fleas by Gamerschlag *et al.* (2008) who reported the existence of length differences between the ITS1 rDNA of the African and the South American *T. penetrans* populations caused by the number of repeats of a repetitive region of 99 bp. Furthermore, these authors detected repetitive sequences within the ITS1 rDNA region of other flea species such as *C. felis*, *Echidnophaga gallinacea*, *P. irritans*, *Spilopsyllus cuniculi*, and *X. cheopis*, highlighting that these repetitive elements could serve as a valuable tool for phylogeographic studies. Our study also agrees with Ghavami *et al.* (2018) who found three repeated units with a length of 98-99 bp and a tandemly repeated sequence within the ITS1 of *P. irritans* populations isolated from Khodabandeh and Mahneshan (Iran). Thus, these authors suggested that the different number and size of repetitive units in ITS1 may be the sign of developed traits establishing plesiomorphic characters among

different populations. In *P. irritans*, the number of these units depends on ecological conditions (McKern *et al.*, 2008; Gamerschlag *et al.* 2008); furthermore, in contrast to other authors such as Vobis *et al.* (2004), Gamerschlag *et al.* (2008), Marrugal *et al.* (2013) and Zurita *et al.* (2015) who not observed great ITS sequences differences among several populations belonging to the same flea species, we found high values of intraspecific variation between both geographical populations in *P. irritans*, especially in ITS1 sequences (Intraspecific similarity ranged from 95.9 % to 96.3 %). The existence of two genetic lineages (Spain and Argentina) was corroborated by ITS2 phylogenetic tree, thus both populations clustered separately based on their geographical origin. Additionally, when ITS1 sequences of different specimens of *P. irritans* isolated from different geographical areas were compared the highest values of nucleotide divergence were observed in specimens from Nearctic and Neotropical areas (United States and Argentina), whereas almost no differences were observed among individuals from Palearctic and Afrotropical regions (Spain and Cameroon) (Table 3). These data might suggest a possible American origin for this flea species since ancestral populations usually exhibit higher genetic diversity values compared to recent populations that have expanded into novel territories (Savolainen *et al.*, 2002).

Cox1 and *cytb* markers have been used in flea studies in the last fifteen years with several purposes. In order to assess the phylogeographic structure of certain populations (Dittmar & Whiting, 2003), to study the phylogenetic diversity of some species (Lawrence *et al.*, 2014), to carry out a molecular characterization of certain species (Zurita *et al.*, 2015; Zurita *et al.*, 2016) or even for the reconstruction of ancestral host affiliation and biogeographic history of fleas (Zhu *et al.*, 2015). At the present study, we amplified c*ox1* partial gene. The obtained sequences showed a low value of intraspecific similarity within *P. irritans* from Spain and Argentina (91.5 % - 92 %), in contrast to

the high values of similarity observed for specimens from the same population (> 99 %). Zurita *et al.* (2016) observed values of similarity around 97 % between two congeneric species of fleas (*C. felis* and *C. canis*) collected from different geographical areas. Recently, Hornok *et al.* (2018) based on *cox1* sequences of *P. irritans* from different hosts, observed two diverged mitochondrial lineages between Croatia and Hungary. Therefore, these authors claimed about the necessity to carry out supplementary studies using a large scale sampling of *P. irritans* from different hosts and geographical areas to conclude in this context. In our study, the comparative study of *cox1* sequences of *P. irritans* isolated from different geographical region showed the lowest values of nucleotide divergence among Palearctic and Australian specimens, whereas, likewise ITS1 analysis, the highest values of nucleotide divergence were observed when these specimens were compared with Argentinean population (Neotropical) (Table 4). These results would support the idea that this species had a South American origin. Historically, DNA barcoding studies on insects and invertebrates have shown maximum intraspecific variation ranging from 3 to 3.9 % (Carew *et al.* 2007). This high degree of polymorphism for intraspecific analysis could be explained attending to wide geographical localities where the samples were collected. Indeed, in certain groups, such as amphibians, when several individuals of the same species come from distant geographical regions, intraspecific variation can exceed the interspecific variation observed between species of the same genus, making it difficult for the delimitation of species with only the sequence of *cox1* (Vences *et al.* 2005). However, in our study, *cytb* sequence analysis confirmed the existence of two highly divergent mitochondrial lineages within *P. irritans* (Spain and Argentina) reforced by ribosomal results. Although we observed a high percentage of intrapopulation similarity in both geographical origins (bit lower in Argentina) based on

447 *cytb* sequences, we noticed greater nucleotide variability in Argentina than in Spain
448 with the existence of a higher number of haplotypes in the South American area. In this
449 case intraspecific similarity observed (90 % to 92.2 %) were similar or even lower than
450 those observed between two different congeneric species such as *C. felis* and *C. canis*
451 (90.6 %) or *Xenopsylla skrjabini* and *Xenopsylla conformis* (92.5 %) (Table 5). These
452 high degrees of mtDNA intraspecific variability could be explained by the fact that
453 generalist flea shows considerably more intraspecific genetic variation than host-
454 specific flea species. For a generalist parasite, greater levels of genetic variability can
455 provide evolutionary potential for local host race formation (Gómez-Díaz *et al.*, 2007).
456 Previous examples have been reported for ticks and lice parasitizing sympatric hosts
457 (McCoy *et al.* 2001; Johnson *et al.* 2002). For this reason, we analyzed the *cox1* and
458 *cytb* intraspecific similarity between Spaniard and Argentinean populations of another
459 generalist flea like *C. felis*, but, surprisingly, we did not observe differences between
460 them. *Cox1*, *cytb* and concatenated phylogenetic trees reinforced the idea of the
461 existence of two geographical genetic lineages within *P. irritans*. Thus, *cox1*
462 phylogenetic tree showed specimens collected from Palearctic and Australian region
463 (Spain, Croatia, China, Hungary and New Zealand) clustered together in the same clade
464 and separated from individuals collected from Neotropical region (Argentina).
465 Furthermore, these results show no significant host dependency since specimens
466 collected from Palearctic and Australian areas were isolated from different hosts (see
467 appendix 1). Likewise, *cox1*, *cytb* and concatenated phylogenetic trees showed two well
468 supported subclades within *P. irritans* based on geographical origins (Spain and
469 Argentina).

470 The phylogenetic analysis carried out on the basis on ribosomal and mitochondrial
471 DNA molecular markers suggests the existence of two genetic lineages (Argentina,

South America and Spain, Europe) of *P. irritans* populations and the minimum spanning network showed all the *cytb* haplotypes from Argentina clustered together and with star-like pattern around H2 haplotype. Based on coalescent theory (Slatkin & Hudson, 1991) this star topology showed that *P. irritans* populations had experienced a significant population expansion. At the centre of the network is haplotype 2, which takes over the highest proportion in the population. This suggests that the haplotype 2 should be the ancestral haplotype. This higher genetic diversity in Argentina would reinforce the idea suggested by Buckland & Sadler (1989) that *P. irritans*, in contrast with other human ectoparasites, could have a South American origin reaching Western Palearctic area through Beringian and Asiatic routes, at any time during the Postglacial. This fact together with a reproductive isolation, could have originated the existence of two cryptic species within *P. irritans*. In spite of that, to confirm a possible South American origin of this species, more taxonomic, phylogenetic and phylogeographic studies of *P. irritans* parasitizing different hosts from different geographical areas are needed.

In conclusion, the present study provides for the first time, comparative morphometric and molecular data of *P. irritans* collected from Spain and Argentina (Palearctic and Neotropical areas). On the basis on morphometric results, we found slight differences between both populations. Although we only assessed two populations of *P. irritans* in this study, our results could suggest the hypothesis that this flea species, in contrast to other generalist fleas, maintain a certain degree of morphological similarity, at least between Western Palearctic and Neotropical areas. Furthermore, based on molecular and phylogenetic data obtained in this work we provided the existence of two well defined geographical genetic lineages within *P. irritans* species suggesting the existence of two cryptic species which could be discriminated by PCR-linked RFLP.

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References

Bandelt, H.J., Forster, P. & Röhl, A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16**, 37–48.

Barrera, A. (1955) Las especies Mexicanas del género *Pulex* Linnaeus (Siph, Pulicidae). *Anales de la Escuela Nacional de Ciencias Biológicas*, **8**, 219–236.

Beaucournu, J.C. & Launay, H. (1990) Les Puces (Siphonaptera) de France et du Bassin méditerranéen occidental, Faune de France, Vol. 76. Fedération Française des Sociétés des Sciences Naturelles, Paris.

Beaucournu, J.C., Le Piver, M. & Guiguen, C. (1993) The present status of the conquest of tropical Africa by *Pulex irritans* Linnaeus, 1758. *Bulletin de la Societe de pathologie exotique*, **86**, 290-294.

Beaucournu, J.C. & Pascal, M. (1998) Origine biogeographique de *Nosopsyllus fasciatus* (Bosc, 1800) (Siphonaptera-Ceratophyllidae) et observations sur son hote primitif. *Biogeographica*, **74**, 132–135.

517 Belthoff, J.R., Bernhardt, S.A., Ball, C.L., Gregg, M., Johnson, D.H., Ketterling, R.,
518 Price, E. & Tinker, J.K. (2015) Burrowing Owls, *Pulex irritans*, and Plague. *Vector*
519 *Borne and Zoonotic Diseases*, **15**, 556–64.

520 Buckland, P.C. & Sadler, J.P. (1989) A biogeography of the human flea, *Pulex irritans*
521 L. (Siphonaptera: Pulicidae). *Journal of Biogeography*, **16**, 115–120.

522 Carew, M.E., Pettigrove, V., Cox, R.L. & Hoffmann, A.A. (2007) DNA identification
523 of urban *Tanytarsini chironomids* (Diptera: Chironomidae). *Journal of the North*
524 *American Benthological Society*, **26**, 587–600.

525 Cassens, I., Van Waerebeek, K., Best, P.B., Crespo, E.A., Reyes, J. & Milinkovitch,
526 M.C. (2003) The phylogeography of dusky dolphins (*Lagenorhynchus obscurus*): a
527 critical examination of network methods and rooting procedures. *Molecular Ecology*,
528 **12**, 1781–1792.

529 Cutillas, C., Callejón, R., de Rojas, M., Tewes, B., Ubeda, J.M., Ariza, C. & Guevara,
530 D.C. (2009) *Trichuris suis* and *Trichuris trichiura* are different nematode species. *Acta*
531 *Tropica*, **111**, 299–307.

532 Dittmar, K. & Whiting, M.F. (2003) Genetic and phylogeographic structure of
533 populations of *Pulex simulans* (Siphonaptera) in Peru inferred from two genes (*CytB*
534 and *CoII*). *Parasitology Research*, **91**, 55–59.

535 Dujardin, J.P. (2002) BAC software. Institut de Recherches pour le Développement
536 (IRD), France, http://www.mpl.ird.fr/morphometrics/bac/index.html.

537 Dujardin, J.P. & Le Pont, F. (2004) Geographical variation of metric properties within
538 the neotropical sandflies. *Infection Genetics and Evolution*, 4, 353–359.

539 Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and
540 high throughput. *Nucleic Acids Research*, **32**, 1792–1797.

541 Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the
542 bootstrap. *Evolution*, **39**, 783–791.

543 Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for
544 amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan
545 invertebrates. *Molecular Marine Biology Biotechnology,* **3**, 294–299.

546 Fontalvo, M.C., Favacho, A.R.M., Araujo, A.C., Santos, N.M.D., Oliveira, G.M.B.,
547 Aguiar, D.M., Lemos, E.R.S. & Horta, M.C. (2017) *Bartonella* species pathogenic for
548 humans infect pets, free-ranging wild mammals and their ectoparasites in the Caatinga
549 biome, Northeastern Brazil: a serological and molecular study. *The Brazilian Journal of*
550 *Infectious Diseases*, **21**, 290–296.

551 Gamerschlag, S., Mehlhorn, H., Heukelbach, J., Feldmeier, H. & D'Haese, J. (2008)
552 Repetitive sequences in the ITS1 region of the ribosomal DNA of *Tunga penetrans* and
553 other flea species (Insecta, Siphonaptera). *Parasitology Research*, **102**, 193–199.

554 Gasser, R.B., Nansen, P. & Guldberg, P. (1996) Fingerprinting sequence variation in
555 ribosomal DNA of parasites by DGGE. *Molecular Cellular Probes*, **10**, 99–105.

556 Ghavami, M.B., Mirzadeh, H., Mohammadi, J. & Fazaeli, A. (2018) Molecular survey
557 of ITS1 spacer and *Rickettsia* infection in human flea, *Pulex irritans*. *Parasitology*
558 *Research*, **117**, 1433–1442.

559 Gómez-Díaz, E., González-Solís, J., Peinado, M.A. & Page, R.D.M. (2007) Lack of
560 host-dependent genetic structure in ectoparasites of *Calonectris* shearwaters. *Molecular*
561 *Ecology*, **16**, 5204–15.

562 Graham, C.B., Eisen, R.J. & Belthoff, J.R. (2016) Detecting Burrowing Owl
563 Bloodmeals in *Pulex irritans* (Siphonaptera: Pulicidae). *Journal of Medical*
564 *Entomology*, **53**, 446–50.

565 Gratz, N.G. (1999) Rodent reservoirs and flea vectors of natural foci of plague, pp. 63–
566 96. In D. T. Dennis, K. L. Gage, N. Gratz, J. D. Poland, and E. Tikhomirov (eds.),
567 Plague manual: Epidemiology, distribution, surveillance and control. World Health
568 Organization, Geneva, Switzerland.

569 Guindon, S. & Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate
570 large phylogenies by maximum likelihood. *Systematic Biology*, **52**, 696–704.

571 Hastriter, M.W., Miller, K.B., Svenson, G.J., Martin, G.J. & Whiting, M.F. (2017) New
572 record of a phoretic flea associated with earwigs (*Dermaptera*, *Arixeniidae*) and a
573 redescription of the bat flea *Lagaropsylla signata* (Siphonaptera, Ischnopsyllidae).
574 *ZooKeys*, **657**, 67–69.

575 Hopkins, G.H.E. & Rothschild, M. (1953) An Illustrated Catalogue of the Rothschild
576 Collection of Fleas in the British Museum (Nat. Hist.). Vol. I. Tungidae and Pulicidae.
577 Cambridge University Press, Cambridge, UK.

578 Hopla, C. E. (1980) A study of the host associations and zoogeography of *Pulex*, pp.
579 185–207. In R. Traub and H. Starcke (eds.), In Proceedings of the International
580 Conference on Fleas Lewis, R. E., J. H. Lewis, & C. Maser. (1988) The fleas of the
581 Pacific Northwest. Oregon State University Press, Corvallis, OR.n Fleas, 1977,
582 Peterborough, UK. A.A. Balkema, Rotterdam.

583 Hornok, S., Beck, R., Farkas, R., Grima, A., Otranto, D., Kontschán, J., Takács, N.,
584 Horváth, G., Szőke, K., Szekeres, S., Majoros, G., Juhász, A., Salant, H., Hofmann-

585 Lehmann, R., Stanko, M. & Baneth, G. (2018) High mitochondrial sequence divergence
586 in synanthropic flea species (Insecta: Siphonaptera) from Europe and the
587 Mediterranean. *Parasites & Vectors*, **11**, 221.

588 Huelsenbeck, J.P. & Rannala, B. (1997) Phylogenetic methods come of age: testing
589 hypotheses in an evolutionary context. *Science*, **276**, 227–232.

590 Johnson, K.P., Williams, B.L., Drown, D.M., Adams, R.J. & Clayton, D.H. (2002) The
591 population genetics of host specificity: genetic differentiation in dove lice (Insecta:
592 Phthiraptera). *Molecular Ecology*, **11**, 25–38.

593 Klingenberg, C.P. (1996) Multivariate allometry. In: Marcus, L.F., Corti, M., Loy,
594 G.J.P., Naylor, A., Slice, D. (Eds.), Advances in Morphometrics, Proceedings of the
595 1993 NATO-ASI on Morphometrics, NATO ASI, Ser. A, Life Sciences. Plenum
596 Publishers, New York, pp. 23–49.

597 Krasnov, B.R. (2008) Functional and Evolutionary Ecology of Fleas. Cambridge
598 University Press, New York (United States of America).

599 Krasnov, B.R., Shenbrot, G. & Khokhlova, I.S. (2015) Historical biogeography of fleas:
600 the former Bering Land Bridge and phylogenetic dissimilarity between the Nearctic and
601 Palearctic assemblages. *Parasitology Research*, **114**, 1677–86.

602 Lawrence, A.L., Brown, G.K., Peters, B., Spielman, D.S., Morin-Adeline, M. &
603 Slapeta, J. (2014) High phylogenetic diversity of the cat flea (*Ctenocephalides felis*) at
604 two mitochondrial DNA markers. *Medical and Veterinary Entomology*, **28**, 330–336.

605 Lewis, R.E. (1998) Resume of the Siphonaptera (Insecta) of the World. *Journal of*
606 *Medical Entomology*, **35**, 377–389.

607 Linardi, P.M. & Santos, J.L.C. (2012) *Ctenocephalides felis felis* vs *Ctenocephalides*
608 *canis* (Siphonaptera: Pulicidae): some issues in correctly identify these species.
609 *Brazilian Journal of Veterinary Parasitology*, **4**, 345–354.

610 López Berrizbeitia, M.F., Hastriter, M.W. & Díaz, M.M. (2016) A New Flea Species of
611 the Genus *Cleopsylla* (Siphonaptera: Stephanocircidae) from Northwestern Argentina.
612 *The Journal of Parasitology*, 102, 514–519.

613 Luchetti, A., Trentini, M., Pampiglone, S., Fiorawanti, M.L. & Mantovani, B. (2007)
614 Genetic variability of *Tunga penetrans* (Siphonaptera, Tungidae) and fleas across South
615 America and Africa. *Parasitology Research*, **100**, 593–598.

616 Marrugal, A., Callejón, R., de Rojas, M., Halajian, A. & Cutillas, C. (2013)
617 Morphological, biometrical and molecular characterization of *Ctenocephalides felis* and
618 *Ctenocephalides canis* isolated from dogs from different geographical regions.
619 *Parasitology Research*, **112**, 2289–2298.

620 McCoy, K.D., Bouliner, T., Tirard, C. & Michalakis, Y. (2001) Host specificity of a
621 generalist parasite: genetic evidence of sympatric host races in the seabird tick *Ixodes*
622 *uriae*. *Journal of Evolutionary Biology*, **14**, 395–405.

623 McKern, J.C., Szalanski, A.L., Austin, J.W. & Gold, R.E. (2008) Genetic diversity of
624 field populations of the cat flea, *Ctenocephalides felis*, and the human flea, *Pulex*
625 *irritans*, in the South Central United States. *Journal of Agricultural and Urban*
626 *Entomology*, **25**, 259–263.

627 Medvedev, S.G. (1998) Classification of fleas (Order Siphonaptera) and its theoretical
628 foundations. *Entomological Review*, **78**, 1080–1093.

629 Monje, L.D., Quiroga, M., Manzoli, D., Couri, M.S., Silvestri, L., Venzal, J.M., Cuervo,
630 P. & Beldomenico, P.M. (2013) Sequence analysis of the internal transcribed spacer 2
631 (ITS2) from *Philornis seguyi* (García, 1952) and *Philornis torquans* (Nielsen, 1913)
632 (Diptera: Muscidae). *Systematic Parasitology*, **86**, 41–53.

633 Palomar, A.M., Cevidanes, A., Portillo, A., Kalema-Zikusoka, G., Chirife, A.D.,
634 Romero, L., Muro, J., Mugisha, L., Oteo, J.A. & Millán, J. (2017) High Prevalence of
635 *Rickettsia* spp. in Dog Fleas (Siphonaptera: Pulicidae) in Rural Uganda. *Journal of*
636 *Medical Entomology*, **54**, 1076–1079.

637 Pardo, A. & Ruiz, M.A. (2002) SPSS 11. Guía para el análisis de datos. Madrid,
638 McGraw-Hill.

639 Posada, D. & Buckley, T.R. (2004) Model selection and model averaging in
640 phylogenetics: advantages of Akaike information criterion and Bayesian approaches
641 over likelihood ratio tests. *Systematic Biology*, **53**, 793–808.

642 Posada, D. (2008) Jmodeltest: phylogenetic model averaging. *Molecular Biology and*
643 *Evolution*, **25**, 1253–1256.

644 Rambaut, A. & Drummond, A. (2007) Tracer v1.6. Available online at
645 http://beast.bio.ed.ac.uk/.

646 Rice, P., Longden, I. & Bleasby, A. (2000) EMBOSS: the European Molecular Biology
647 Open Software Suite. *Trends in Genetics*, **16**, 276–277.

648 Rohlf, F.J. & Marcus, L.F. (1993) A revolution in morphometrics. *Trends in Ecology &*
649 *Evolution*, **8**, 129–132.

650 Ronquist, F. & Huelsenbeck, J.P. (2003) MrBAYES 3: Bayesian phylogenetic inference
651 under mixed models. *Bioinformatics*, **19**, 1572–1574.

652 Rothschild, M. (1973) Report on a femal *Pulex irritans* in a tenth century Viking pit.
653 *Proceedings of the Royal Society of London*, **38**, 29.

654 Savolainen, P., Zhang, Y.P., Luo, J., Lundeberg, J., Leitner, T. (2002) Genetic evidence
655 for an East Asian origin of domestic dogs. *Science*, **298**, 1610–1613.

656 Slatkin, M. & Hudson, R.R. (1991) Pairwise comparisons of mitochondrial DNA
657 sequences in stable and exponentially growing populations. *Genetics*, **129**, 555–562.

658 Smit, F.G.A.M. (1958) A preliminary note on the occurrence of *Pulex irritans* and
659 *Pulex simulans* baker in North America. *The Journal of Parasitology*, **44**, 523–526.

660 Stothard, P. (2000) The sequence manipulation suite: JavaScript programs for analyzing
661 and formatting protein and DNA sequences. *Biotechniques*, **28**, 1102–1104.

662 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011)
663 MEGA5: molecular evolutionary genetics analysis using maximum likelihood,
664 evolutionary distance, and maximum parsimony methods. *Molecular Biology and*
665 *Evolution*, **28**, 2731–2739.

666 Traub, R. (1980) The zoogeography and evolution of some fleas, lice and mammals. –
667 In: Traub, R. and Starke, H. (eds), In Proceedings of the International Conference on
668 Fleas, Ashton Wold, Peterborough, UK, June 1977. A. A. Balkema, pp. 93–172.

669 Valero, M.A., Perez-Crespo, I., Periago, M.V., Khoubbane, M. & Mas-Coma, S. (2009)
670 Fluke egg characteristics for the diagnosis of human and animal fascioliasis by *Fasciola*
671 *hepatica* and *F. gigantica*. *Acta Tropica*, **111**, 150–159.

672 Van der Mescht, L., Matthee, S. & Matthee, C.A. (2015) Comparative phylogeography
673 between two generalist flea species reveal a complex interaction between parasite life
674 history and host vicariance: parasite-host association matters. *BMC Evolutionary*
675 *Biology*, **15**, 105.

676 Vences, M., Thomas, M., Van der Meijden, A., Chiari, Y. & Vieites, D.R. (2005)
677 Comparative Performance of the 16S rRNA Gene in DNA Barcoding of Amphibians.
678 *Frontiers in Zoology*, **2**, 5.

679 Vobis, M., D'Haese, J., Mehlhorn, H., Mencke, N., Blagburn, B.L., Bond, R., Denholm,
680 I., Dryden, M.W., Payne, P., Rust, M.K., Schroeder, I., Vaughn, M.B. & Bledsoe, D.
681 (2004) Molecular phylogeny of isolates of *Ctenocephalides felis* and related species
682 based on analysis of ITS1, ITS2 and mitochondrial 16S rDNA sequences and random
683 binding primers. *Parasitology Research*, **94**, 219–226.

684 Whiting, M.F. (2002) Mecoptera is paraphyletic: multiple genes and phylogeny of
685 Mecoptera and Siphonaptera. *Zoologica Scripta*, **31**, 93–104.

686 Whiting, M.F., Whiting, A.S., Hastriter, M.W. & Dittmar, K. (2008) A molecular
687 phylogeny of fleas (Insecta: Siphonaptera): origins and host associations. *Cladistics*, **24**,
688 677–707.

689 Yssouf, A., Socolovschi, C., Leulmi, H., Kernif, T., Bitam, I., Audoly, G., Almeras, L.,
690 Raoult, D. & Parola, P. (2014) Identification of flea species using MALDITOF/MS.
691 *Comparative Immunology, Microbiology and Infectious Diseases*, **37**, 153–157.

692 Zagoskin, M.V., Lazareva, V.I., Grishanin, A.K. & Mukha, D.V. (2014) Phylogenetic
693 information content of Copepoda ribosomal DNA repeat units: ITS1 and ITS2 impact.
694 *BioMed Research International*, ID: 926342.

695 Zhu, Q., Hastriter, M.W., Whiting, M.F. & Dittmar, K. (2015) Fleas (Siphonaptera) are
696 Cretaceous, and evolved with Theria. *Molecular Phylogenetics and Evolution*, **90**, 129–
697 139.

698 Zurita, A., Callejón, R., de Rojas, M., Gómez-López, M.S. & Cutillas, C. (2015)
699 Molecular study of *Stenoponia tripectinata tripectinata* (Siphonaptera:
700 Ctenophthalmidae: Stenoponiinae) from the Canary Islands: taxonomy and phylogeny.
701 *Bulletin of Entomological Research*, **104**, 704–711.

702 Zurita, A., Callejón, R., de Rojas, M., Halajian, A. & Cutillas, C. (2016)
703 *Ctenocephalides felis* and *Ctenocephalides canis*: introgressive hybridization?.
704 *Systematic Entomology*, **41**, 567–579.

705 Zurita, A., Callejón, R., de Rojas, M. & Cutillas, C. (2018) Morphological and
706 molecular study of the genus *Nosopsyllus* (Siphonaptera: Ceratophyllidae). *Nosopsyllus*
707 *barbarus* (Jordan & Rothschild 1912) as a junior synonym of *Nosopsyllus fasciatus*
708 (Bosc, d'Antic 1800). *Insect Systematic and Evolution*, **49**, 81–101.

709 **Figure captions**

710 Fig. 1. Morphological specific characteristics of *Pulex irritans*. a- Head with a vestigial
711 genal comb; b- Club of antenna; c- Genal, pre-ocular and post-antennal setae of head; d-
712 Sternite VII of females; e- Spermatheca of females; f- Row of small spines inside of
713 hind coxa of females; g- Row of small spines inside of hind coxa of males; h- Clasper of
714 males, P1 (Process 1), P2 (Process 2) and P3 (Process 3); i- Crochet of males (cr)
715 expanded apically and dorsal aedeagal sclerite (das) of males long and slender.

716 Fig. 2. Factor map corresponding to *Pulex irritans* adults from Spain and South
717 America. Samples are projected onto the first (PC1, 71 %) and second (PC2, 14 %)
718 principal components. Each group is represented by its perimeter.

719 Fig. 3. Alignment of the parcial ITS1 rDNA sequences of *Pulex irritans* from Spain and
720 Argentina. In red and green bold type the 86 bp repeat unit. Vertical box in blue bold
721 type indicates the polymorphic sites. Gaps generated by alignment (marked by a dash).

722 Fig. 4. Phylogenetic tree of *Pulex irritans* from different geographical origins (see Table
723 1) based on partial cytochrome c-oxidase 1 (*cox1*) gene of mitochondrial DNA
724 sequences using the Bayesian (B) and Maximum Likelihood (ML) methods and
725 Bayesian topology. The percentage of replicate trees in which the associated taxa
726 clustered together in the bootstrap test (1,000 replicates) is shown on the branches
727 (B/ML). Bootstrap values lower than 60 % are not shown. The Bayesian Posterior
728 Probabilities (BPP) is percentage converted.

729 Fig. 5. Phylogenetic tree of *Pulex irritans* from different geographical origins (see Table
730 1) based on partial cytochrome b (*cytb*) gene of mitochondrial DNA using the Bayesian
731 (B) and Maximum Likelihood (ML) methods and Bayesian topology. The percentage of
732 replicate trees in which the associated taxa clustered together in the bootstrap test (1,000
733 replicates) is shown on the branches (B/ML). Bootstrap values lower than 60 % are not
734 shown. The Bayesian Posterior Probabilities (BPP) is percentage converted.

735 Fig. 6. A minimum spanning network constructed using 20 haplotypes of mitochondrial
736 *cytb* partial gene sequences of *Pulex irritans*. The sizes of the circles are proportional to
737 the number of haplotypes represented and the numbers correspond to the mutational
738 steps observed between haplotypes. H1 (2): *P. irritans* from Argentina; H2 (3): *P.*
739 *irritans* from Argentina; H3 (1): *P. irritans* from Argentina; H4 (1) *P. irritans* from

740 Argentina; H5 (1): *P. irritans* from Argentina; H6 (1): *P. irritans* from Argentina; H7
741 (1): *P. irritans* from Argentina; H8 (10): *P. irritans* from Spain; H9 (1): *P. irritans* from
742 Spain.

743 Fig. 7. Phylogenetic tree of *Pulex irritans* from different geographical origins (see Table
744 1) based on concatenated Internal Transcribed Spacer 2 (ITS2), partial cytochrome c-
745 oxidase 1 (*cox1*) and cytochrome b (*cytb*) gene of mitochondrial DNA inferred using the
746 Bayesian (B) and Maximum Likelihood (ML) methods and Bayesian topology. The
747 percentage of replicate trees in which the associated taxa clustered together in the
748 bootstrap test (1,000 replicates) is shown on the branches. The Bayesian Posterior
749 Probabilities (BPP) are percentage converted.

750 Fig. S1. Phylogenetic tree of *Pulex irritans* from different geographical origins (see
751 Table 1) based on the Internal Transcribed Spacer 2 (ITS2) sequences using the
752 Bayesian (B) and Maximum Likelihood (ML) methods and Bayesian topology. The
753 percentage of replicate trees in which the associated taxa clustered together in the
754 bootstrap test (1,000 replicates) is shown on the branches (B/ML). Bootstrap values
755 lower than 60 % are not shown. The Bayesian Posterior Probabilities (BPP) is
756 percentage converted.

Table 1. GenBank accession numbers of ITS1, ITS2 and partial *cytb*, *cox1* gene sequences of individuals of *P. irritans* and *C. felis* obtained in this study.

ITS1					
Location/Country/Sample ID	**Species/Gender**	**Host**	**Number of fleas**	**Base pairs (bp)**	**Accession number**
Seville/Spain/PI1	*P. irritans* /1♂	-	1	962	LT797452
Seville/Spain/PI1 (Clone 3)	*P. irritans*	-	-	968	LT853871
Seville/Spain/PI1 (Clone 1-2, 4)	*P. irritans*	-	-	968	LT853872
Seville/Spain/PI2	*P. irritans* /1♀	-	1	876	LT797453
Seville/Spain/PI4	*P. irritans* /1♀	-	1	876	LT797454
Seville/Spain/PI4 (Clone 2)	*P. irritans*	-	-	882	LT853866
Seville/Spain/PI4 (Clone 3)	*P. irritans*	-	-	882	LT853867
Seville/Spain/PI4 (Clone 5)	*P. irritans*	-	-	882	LT853869
Seville/Spain/PI4 (Clone 6-7)	*P. irritans*	-	-	881	LT853868
Seville/Spain/PI4 (Clone 4, 8)	*P. irritans*	-	-	882	LT853870
Seville/Spain/PI5	*P. irritans* /1♀	-	1	876	LT797455
Seville/Spain/PI6	*P. irritans* /1♂	-	1	962	LT797456
Seville/Spain/PI7	*P. irritans* /1♀	-	1	876	LT797457
Seville/Spain/PI8	*P. irritans* /1♀	-	1	876	LT797458
Seville/Spain/PI10	*P. irritans* /1♂	-	1	876	LT797459
Seville/Spain/PI11	*P. irritans* /1♀	-	1	876	LT797460
Seville/Spain/PI12	*P. irritans* /1♂	-	1	962	LT797461
Seville/Spain/PI13	*P. irritans* /1♀	-	1	876	LT797462
Seville/Spain/PI14	*P. irritans* /1♂	-	1	876	LT797463
Santa Cruz/Argentina/ PI26-35	*P. irritans*/6♂ 5♀	*L. culpaeus* and *L. griseus*	10	796	LT797464
Santa Cruz/Argentina/PI32 (Clone 1)	*P. irritans*	*L. culpaeus and L. griseus*	-	796	LT853873
Santa Cruz/Argentina/PI32 (Clone 3)	*P. irritans*	*L. culpaeus and L. griseus*	-	796	LT853874
Santa Cruz/Argentina/PI32 (Clone 2, 6, 8)	*P. irritans*	*L. culpaeus and L. griseus*	-	796	LT853875
La Plata/Argentina/668, 670	*C. felis*/2♀	*Canis lupus familiaris*	2	668	LT853877
ITS2					
Location/Country/Sample ID	**Species/Gender**	**Host**	**Number of fleas**	**Base pairs (bp)**	**Accession number**
Seville/Spain/PI1	*P. irritans* /1♂	-	1	322	LT797448
Seville/Spain/PI6	*P. irritans* /1♂	-	1	322	LT797449
Seville/Spain/ PI2,4,5,7,8,10-14	*P. irritans*/3♂ 7♀	-	10	322	LT797450
Santa Cruz/Argentina/ PI26-35	*P. irritans*/6♂ 5♀	*L. culpaeus and L. griseus*	10	324	LT797451
La Plata/Argentina/668, 670	*C. felis*/2♀	*Canis lupus familiaris*	2	327	LT853876
Cox1					
Location/Country/ID	**Species/Gender**	**Host**	**Number of fleas**	**Base pairs (bp)**	**Accession number/Haplo type**
Seville/Spain/PI2	*P. irritans* /1♀	-	1	658	LT797468/H4
Seville/Spain/PI6	*P. irritans* /1♂	-	1	658	LT797469/H5
Seville/Spain/PI1, 4-5, 7-8, 10-14	*P. irritans*/4♂ 6♀	-	10	658	LT797470/H6
Santa Cruz/Argentina/ PI30	*P. irritans* /1♀	*L. culpaeus*	1	658	LT797465/H1
Santa Cruz/Argentina/ PI33	*P. irritans* /1♂	*L. griseus*	1	658	LT797466/H2
Santa Cruz/Argentina/ PI26, 27, 35	*P. irritans* /3♀	*L. culpaeus*	3	658	LT797467/H3
La Plata/Argentina/668	*C. felis*/2♀	*Canis lupus familiaris*	2	601	LT853879
Cytb					
Location/Country/ID	**Species/Gender**	**Host**	**Number of fleas**	**Base pairs (bp)**	**Accession number/Haplo type**
Seville/Spain/PI2	*P. irritans* /1♀	-	1	374	LT797473/H9
Seville/Spain/PI1, PI4-8, PI10-11, PI13-14	*P. irritans*/4♂ 6♀	-	10	374	LT797474/H8
Santa Cruz/Argentina/PI26, PI32	*P. irritans* /2♀	*L. culpaeus*	2	374	LT797475/H1
Santa Cruz/Argentina/PI27, PI29, PI35	*P. irritans* /3♀	*L. culpaeus*	3	374	LT797476/H2
Santa Cruz/Argentina/ PI28	*P. irritans* /1♀	*L. culpaeus*	1	374	LT797477/H3
Santa Cruz/Argentina/ PI30	*P. irritans* /1♀	*L. culpaeus*	1	374	LT797478/H4
Santa Cruz/Argentina/ PI31	*P. irritans* /1♀	*L. culpaeus*	1	374	LT797479/H5
Santa Cruz/Argentina/ PI33	*P. irritans* /1♂	*L. griseus*	1	374	LT797480/H6
Santa Cruz/Argentina/ PI34	*P. irritans* /1♀	*L. culpaeus*	1	374	LT797481/H7
La Plata/Argentina/668, 670	*C. felis*/2♀	*Canis lupus familiaris*	2	374	LT853878

Table 2. Morphometric analysis of ten specimens of *P. irritans* from Argentina (seven females and three males) and fourteen specimens of *P. irritans* from Spain (six males and eight females) assessed in this study. Individuals from both sexes have been pooled in both populations. TLF = total female length, TLM = total male length, TWF = total female width, TWM = total male width, HLF = total length of the female head, HLM = total length of the male head, HWF = total width of the female head, HWM = total width of the male head, EL = total length of the spermatheca, EW = total width of the spermatheca, CRL = total length of the extended region of the crochet, DASL = total length of the dorsal aedeagal sclerite, PROL= total length of the prothorax, MESL = total length of the mesothorax, METL = total length of the metathorax, MAX = maximum, MIN = minimum, SD = standard deviation. X = arithmetic mean, CV = Coefficient of Variation (percentage converted). In black bold type parameters which showed certain differences between both geographical origins.

	Pulex irritans (Spain)					*Pulex irritans* (Argentina)				
	MIN	MAX	X	SD	CV	MIN	MAX	X	SD	CV
TLF(mm)	1.8	2.1	1.9	0.1	5	1.7	2.5	2.1	0.2	9
TLM(mm)	1.3	2.1	1.8	0.3	17	1.4	1.8	1.6	0.2	12
TWF(mm)	1.1	1.2	1.1	0.1	9	1.0	1.3	1.1	0.1	9
TWM(mm)	0.8	1.2	1.0	0.2	20	0.7	0.9	0.8	0.1	12
HLF(µm)	**398**	**597**	**458**	**40**	**8**	**352**	**469**	**416**	**38**	**9**
HLM(µm)	**380**	**498**	**418**	**47**	**11**	**322**	**387**	**354**	**46**	**13**
HWF(µm)	**234**	**293**	**259**	**25**	**9**	**205**	**264**	**237**	**22**	**9**
HWM(µm)	**205**	**293**	**250**	**32**	**12**	**176**	**234**	**205**	**41**	**20**
EL(µm)	82	147	119	21	17	70	147	110	26	23
EW(µm)	53	59	57	3	5	47	64	57	6	10
CRL(µm)	26	40	34	5	14	28	33	30	4	13
DASL(µm)	82	110	97	10	10	80	94	87	10	11
PROL(µm)	**87**	**152**	**111**	**16**	**14**	**70**	**137**	**100**	**24**	**24**
MESL(µm)	**106**	**167**	**135**	**19**	**14**	**88**	**152**	**124**	**25**	**20**
METL(µm)	**134**	**182**	**162**	**15**	**9**	**100**	**182**	**145**	**24**	**16**

Table 3. Intrapopulation* and intraspecific similarity observed amog all the ITS1 sequences of *Pulex irritans* from different geographical areas obtained in this work and from Genbank database. Values are given in percentages.

ITS1	*P. irritans* from Spain LT797452- LT797463	*P. irritans* from Argentina LT797464	*P. irritans* from United States GQ387496. Host unknown	*P. irritans* from Cameroon EU169198. Human host	*P. irritans* from Iran KX822017. Host unknown
P. irritans from Spain LT797452- LT797463	99.5-100*				
P. irritans from Argentina LT797464	95.9-96.3	100*			
P. irritans from United States GQ387496. Host unknown	94.0-95.4	94.4	-		
P. irritans from Cameroon EU169198. Human host	99.5-99.8	96.2	95.1	-	
P. irritans from Iran KX822017. Host unknown	99.6-99.9	96.5	93.9	99.6	-

Table 4. Intrapopulation (*), intraspecific and interspecific similarity observed among all the partial *cox1* mtDNA gene sequences of *Pulex irritans* from different geographical areas obtained in this work and other Pulicidae species from GenBank database. Values are given in percentages. (PI = *Pulex irritans*).

COX1	*PI*/Seville (Spain)/ LT797468-70 (H4-H6)	*PI*/Santa Cruz (Argentina)/ LT797465-67 (H1-H3)	*P. irritans*/Spain/ Badger/ KF479246	*P. irritans*/Spain/ human/ KF479247	*P. irritans*/New Zealand/dog/ KY048351	*P. irritans*/Hungary/ fox/ MG668624	*P. irritans*/Hungary/ badger/ MG668626	*P. irritans*/Hungary/ jackal/ MG668627	*P. irritans*/China/ polecat/ MF000666	*P. irritans*/Croatia/ human/ MG668622	*C. felis*/ Argentina/ LT853879	*C. felis*/Spain/ LN827896	*C. canis*/ Iran/LN827901	*E. gallinacea*/ Australia/ JN008921	*E.. iberica*/ Spain/ KF479239
PI/Seville (Spain)/LT797468-70 (H4-H6)	*99.6-99.8														
PI/Santa Cruz (Argentina)/ LT797465-67 (H1-H3)	91.4-92.0	*99.2-99.8													
P. irritans/Spain/badger/ KF479246	96.0-96.2	92.8-93.0	-												
P. irritans/Spain/human/ KF479247	99.5-99.8	91.8-92.2	96.4	-											
P. irritans/New Zealand/dog KY048351	99.8-100	91.5-91.8	96.0	99.7	-										
P. irritans/Hungary/fox/ MG668624	96.0-96.2	93.2-93.4	98.6	96.4	96.0	-									
P. irritans/Hungary/badger/ MG668626	96.0-96.2	93.2-93.4	98.6	96.4	96.0	100	-								
P. irritans/Hungary/jackal/ MG668627	96.0-96.2	93.2-93.4	98.6	96.4	96.0	100	100	-							
P. irritans/China/polecat/ MF000666	99.6-99.8	91.4-91.8	96.2	99.4	99.8	96.2	96.2	96.2	-						
P. irritans/Croatia/human/ MG668622	99.4-99.6	91.2-91.6	95.6	99.2	99.6	95.6	95.6	95.6	99.4	-					
C. felis/Argentina/ LT853879	85.7-86.0	84.9-85.2	85.9	85.9	85.9	87.3	87.3	87.3	86.5	86.3	-				
C. felis/Spain/LN827896	85.7-86.0	84.9-85.2	85.9	85.9	85.9	87.3	87.3	87.3	86.5	86.3	100	-			
C. canis/Iran/LN827901	86.4-86.5	85.2	86.4	86.7	86.4	87.3	87.3	87.3	86.9	86.7	97.7	97.7	-		
E. gallinacea/ Australia/JN008921	87.9-88.0	87.5	88.9	88.0	87.9	88.5	88.5	88.5	88.3	88.1	85.7	85.7	86.0	-	
E.. iberica/Spain/KF479239	88.7-88.9	89.2-89.5	89.9	88.9	88.7	89.6	89.6	89.6	88.7	88.9	86.7	86.7	87.2	93.5	-

Table 5. Intrapopulation (*), intraspecific and interspecific similarity observed among all the partial *cytb* mtDNA gene sequences of *Pulex irritans* from different geographical areas obtained in this work and other Pulicidae species from GenBank database. Values are given in percentages. (PI = *Pulex irritans*).

CYTB	*PI*/Seville (Spain)/ LT797473-74 (H8-H9)	*PI*/Santa Cruz (Argentina)/ LT797475-76 (H1-H7)	*A. erinacei*/ Spain/ LT604120	*A. erinacei*/ Corse (France)/ LT627350	*C. felis*/Spain/ LN897470	*C. felis*/ Argentina/ LT853878	*C. canis*/Iran/ LN897471	*X. conformis*/ KM890723	*X. skrjabini*/ KM890718	*X. cheopis*/ Canary Islands/ LT604122	*S. cuniculi*/ KM890622	*S. girardi*/ KM890686	*E. oschanini*/ KM890719
PI/Seville (Spain)/ LT797473-74 (H8-H9)	*99.7-100												
PI/Santa Cruz (Argentina)/ LT797475-81 (H1-H7)	90.9-92.2	*97.8-99.7											
A. erinacei/Spain/ LT604120	84.9-85.2	82.5-83.6	-										
A. erinacei/Corse (France)/ LT627350	85.5-85.8	82.5-83.6	98.9	-									
C. felis/Spain/ LN897470	82.0-82.3	80.6-81.2	84.7	85.2	-								
C. felis/Argentina/ LT853878	82.0-82.3	80.6-81.2	84.7	85.2	100	-							
C. canis/Iran/ LN897471	82.3	79.8-80.4	85.5	86.0	90.6	90.6	-						
X. conformis/ KM890723	80.1-80.4	78.2-78.8	82.0	82.5	83.9	83.9	83.6	-					
X. skrjabini/ KM890718	79.8-80.1	77.7-78.2	81.7	82.5	84.4	84.4	82.8	92.5	-				
X. cheopis/Canary Islands/ LT604122	75.8	72.6-73.4	79.8	80.1	82.0	82.0	81.2	79.6	80.9	-			
S. cuniculi/ KM890622	81.5	81.7-82.3	82.3	82.8	83.1	83.1	83.9	82.3	81.7	80.1	-		
S. girardi/ KM890686	80.6-80.9	78.5-79.8	83.6	84.7	84.9	84.9	84.1	84.9	82.8	80.1	78.5	-	
E. oschanini/KM890719	84.9-85.2	81.7-82.3	84.1	84.7	83.6	83.6	83.9	83.9	83.1	78.5	82.0	79.3	-

Table S1**.** PCR mix, primers and conditions used for each molecular marker sequenced in this study.

	ITS1	**ITS2**	***Cytb***	***cox1***
		PCR Mix		
PCR Buffer (5X)	10 µl	10 µl	10 µl	10 µl
dNTPs (10mM)	2 µl	1 µl	1 µl	1 µl
$MgCl_2$ (25 mM)	6 µl	6 µl	4 µl	4 µl
Forward Primer (10 µM)	5 µl	5 µl	5 µl	5 µl
Reverse Primer (10 µM)	5 µl	5 µl	5 µl	5 µl
Template DNA	5 µl	5 µl	5 µl	5 µl
goTaq DNA polymerase	0,5 µl	0,5 µl	0,5 µl	0,5 µl
Autoclaved distilled water to	100 µl	50 µl	50 µl	50 µl
		PCR Primers		
Forward Primer	NC5 (Gasser *et al.*, 1996)	senITS2 (Vobis *et al.*, 2004)	CytbF (Dittmar &Whiting, 2003)	LCO1490 (Folmer *et al.*, 1994)
Reverse Primer	ITS1rev (Marrugal *et al.*., 2013)	ITS2R (Luchetti *et al.*, 2007)	A5F (Dittmar &Whiting, 2003)	HCO2198 (Folmer *et al.*, 1994)
		PCR Conditions		
Initial Denaturing	94 ºC for 5´	94 ºC for 5´	95 ºC for 12´	96 ºC for 2´
Number of cycles	35	35	30	40
Denaturing	94 ºC for 30´´	94 ºC for 60´´	95 ºC for 30´´	94 ºC for 30´´
Annealing	58 ºC for 30´´	55 ºC for 60´´	40 ºC for 30´´	50 ºC for 30´´
Primer extension	72 ºC for 90´´	72 ºC for 60´´	68 ºC for 2´	72 ºC for 60´´
Final extension	72 ºC for 5´	72 ºC for 10´	68 ºC for 7´	72 ºC for 7´

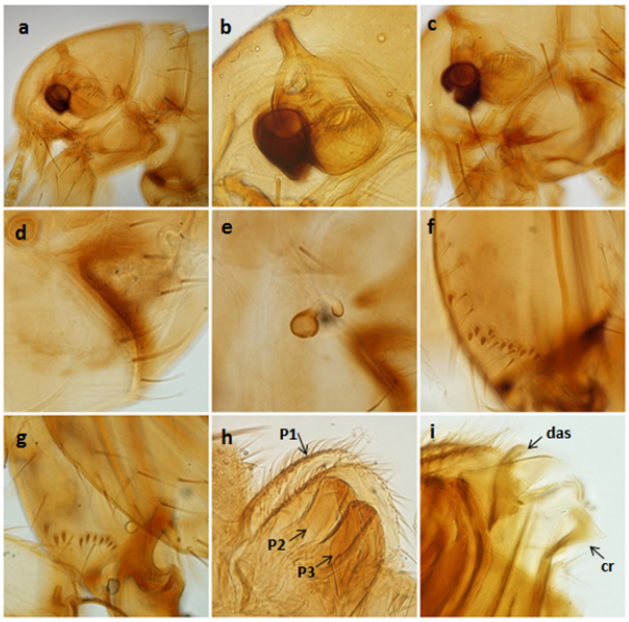

Figure 1

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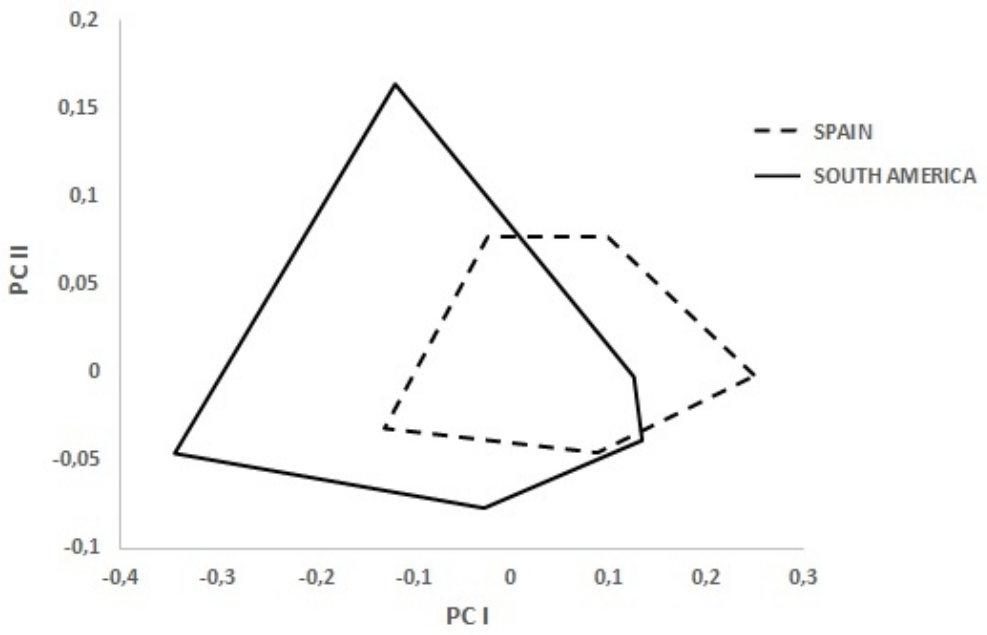

Figure 2

138x90mm (96 x 96 DPI)

Figure 3

210x297mm (200 x 200 DPI)

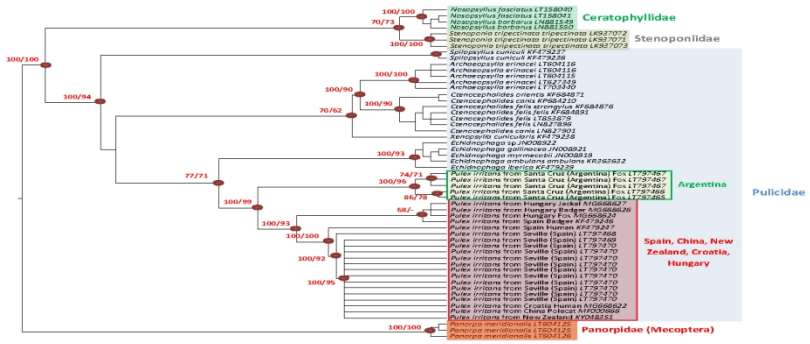

Figure 4

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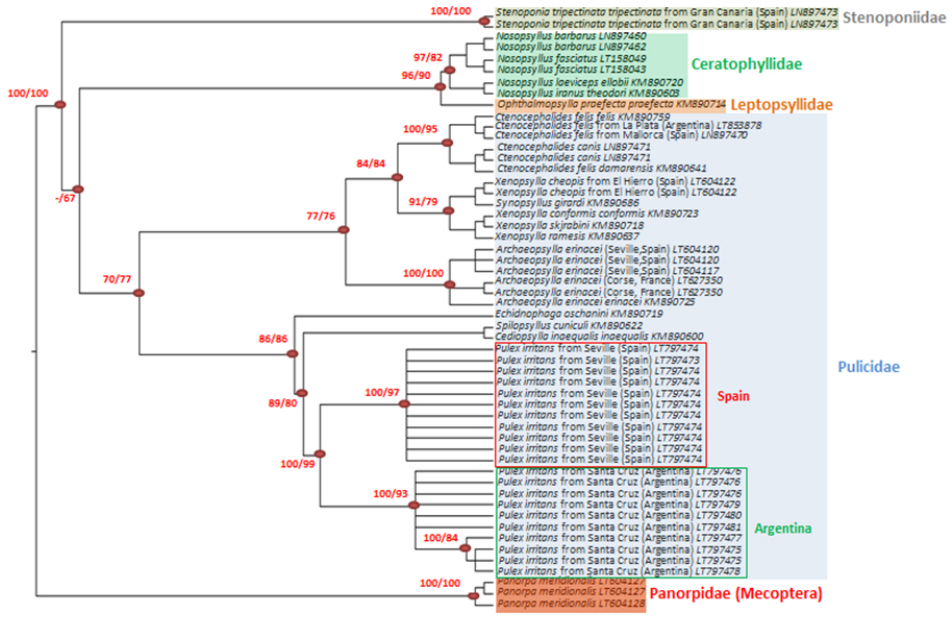

Figure 5

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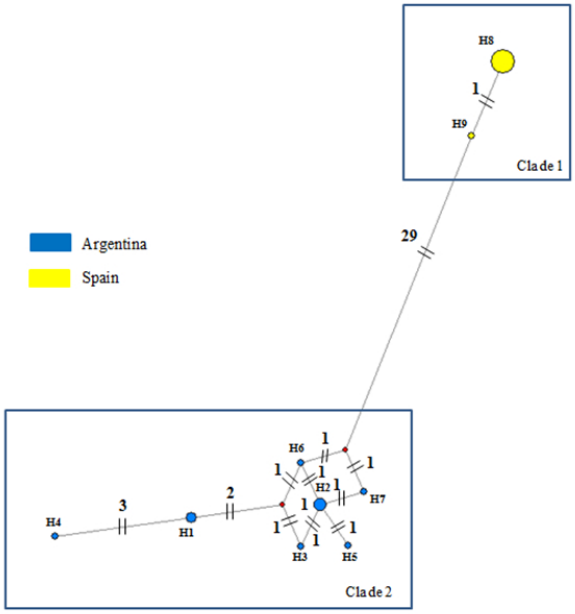

Figure 6

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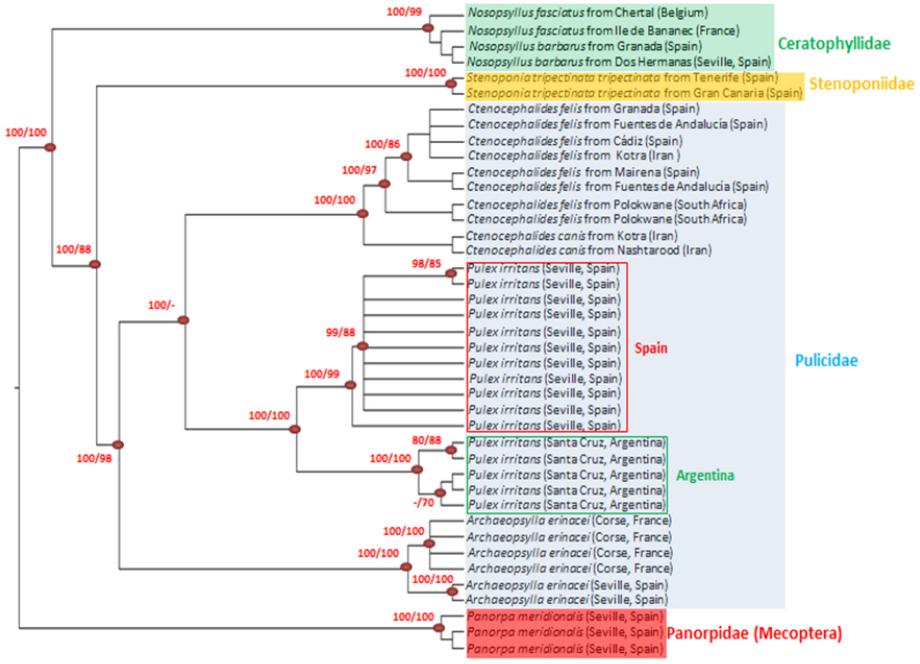
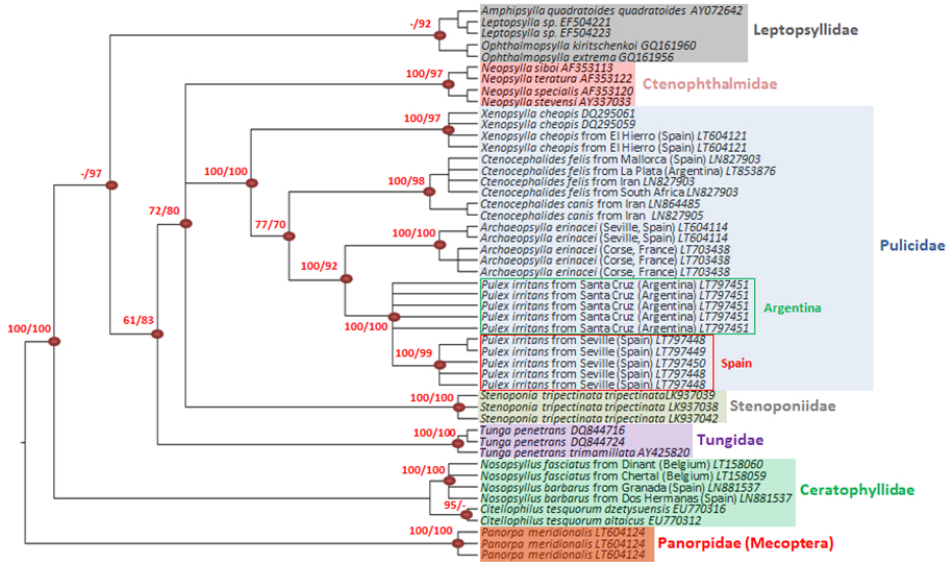


Figure 7

254x190mm (96 x 96 DPI)



254x190mm (96 x 96 DPI)

Appendix 1

List of taxa used in the analysis, including GenBank accession numbers and host information.

Species	Family	Host	Accession number	Gen Region	Sequence length
Pulex irritans	Pulicidae	*Homo sapiens*	EU169198	ITS1	929
Pulex irritans	Pulicidae	*Homo sapiens*	GQ387496	ITS1	948
Pulex irritans	Pulicidae	Unknown	KX822017	ITS1	1,208
Ophthalmopsylla kiritschenkoi	Leptopsyllidae	Unknown	GQ161960	ITS2	474
Ophthalmopsylla extrema	Leptopsyllidae	Unknown	GQ161956	ITS2	466
Amphipsylla quadratoides quadratoides	Leptopsyllidae	Unknown	AY072642	ITS2	497
Leptopsylla sp.	Leptopsyllidae	Unknown	EF504221	ITS2	459
Leptopsylla sp.	Leptopsyllidae	Unknown	EF504223	ITS2	449
Neopsylla siboi	Ctenophthalmidae	Unknown	AF353113	ITS2	479
Neopsylla teratura	Ctenophthalmidae	Unknown	AF353122	ITS2	479
Neopsylla stevensi	Ctenophthalmidae	Unknown	AY337033	ITS2	479
Neopsylla specialis	Ctenophthalmidae	Unknown	AF353120	ITS2	479
Xenopsylla cheopis	Pulicidae	*Rattus* sp.	DQ295061	ITS2	356
Xenopsylla cheopis	Pulicidae	*Rattus* sp.	DQ295059	ITS2	356
Xenopsylla cheopis	Pulicidae	*Rattus sp.*	LT604121	ITS2	358
Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LN827903	ITS2	327
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN827905	ITS2	327
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN864485	ITS2	327
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT703438	ITS2	360
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT604114	ITS2	361
Tunga penetrans	Tungidae	*Homo sapiens*	DQ844716	ITS2	471
Tunga penetrans	Tungidae	*Homo sapiens*	DQ844724	ITS2	473
Tunga trimamillata	Tungidae	Unknown	AY425820	ITS2	470
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937042	ITS2	332
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937039	ITS2	332
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937038	ITS2	332
Citellophilus tesquorum dzetysuensis	Ceratophyllidae	Unknown	EU770316	ITS2	332
Citellophilus tesquorum altaicus	Ceratophyllidae	Unknown	EU770312	ITS2	332
Nospsyllus fasciatus	Ceratophyllidae	*Apodemus sylvaticus*	LT158059	ITS2	318
Nosopsyllus fasciatus	Ceratophyllidae	Muridae	LT158060	ITS2	318
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp.	LN881537	ITS2	318
Panorpa meridionalis	Panorpidae	-	LT604124	ITS2	1,121
Echidnophaga gallinacea	Pulicidae	*Oryctolagus cuniculus*	JN008921	*Cox1*	650
Echidnophaga myrmecobii	Pulicidae	*Oryctolagus cuniculus*	JN008919	*Cox1*	649
Echidnophaga iberica	Pulicidae	*Oryctolagus cuniculus*	KF479239	*Cox1*	658
Echidnophaga sp.	Pulicidae	Mammal	JN008922	*Cox1*	654
Echidnophaga ambulans ambulans	Pulicidae	*Tachyglossus aculeatus*	KR363632	*Cox1*	601
Xenopsylla cunicularis	Pulicidae	*Oryctolagus cuniculus*	KF479238	*Cox1*	658
Pulex irritans	Pulicidae	*Meles meles*	KF479246	*Cox1*	658
Pulex irritans	Pulicidae	*Homo sapiens*	KF479247	*Cox1*	658
Pulex irritans	Pulicidae	*Canis lupus familiaris*	KY048351	*Cox1*	658
Pulex irritans	Pulicidae	Jackal	MG668627	*Cox1*	489
Pulex irritans	Pulicidae	Badger	MG668626	*Cox1*	489
Pulex irritans	Pulicidae	Fox	MG668624	*Cox1*	489
Pulex irritans	Pulicidae	*Homo sapiens*	MG668622	*Cox1*	489
Pulex irritans	Pulicidae	*Vormela peregusna*	MF000666	*Cox1*	672
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	KF479236	*Cox1*	658
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	KF479237	*Cox1*	658
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT604116	*Cox1*	658
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT604115	*Cox1*	658
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT627349	*Cox1*	658
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT703440	*Cox1*	658
Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LN827896	*Cox1*	600

Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LT853879	*Cox1*	600
Ctenocephalides felis felis	Pulicidae	*Felis catus*	KF684891	*Cox1*	601
Ctenocephalides felis strongylus	Pulicidae	*Canis lupus familiaris*	KF684876	*Cox1*	601
Ctenocephalides orientis	Pulicidae	*Canis lupus familiaris*	KF684871	*Cox1*	601
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	KP684210	*Cox1*	658
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN827901	*Cox1*	600
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937072	*Cox1*	677
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937071	*Cox1*	677
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937073	*Cox1*	677
Nosopsyllus fasciatus	Ceratophyllidae	*Crocidura russula*	LT158040	*Cox1*	658
Nosopsyllus fasciatus	Ceratophyllidae	*Apodemus sylvaticus*	LT158041	*Cox1*	658
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp	LN881549	*Cox1*	658
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp	LN881550	*Cox1*	658
Panorpa meridionalis	Panorpidae	-	LT604125	*Cox1*	658
Panorpa meridionalis	Panorpidae	-	LT604126	*Cox1*	658
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LN897473	*Cytb*	374
Ophthalmopsylla praefecta praefecta	Leptopsyllidae	Unknown	KM890714	*Cytb*	369
Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LN897470	*Cytb*	374
Ctenocephalides felis felis	Pulicidae	Unknown	KM890759	*Cytb*	369
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN897471	*Cytb*	374
Ctenocephalides felis damarensis	Pulicidae	Unknown	KM890641	*Cytb*	369
Xenopsylla cheopis	Pulicidae	*Rattus* sp.	LT604122	*Cytb*	374
Archaeopsylla erinacei erinacei	Pulicidae	Unknown	KM890725	*Cytb*	369
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT604120	*Cytb*	374
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT604117	*Cytb*	374
Archaeopsylla erinacei	Pulicidae	*Erinaceus europaeus*	LT627350	*Cytb*	374
Synopsyllus girardi	Pulicidae	Unknown	KM890686	*Cytb*	369
Xenopsylla conformis conformis	Pulicidae	Unknown	KM890723	*Cytb*	369
Xenopsylla skjrabini	Pulicidae	Unknown	KM890718	*Cytb*	369
Xenopsylla ramesis	Pulicidae	Unknown	KM890637	*Cytb*	342
Echidnophaga oschanini	Pulicidae	Unknown	KM890719	*Cytb*	369
Spilopsyllus cuniculi	Pulicidae	Unknown	KM890622	*Cytb*	369
Cediopsylla inaequalis inaequalis	Pulicidae	Unknown	KM890600	*Cytb*	369
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp	LN897460	*Cytb*	374
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp	LN897462	*Cytb*	374
Nosopsyllus fasciatus	Ceratophyllidae	Muridae	LT158049	*Cytb*	374
Nosopsyllus fasciatus	Ceratophyllidae	*Apodemus sylvaticus*	LT158043	*Cytb*	374
Nosopsyllus iranis theodori	Ceratophyllidae	*Gerbillus dasyurus*	KM890603	*Cytb*	369
Nosopsyllus laeviceps ellobii	Ceratophyllidae	Unknown	KM890720	*Cytb*	369
Panorpa meridionalis	Panorpidae	-	LT604127	*Cytb*	374
Panorpa meridionalis	Panorpidae	-	LT604128	*Cytb*	374