ORIGINAL ARTICLE

Combination of nuclear and mitochondrial markers as a useful tool to identify *Ctenophthalmus* species and subspecies (Siphonaptera: Ctenophthalmidae)

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Abstract

Ctenophthalmus is considered the largest genus within the Order Siphonaptera. From a morphological point of view, only males of this genus can be identified at species and subspecies levels using morphological keys, whereas there are no morphological criteria in order to classify females at these taxonomical levels. Furthermore, the amount of available molecular and phylogenetic data for this genus is quite scarce so far. The main objective of this work was to assess the utility of the combination of nuclear and mitochondrial markers with respect to their ability to differentiate among different subspecies within the *Ctenophthalmus* genus. With this purpose, we carried out a comparative morphological and molecular study of three different subspecies (*Ctenophthalmus baeticus arvernus*, *Ctenophthalmus nobilis dobyi*, and *Ctenophthalmus andorrensis catalaniensis*) in order to clarify and discuss its taxonomic status. In addition, our study complemented the molecular data previously provided for *Ctenophthalmus baeticus boisseauorum* and *Ctenophthalmus apertus allani* subspecies. We sequenced five different molecular markers: *EF1-α*, ITS1, ITS2, *cox*1, and *cyt*b. Our results confirmed that morphological data by themselves are not able to discriminate among *Ctenophthalmus* female taxa; however, the combination of the nuclear marker *EF1-α* together with mtDNA markers *cyt*b and *cox*1 constituted a useful taxonomical and phylogenetic tool to solve this issue. Based on these results, we consider that the use of this molecular approach should be gradually used within *Ctenophthalmus* genus in order to complement its classical taxonomy and clarifying the complex taxonomy of other congeneric species of fleas.

Keywords Siphonaptera · *Ctenophthalmus* · Taxonomy · Mitochondrial DNA · Nuclear DNA

Introduction

The family Ctenophthalmidae has been considered a "catch-all" for a wide range of divergent taxa within the Order Siphonaptera (Whiting et al. 2008; Keskin 2019). In this sense, some authors recently reported that some subfamilies included within Ctenophthalmidae such as Stenoponiinae should be considered a monophyletic family (Stenoponiidae) (Zurita et al. 2015). *Ctenophthalmus* is considered the largest genus within the Order Siphonaptera; thus, this genus comprises fifteen different subgenera distributed throughout the world out of which the *Ctenophthalmu*s subgenus is the largest one being distributed along the Palearctic region (Hopkins and Rothschild 1966; Beaucournu and Loverlec 2014). Only in the Western Palearctic Zone (European region) 143 taxa (57 species and 86 subspecies) have been described so far (Beaucournu and Loverlec 2014).

From a taxonomical point of view, it is noteworthy that *Ctenophthalmus* genus has been intensively studied using morphological features. Nevertheless, specialized entomologists have not been able to find standard criteria in order to discriminate among *Ctenophthalmus* female species so far; thus, there is no specific taxonomical key to identify *Ctenophthalmus* females based on morphological traits (Beaucournu and Launay 1990; Zurita et al. 2020). On the other hand, males of *Ctenophthalmus* can be easily distinguishable based on their complex genitalia. In this context, it is usual to find some taxonomical keys for this genus providing a deep level of classification to subspecies level exclusively based on males (Beaucournu and Launay 1990). For example, Beaucournu and Loverlec (2014) reported eight different

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subspecies in *Ctenophthalmus apertus*: *C. apertus apertus*, *C. apertus allani*, *C. apertus azevedoi*, *C. apertus gilcolladoi*, *C. apertus gosalbezi*, *C. apertus meylani*, *C. apertus queirozi*, and *C. apertus personatus*. It should be highlighted that all these subspecies were found in the southwestern of Europe (south of France, Spain, and Portugal) as well as they share the same hosts (*Pitymys duodecimcostatus*, *Apodemus sylvaticus*, *Mus spretus*, *Mustela nivalis*, *Crocidura russula*, or *Arvicola sapidus*).

Furthermore, other *Ctenophthalmus* species have more than three subspecies described. For example, *Ctenophthalmus baeticus* has six known subspecies, whereas six and three different subspecies have been described for *Ctenophthalmus andorrensis* and *Ctenophthalmus nobilis*, respectively (Beaucournu and Launay 1990; Beaucournu and Loverlec 2014). In addition, all these subspecies share the same geographical area of distribution (southwestern of Europe) and the same hosts previously cited for *C. apertus* subspecies.

Unlike classical taxonomy, there is a severe shortage of molecular and phylogenetic data for this genus. Recently, Zurita et al. (2020) provided, for the first time, morphometric and molecular data of two *Ctenophthalmus* subspecies belonging to *Ctenophthalmus* subgenus (*C. baeticus boisseauorum* and *C. a. allani*). These authors observed morphological differences in males, which did not correspond with molecular and phylogenetic data, suggesting that both subspecies could be considered synonymous taxa due to a phenotypic plasticity phenomenon. In addition, they claimed for the necessity to provide complementary molecular and phylogenetic data for this genus in order to clarify the taxonomic position of several *Ctenophthalmus* species and subspecies.

The main objective of this work was to assess the utility of the combination of nuclear and mitochondrial markers with respect to their ability to differentiate among different subspecies within the *Ctenophthalmus* genus. For this purpose, we carried out a comparative molecular study of three different western palearctic subspecies belong to three different species of the *Ctenophthalmus* genus: *Ctenophthalmus baeticus arvernus* (Jordan 1931), *Ctenophthalmus nobilis dobyi* (Beaucournu 1962), and *Ctenophthalmus andorrensis catalaniensis* (Beaucournu 1973), in order to clarify the taxonomic status of these taxa. Within *Ctenophthalmus* genus, all these taxa are classified into *Ctenophthalmus* subgenus, occurring in the same region and occurring on the same hosts. In order to assess the consistency of genetic and morphological identification, we amplified and sequenced five different molecular markers: the nuclear *Elongation Factor 1 alpha* (*EF1-α*), Internal Transcribed Spacers (ITSs) 1 and 2 of ribosomal DNA (rDNA), and the mtDNA partial *cytochrome c oxidase* subunit 1 (*cox*1) and *cytochrome* b (*cyt*b) gene fragments. We complete our study providing new molecular data

for *C. baeticus boisseauorum* and *C. a. allani* subspecies specimens provided by Zurita et al. (2020). Although these five subspecies by themselves do not represent the whole *Ctenophthalmus* genus, this study expects to provide, for the first time, useful molecular tools in order to clarify the complex taxonomy of this flea group.

Material and methods

Collection of samples

All the samples were collected from rodents *Apodemus sylvaticus* and *Crocidura russula* from Py (eastern Pyrenees, south of France) (42° 29′ 45″ N, 2° 21′ 03″ E) during two different periods (July 2016 and July 2019) (Table 1). Rodents were trapped using live traps. Afterward, rodents were exhaustively examined for fleas by combing through an inspection of head, neck, body, sides, tail, and ventral regions of each animal. Fleas obtained were kept in Eppendorf tubes with 96% ethanol for subsequent identification and DNA extraction.

Morphological identification

For morphological analysis, all whole specimens were examined and photographed under an optical microscope. Subsequently, eighteen samples (13 males and 5 females) corresponding to three different subspecies (*C. b. arvernus*, *C. n. dobyi*, and *C. a. catalaniensis*) were put away for molecular purposes (only males could be classified at subspecies level). On the other hand, one male for each species and five *Ctenophthalmus* sp. females were cleared with 10% KOH, prepared, and mounted on glass slides using conventional procedures with EUKITT mounting medium (O. Kindler GmbH & Co., Freiburg, Germany) (Lewis 1993). Once mounted, they were examined and photographed again for a deeper morphological analysis using a CX21 microscope (Olympus, Tokyo, Japan). Diagnostic morphological characters of all the samples were studied by comparison with figures, keys, and descriptions reported by Hopkins and Rothschild (1966) and Beaucournu and Launay (1990).

Molecular and phylogenetic study

As well as the three just cited *Ctenophthalmus* subspecies, we amplified *EF1-α* sequences from individuals of *C. b. boisseauorum*, *C. a. allani*, *Ctenocephalides felis*, *Ctenocephalides canis*, *Nosopsyllus barbarus*, *Nosopsyllus fasciatus*, *Archaeopsylla erinacei*, *Pulex irritans*, *Stenoponia tripectinata tripectinata*, and *Panorpa meridionalis*, only for phylogenetic comparative purposes.

Table 1 GenBank accession numbers of ITS1, ITS2, *EF1-α*, and partial *cytb* and *cox1* gene sequences of individuals of *Ctenophthalmus* sp.; CND, *C. nobilis dobyi*; CBA, *C. baeticus arvernus*, and CAC, *C. andorrensis catalaniensis* obtained in this study. In addition, for this study, we provided GenBank accession numbers of *EF1-α* from individuals of CBB, *C. baeticus boisseauorum*; CAA, *C. apertus allani*; *Ctenocephalides felis*; *Cenocephalides canis*; NB, *Nosopsyllus barbarus*; NF, *Nosopsyllus fasciatus*; AE, *Archaeopsylla erinacei*; PI, *Pulex irritans*; *Stenoponia tripectinata tripectinata*; and PM, *Panorpa meridionalis*

Species/gender	Sample ID/geographical area	Host	Number of fleas	Base pairs (bp)	Accession number
ITS1					
C. a. catalaniensis /male	CAC2-5/Py, France	*Apodemus sylvaticus*	4	889	LR760744
C. b. arvernus/male	CBA1, CBA2/Py, France	*Apodemus sylvaticus*	2	888	LR760746
C. n. dobyi/male	CND2-8/Py, France	*Apodemus sylvaticus*	7	888	LR760747
Ctenophthalmus sp./female	CT35/Py, France	*Apodemus sylvaticus*	1	889	LR760745
Ctenophthalmus sp./female	CT36, CT34/Py, France	*Crocidura russula*	2	889	LR760742
Ctenophthalmus sp./female	CT33/Py, France	*Apodemus sylvaticus*	1	888	LR760741
Ctenophthalmus sp./female	CT37/Py, France	*Apodemus sylvaticus*	1	888	LR760743
ITS2					
C. a. catalaniensis /male	CAC2-5/Py, France	*Apodemus sylvaticus*	4	492	LR760738
C. b. arvernus/male	CBA1, CBA2/Py, France	*Apodemus sylvaticus*	2	492	LR760739
C. n. dobyi/male	CND2-8/Py, France	*Apodemus sylvaticus*	7	492	LR760740
Ctenophthalmus sp./female	CT33-37/Py, France	*Apodemus sylvaticus*, *Crocidura russula*	5	492	LR760737
Cox1					
C. a. catalaniensis /male	CAC2- CAC3/Py, France	*Apodemus sylvaticus*	2	453	LR760763
C. a. catalaniensis /male	CAC4-CAC5/Py, France	*Apodemus sylvaticus*	2	453	LR760764
C. b. arvernus/male	CBA1, CBA2/Py, France	*Apodemus sylvaticus*	2	453	LR760761-62
C. n. dobyi/male	CND2-8/Py, France	*Apodemus sylvaticus*	7	453	LR760754-60
Ctenophthalmus sp./female	CT33-37/Py, France	*Apodemus sylvaticus*, *Crocidura russula*	5	453	LR760749-53
Cytb					
C. a. catalaniensis/male	CAC 2-5/Py, France	*Apodemus sylvaticus*	4	374	LR760789
C. b. arvernus/male	CBA1/Py, France	*Apodemus sylvaticus*	1	374	LR760787
C. b. arvernus/male	CBA2/Py, France	*Apodemus sylvaticus*	1	374	LR760788
C. n. dobyi/male	CND2, CND3	*Apodemus sylvaticus*	2	374	LR760783
C. n. dobyi/male	CND4, CND6, CND7/Py, France	*Apodemus sylvaticus*	3	374	LR760784
C. n. dobyi/male	CND5/Py, France	*Apodemus sylvaticus*	1	374	LR760785
C. n. dobyi/male	CND8/Py, France	*Apodemus sylvaticus*	1	374	LR760786
Ctenophthalmus sp./female	CT34, CT35, CT36/Py, France	*Apodemus sylvaticus*, *Crocidura russula*	3	374	LR760782
Ctenophthalmus sp./female	CT33/Py, France	*Apodemus sylvaticus*	1	374	LR760780
Ctenophthalmus sp./female	CT37/Py, France	*Apodemus sylvaticus*	1	374	LR760781
EF1-α					
C. a. catalaniensis /male	CAC2-5/Py, France	*Apodemus sylvaticus*	4	976	LR760808-11
C. b. arvernus/male	CBA1, CBA2/Py, France	*Apodemus sylvaticus*	2	976	LR760806-07

Table 1 (continued)

Species/gender	Sample ID/geographical area	Host	Number of fleas	Base pairs (bp)	Accession number
C. n. dobyi/male	CND2-7/Py, France	*Apodemus sylvaticus*	6	976	LR760800-05
Ctenophthalmus sp./female	CT33-37/Py, France	*Apodemus sylvaticus, Crocidura russula*	5	976	LR760812-16
Ctenophthalmus sp./female	CT26, CT28-31/Asturias, Spain	*Arvicola scherman*	5	976	LR743659-63
C. b. boisseauorum/male	CBB23-24, CBB26, CBB28, CBB31-34/Asturias, Spain	*Arvicola scherman*	8	976	LR743651-58
C. a. allani/male	CAA1, CAA5-7, CAA16-17, CAA76-77/Asturias, Spain	*Arvicola scherman*	8	976	LR743643-50
C. felis/male and female	481, 645/Spain, 491, 589/South Africa	*Canis lupus familiaris*	4	976	LR743994-97
C. canis/female	212/Irán	*Canis lupus familiaris*	1	976	LR743998
N. barbarous/male	NB13/Canary Island, Spain	*Rattus* sp.	1	976	LR744000
N. fasciatus/male	NF50/France	*Crocidura russula*	1	975	LR743999
A. erinacei/male and female	AE16, AE19/Spain, AE20/France	*Erinaceus europaeus*	3	976	LR744008-10
P. irritans/male and female	PI13-14/Spain, PI33, PI35/Argentina	Human, red fox	4	976	LR744002-05
S. t. tripectinata	9AZ, 25AZ/ Canary Island, Spain	*Mus musculus*	2	976	LR744006-07
P. meridionalis	PM1, PM4-5/Spain	-	3	976	LR744012-14

Genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol and was checked using an electrophoresis in a 0.8% agarose gel infused with SYBR Safe.

All molecular markers sequenced in the present study (*EF1-α*, ITS1 and ITS2 rDNA, *cox*1, and *cyt*b mtDNA) were amplified by a polymerase chain reaction (PCR) using a thermal cycler (Eppendorf AG; Eppendorf, Hamburg, Germany). PCR mix, PCR conditions, and PCR primers are summarized in the Supporting information (Table S1).

Initially, we amplified a 658-bp fragment of *cox*1, the so-called barcoding fragment that can serve as the core of a global bioidentification system for animals (Hebert et al. 2003). For this purpose, we used the generic invertebrate amplification primers LC01490 and HC02198 (Folmer et al. 1994); however, we did not obtain reliable results owing to co-amplification of nonspecific products. For that reason, we finally used Kmt6 primer (Zhu et al. 2015) as a forward to amplify the *cox*1 partial gene (453 pb), whereas HC02198 remained as reverse primer for this partial gene. The *EF1-α*, ITS1, ITS2, *cox*1, and *cyt*b partial gene sequences obtained from all specimens analyzed were deposited in the GenBank database (Table 1).

The PCR products were checked on SYBR Safe stained 2% Tris–borate–ethylenediaminetetraacetic acid 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, USA). Once purified, the products were sequenced by Stab Vida (Lisbon, Portugal). To obtain a nucleotide sequence alignment file, the MUSCLE alignment method (Edgar 2004) was used in MEGA, version 5.2 (Tamura et al. 2011). To assess the similarity among all marker sequences of all specimens analyzed in the present study and other flea species, the number of base differences per sequence was assessed using the number of differences method of MEGA, version 5.2 (Tamura et al. 2011).

Phylogenetic trees were inferred using nucleotide data and performed using two methods: maximum likelihood (ML) and Bayesian inferences (BI). Maximum likelihood trees were generated using the PHYML package from Guindon and Gascuel (2003), whereas Bayesian inferences were generated using MRBAYES, version 3.2.6 (Ronquist and Huelsenbeck 2003). JMODELTEST (Posada 2008) was used to determinate the best-fit substitution model for the parasite data (*EF1-α*, ITS2, *cox*1, and *cyt*b). Models of evolution were chosen for subsequent analyses according to the Akaike information criterion (Huelsenbeck and Rannala 1997; Posada and Buckley 2004). To investigate the dataset containing the concatenation of four markers (*EF1-α*, ITS2, *cox*1, and *cyt*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 a general time-reversible model with gamma-distributed rate variation GTR+G (ITS2), a Tamura-

Nei model with gamma-distributed rate variation and a proportion of invariable sites, TrN+I+G (*cox*1) and a general time-reversible model with gamma-distributed rate variation and a proportion of invariable sites, GTR+I+G (*EF1-α*, *cyt*b). 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 Bayesian inference, were *nst* =6 with gamma rates (ITS2) and *nst* =6 with invgamma rates (*EF1-α*, *cox*1, and *cyt*b). For BI, the standard deviation of split frequencies was used to determine whether the number of generations completed was enough; the chain was sampled every 500 generations and each dataset was run for 10 million generations. Adequacy of sampling and run convergence was assessed using the effective sample size diagnostic in tracer, version 1.6 (Rambaut and 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) comprise the significance scores for the nodes.

The phylogenetic analyses, based on single markers *EF1-α*, ITS2, *cox*1, and *cyt*b sequences, were carried out using our sequences and those obtained from GenBank database (see Table S2). Phylogenetic trees were rooted including *Panorpa meridionalis* (Mecoptera: Panorpidae) as outgroup. This choice was based on the combination of morphological and molecular data obtained in previous studies, which provided compelling evidence for a sister group relationship between Mecoptera and Siphonaptera (Whiting 2002; Whiting et al. 2008). The ITS1 sequence of *P. meridionalis* or other species of Mecoptera was not available either by amplification of different individuals or in any public database. Thus, no phylogenetic tree with other Siphonaptera species based on ITS1 sequences was constructed, discarding this marker for the concatenated dataset.

Results

Morphological and biometrical results

All the specimens studied in this work showed morphological characteristics expected for the genera *Ctenophthalmus*. All these characters were described in detail by Zurita et al. (2020).

Males could be easily discriminated among three subspecies (*C. a. catalaniensis*, *C. b. arvernus*, and *C. n. dobyi*).

Males of *C. a. catalaniensis* showed specific morphological characters:

• Apex of the distal arm of IX sternum without an apical slot (Fig. 1A).

• Distal arm of IX sternum with parallel margins (Fig. 1A).

• Dorsal process basimere is slightly longer than wide with two long setae with the same length each other (Fig. 1B).

• Ventral process basimere is as longer as wide showing a small apical slot close to the beginning of the dorsal margin (Fig. 1C).

• Telomere with a narrow apex, carrying one curved long seta located near to the apex in dorsal margin. (Fig. 1D).

Males of *C. b. arvernus* showed specific morphological characters:

• Arm of the distal branch of IX sternum without an apical slot (Fig. 2A).

• Apical part of distal arm of IX sternum with parallel margins (Fig. 2A).

• Dorsal process basimere is significantly longer than wide with only one long seta (Fig. 2B).

• A narrow ventral process basimere, longer than dorsal processus basimere and with an apical slot on the apex (Fig. 2C).

Males of *C. n. dobyi* showed specific morphological characters:

• Distal arm of IX sternum thumb-shaped without an apical slot in the apex (Fig. 2D).

• Dorsal process basimere is significantly longer than wide with three setae two of them long with the same length (Fig. 2E).

• Ventral process basimere is as longer as wide with a big posterior lobe with a triangular shape (Fig. 2F).

The morphology of females was studied focusing on the spermatheca's shape and the chaetotaxy and shape of the margin of the sternum VII; however, we did not find any morphological criteria to discriminate among females of *Ctenophthalmus* sp. collected for this study. The spermatheca always showed a hilla shorter and narrower than bulga showing a small prominence at the end in some specimens. According to chaetotaxy, all specimens showed the presence of six setae with different degree of development in sternum VII. In addition, the presence of three strong setae was common, longer than the other ones, which appeared very close to each other. With all these morphological results, we were not able to set up any taxonomical differentiation for female discrimination. All these female morphological traits were described in detail by Zurita et al. (2020) (see pictures of female in this paper).

Molecular results

EF1-α, ITS1, and ITS2 analysis

The length of the ITS1 sequences of *Ctenophthalmus* specimens ranged from 888 base pairs (bp) (*C. b. arvernus* and *C. n. dobyi* males and some *Ctenophthalmus* sp. females) to 889 bp (*C. a. catalaniensis* males and some *Ctenophthalmus* sp. females). On the other hand, the length of the ITS2 and
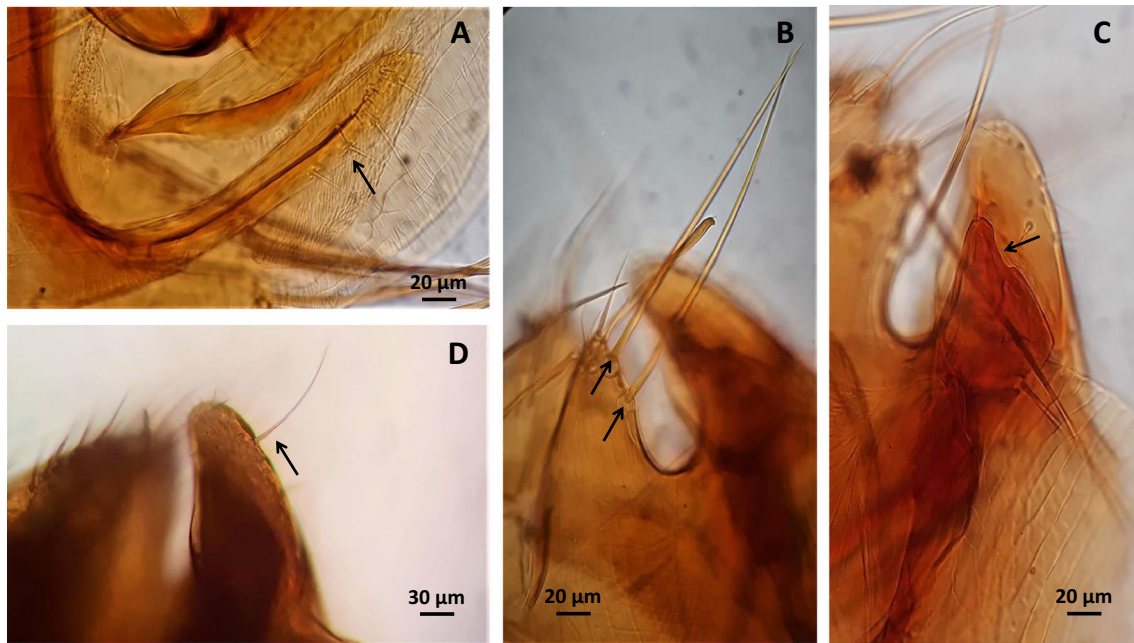
Fig. 1 Morphological characteristics of males of *C. a. catalaniensis*. A—Distal arm of IX sternum (arrowed); B—dorsal process basimere with two long setae (arrowed); C—ventral process basimere with an apical slot (arrowed); D—telomere carrying one curved long setae (arrowed)

EF1-α fragment was 492 bp and 976 bp, respectively, for all the specimens (Table 1). The intrageneric similarity ranged from 99.6 to 100% for ITS1 and ITS2 with a maximum of two different base pairs among all the sequences analyzed for both markers, including *C. b. boisseauorum* and *C. a. allani* (table not shown). According to *EF1-α*, the similarity observed for each *Ctenophthalmus* subspecies ranged from

96.7% (*C. a. allani*) to 99.5% (*C. a. allani* and *C. n. dobyi*). Furthermore, if we compare all the *EF1-α* sequences from *Ctenophthalmus* spp. provided in this study, they showed high level of similarity with each other with values about 97–99%. On the other hand, when we compared our sequences with those *Ctenophthalmus* species and subspecies retrieved from
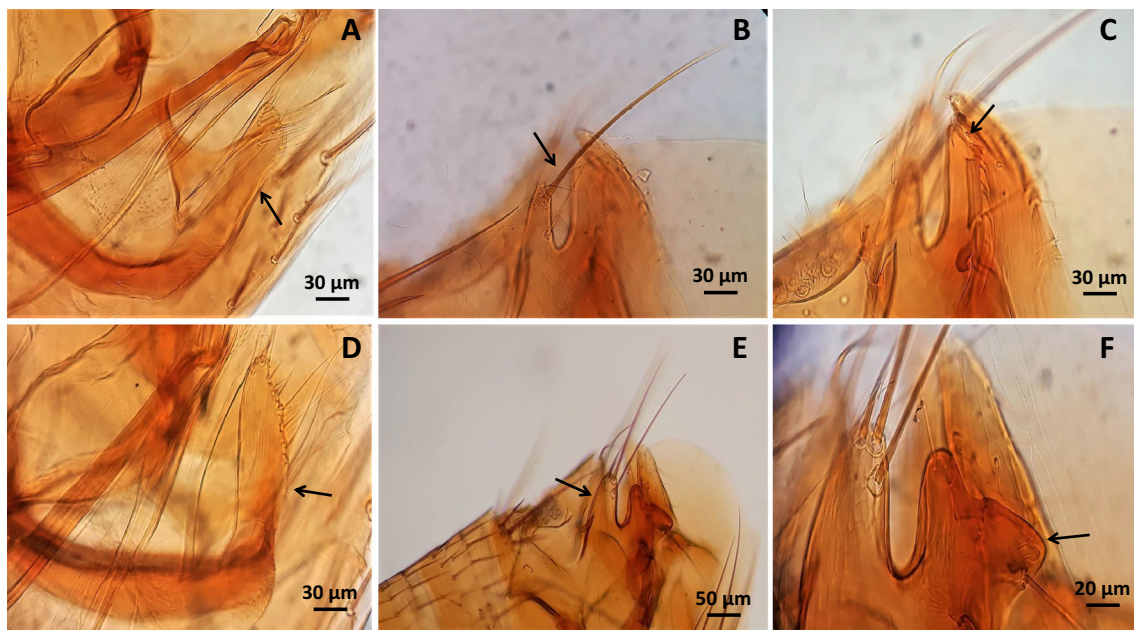

Fig. 2 Morphological characteristics of males of *C. b. arvernus* and *C. n. dobyi*. A—*C. b. arvernus* distal arm of IX sternum (arrowed); B—*C. b. arvernus* dorsal process basimere with only one long setae (arrowed); C—*C. b. arvernus* ventral process basimere with an apical slot (arrowed); D—*C. n. dobyi* distal arm of IX sternum (arrowed); E—*C. n. dobyi* dorsal process basimere (arrowed) with two long setae; F—*C. n. dobyi* ventral process basimere with a triangular posterior lobe (arrowed)

Table 2 Intra-subspecific* and inter-subspecific similarity observed among all the *EF1-α* gene sequences of different species and subspecies belonging to *Ctenophthalmus* sp. obtained in this work and retrieved from GenBank database. Values are given in percentages

EF-1α	C. a. allani	C. b. boisseauorum	C. n. dobyi	C. a. catalaniensis	C. b. arvernus	C. cryptotis KM890508	C. pseudagyrtes AF423842	C. c. congeneroides KM890489	C. sanborni EU336280
C. a. allani	96.7–99.5*								
C. b. boisseauorum	96.5–98.3	97.5–99.2*							
C. n. dobyi	96.8–98.3	97.5–98.7	98.3–99.5*						
C. a. catalaniensis	96.8–98.0	97.4–98.4	98.0–99.3	98.5–99.1*					
C. b. arvernus	96.6–97.5	97.5–98.4	98.3–99.0	98.1–98.9	99.0*				
C. cryptotis KM890508	90.2–90.8	90.4–90.8	90.4–91.1	90.1–90.9	91.0–91.1	-			
C. pseudagyrtes AF423842	90.3–91.2	90.7–91.4	90.7–91.6	90.4–91.0	91.4–91.5	96.5	-		
C. c. congeneroides KM890489	91.2–91.8	91.1–92.2	91.6–92.0	91.0–91.9	92.2–92.3	93.7	93.2	-	
C. sanborni EU336280	90.3–90.8	90.2–90.8	90.0–91.2	90.1–90.9	90.9–91.0	96.7	96.2	92.9	-

GenBank database, these values appeared quite lower (about 91–92%) (see Table 2).

Partial *cox*1 and *cyt*b mtDNA gene analysis

The partial gene *cox*1 and *cyt*b mtDNA sequences of *Ctenophthalmus* male and female specimens amplified in our study were 453 bp and 374 bp in length, respectively (Table 1). Intra-subspecific percentage of similarity observed for both markers ranged from 97.4% (*cox*1 of *C. n. dobyi*) to 100% (*cyt*b of all subspecies excepting *C. b. arvernus*) (see Tables 3 and 4). Based on the comparison of *cox*1 and *cyt*b sequences, we observed that sequences of those taxa belong to *Ctenopthalmus* subgenus (*C. n. dobyi*, *C. a. catalaniensis*, *C. b. arvernus C. a. allani*, and *C. b. boisseauorum*) showed each other high percentages of inter-subspecific similarity, ranging from 95.8 to 100% (Tables 3 and 4). In contrast to that, when these five subspecies were compared with another

Table 3 Intra-subspecific* and inter-subspecific similarity observed among all the partial *cox*1 mtDNA sequences of different species and subspecies belonging to *Ctenophthalmus* sp. obtained in this work and retrieved from GenBank database. Values are given in percentages

Cox1	C. a. allani LR594440–LR594446	C. b. boisseauorum LR594447–LR594451, LR594456	C. n. dobyi	C. a. catalaniensis	C. b. arvernus	C. c. cabirus MH142441	C. cryptotis KM890939	C. c. congeneroides KM890918	C. d. dolichus MF000657
C. a. allani LR594440–LR594446	98.7–100*								
C. b. boisseauorum LR594447–LR594451, LR594456	98.7–100	99.3–100*							
C. n. dobyi	95.8–98.5	96.2–98.9	97.4–99.9*						
C. a. catalaniensis	98.2–98.9	98.5–99.3	96.2–99.1	99.6–100*					
C. b. arvernus	98.9–99.6	99.1–99.6	96.0–98.2	98.5–99.3	99.3*				
C. c. cabirus MH142441	85.7–86.3	86.1–86.3	85.4–86.5	86.1–86.3	85.4–85.9	-			
C. cryptotis KM890939	89.4–90.1	89.6–90.1	88.3–89.6	89.8–90.1	89.4–89.8	85.9	-		
C. c. congeneroides KM890918	88.1–88.5	88.1–88.5	87.1–88.3	87.4–87.6	87.4–87.6	87.7	88.1	-	
C. d. dolichus MF000657	86.8–87.4	86.8–87.4	87.0–87.6	86.5–87.0	87.0–87.6	85.0	90.1	87.4	-

Table 4 Intra-subspecific* and inter-subspecific similarity observed among all the partial *cyt*b mtDNA sequences of different species and subspecies belonging to *Ctenophthalmus* sp. obtained in this work and retrieved from GenBank database. Values are given in percentages

Cytb	C. a. allani LR594464– LR594467	C. b. boisseauorum LR594468– LR594477	C. n. dobyi	C. a. catalaniensis	C. b. arvernus	C. cryptotis KM890672	C. c. congeneroides KM890651	C. sanborni KM890607
C. a. allani LR594464–LR594467	98.7–100*							
C. b. boisseauorum LR594468–LR594477	98.4–100	98.7–100*						
C. n. dobyi	96.5–98.4	96.5–97.9	99.2–100*					
C. a. catalaniensis	98.9–99.5	98.9–99.5	97.6–98.4	100*				
C. b. arvernus	99.2–100	98.4–100	96.8–98.1	99.2	99.5*			
C. cryptotis KM890672	86.1–86.4	86.1–86.6	86.1–86.6	86.6	85.8–86.4	-		
C. c. congeneroides KM890651	85.3–86.1	85.3–85.8	86.4–86.9	85.8	85.6–86.1	85.6	-	
C. sanborni KM890607	85.0–85.3	84.8–85.6	84.5–84.8	85.3	85.3	88.2	85.0	-

congeneric taxa, retrieved from GenBank database, the similarity percentages were always lower than 90% for both markers (Tables 3 and 4).

Phylogenetic results

The phylogenetic trees inferred from each single marker (ITS2, *EF1-α*, *cox*1, and *cyt*b) sequences (see Table S2 showed all the *Ctenophthalmus* specimens (males and females) assessed in this study clustered together in polytomy with high BPP and bootstrap values (Fig. S1-S4). Regarding *EF1-α*, *cox*1, and *cyt*b phylogenetic analysis, all these *Ctenophthalmus* subspecies comprised an independent clade separated from the remaining *Ctenophthalmus* congeneric species (Fig. S2–S4). In addition, for *EF1-α* phylogenetic analysis, we noticed a low supported subclade (64/88 BPP and bootstrap values) clustering *C. b. boisseauorum* and *C. a. allani* (collected from Asturias, Spain) (Fig. S2). On the other hand, *cox*1 phylogenetic tree showed *C. a. catalaniensis* and *C. n. dobyi* closely related to each other setting up a phylogenetic subclade (Fig. S3), whereas for *cyt*b phylogenetic analysis, *C. n. dobyi* clustered distant from *C. b. boisseauorum*, *C. a. allani*, *C. a. catalaniensis*, and *C. b. arvernus* (Fig. S4).

The concatenated dataset of *EF1-α*, ITS2, partial *cyt*b and *cox*1 gene sequences included 2200 aligned sites and 41 taxa, including five different *Ctenophthalmus* subspecies (*C. n. dobyi*, *C. b. boisseauorum*, *C. a. allani C. a. catalaniensis*, and *C. b. arvernus*) and outgroups (*A. erinacei*). Phylogenetic analysis of the concatenated dataset yielded a tree with strongly supported nodes (Fig. 3). This analysis was not in concordance with all trees constructed based on the single markers since *Ctenophthalmus* subspecies appeared clearly separated in different clades (Fig. 3). Therefore, male specimens of *C. n. dobyi* clustered together and phylogenetically distant from the other four subspecies, whereas *C. b. boisseauorum* and *C. a. allani* appeared closely related comprising two main subclades (100/73 BPP and bootstrap values). Nevertheless, two specimens of *C. b. boisseauorum* clustered within "*C. a. allani* subclade." On the other hand, *C. b. arvernus* taxa set up an independent clade phylogenetically close to "*C. b. boisseauorum* and *C. a. allani* complex" (Fig. 3). *C. a. catalaniensis* specimens did not set up any specific clade neither appear phylogenetically related with any *Ctenophthalmus* subspecies. Lastly, *Ctenophthalmus* female specimens clustered separately each other appearing dispersed in the different clades throughout concatenated phylogenetic tree (Fig. 3).

Discussion

The accurate taxonomic identification of an organism constitutes the cornerstone of most aspects of arthropods science. The lack of molecular data in the taxonomic characterization of arthropods can result in many difficulties, especially in certain groups as Siphonaptera that have demonstrated both cryptic and polymorphic species (Lawrence et al. 2014; Zurita et al. 2018a; Zurita et al. 2019). The necessity to study, comparatively, morphological and molecular approaches within Siphonaptera Order has been reported by several authors in the last years (Whiting et al. 2008; Marrugal et al. 2013; Lawrence et al. 2014; Zurita et al. 2018b). *Ctenophthalmus* genus is considered the largest one within Siphonaptera Order (Beaucournu and Loverlec 2014); however, only morphological data are available in order to identify and discriminate species and subspecies of this group. Recently, Zurita et al.
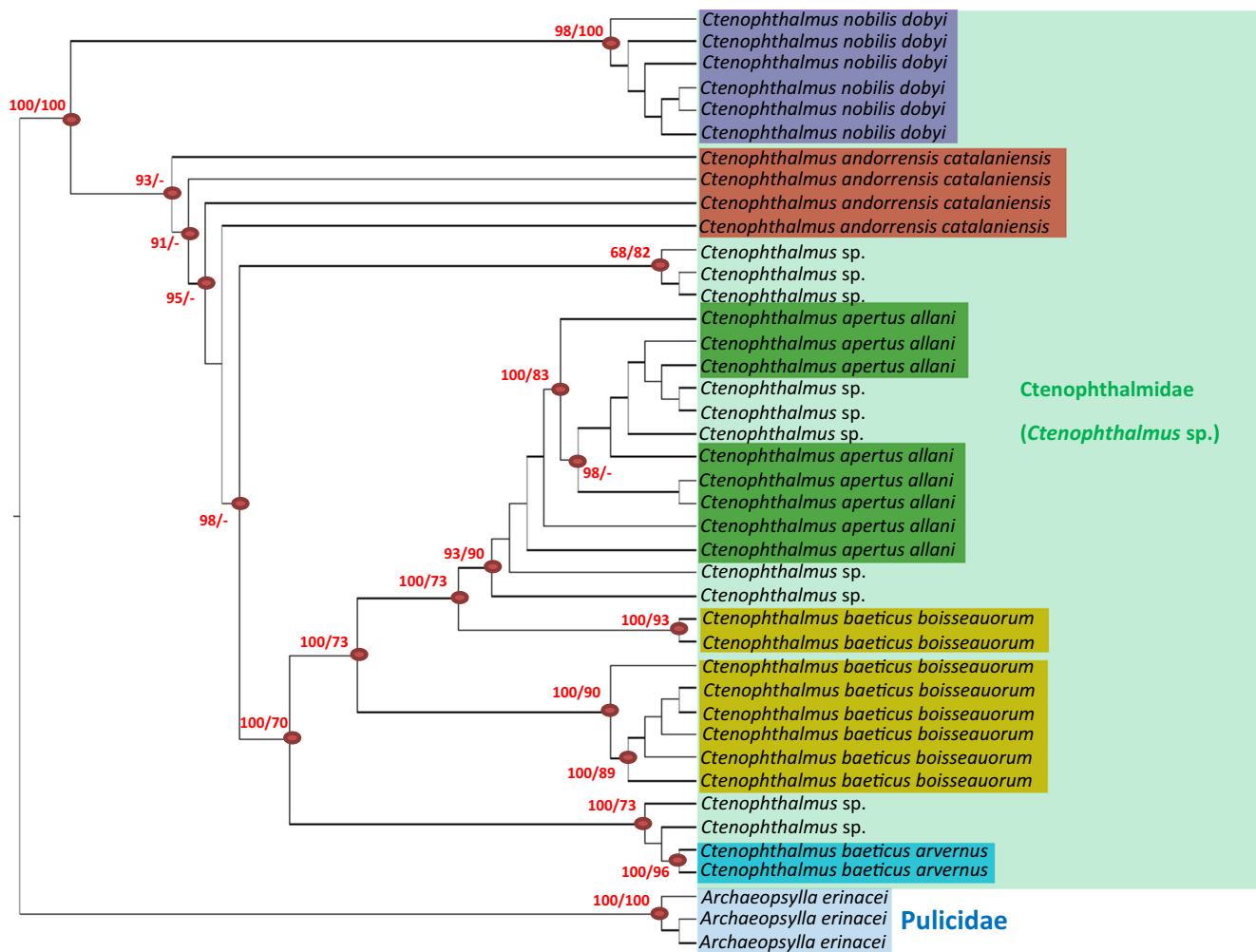
Fig. 3 Phylogenetic tree of males of *C. a. catalaniensis*, *C. b. arvernus*, *C. n. dobyi*, *C. b. boisseauorum*, and *C. a. allani* and *Ctenophthalmus* sp. females assessed in this study (see Table 1). This analysis was based on concatenated sequences of *Elongation Factor 1 alpha* (*EF1-α*), Internal Transcribed Spacer 2 (ITS2), partial *cytochrome c-oxidase* subunit 1 (*cox1*), and *cytochrome* b (*cytb*) gene of mitochondrial DNA inferred using the Bayesian inference (BI) and maximum likelihood (ML) methods and Bayesian topology. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown on the branches (BPP/Bootstrap). The Bayesian Posterior Probabilities (BPP) are percentage converted

(2020) claimed for further molecular and phylogenetic studies for this genus, especially aimed to discriminate among female specimens, which could not be morphologically identified at species and subspecies levels. Furthermore, morphological keys so far available for *Ctenophthalmus* male identification are difficult to use, remaining problematic or impossible for non-specialists (Beaucournu and Launay 1990).

Our results agree with Beaucournu and Loverlec (2014) in relation to the geographical distribution and main hosts of *C. n. dobyi*, *C. a. catalaniensis*, and *C. b. arvernus*. Thus, these three subspecies were found parasitizing *Apodemus* sp. in the eastern Pyrenees, demonstrating that these taxa are well settled in this geographical area. Furthermore, it is important to highlight that the distribution of *C. n. dobyi* is enclosed only to the eastern Pyrenees region whereas the other two subspecies (*C. a. catalaniensis* and *C. b. arvernus*) could be also found parasitizing rodents from any part of the Iberian

Peninsula or the south of France (*C. b. arvernus*) (Beaucournu and Launay 1990; Beaucournu and Loverlec 2014).

From a morphological point of view, our results confirmed the high levels of morphological variation observed in *Ctenophthalmus* male genitalia previously observed by several authors (Hopkins and Rothschild 1966; Beaucournu and Launay 1990; Gómez et al. 2003; Zurita et al. 2020). Thus, we identified three different subspecies (*C. a. catalaniensis*, *C. b. arvernus*, and *C. n. dobyi*); however, morphological study of females did not show any morphological specific pattern based on the spermatheca and the chaetotaxy and shape of the sternum VII. The lack of variability found in these regions agrees with Zurita et al. (2020) who also provided biometrical data for males of *C. b. boisseauorum* and *C. a. allani* even for *Ctenophthalmus* females, but this tool was not useful in order to identify female species. For this reason,

together with the lack of male specimens of some subspecies, we discarded biometrical analysis at the present work, focusing our efforts to assess the utility of different molecular markers to discriminate among different *Ctenophthalmus* species.

The combination of nuclear and mitochondrial markers to solve taxonomical problems of identification in different arthropods groups has demonstrated to be a useful tool in several cases (Xiao et al. 2010; Derocles et al. 2012; Thielsch et al. 2017). Thus, the concatenation of both groups of markers has been widely implemented for taxonomic and phylogenetic studies within Siphonaptera Order in the last 15 years (Whiting et al. 2008; Zurita et al. 2015; Hornok et al. 2018; Zurita et al. 2019). Currently, mitochondrial DNA *cox*1 partial gene is considered the DNA barcoding used to identify several arthropods species using nucleotide sequences from a homologous fragment of this gene (Xiao et al. 2010). This approach provided by Hebert et al. (2003) can be both fast and cost-effective, and the obtained data are easily standardized and compared across taxa (Xiao et al. 2010); however, the exclusive use of *cox*1 barcoding shows some limitations. For example, we can find technical and analytical problems with *cox*1 marker due to the presence of mitochondrial genes integrated into the nuclear genomes (Bensasson et al. 2001). Other limitation of barcoding DNA is the fact that the patterns of diversity and evolution of this gene can be strongly influenced by *Wolbachia* endosymbiont. This endosymbiont has been widely amplified in fleas (Dittmar and Whiting 2003; Heukelbach et al. 2004) reporting some cases of introgressive hybridization such as those discovered by Zurita et al. (2016) between *Ctenocephalides felis* and *Ctenocephalides canis*. For this reason, additional evidence, like the analyses of nuclear markers, or morphology, is mandatory (Thielsch et al. 2017).

Zurita et al. (2020) combined ITS1 and ITS2 rDNA together with *cox*1 and *cyt*b mtDNA in order to establish molecular and phylogenetic specific pattern of discrimination between *C. b. boisseauorum* and *C. a. allani* collected from the same geographical origin and the same host. Nevertheless, these authors found that morphological differences observed in males of *C. b. boisseauorum* and *C. a. allani* did not correspond with molecular and phylogenetic data, suggesting that both subspecies could be considered synonymous taxa. In the present work, we include the study of *EF1-α* as complementary nuclear marker of *C. a. catalaniensis*, *C. b. arvernus*, *C. n. dobyi*, *C. b. boisseauorum*, and *C. a. allani*. This marker has been widely used in fleas' phylogeny for several purposes such as the evaluation of the molecular diversification among different biogeographical lineages (Van der Mescht et al. 2015), or to assess the levels of genetic diversity within *C. felis* (Lawrence et al. 2019). Furthermore, *EF1-α*, *cox*1, or *cyt*b nucleotide markers have showed high level of nucleotide divergence among different geographical population of fleas (Van der Mescht et al. 2015; Hornok et al. 2018; Zurita et al. 2019).

In our study, nucleotide similarity observed for each single nuclear and mitochondrial marker did not correspond with the morphological differences observed among males of these *Ctenophthalmus* subspecies analyzed in our study. Therefore, intra- and inter-subspecific percentages of similarity observed overlapped each other among *Ctenophthalmus* taxa (*C. n. dobyi*, *C. a. catalaniensis*, *C. b. arvernus*, *C. b. boisseauorum*, and *C. a. allani*). On the other hand, these five subspecies, classified into *Ctenophthalmus* subgenus, showed higher level of mitochondrial and nuclear nucleotide divergence with other congeneric species belonging to another subgenus such as *C. sanborni* and *C. pseudagyrtes* (*Nearctoctenophthalmus* subgenus), *C. calceatus* (*Ethioctenophthalmus* subgenus), *C. dolichus* (*Paractenophthalmus* subgenus), or *C. congeneroides* (*Euctenophthalmus* subgenus). This nucleotide pattern of divergence was corroborated by phylogenetic analysis separately based on each single marker (ITS2, *EF1-α*, *cox*1, and *cyt*b). Therefore, all the subspecies belonging to *Ctenophthalmus* subgenus appeared closely related to each other setting up a monophyletic clade clearly separated from the remaining congeneric species. At this point, we should remind the fact that *Ctenophthalmus* subgenus mainly has a Western Palearctic distribution; however, those species retrieved from GenBank in our study belong to different subgenus distributed outside European region (Eastern Palearctic, Nearctic, Neotropical, and Afrotropical area) (Hopkins and Rothschild 1966; Peus 1977; Beaucournu and Loverlec 2014).

In contrast to these results based on each single marker, the concatenated phylogenetic analysis was in concordance with morphological differences observed among male specimens. Thus, the combination of certain nuclear and mitochondrial markers could be considered a useful taxonomic tool in order to infer phylogenetic relationships within *Ctenophthalmus* genus and to discriminate at species and subspecies levels within this group of fleas. Based on concatenated dataset, we could notice a clear phylogenetic distance between *C. n. dobyi* and the other *Ctenophthalmus* subspecies assessed in this study. Furthermore, it seems that the phylogenetic synonym between *C. b. boisseauorum* and *C. a. allani* observed by Zurita et al. (2020) could be solved by the use of combinative analysis of *EF1-α* and mitochondrial markers since most specimens of both subspecies appeared clearly separated in two subclades. Despite that, in the light of our results, a new phylogenetic issue should be inquired into *C. baeticus* and *C. apertus* complex in order to solve why *C. a. allani* still appear phylogenetically closer to *C. b. boisseauroum* than other conspecific taxa, such as *C. b. arvernus*. This fact could be a possible phylogeographic phenomenon due to an isolation process of these subspecies (collected from Asturias). Pyrenees Mountain could constitute a geographical obstacle for *Ctenophthalmus* subspecies and its hosts (*Arvicola sherman*) collected from Asturias since it has

been demonstrated that the presence of certain geographical barriers can induce lower levels of genetic variation in an isolated population of fleas (Lin et al. 2014; Zurita et al. 2015).

To summarize, this work constitutes the first study that provides a combination of morphological, molecular, and phylogenetic comparative data of three subspecies (*C. n. dobyi*, *C. a. catalaniensis*, and *C. b. arvernus*) in order to assess their taxonomic and phylogenetic relationships. In concordance with previous authors (Hopkins and Rothschild 1966; Beaucournu and Launay 1990; Zurita et al. 2020), we conclude that just the study of morphological traits in *Ctenophthalmus* genus is not able to discriminate at species and subspecies levels in females; therefore, molecular and phylogenetic data are needed in order to solve this taxonomical issue. We evaluated the efficiency of five different molecular markers (ITS1, ITS2, *EF1-α*, *cox*1, and *cyt*b) to discriminate among *Ctenophthalmus* subspecies. In this sense, although ITS1 and ITS2 have demonstrated their utility to discriminate among congeneric species in fleas (Vobis et al. 2004; Marrugal et al. 2013), we suggest that these two rDNA markers should be discarded for specific identification within *Ctenophthalmus* species. We based this idea on the lack of nucleotide divergence observed for these two markers for all the subspecies analyzed in this study (with a maximum of two different base pairs). On the other hand, the use of these five molecular markers individually could not discriminate among all these subspecies; however, this paper demonstrates, by the first time, that the combination of the nuclear marker *EF1-α* together with mtDNA markers *cyt*b and *cox*1 could constitute a useful taxonomical and phylogenetic tool in order to discriminate among different *Ctenophthalmus* species and subspecies. This molecular approach, provided in this study, is especially relevant for female specimens belonging to this genus, which cannot be classified with morphological techniques or even for a possible discrimination between *C. a. allani* and *C. b. boisseauroum*, which had recently been suggested as synonymous taxa (Zurita et al. 2020). Lastly, although we just could include five different *Ctenophthalmus* taxa, we consider that the use of these molecular markers together should be gradually used within *Ctenophthalmus* genus where most taxa have been only described from a morphological point of view. Thus, it would be necessary to complement these classic taxonomical data with phylogenetic studies based on molecular data in order to clarify the complex taxonomy of not only *Ctenophthalmus* genus but also the whole Ctenophthalmidae family.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13127-021-00500-4.

Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets generated during and/or analyzed during the current study are also available from the corresponding author on reasonable request.

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