Ctenophthalmus baeticus boisseauorum (Beaucournu, 1968) and *Ctenophthalmus apertus allani* (Smit, 1955) (Siphonaptera: Ctenophthalmidae) as synonymous taxa. Morphometric, phylogenetic and molecular characterization.

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Abstract

The family Ctenophthalmidae (Order Siphonaptera) has been considered as a ``catchall´´ for a wide range of divergent taxa showing a paraphyletic origin. In turn, *Ctenophthalmus* sp. (Ctenophthalmidae) includes 300 valid described taxa. Within this genus, males are easily distinguishable basing on the size, shape and chaetotaxy of their genitalia; however, females show slight morphological differences each other. The main objective of this work was to carry out a comparative morphometric, phylogenetic and molecular study of two different subspecies: *Ctenophthalmus baeticus boisseauorum* and *Ctenophthalmus apertus allani* in order to clarify and discuss its taxonomic status. From a morphological and biometrical point of view we found clear differences between modified abdominal segments of males of both subspecies and slight differences in the margin of sternum VII of all female specimens which did not correspond with molecular and phylogenetic results based on four different molecular markers (Internal Transcribed Spacer 1 and 2 of ribosomal DNA, and the partial cytochrome *c* oxidase subunit 1 and cytochrome *b* of mitochondrial DNA). Thus, we observed a phenotypic plasticity between both subspecies, which did not correspond with a real genotypic variability nor different environmental or ecological conditions. Basing on these results we could consider that there are no solid arguments to consider these two "morphosubspecies" as two different taxa. We propose that *C. b. boisseauorum* should be considered as a junior synonym of *C. a. allani*.

Introduction

In the last years, the number of taxonomic studies of fleas based on molecular and phylogenetical data is increasing; however, most genera, species and subspecies have been described just using morphological criteria. Ctenophthalmidae family has been considered as a ``catchall´´ for a wide range of divergent taxa showing a paraphyletic origin (Whiting *et al*., 2008). The family Ctenophthalmidae (sensu Lewis, 1993) consists of nine subfamilies and 17 described tribes, with 42 genera and 664 species (Whiting *et al*., 2008). This high number of species corresponds approximately with one-quarter of flea species described up until now.

Morphological identification of fleas is essentially based on the shape and structure of their complex genitalia and the distribution of setae, spines and ctenidia (Beaucournu & Launay, 1990). The modifications of the terminal abdominal segments of the male are much more complicated than in females. From a taxonomic point of view, the most important organ of male genitalia is the aedeagus. It is an extremely complex structure of obscure derivation and is seldom used in identification. Furthermore, associated structures derived from the terminal tergites and sternites are used too for taxonomic discrimination (Lewis, 1993). Sternum VIII of males, although it can be reduced in some species, have a great importance in terms of specific identification due to it encloses the remaining genital structures and it may bear modifications that are useful in identification, such as spicules and a characteristic chaetotaxy (Lewis, 1993). On the other hand, in females, sternum VII and VIII are usually well developed covering most if not all the terminal portion of the abdomen (Linardi, 2000). In most cases, the configuration, shape and chaetotaxy of the sternum VII caudal margin can be useful in taxonomic discrimination. Together with sternum VII, the spermatheca of females is considered the most important taxonomic character in order to identify and classify female fleas at

different taxonomical levels (Lewis, 1993; Beaucournu & Launay, 1990). The spermatheca is usually placed within sternum VII and is divided into a heavily sclerotized bulga and a less sclerotized finger-like projection, the hilla (Linardi, 2000). From a taxonomical point of view, in recent years, some species of the Ctenophthalmidae family have been studied mainly based on the morphological features mentioned above (Sanchez & Lareschi, 2014; Acosta & Hastriter, 2017; Keskin, 2019; Keskin & Beaucournu, 2019a) including the descriptions of two new species and a new subspecies of the genus *Ctenophthalmus* (Keskin & Beaucournu, 2019b)

Despite using these morphological structures as useful taxonomical tools, there are many cases where the specific identification of females can be more complicated, especially when they are isolated without males to compare them to. This is the case of the genus *Ctenophthalmus* whose males are easily distinguishable basing on the size, shape and chaetotaxy of their genitalia; however, females show slight morphological differences each other (Beaucournu & Launay, 1998). Therefore, the specific and subspecific determination within the genus *Ctenophthalmus* has been exclusively based on the male morphological characters due to the lack of morphological differences among females. These morphological differences of most species were so small and intraspecific variation so great that it seemed useless to attempt to make a taxonomical key for this sex (Lewis, 1993; Beaucournu & Launay, 1990).

Due to the inability of systematists to homologize characters adequately across fleas and outgroup taxa, different taxonomic studies have revealed the necessity to carry out an exhaustive revision in flea taxonomy combining morphological, molecular and phylogenetic data specially focused to species and subspecies level (Whiting *et al*., 2008; Zurita *et al*., 2018a, 2018b). This necessity is due to the fact that fleas show a high degree of morphological specializations associated with ectoparasitism. Therefore, fleas appear

to have many instances of parallel evolution of morphology, probably associated with multiple invasions of similar hosts, which further obscures homology (Holland 1964). This fact has been observed in different flea taxa in the last years, Marrugal *et al*. (2013) noticed that *Ctenocephalides felis* showed a certain degree of phenotypic plasticity which did not correspond with molecular differences. Recently, Zurita *et al*. (2018a) found that some morphological diagnostic characters historically used to discriminate between two congeneric species (*Nosopsyllus fasciatus* and *Nosopsyllus barbarus*) should be revised. Based on these precedents, the main objective of this work was to carry out a comparative morphometric, phylogenetic and molecular study of two different subspecies belonging to genus *Ctenophthalmus*: *Ctenophthalmus baeticus boisseauorum* (Beaucournu, 1968) and *Ctenophthalmus apertus allani* (Smit, 1955) in order to clarify the taxonomic status of these two subspecies. These species were chosen due to their morphological similarities as well as the fact that their shared the same host and were collected from the same geographical area. In order to carry out this work, Internal Transcribed Spacer (ITS) 1 and ITS2 of ribosomal DNA (rDNA) and the partial cytochrome *c* oxidase subunit 1 (*cox1*) and cytochrome *b* (*cytb*) of mitochondrial DNA (mtDNA) genes were sequenced and assessed.

Material and methods

Collection of samples

A total of eighty fleas were collected from rodents *Arvicola scherman* (Arvicolinae) from Asturias (North of Spain) (43°20′00″N 6°00′00″O) (Table 1). These fleas were obtained and previously classified with the assistance of colleagues (see Acknowledgements). Fleas obtained were kept in Eppendorf tubes with 70% ethanol for subsequent identification and DNA extraction.

Morphological identification and biometrical study

For morphological analysis, whole specimens were examined and photographed under an optical microscope. Subsequently, thirty fleas were put away for molecular purposes, whereas the rest of samples (fifty fleas) 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 & Rothschild (1953) and Beaucournu & Launay (1990). After morphological identification thirty males and twenty females were measured according to 16 different parameters for males and 12 different parameters for females (Tables 2 and 3). Descriptive univariate statistics (arithmetic means, standard deviation and coefficient of variation) for all parameters were determined using SPSS program version 24 (IBM Corp., Armonk, NY, U.S.A.) (Pardo & Ruiz, 2002). Furthermore, to assess phenotypic variations among the samples, morphometric data were explored using multivariate analysis in 9 measurements (LDBS9, WDBS9, WDPB, WVPB, DSETDPB, TL (Excluding PROTW, MESOW, METW), PROTW, MESOW, METW) in males (see

Table 2) and 11 measurements (BULGAL, BULGAW, APEHILL, DBMV, PS7L, TW, HL, HW, PROTW, MESOW, METW) in females (see Table 3) by principal component analysis (PCA), consisting in a method for summarizing most of the variations in a multivariate dataset in few dimensions (Dujardin & Le Pont, 2004). Phenotypic analyses were conducted using BAC v.2 software (Dujardin, 2002; Valero *et al*., 2009; García-Sánchez *et al*., 2019).

Molecular study

A total of thirty fleas were molecularly analyzed. We previously selected ten males of each subspecies (*C. b. boisseauorum* and *C. a. allani*) and ten females previously classified as *Ctenophthalmus* sp.

For DNA amplification each specimen (only those isolated for molecular purposes) were transferred to a 1.5 mL tube containing 180 µL of G2 lysis buffer (Qiagen, Hilden, Germany), and 20 µL of proteinase K (Qiagen, Hilden, Germany), and incubated at 56º C overnight. DNA extraction was performed with an EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer recommendations. Flea DNAs were then eluted in 100 µL of Tris EDTA buffer using the DNA extracting EZ1 Advanced XL Robot (Qiagen, Hilden, Germany). The DNA was either immediately used or stored at −20° C until molecular analysis. The DNA extracting EZI Advanced XL Robot was disinfected after each batch of extraction as per the manufacturer's recommendations, to avoid cross-contamination. All molecular markers sequenced in the present study (ITS1 and ITS2 rDNA, *cox1* and *cytb* 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). In the case of *cox1*, we initially tried to obtain a 658 bp fragment of this marker, the so-called barcoding fragment which can serve as the core of a global bioidentification system

for animals (Hebert *et al*., 2003). For this purpose, we initially used the generic invertebrate amplification primers LCO1490 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 *cox1* partial gene (453 pb) whereas, HC02198 remained as reverse primer for this partial gene. The ITS1, ITS2, *cox1* and *cytb* partial gene sequences obtained from all specimens analysed 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, U.S.A.). 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 analysed in the present study and other flea species, the number of base differences per sequence with respect to the sequences under investigation 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 & Gascuel (2003), whereas Bayesian Inferences were generated using MRBAYES, version 3.2.6 (Ronquist & Huelsenbeck, 2003). JMODELTEST (Posada, 2008) 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). To investigate the dataset

189 containing the concatenation of three markers (ITS2, *cox1* and *cytb*), analyses based on
190 BI were partitioned by gene and models for individual genes within partitions were those
191 selected by JMODELTEST. For ML inference, best-fit nucleotide substitution models
192 included a general time-reversible model with gamma-distributed rate variation GTR+G
193 (ITS2) and a Tamura-Nei model with gamma-distributed rate variation and a proportion
194 of invariable sites, TrN+I+G (*cox1* and *cytb*). Support for the topology was examined
195 using bootstrapping (heuristic option) (Felsenstein, 1985) over 1000 replications to assess
196 the relative reliability of clades. The commands used in MRBAYES, version 3.2.6 for
197 Bayesian inference were *nst* =6 with gamma rates (ITS2) and *nst* =6 with invgamma rates
198 (*cox1* and *cytb*). For BI, the standard deviation of split frequencies was used to determine
199 whether the number of generations completed was sufficient; the chain was sampled
200 every 500 generations and each dataset was run for 10 million generations. Adequacy of
201 sampling and run convergence were assessed using the effective sample size diagnostic
202 in tracer, version 1.6 (Rambaut & Drummond, 2007). Trees from the first million
203 generations were discarded based on an assessment of convergence. Burn-in was
204 determined empirically by examination of the log likelihood values of the chains. The
205 Bayesian posterior probabilities (BPP) comprise the percentage converted.
206 The phylogenetic analyses, based on ITS2, *cox1* and *cytb* sequences were carried out
207 using our sequences and those obtained from GenBank database (see Table S2).
208 Phylogenetic trees based on concatenated sequences of ITS2, *cox1* and *cytb* were rooted
209 including *Panorpa meridionalis* (Mecoptera: Panorpidae) as outgroup. This choice was
210 based on the combination of morphological and molecular data obtained in previous
211 studies, which provided compelling evidence for a sister group relationship between
212 Mecoptera and Siphonaptera (Whiting, 2002; Whiting *et al.*, 2008). The ITS1 sequence
213 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, and this molecular marker was also 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 in the GenBank database.

Results

Morphological and biometrical results

All the specimens studied in this work showed morphological characteristics expected for the genera *Ctenophthalmus* sp:

- Labial palp with no more than four segments.
- Presence of pronotal ctenidia (Fig. 1A).
- Antennae with nine well visible segments. Basal segments of the antennae not fused (Fig. 1B).
- Genal ctenidia with three cone-shaped setae horizontally inserted with a sharped apex (Fig. 1B).

Males could be easily discriminated between the two subspecies (*C. b. boisseauorum* and *C. a. allani*).

Males of *C. b. boisseauorum* showed different specific morphological characters:

- Apex of the distal branch of IX sternum without an apical slot (Fig. 1C).
- Distal branch of IX sternum with parallel margins (Fig. 1C).
- Dorsal processus basimere significantly longer than it is wide with two long setae showing different length each other (Fig. 1D).
- Ventral processus basimere significantly longer than it is wide showing an apical slot (Fig. 1D).

Males of *C. a. allani* showed different specific morphological characters:

- Apex of the distal branch of IX sternum with a small apical slot (Fig. 1E).
- Apical part of distal branch of IX sternum with parallel margins (Fig. 1E).
- Dorsal processus basimere significantly longer than it is wide with two long setae with the same length each other (Fig. 1F).

- Ventral processus basimere cone-shaped or digitiform without any slot on the apex (Fig. 1F).

Since there are no criteria to discriminate females belonging to *Ctenophtahlmus* sp., we considered all the females as two main groups: The first group included females isolated together with *C. b. boisseauorum* males from the same host, whereas, the second group included females isolated together with *C. a. allani* males from the same host. In spite of the non-existence of discriminative taxonomical characters, the spermatheca and the chaetotaxy and shape of the margin of the sternum VII in females have remained as the most reliable and variable characters in order to carry out a specific classification within Order Siphonaptera. For this reason, we focused on these regions in a deeper way. The spermatheca appeared very similar in all females' specimens assessed without any morphological discriminative pattern between both groups (Fig. 2). Thus, the spermatheca always showed a hilla shorter and narrower than bulga. Furthermore, we could notice a small prominence at the end of the bulga in some specimens from both female groups (Fig. 2D and 2F) which sometimes could appear less prominent (Fig. 2B and 2C). Likewise, morphological analysis based on the spermatheca, our results did not show any morphological specific pattern in order to discriminate among all the female specimens analyzed based on the chaetotaxy and shape of the sternum VII. Thus, we noticed aleatory appearances and shapes for the margin of sternum VII in females (Fig. 3). Some females of both groups showed two well developed apical lobes of variable size which subtended two little sinus of variable size on the posterior margin of VII sternum (Fig. 3A-3G), whereas other females from both groups showed only one well developed apical lobe (Fig. 3H-3K) together with a deep sinus (Fig. 3I-3K). According to chaetotaxy, no significant differences were observed between both females' groups. Therefore, all specimens assessed showed the presence of six setae with different degree

of development (Fig. 4). The distribution of these setae changed among all the specimens analyzed; however, it was common the presence of three strong setae, longest than the other ones, which appeared very close each other (Fig. 4A-4F). With all these variable morphological results, we were not able to set up any taxonomical key or similar for female discrimination.

Biometrical results showed significant differences between males of both subspecies (*C. b. boisseauorum* and *C. a. allani*) based on different parameters such as TL, LDBS9, WDBS9, WDPB, WVPB, DSETDPB, MESOW, METW (see Table 2). Males of *C. b. boisseauorum* showed a wider distal branch of the IX sternum, a wider ventral processus basimere and more distance between the two setae present on the dorsal processus basimere than *C. a. allani* males. According to sex differentiation, females generally appeared longer and with a wider head than males (Table 3). Only MESOW (width of mesothorax) appeared as a differential significant statistic value between both female groups; although in some individuals this parameter overlapped between these groups (Table 3). Additionally, these data were compared with the results obtained by PCA consisting in the regression of each character separately on the within group first principal component (PC1). Therefore, male variables significantly correlated with PC1, contributing 73 % to the overall variation. Both male populations appeared separated from each other, with no overlapping areas between *C. b. boisseauorum* and *C. a. allani* (Fig. 5A). The factor map (Fig. 5A) clearly showed a bigger global size in the male population of *C. a. allani*.

Furthermore, female variables significantly correlated with PC1, contributing 67 % to the overall variation. In this case, the factor map (Fig. 5B) showed an overlapping area without remarkable global size differences between both female groups.

Molecular results

293 *ITS1 and ITS2 analysis*

294 The length of the ITS1 sequences of all the *Ctenophthalmus* specimens ranged from 888
295 base pairs (bp) (*C. a. allani* males) to 889 bp (*C. b. boisseauorum* males and
296 *Ctenophthalmus* sp. females) (Table 1), whereas, the length of the ITS2 fragment was 492
297 bp for all the specimens. The intrageneric similarity ranged from 99.9 % to 100 %. The
298 ITS2 sequences showed a intrageneric similarity ranged from 99.6 % to 100 % with a
299 maximum of two different base pairs among all the sequences analyzed.

300 The phylogenetic tree inferred from ITS2 sequences of *C. b. boisseauorum* and *C. a.*
301 *allani* and other ITS2 sequences retrieved from GenBank (see Table S2) showed all the
302 *Ctenophthalmus* species and subspecies clustered together in polytomy with high
303 bootstrap and BPP values (100/100) without any specific phylogenetic pattern of
304 distribution. Furthermore, this genus appeared close related with *Tunga penetrans*
305 (Tungidae) sharing clade with other species of Ctenophthalmidae (Fig. S1).

306 *Partial* cox1 *mtDNA gene analysis*

307 The partial gene *cox1* mtDNA sequences of *C. b. boisseauorum and C. a. allani* males
308 and *Ctenophthalmus* sp. females were 453 bp in length (Table 1). The similarity observed
309 among *cox1* sequences of *C. a. allani* ranged from 98.7 % to 100 %, whereas this value
310 ranged from 99.3 % to 100 % for *C. b. boisseauorum* (Table 4). Similar values were
311 observed when we calculated the similarity between males from both subspecies and
312 *Ctenophthalmus* sp. females, thus we noticed overlapped percentages between them with
313 a minimum value of 98.2 % (*Ctenophthalmus* sp. females - *C. a. allani* males) and with
314 a maximum value of 100 % (*Ctenophthalmus* sp. females - *C. b. boisseauorum* males; *C.*
315 *b. boisseauorum* males - *C. a. allani* males) (Table 4). In contrast to that, these similarity
316 percentage values were considerably lower when we compared these sequences with
317 partial gene *cox1* sequences from other congeneric species. Therefore, these percentage

values ranged from 86.5 % (*Ctenophthalmus* sp. females - *Ctenophthalmus cryptotis*) to 90.3 % (*Ctenophthalmus* sp. females – *Ctenophthalmus dolichus dolichus*). On the other hand, the lowest value of similarity was observed between *C. dolichus dolichus* and *Ctenophthalmus calceatus cabirus* (85.0 %) (Table 4).

Phylogenetic tree topology revealed a clade (BPP and bootstrap values: 67/87) clustering all *Ctenophthalmus* species, excluding one *Ctenophthalmus* sp. sequence (AN: KM891003). Within this clade, we observed a highly supported subclade (92/89 - BPP and bootstrap values) corresponding to our sequences appearing in polytomy. Furthermore, Ctenophthalmidae family appeared in polytomy with other flea families (Fig. S2).

Partial cytb *mtDNA gene analysis*

The length of the *cytb* mtDNA sequences of the all *Ctenophthalmus* sp. specimens obtained in this study was 374 (Table 1). The similarity observed among the partial *cytb* sequences of males of both subspecies (*C. b. boisseauorum* and *C. a. allani*) ranged from 98.7 % to 100 %, whereas the percentage of similarity obtained when we compared all the *Ctenophthalmus* sp. females *cytb* sequences each other ranged from 98.4 % to 100 % (Table 5). Similar results were observed when we obtained the similarity between males of both subspecies together with *Ctenophthalmus* sp. females, thus these values ranged from 98.4 % (*Ctenophthalmus* sp. females - *C. a. allani* males - *C. b. boisseauorum* males) to 100 % (*Ctenophthalmus* sp. females - *C. a. allani males*; *C. b. boisseauorum* males - *C. a. allani* males) (Table 5). Additionally, we also calculated the interspecific similarity between the *cytb* sequences obtained in this study and those from other species belonging to the same genus (*C. cryptotis*, *Ctenophthalmus congeneroides congeneroides* and *Ctenophthalmus sanborni*). Our analysis revealed lower values out of which none

exceeded 86.6 %, with a minimum percentage value of 84.8 %(*C. b. boisseauorum* males – *C. sanborni*).

The phylogenetic tree inferred from partial *cytb* gene sequences revealed a well supported clade (100/88 - BPP and bootstrap values) comprising all the species belonging to *Ctenophthalmus* genus (Fig. S3). Within this clade, we noticed a highly supported subclade (100/95 - BPP and bootstrap values) clustering all the partial *cytb* mtDNA sequences of *C. b. boisseauorum* and *C. a. allani* males and *Ctenophthalmus* sp. females without any specific phylogenetic pattern of distribution (Fig. S3). On the other hand, all the different flea families appeared in polytomy in the same clade (Pulicidae, Ctenophthalmidae, Ceratophyllidae, Stephanocircidae, Pygiopsyllidae, Stivaliidae and Stenoponiidae) (Fig. S3).

The concatenated dataset of ITS2, partial *cytb* and *cox1* gene sequences included 1,405 aligned sites and 55 taxa, including outgroups. Phylogenetic analyses of the concatenated dataset yielded a tree with branches that were strongly supported (Fig. 6). The analysis based on the concatenated dataset showed all species belonging to *Ctenophthalmus* genera obtained in this work presenting a monophyletic origin and clustering together in a highly supported clade not showing any specific phylogenetic pattern of distribution (Fig. 6). In addition, differente families such as Ceratophyllidae, Pulicidae and Stenoponiidae appeared separated from Ctenophthalmidae (Fig. 6).

Discussion

Morphological data combined with the modern molecular approaches have become a major source for phylogenetic inference in taxonomical studies (Bybee *et al*., 2010). Nevertheless, probably due to the high level of morphological diversity observed in the Order Siphonaptera the number of combined analyses of molecular and morphological data are still unusual in this Order. This work constitutes the first study that provides a combination of morphological, biometrical, molecular and phylogenetic comparative data of two subspecies (*C. b. boisseauorum* and *C. a. allani*) belonging to *Ctenophthalmus* genus in order to assess their taxonomic and phylogenetic relationships. It should be highlighted that genus *Ctenophthalmus* includes aproximately 300 valid taxa (Beaucournu & Lorvelec, 2014) representing the most abundant flea genus in Europe (Beaucournu & Launay, 1990).

Gómez *et al*. (2003) reported some notes about the morphological variability of *Ctenophthalmus* sp. in Spain. These authors argued that even seven different subspecies of *Ctenophthalmus (C.) apertus* had been described in Spain: *C. (C.) apertus apertus, C. (C.) apertus allani, C. (C.) apertus azevedoi, C. (C.) apertus gilcolladoi, C. (C.) apertus gosalhezi, C. (C.) apertus meylani* and *C. (C.) apertus personatus*, having each of these species their own geographic distribution. Therefore, they placed *C. a. allani* in the north of Spain at the cities of León, Oviedo, Santander and Zamora (Beaucournu & Launay 1990; Gómez *et al*., 2003). These locations agree with our results since our specimens classified as *C. a. allani* were isolated from Asturias (north of Spain). In addition, previous authors (Beaucournu & Launay 1990; Beaucournu & Lorvelec 2014) have just placed *C. b. boisseauorum* in different geographical areas of the north of Spain. The morphological analysis carried out by Hopkins & Rothschild (1966) and Beaucournu & Launay (1978, 1990) reported that several specimens of each *"apertus"* subspecies

386 evidenced great variability in male modified abdominal segments as well as in female
387 sternum VII; however, these authors only provided a taxonomical keys for males.
388 Beaucournu & Launay (1978, 1990) speculated about the possibility that this
389 morphological variability was possibly due to interbreeding of two subspecies which have
390 sympatric distribution, but finally, they supported that this fact were just different
391 morphotypes as a consequence of the wide morphological intraspecific *"apertus"*
392 variations. The higher degree of morphological variation observed in males could be
393 explained because in temporary parasites, males mostly have a shorter life period and are
394 more active in terms of looking for new hosts. Thus, males leave earlier from their hosts
395 (Marshall, 1981), whereas, females need blood to produce their eggs, leaving their hosts
396 later (Dryden, 1993). Attending to our morphological results we could discriminate
397 between males of *C. b. boisseauorum* and *C. a. allani* generally based on the width of the
398 ventral processus basimere and in the total distance between the two setae present on the
399 dorsal processus basimere which showed different length in *C. b. boisseauorum*. Unlike
400 males, females showed an aleatory high degree of polymorphism based on the shape of
401 margin of the sternum VII. These characters did not correspond with any subspecific
402 morphological pattern between the two groups of *Ctenophthalmus* females analysed in
403 this study. Márquez & Soringuer (1987) observed a great variability in the margin of
404 sternum VII in females of *C. a. meylani* noticing that some specimens showed
405 morphological characteristic similar to the subspecies *C. a. queirozi*. These authors
406 argued that in each population could exist a great morphological variability in females
407 associated with different ecological traits which would be responsible to the selection of
408 one specific morphotype. Nevertheless, in our study the variability observed in the shape
409 of the margin of the sternum VII was similar in both female groups isolated from the same
410 host and from the same geographical origin.

411 In spite of that, Marquez & Soringuer (1987) found some differences in this region in
412 terms of number of setae from one population of C. *a. meylani* isolated from Granada,
413 Córdoba and Jaén (Spain). Nevertheless, most specimens analyzed by these authors
414 showed six main setae in sternum VII agreeing on our results. In this sense, the chaetotaxy
415 of sternum VII of females was assessed in our study in order to find new possible
416 morphological variations which allow us to discriminate between females of
417 *Ctenophthalmus* genus. Nevetheless, both characters appeared hardly identical (with
418 slighty differences in spermatheca of some specimens) even between the two female
419 groups of this study. These results would be in agreement with Beaucournu & Launay
420 (1990) who did not find clear differences in this region in *Ctenophthalmus* genus. These
421 taxonomical results were corroborated by PCA and biometrical analysis but were not in
422 concordance with molecular and phylogenetic results, specially based on male specimens
423 which showed a high degree of nucleotide similarity.
424 ITS1 and ITS2 have been reported as two useful markers in order to infer phylogenetic
425 studies in flea taxonomy, being used with several purposes: molecular characterization of
426 several flea species (Vobis *et al*., 2004), molecular discrimination among congeneric
427 species (Marrugal *et al*., 2013; Zurita *et al*., 2016), molecular characterization of different
428 geographical lineages from the same species (Luchetti *et al*., 2007; Ghavami *et al*., 2018)
429 or even molecular discrimination among possible cryptic species (Zurita *et al*., 2019).
430 In our study, we observed a high similarity (99.6 % - 100 %) between *C. b. boisseauorum*
431 and *C. a. allani* based on ITSs sequences analysis. These results did not correspond with
432 the morphological differences observed between both subspecies agreeing with Zurita *et*
433 *al*. (2018a) who did not observe substantial nucleotide differences when they compared
434 ITS1 and ITS2 sequences of *N. barbarus* and *N. fasciatus* supporting the idea that *N.*
435 *barbarus* should be considered a junior synonym of *N. fasciatus*.

Even in a longer way to ITSs sequences, mitochondrial markers have been widely used for estimating molecular phylogenies in fleas in the last years (Lawrence *et al*., 2014; Zurita *et al*., 2018a, b; Hornok *et al*., 2018). The *cox1* gene has widely showed enough interspecific nucleotide variability among different groups of arthropods in order to discriminate between species and subspecies, even, which they appeared morphologically similar (Paz *et al*., 2011). Thus, sequencing this gen represents one of the best options for phylogenetic study at these taxonomical level of any group of insects including fleas since it is generally considered the potential 'barcode' for insect identification (Hebert *et al*., 2003). *Cytb* partial gene has also been widely used in order to infer phylogenetic relationships among different closed flea taxa (Dittmar & Whiting, 2003; Zurita *et al*., 2019). In the most recent published articles, flea DNA barcoding data have shown a maximum of intraspecific and interspecific similarity ranging from 91.5 % to 97 % (Zurita *et al*., 2019). Analyzing all these studies, it seems obvious that *cytb* and *cox1* (likewise ITS1 and ITS2) are easily able to discriminate themselves between two close related flea species, among different cryptic species or even to reveal the existence of different geographical lineages within the same species. Nevertheless, we noticed a high degree of similarity between *C. b. boisseauorum* and *C. a. allani* based on mitochondrial DNA markers (98.2 % - 100 %), whereas *cytb* and *cox1* were able to discriminate between this two subspecies and other congeneric ones such as *C. cryptotis*, *C. c. congeneroides*, *C. sanborni* or *C. d. dolichus* (84.8 % - 90.3 %). Likewise ITS analysis, morphological differences observed between males from both subspecies did not correspond with substantial nucleotide differences in *cox1* and *cytb* sequences. These results could suggest the idea that *C. b. boisseauorum* and *C. a. allani* were the same taxon or even consider *C. b. boisseauorum* as a junior synonym of *C. a. allani*.

This idea, reinforce the results reported by concatenated phylogenetic tree and all trees constructed on the basis of the single markers. Thus, in all of them we observed both subspecies clustering together in the same well supported clades without any specific distribution pattern and separated from other *Ctenophthalmus* species suggesting that there are no phylogenetic reasons to consider these two morphosubspecies (*C. b. boisseauorum* and *C. a. allani*) as two different taxa. In spite of these results, complementary phylogenetic and molecular studies are necessary to confirm a case of synonymy between C. *apertus* and C. *baeticus*. Therefore, we should take into account that several subspecies have been described for *C. apertus* and *C. baeticus* species which should be molecularly studied before to confirm the existence of phenotypic differences which did not correspond with a real genotypic variability between both species.

In conclusion, for the first time, the present study provides comparative morphometric, phylogenetic and molecular data for two *Ctenophthalmus* subspecies (*C. b. boisseauorum* and *C. a. allani*). From a morphological point of view, we can conclude that the spermatheca, the outline of VII sternum and the chaetotaxy of this region in females are not useful tools in order to discriminate between both subspecies. This idea is in agreement with Beaucournu & Launay (1990) who considered the outline of VII sternum as aleatory and not reliable for taxonomic studies within this genus whereas both spermatheca and chaetotaxy of sternum VII appeared hardly identical among all the females belonging to these two subspecies. On the other hand, although males of both subspecies could be differentiated based on morphological traits, these morphological differences did not correspond with molecular and phylogenetic data. For that reason, this work brings to light by the first time, the necessity to carry out a progressive taxonomical revision within not only *Ctenophthalmus* genus if not in the whole Ctenophthalmidae family, which has remained as the ``catchall´´ for a large number of divergent taxa

(Whiting *et al.*, 2008; Zurita *et al*., 2015; Keskin, 2019; Keskin & Beaucournu, 2019b). Within this family, a wide range of different taxa have been only described from a morphological point of view, for that reason 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 the Ctenophthalmidae family.

In addition, it is known that phenotypic polymorphism is generally due to genetic and environmental sources of variation (Fusco & Minelli, 2010). In this sense, complementary data and rigorous and statistical analysis related to ecological conditions and intrinsic characteristics of the host would be needed. These extra data would help us to confirm possible cases of phenotypic plasticity within *Ctenophthalmus* genus especially referring to modified abdominal segments of males and the outline of VII sternum in females.

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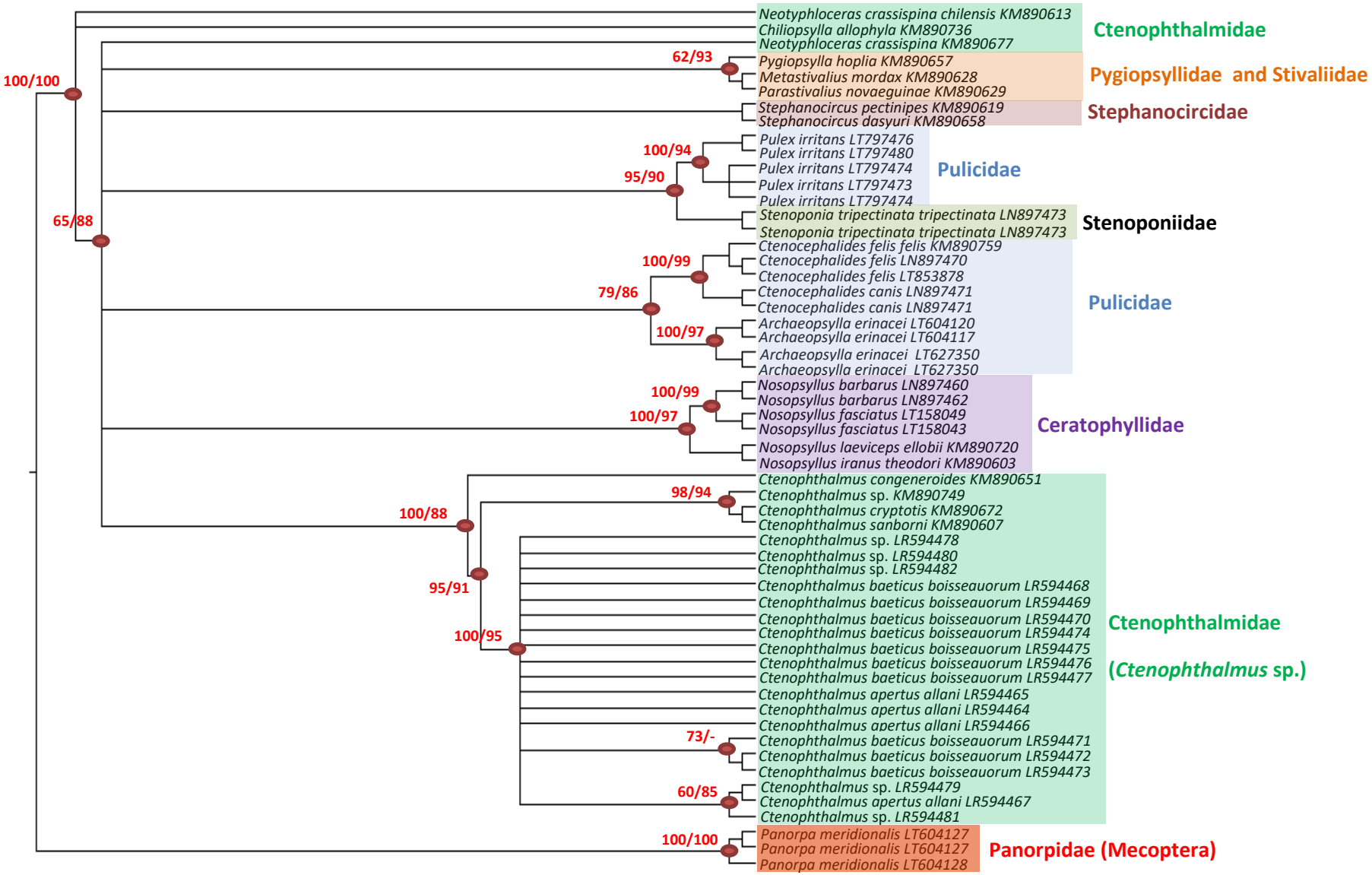
658 **Figure captions**

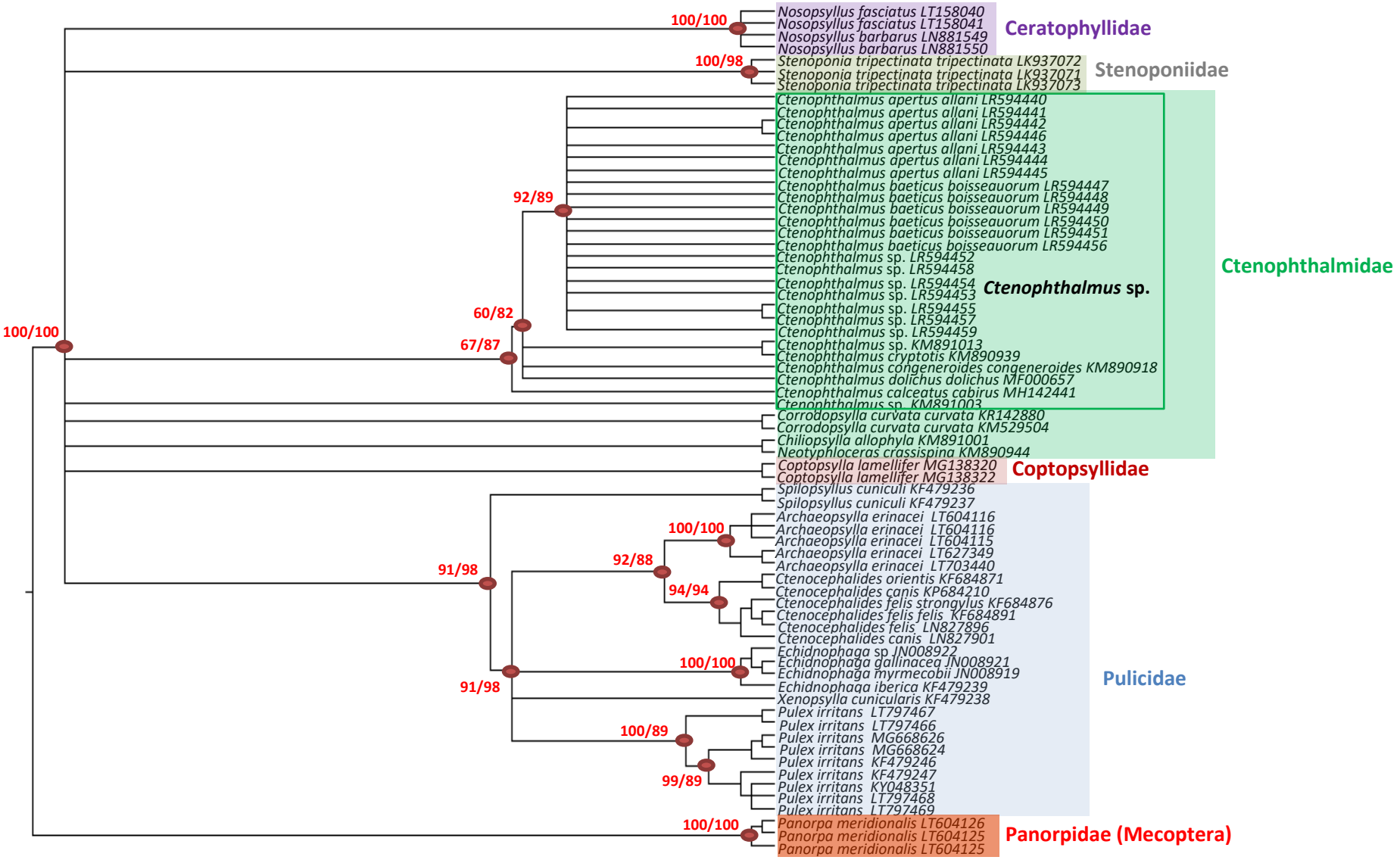
659 Figure 1: Morphological characteristics of *Ctenophthalmus* sp, *Ctenophthalmus baeticus*
660 *boisseauorum* and *Ctenophthalmus apertus allani*. A- Pronotal ctenidia (black arrow) of
661 *Ctenophthalmus* sp.; B- Head with antennae (black arrow) and genal ctenidia of

662 *Ctenophthalmus* sp (blue arrow); C- Male distal branch of IX sternum (black arrow) of
663 *C. b. boisseauorum*; D- Dorsal processus basimere (black arrow) and ventral processus
664 basimere (blue arrow) of males of *C. b. boisseauorum*; E- Male distal branch of IX
665 sternum (black arrow) of *C. a. allani*; F- Dorsal processus basimere (black arrow) and
666 ventral processus basimere (blue arrow) of males of *C. a. allani.*
667 Figure 2: Spermatheca of females of *Ctenophthalmus* sp. analyzed in this study. A small
668 prominence at the end of the bulga is arrowed in figures 2D and 2F.
669 Figure 3: Variability observed in the shape of the margin of sternum VII of
670 *Ctenophthalmus* sp. females.
671 Figure 4: Variability observed in chaetotaxy of sternum VII of females belonging to
672 *Ctenophthalmus* sp. assessed in this study.
673 Figure 5: A. Factor map corresponding to adult *C. b. boisseauorum* (CBBM) and *C. a.*
674 *allani* (CAAM) males from Asturias (Spain). Samples are projected onto the first (PC1,
675 73%) and second (PC2, 9%) principal components. Each group is represented by its
676 perimeter. B. Factor map corresponding to adult *Ctenophthalmus* sp. females from
677 Asturias (Spain). Samples are projected onto the first (PC1, 67%) and second (PC2, 18%)
678 principal components. Each group is represented by its perimeter. CTH1: Females of
679 *Ctenophthalmus* sp. isolated together with *C. b. boisseauorum* males from the same host;
680 CTH2: Females of *Ctenophthalmus* sp. isolated together with *C. a. allani* males from the
681 same host.
682 Figure 6: Phylogenetic tree of *Ctenophthalmus sp.*, *Ctenophthalmus baeticus*
683 *boisseauorum* and *Ctenophthalmus apertus allani* assessed in this study (see Table 1)
684 based on concatenated Internal Transcribed Spacer 2 (ITS2), partial cytochrome c-
685 oxidase subunit 1 (*cox1*) and cytochrome b (*cytb*) gene of mitochondrial DNA inferred
686 using the Bayesian Inference (BI) and Maximum Likelihood (ML) methods and Bayesian

687 topology. The percentage of replicate trees in which the associated taxa clustered together
688 in the bootstrap test (1,000 replicates) is shown on the branches. The Bayesian Posterior
689 Probabilities (BPP) are percentage converted.
690 Figure S1: Phylogenetic tree of *Ctenophthalmus* sp., *Ctenophthalmus baeticus*
691 *boisseauorum* and *Ctenophthalmus apertus allani* assessed in this study (see Table 1)
692 based on the Internal Transcribed Spacer 2 (ITS2) sequences using the Bayesian Inference
693 (BI) and Maximum Likelihood (ML) methods and Bayesian topology. The percentage of
694 replicate trees in which the associated taxa clustered together in the bootstrap test (1,000
695 replicates) is shown on the branches (B/ML). Bootstrap values lower than 60% are not
696 shown. The Bayesian Posterior Probabilities (BPP) is percentage converted.
697 Figure S2: Phylogenetic tree of *Ctenophthalmus* sp., *Ctenophthalmus baeticus*
698 *boisseauorum* and *Ctenophthalmus apertus allani* assessed in this study (see Table 1)
699 based on partial cytochrome c-oxidase 1 (*cox1*) gene of mitochondrial DNA sequences
700 using the Bayesian Inference (BI) and Maximum Likelihood (ML) methods and Bayesian
701 topology. The percentage of replicate trees in which the associated taxa clustered together
702 in the bootstrap test (1,000 replicates) is shown on the branches (B/ML). Bootstrap values
703 lower than 60% are not shown. The Bayesian Posterior Probabilities (BPP) is percentage
704 converted.
705 Figure S3: Phylogenetic tree of *Ctenophthalmus* sp., *Ctenophthalmus baeticus*
706 *boisseauorum* and *Ctenophthalmus apertus allani* assessed in this study (see Table 1)
707 based on partial cytochrome b (*cytb*) gene of mitochondrial DNA using the Bayesian
708 Inference (BI) and Maximum Likelihood (ML) methods and Bayesian topology. The
709 percentage of replicate trees in which the associated taxa clustered together in the
710 bootstrap test (1,000 replicates) is shown on the branches (B/ML). Bootstrap values lower

than 60% are not shown. The Bayesian Posterior Probabilities (BPP) is percentage converted.


Neotyphloceras crassispina chilensis KM890613
Chiliopsylla allophyla KM890736
Neotyphloceras crassispina KM890677
Ctenophthalmidae
62/93
Pygiopsylla hoplia KM890657
Metastivalius mordax KM890628
Parastivalius novaeguinae KM890629
Pygiopsyllidae and Stivaliidae
Stephanocircus pectinipes KM890619
Stephanocircus dasyuri KM890658
Stephanocircidae
100/100
100/94
Pulex irritans LT797476
Pulex irritans LT797480
95/90
Pulex irritans LT797474
Pulex irritans LT797473
Pulex irritans LT797474
Pulicidae
65/88
Stenoponia tripectinata tripectinata LN897473
Stenoponia tripectinata tripectinata LN897473
Stenoponiidae
Ctenocephalides felis felis KM890759
100/99
Ctenocephalides felis LN897470
Ctenocephalides felis LT853878
79/86
Ctenocephalides canis LN897471
Ctenocephalides canis LN897471
100/97
Archaeopsylla erinacei LT604120
Archaeopsylla erinacei LT604117
Archaeopsylla erinacei LT627350
Archaeopsylla erinacei LT627350
Pulicidae
100/99
Nosopsyllus barbarus LN897460
Nosopsyllus barbarus LN897462
100/97
Nosopsyllus fasciatus LT158049
Nosopsyllus fasciatus LT158043
Nosopsyllus laeviceps ellobii KM890720
Nosopsyllus iranus theodori KM890603
Ceratophyllidae
Ctenophthalmus congeneroides KM890651
98/94
Ctenophthalmus sp. KM890749
100/88
Ctenophthalmus cryptotis KM890672
Ctenophthalmus sanborni KM890607
Ctenophthalmus sp. LR594478
Ctenophthalmus sp. LR594480
Ctenophthalmus sp. LR594482
95/91
Ctenophthalmus baeticus boisseauorum LR594468
Ctenophthalmus baeticus boisseauorum LR594469
Ctenophthalmus baeticus boisseauorum LR594470
100/95
Ctenophthalmus baeticus boisseauorum LR594474
Ctenophthalmus baeticus boisseauorum LR594475
Ctenophthalmus baeticus boisseauorum LR594476
Ctenophthalmus baeticus boisseauorum LR594477
Ctenophthalmus apertus allani LR594465
Ctenophthalmus apertus allani LR594464
Ctenophthalmus apertus allani LR594466
73/-
Ctenophthalmus baeticus boisseauorum LR594471
Ctenophthalmus baeticus boisseauorum LR594472
Ctenophthalmus baeticus boisseauorum LR594473
60/85
Ctenophthalmus sp. LR594479
Ctenophthalmus apertus allani LR594467
Ctenophthalmus sp. LR594481
Ctenophthalmidae
(Ctenophthalmus sp.)
100/100
Panorpa meridionalis LT604127
Panorpa meridionalis LT604127
Panorpa meridionalis LT604128
Panorpidae (Mecoptera)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Nosopsyllus fasciatus LT158041
Nosopsyllus barbarus LN881549
Nosopsyllus barbarus LN881550
Ceratophyllidae
Stenoponia tripectinata tripectinata LK937072
Stenoponia tripectinata tripectinata LK937071
Stenoponia tripectinata tripectinata LK937073
Stenoponiidae
Ctenophthalmus apertus allani LR594440
Ctenophthalmus apertus allani LR594441
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Ctenophthalmus apertus allani LR594443
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Ctenophthalmus apertus allani LR594445
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Neotyphloceras crassispina KM890944
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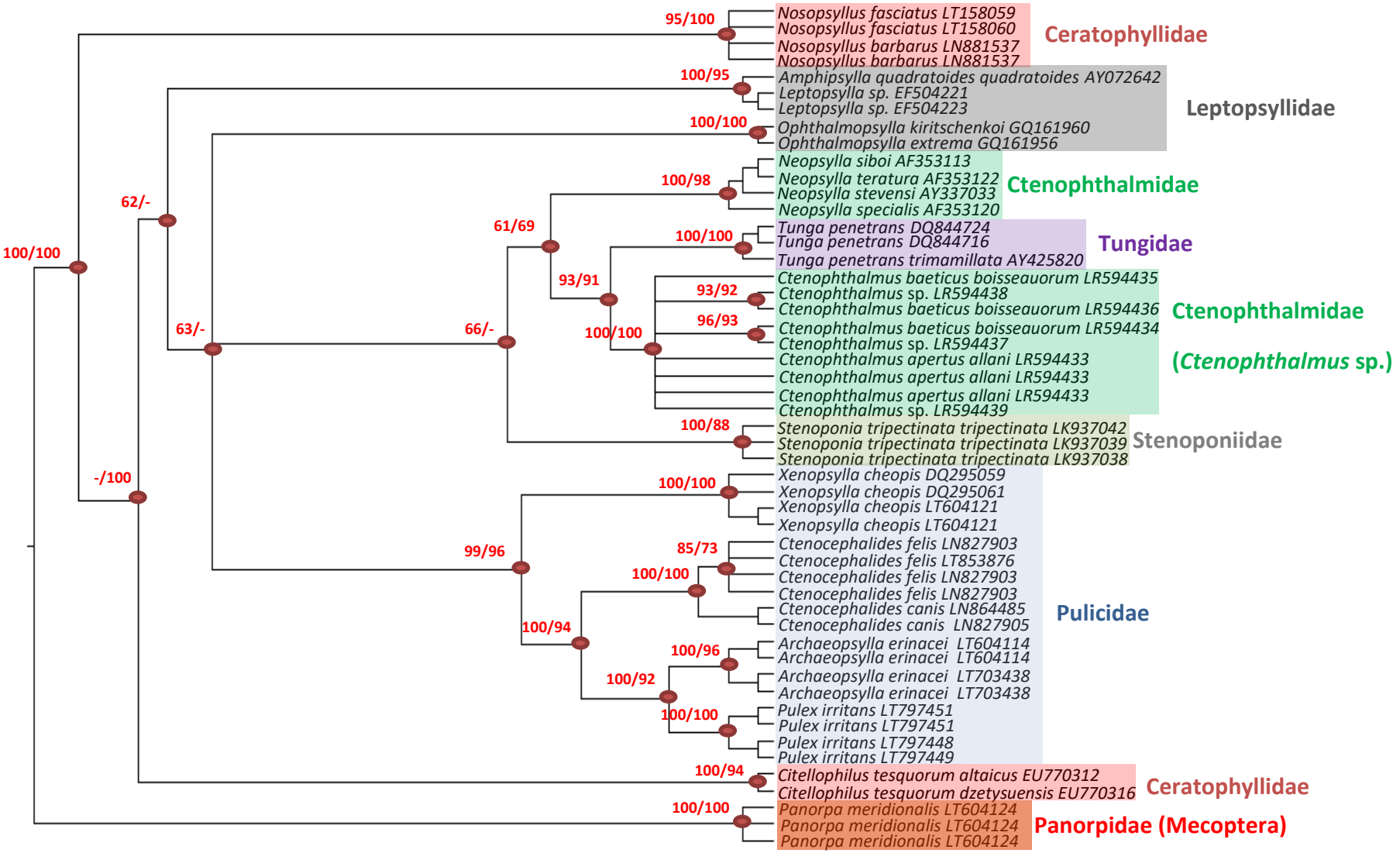
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100/100
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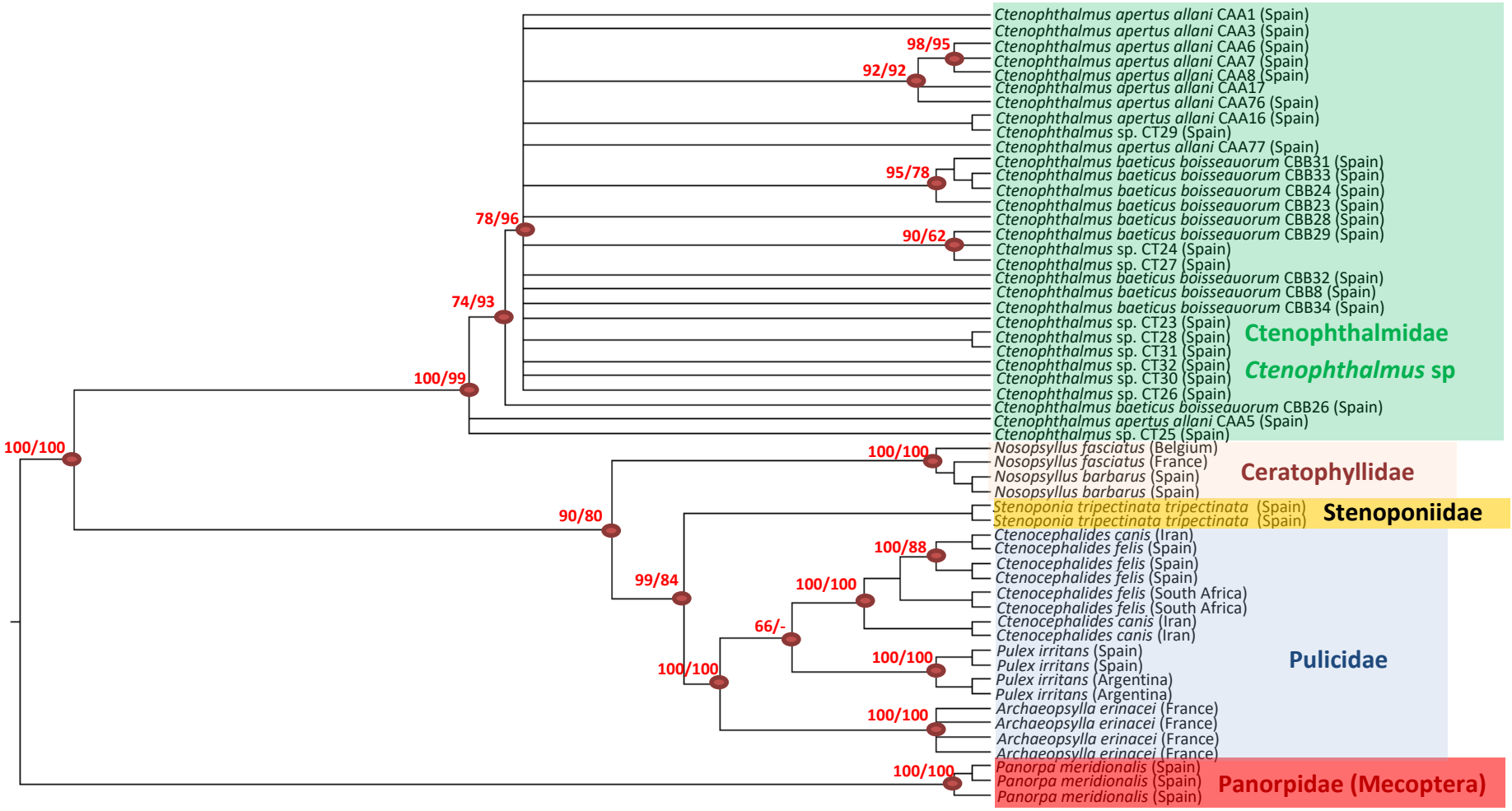
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Ctenophthalmus sp. CT24 (Spain)
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Ctenophthalmus sp
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Pulicidae
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Panorpidae (Mecoptera)
98/95
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78/96
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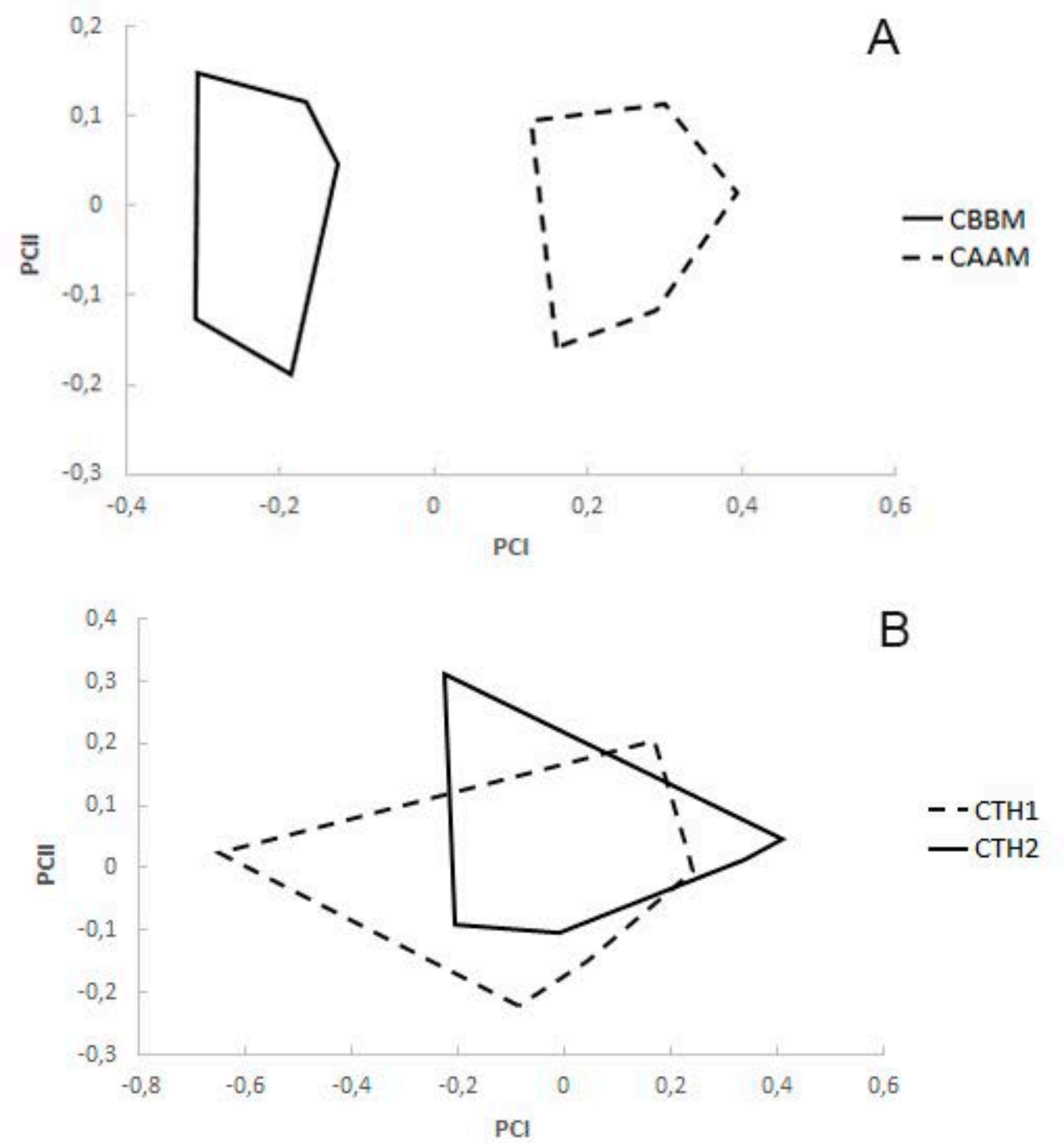
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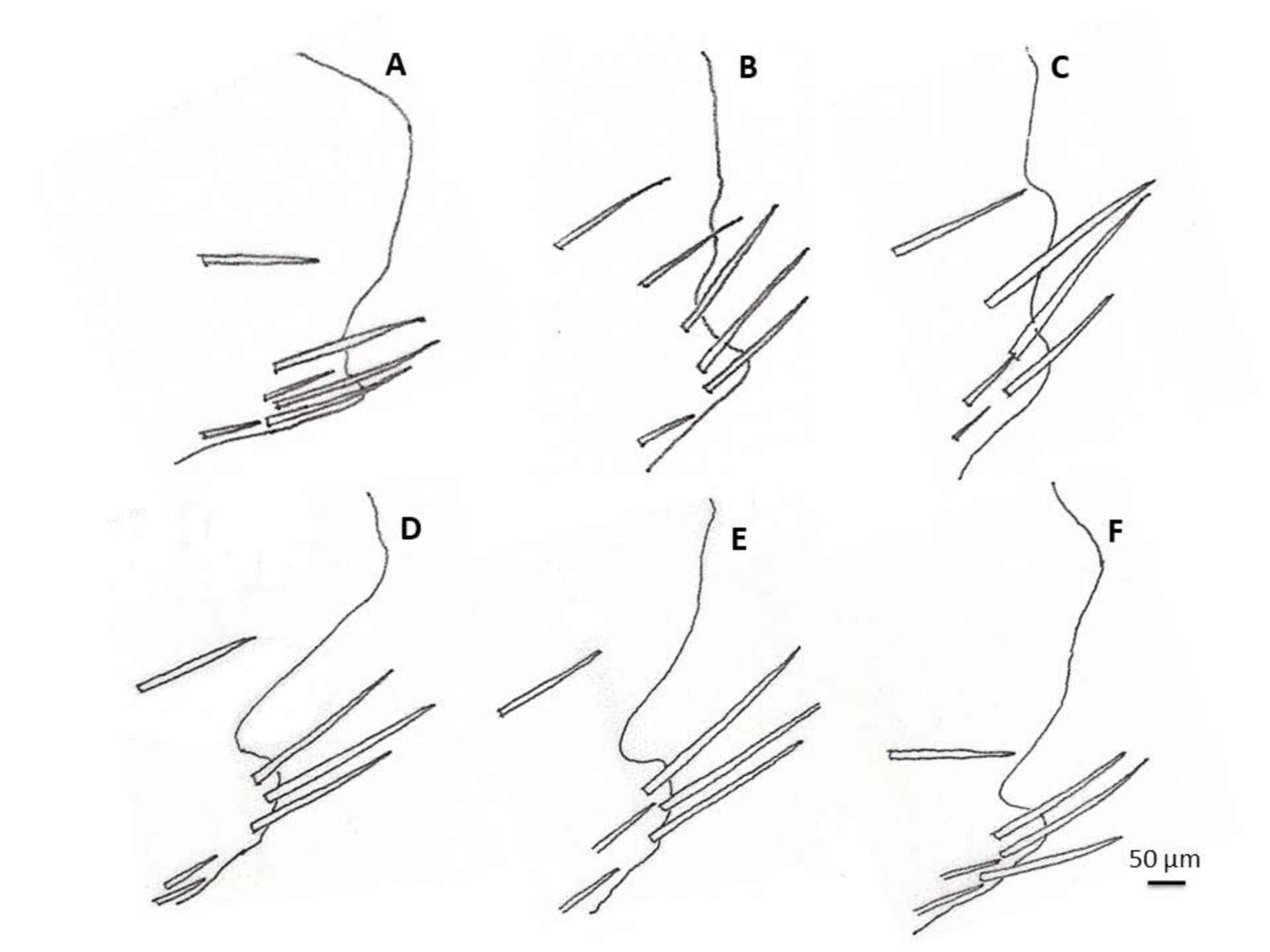
CTH2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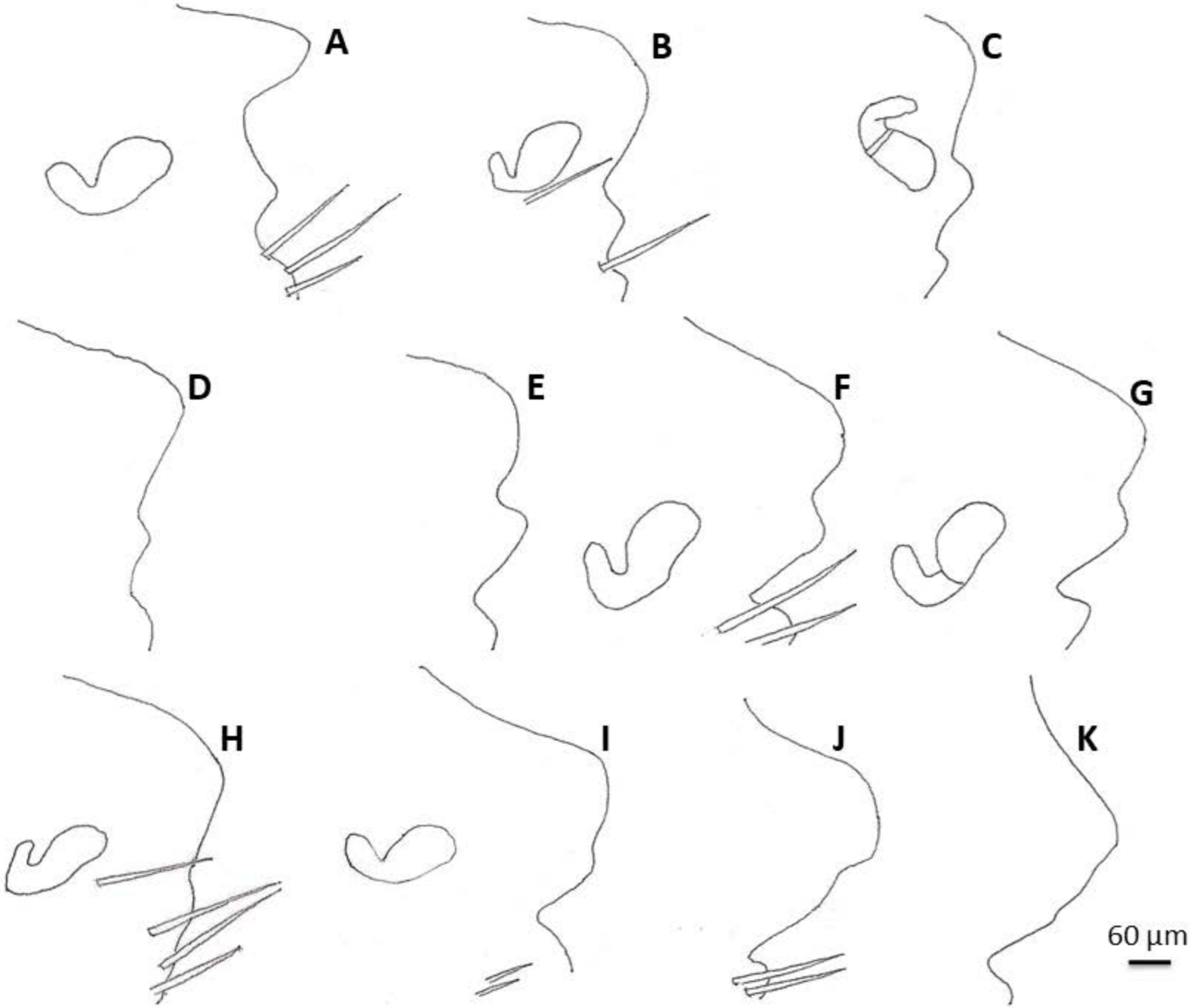
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G
H
I
J
K
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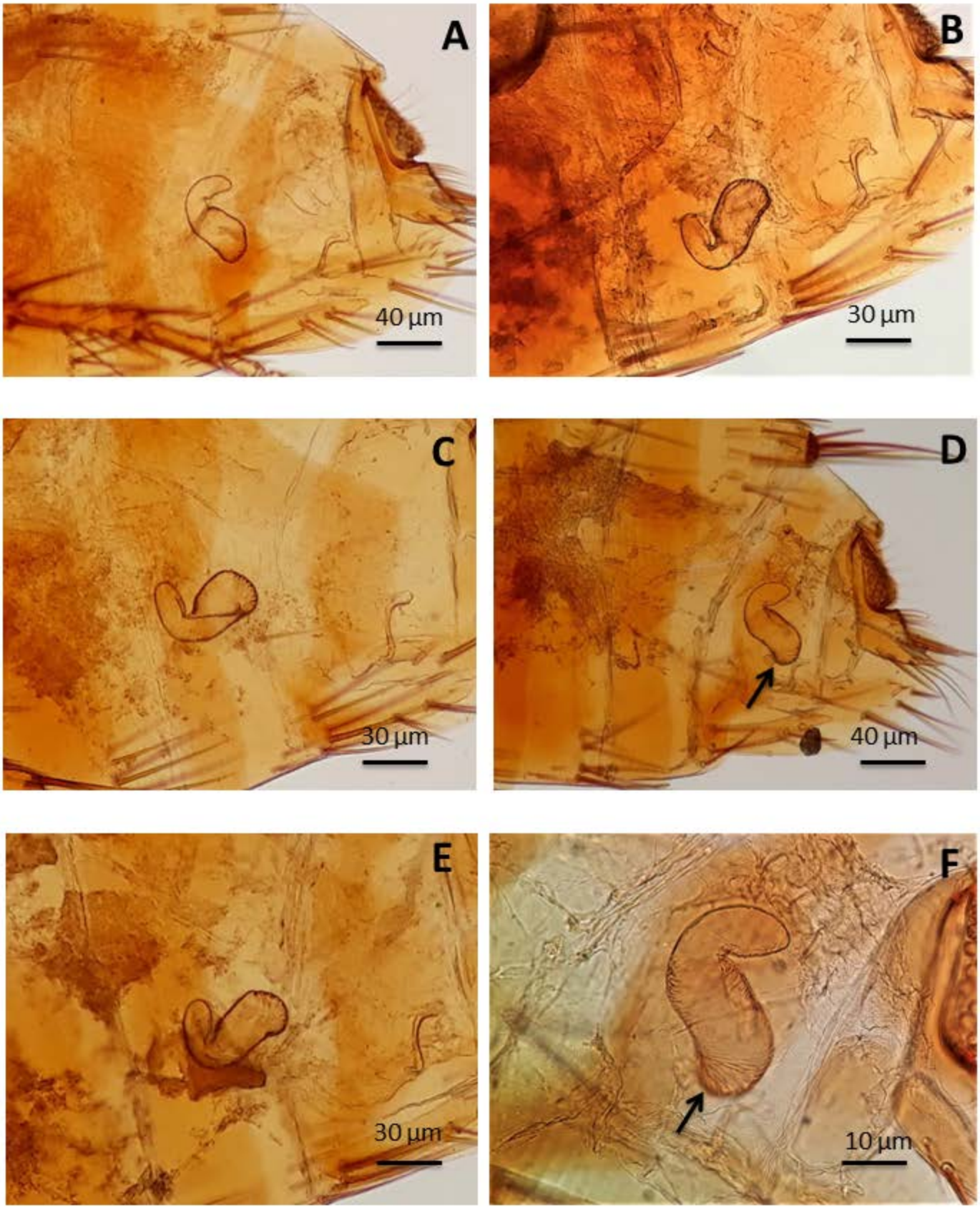
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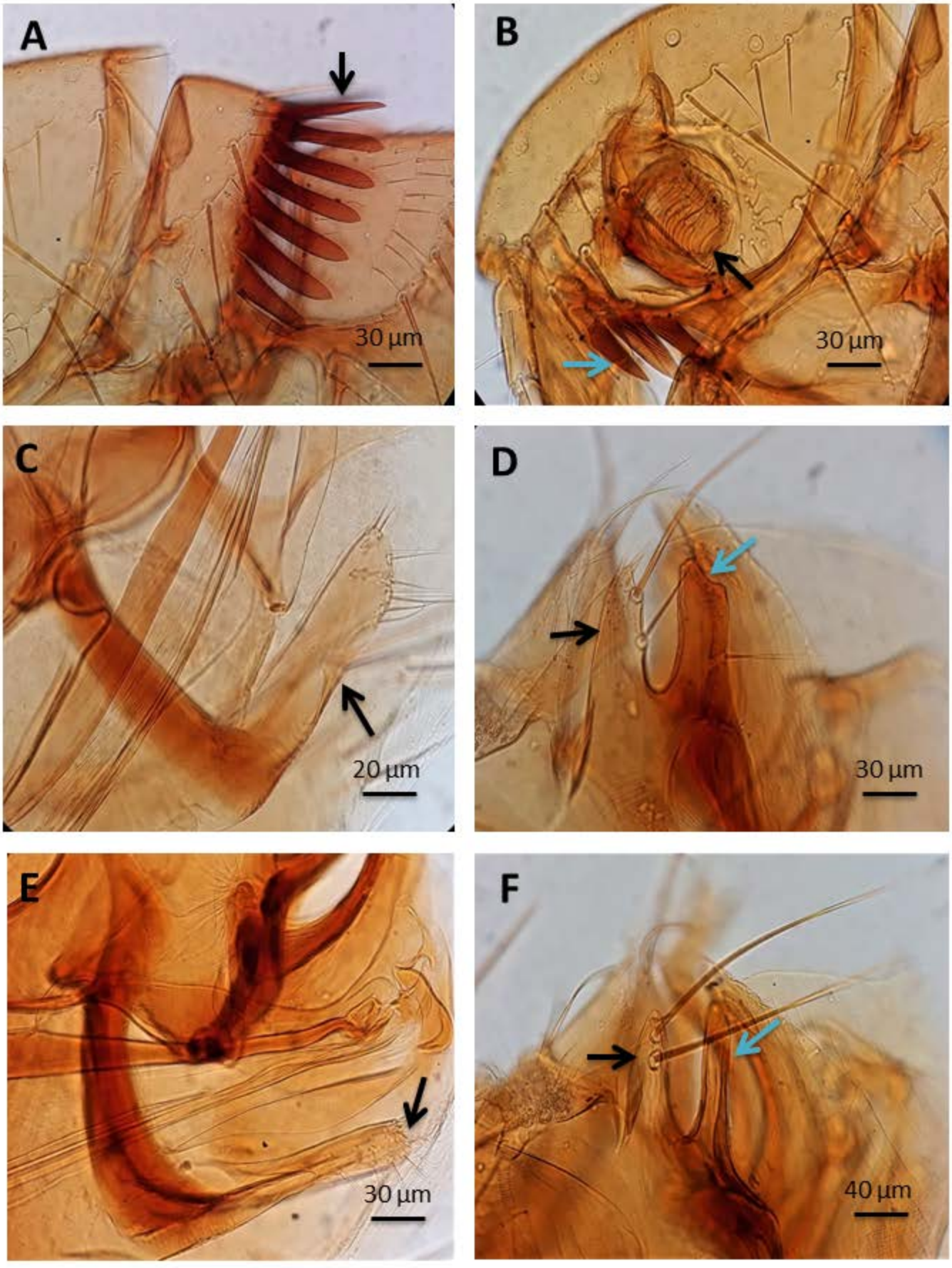
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E
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Table 1. GenBank accession numbers of ITS1, ITS2 and partial *cytb*, *cox1* gene sequences of individuals of *Ctenophthalmus* sp. (CT), *C. baeticus boisseauorum* (CBB) and *C. apertus allani* (CAA) obtained in this study.

ITS1					
Species/Gender	**Sample ID**	**Host**	**Number of fleas**	**Base pairs (bp)**	**Accession number**
C. a. allani/male	CAA17,76,77	*Arvicola scherman*	3	888	LR594427
C. a. allani/male	CAA8, 33, 5-7, 13, 16	*Arvicola scherman*	7	888	LR594428
C. b. boisseauorum/male	CBB26, 32, 34	*Arvicola scherman*	3	889	LR594429
C. b. boisseauorum/male	CBB 9, 23-24, 28-29, 31, 33	*Arvicola scherman*	7	889	LR594430
Ctenophthalmus sp./female	CT24, 30-32	*Arvicola scherman*	4	889	LR594431
Ctenophthalmus sp./female	CT23, 25-29	*Arvicola scherman*	6	889	LR594432
ITS2					
C. a. allani/male	CAA1, 3, 5-8, 16-17, 76-77	*Arvicola scherman*	10	492	LR594433
C. b. boisseauorum/male	CBB26, 28, 32, 34	*Arvicola scherman*	4	492	LR594434
C. b. boisseauorum/male	CBB9, 23	*Arvicola scherman*	2	492	LR594435
C. b. boisseauorum/male	CBB24, 29, 31, 33	*Arvicola scherman*	4	492	LR594436
Ctenophthalmus sp./female	CT23	*Arvicola scherman*	1	492	LR594437
Ctenophthalmus sp./female	CT24, 27	*Arvicola scherman*	2	492	LR594438
Ctenophthalmus sp./female	CT25, 26, 28-32	*Arvicola scherman*	7	492	LR594439
Cox1					
C. a. allani/male	CAA1	*Arvicola scherman*	1	453	LR594440
C. a. allani/male	CAA3	*Arvicola scherman*	1	453	LR594441
C. a. allani/male	CAA5	*Arvicola scherman*	1	453	LR594442
C. a. allani/male	CAA16	*Arvicola scherman*	1	453	LR594443
C. a. allani/male	CAA17, 76	*Arvicola scherman*	2	453	LR594444
C. a. allani/male	CAA77	*Arvicola scherman*	1	453	LR594445
C. a. allani/male	CAA6-8	*Arvicola scherman*	3	453	LR594446
C. b. boisseauorum/male	CBB24	*Arvicola scherman*	1	453	LR594447
C. b. boisseauorum/male	CBB26	*Arvicola scherman*	1	453	LR594448
C. b. boisseauorum/male	CBB28	*Arvicola scherman*	1	453	LR594449
C. b. boisseauorum/male	CBB29	*Arvicola scherman*	1	453	LR594450
C. b. boisseauorum/male	CBB34	*Arvicola scherman*	1	453	LR594451
C. b. boisseauorum/male	CBB8, 23, 31-33	*Arvicola scherman*	5	453	LR594456
Ctenophthalmus sp./female	CT23-24	*Arvicola scherman*	2	453	LR594452
Ctenophthalmus sp./female	CT25	*Arvicola scherman*	1	453	LR594453
Ctenophthalmus sp./female	CT26	*Arvicola scherman*	1	453	LR594454
Ctenophthalmus sp./female	CT27	*Arvicola scherman*	1	453	LR594455
Ctenophthalmus sp./female	CT28	*Arvicola scherman*	1	453	LR594457
Ctenophthalmus sp./female	CT29	*Arvicola scherman*	1	453	LR594458
Ctenophthalmus sp./female	CT30-32	*Arvicola scherman*	3	453	LR594459
Cytb					
C. a. allani/male	CAA5	*Arvicola scherman*	1	374	LR594464
C. a. allani/male	CAA3	*Arvicola scherman*	1	374	LR594465
C. a. allani/male	CAA16	*Arvicola scherman*	1	374	LR594466
C. a. allani/male	CAA1, 6-8, 17, 76-77	*Arvicola scherman*	7	374	LR594467
C. b. boisseauorum/male	CBB9	*Arvicola scherman*	1	374	LR594468
C. b. boisseauorum/male	CBB26	*Arvicola scherman*	1	374	LR594469
C. b. boisseauorum/male	CBB29	*Arvicola scherman*	1	374	LR594470
C. b. boisseauorum/male	CBB31	*Arvicola scherman*	1	374	LR594471
C. b. boisseauorum/male	CBB23	*Arvicola scherman*	1	374	LR594472
C. b. boisseauorum/male	CBB24	*Arvicola scherman*	1	374	LR594473
C. b. boisseauorum/male	CBB28	*Arvicola scherman*	1	374	LR594474
C. b. boisseauorum/male	CBB32	*Arvicola scherman*	1	374	LR594475
C. b. boisseauorum/male	CBB33	*Arvicola scherman*	1	374	LR594476
C. b. boisseauorum/male	CBB34	*Arvicola scherman*	1	374	LR594477
Ctenophthalmus sp./female	CT25	*Arvicola scherman*	1	374	LR594478
Ctenophthalmus sp./female	CT30, 32	*Arvicola scherman*	2	374	LR594479
Ctenophthalmus sp./female	CT23-24	*Arvicola scherman*	2	374	LR594480
Ctenophthalmus sp./female	CT28, 31	*Arvicola scherman*	2	374	LR594481
Ctenophthalmus sp./female	CT26-27, 29	*Arvicola scherman*	3	374	LR594482

Table 2. Biometrical data of males of *Ctenophthalmus baeticus boisseauorum* and *Ctenophthalmus apertus allani* analyzed in this study.

	Ctenophthalmus baeticus boisseauorum /males					*Ctenophthalmus apertus allani*/males				
	MIN	MAX	Mean	SD	VC	MIN	MAX	Mean	SD	VC
TL(mm)†	1.7	2.2	2.0	0.2	10	1.4	2.0	1.8	0.2	11
TW(mm)	0.5	0.7	0.6	0.1	16	0.5	0.7	0.6	0.1	16
HL(µm)	234	316	291	20	7	246	311	284	19	7
HW(µm)	176	205	188	6	3	170	199	183	9	5
LDBS9(µm)†	165	204	187	11	6	197	216	208	7	3
WDBS9(µm)†	31	66	42	8	19	16	28	23	4	17
LPBS9(µm)	169	204	186	11	6	129	212	175	19	11
LDPB(µm)	61	85	75	7	9	63	85	75	6	8
WDPB(µm)†	33	47	40	4	10	26	42	35	5	14
LVPB(µm)	68	89	79	7	9	73	89	85	5	6
WVPB(µm)†	31	47	41	5	12	19	26	22	2	9
DSETDPB(µm)†	21	42	28	5	18	12	21	17	3	18
WBB(µm)	75	106	85	8	9	68	92	79	6	8
PROTW(µm)	71	101	87	8	9	78	94	82	4	5
MESOW(µm)†	85	200	162	26	16	122	200	161	23	14
METW(µm)†	87	118	107	8	7	78	99	89	6	7

TL = total length, TW = total width, HL = total length of the head, HW = total width of the head, LDBS9 = total length of the distal branch of the IX sternum, WDBS9 = total width of the distal branch of the IX sternum, LPBS9 = total length of the proximal branch of the IX sternum, LDPB = total length of the dorsal processus basimere, WDPB = total width of the dorsal processus basimere, LVPB = total length of the ventral processus basimere, WVPB = total width of the ventral processus basimere DSETDPB = Distance between the two setae of the dorsal processus basimere, WBB = total width of the basimere basis, PROTW= total width of the prothorax, MESOW = total width of the mesothorax, METW = total width of the metathorax, MAX = maximum, MIN = minimum, SD = standard deviation, Mean = arithmetic mean, VC = coefficient of variation (percentage converted), † = Significant differences between *C. b. boisseauorum* and *C. a. allani* males ($P<0.005$).

Table 3. Biometrical data of females of *Ctenophthalmus* sp. analyzed in this study.

	Ctenophthalmus sp./females (isolated together with *C. b. boisseauorum* males from the same host)					*Ctenophthalmus* sp./females (isolated together with *C. a. allani* males from the same host)				
	MIN	MAX	Mean	SD	VC	MIN	MAX	Mean	SD	VC
TL(mm)	2.1	2.6	2.4	0.1	4	1.8	2.7	2.1	0.3	14
TW(mm)	0.7	0.8	0.8	0.1	13	0.6	0.8	0.7	0.1	14
HL(µm)	251	281	270	11	4	251	293	275	14	5
HW(µm)	199	246	225	18	8	234	287	246	17	7
BULGAL(µm)	63	89	79	9	11	71	96	78	9	11
BULGAW(µm)	42	61	50	6	12	45	59	52	5	10
APEHILL(µm)	35	59	46	6	13	40	52	46	5	11
DBMV(µm)	94	235	159	38	24	85	188	148	36	24
PS7L(µm)	12	94	56	26	46	35	141	69	43	62
PROTW(µm)	89	118	102	8	8	82	110	97	9	9
MESOW(µm)†	153	223	195	22	11	118	216	182	31	17
METW(µm)	94	129	117	12	10	99	118	107	6	6

TL = total length, TW = total width, HL = total length of the head, HW = total width of the head, BULGAL = total length of the bulga, BULGAW = total width of the bulga, APEHILL = total length of the apex of the hilla, DBMV = distance from bulga to ventral margin of the body, PS7L = total length of the VII sternum prominence, PROTW= total width of the prothorax, MESOW = total width of the mesothorax, METW = total width of the metathorax, MAX = maximum, MIN = minimum, SD = standard deviation, Mean = arithmetic mean, VC = coefficient of variation (percentage converted), † = Significant differences between the two groups of females ($P<0.005$).

Table 4. Similarity observed among all the partial *cox1* mtDNA gene sequences of different species belonging to *Ctenophthalmus* sp. obtained in this work and retreived from Genbank database. Values are given in percentages.

COX1	*C. a. allani*/males LR594440-LR594446	*C. b. boisseauorum*/males LR594447-LR594451, LR594456	*Ctenophthalmus* sp./females LR594452-LR594459	*C. calceatus cabirus* MH142441	*C. cryptotis* KM890939	*C. congeneroides congeneroides* KM890918	*C. dolichus dolichus* MF000657
C. a. allani/males LR594440- LR594446	98.7-100						
C. b. boisseauorum/males LR594447- LR594451, LR594456	98.7-100	99.3-100					
Ctenophthalmus sp./females LR594452- LR594459	98.2-99.8	98.9-100	98.7-100				
C. calceatus cabirus MH142441	85.7-86.3	86.1-86.3	85.4-86.3	-			
C. cryptotis KM890939	89.4-90.1	89.6-90.1	86.5-87.4	85.9	-		
C. congeneroides congeneroides KM890918	88.1-88.5	88.1-88.5	87.9-88.5	87.7	88.1	-	
C. dolichus dolichus MF000657	86.8-87.4	86.8-87.4	89.4-90.3	85.0	90.1	87.4	-

Table 5. Similarity observed amog all the partial *cytb* mtDNA gene sequences of different species belonging to *Ctenophthalmus* sp. obtained in this work and retreived from Genbank database. Values are given in percentages.

CYTB	*C. a. allani*/males LR594464-LR594467	*C. b. boisseauorum*/males LR594468-LR594477	*Ctenophthalmus* sp./females LR594478-LR594482	*C. cryptotis* KM890672	*C. congeneroides congeneroides* KM890651	*C. sanborni* KM890607
C. a. allani/males LR594464- LR594467	98.7-100					
C. b. boisseauorum/males LR594468-LR594477	98.4-100	98.7-100				
Ctenophthalmus sp./females LR594478- LR594482	98.4-100	98.4-99.7	98.4-100			
C. cryptotis KM890672	86.1-86.4	86.1-86.6	86.1-86.4	-		
C. congeneroides congeneroides KM890651	85.3-86.1	85.3-85.8	85.6-86.1	85.6	-	
C. sanborni KM890607	85.0-85.3	84.8-85.6	85.0-85.6	88.2	85.0	-

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

	ITS1	**ITS2**	***Cytb***	***cox1***
		PCR Mix		
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 G2 Green Master Mix DNA polymerase	25 µl	25 µl	25 µl	25 µl
Autoclaved distilled water to	50 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)	Kmt6 (Zhu *et al*., 2015)
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´

Table S2. List of taxa used in the analysis, including GenBank accession numbers and host information.

Species	Family	Host	Accession number	Gen Region	Sequence length
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 felis	Pulicidae	*Canis lupus familiaris*	LT853876	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
Pulex irritans	Pulicidae	*Lycalopex culpaeus*	LT797451	ITS2	324
Pulex irritans	Pulicidae	-	LT797448	ITS2	322
Pulex irritans	Pulicidae	-	LT797449	ITS2	322
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
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	Fox	MG668624	*Cox1*	489
Pulex irritans	Pulicidae	-	LT797468	*Cox1*	658
Pulex irritans	Pulicidae	-	LT797469	*Cox1*	658
Pulex irritans	Pulicidae	*Lycalopex griseus*	LT797466	*Cox1*	658
Pulex irritans	Pulicidae	*Lycalopex culpaeus*	LT797467	*Cox1*	658
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 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
Coptopsylla lamellifer	Coptopsyllidae	Rodent	MG138322	*Cox1*	658
Coptopsylla lamellifer	Coptopsyllidae	Rodent	MG138320	*Cox1*	658
Neotyphloceras crassispina	Ctenophthalmidae	*Abrocoma bennetti*	KM890944	*Cox1*	1,197
Chiliopsylla allophyla	Ctenophthalmidae	Unknown	KM891001	*Cox1*	1,244
Corrodopsylla curvata curvata	Ctenophthalmidae	Unknown	KR142880	*Cox1*	638
Corrodopsylla curvata curvata	Ctenophthalmidae	Unknown	KM529504	*Cox1*	615
Ctenophthalmus sp.	Ctenophthalmidae	Unknown	KM891003	*Cox1*	630
Ctenophthalmus calceatus cabirus	Ctenophthalmidae	*Lemmniscomys striatus*	MH142441	*Cox1*	659
Ctenophthalmus dolichus dolichus	Ctenophthalmidae	*Rattus* sp.	MF000657	*Cox1*	657
Ctenophthalmus congeneroides congeneroides	Ctenophthalmidae	Unknown	KM890918	*Cox1*	1,182
Ctenophthalmus cryptotis	Ctenophthalmidae	Unknown	KM890939	*Cox1*	1,218
Panorpa meridionalis	Panorpidae	-	LT604125	*Cox1*	658
Panorpa meridionalis	Panorpidae	-	LT604126	*Cox1*	658
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LN897473	*Cytb*	374
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	Pulicidae	*Canis lupus familiaris*	LT853878	*Cytb*	374
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
Pulex irritans	Pulicidae	*Lycalopex culpaeus*	LT797476	*Cytb*	374
Pulex irritans	Pulicidae	*Lycalopex griseus*	LT797480	*Cytb*	374
Pulex irritans	Pulicidae	-	LT797473	*Cytb*	374
Pulex irritans	Pulicidae	-	LT797474	*Cytb*	374
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
Stephanocircus dasyuri	Stephanocircidae	Unknown	KM890619	*Cytb*	369
Stephanocircus pectinipes	Stephanocircidae	Unknown	KM890658	*Cytb*	369
Pygiopsylla hoplia	Pygiopsyllidae	Unknown	KM890657	*Cytb*	369
Metastivalius mordax	Stivaliidae	Unknown	KM890628	*Cytb*	369
Parastivalius novaeguinae	Stivaliidae	Unknown	KM890629	*Cytb*	369
Neotyphloceras crassispina chilensis	Ctenophthalmidae	Unknown	KM890613	*Cytb*	369
Neotyphloceras crassispina	Ctenophthalmidae	Unknown	KM890677	*Cytb*	369
Chiliopsylla allophyla	Ctenophthalmidae	Unknown	KM890736	*Cytb*	369
Ctenophthalmus congeneroides	Ctenophthalmidae	Unknown	KM890651	*Cytb*	369
Ctenophthalmus cryptotis	Ctenophthalmidae	Unknown	KM890672	*Cytb*	369
Ctenophthalmus sanborni	Ctenophthalmidae	Unknown	KM890607	*Cytb*	330
Ctenophthalmus sp.	Ctenophthalmidae	Unknown	KM890749	*Cytb*	369
Panorpa meridionalis	Panorpidae	-	LT604127	*Cytb*	374
Panorpa meridionalis	Panorpidae	-	LT604128	*Cytb*	374