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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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Research Paper

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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 with 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 and 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 great importance in terms of specific identification because 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 (Beaucournu and Launay, 1990; Lewis, 1993). 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 and Lareschi, 2014; Acosta and Hastriter, 2017; Keskin, 2019; Keskin and Beaucournu, 2019*a*) including the descriptions of two new species and a new subspecies of the genus *Ctenophthalmus* (Keskin and Beaucournu, 2019*b*)

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 based on the size, shape, and chaetotaxy of their genitalia; however, females show slight morphological differences on each other (Beaucournu & Launay, 1990). 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 was so great that it seemed useless to attempt to make a taxonomical key for this sex (Beaucournu and Launay, 1990; Lewis, 1993).

Due to the inability of systematics 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*., 2018*a*, 2018*b*). 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 the 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*. (2018*a*) 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 they 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 (*cox*1) and *cytochrome* b (*cyt*b) 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, 30 fleas were put away for molecular purposes, whereas the rest of the samples (50 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 and Rothschild (1953) and Beaucournu and Launay (1990). After morphological identification, 30 males and 20 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, USA) (Pardo and Ruiz, 2002). Furthermore, to assess phenotypic variations among the samples, morphometric data were explored using multivariate analysis in nine 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, consisting in a method for summarizing most of the variations in a multivariate dataset in few dimensions (Dujardin and 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 30 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) was transferred to a 1.5 ml tube containing 180 µl of G2 lysis buffer (Qiagen, Hilden, Germany) and 20 µl of proteinase K (Qiagen), and incubated at 56°C overnight. DNA extraction was performed with an EZ1 DNA Tissue Kit (Qiagen) according to the manufacturer recommendations. Flea DNAs were then eluted in 100 µl of Tris EDTA buffer using the DNA extracting EZ1 Advanced XL Robot (Qiagen). 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, *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). In the case of *cox*1, 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

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Table 1. GenBank accession numbers of ITS1, ITS2 and partial *cytb*, *cox*1 gene sequences of individuals of *Ctenophthalmus* sp. (CT), *C. baeticus boisseauorum* (CBB), and *C. apertus allani* (CAA) obtained in this study

Species/gender	Sample ID	Host	Number of fleas	Base pairs (bp)	Accession number
ITS1					
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
*Cox*1					
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
*Cyt*b					
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

(*Continued*)

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Table 1. (*Continued.*)

Species/gender	Sample ID	Host	Number of fleas	Base pairs (bp)	Accession number
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 ($P < 0.005$).

† = Significant differences between C. b. boisseauorum and C. a. allani males ($P < 0.005$).

LCO1490 and HC02198 (Folmer *et al.*, 1994); however, we did not obtain reliable results owing to co-amplification of non-specific 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 a reverse primer for this partial gene. The ITS1, ITS2, *cox*1, and *cyt*b partial gene sequences obtained from all specimens analyzed were deposited in the GenBank database (table 1).

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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 ($P < 0.005$).
† = Significant differences between C. b. boisseauorum and C. a. allani males ($P < 0.005$).

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 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). ML trees were generated using the PHYML package from Guindon and Gascuel (2003), whereas BI 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 (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 three markers (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 γ-distributed rate variation GTR + G (ITS2) and a Tamura–Nei model with γ-distributed rate variation and a proportion of invariable sites, TrN + I + G (*cox*1 and *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 BI were *nst* = 6 with γ rates (ITS2) and *nst* = 6 with invgamma rates (*cox*1 and *cyt*b). For BI, the standard deviation of split frequencies was used to determine whether the number of generations completed was sufficient; the chain was sampled every 500 generations and each dataset was run for 10 million generations. Adequacy of sampling and run convergence was assessed using the effective sample size diagnostic in the 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 the examination of the log-likelihood values of the chains. The Bayesian posterior probabilities (BPP) comprise the percentage converted.

The phylogenetic analyses, based on ITS2, *cox*1, and *cyt*b sequences, were carried out using our sequences and those obtained from the GenBank database (see table S2). Phylogenetic trees based on concatenated sequences of ITS2, *cox*1, and *cyt*b 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, 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, *cox*1, and *cyt*b 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:

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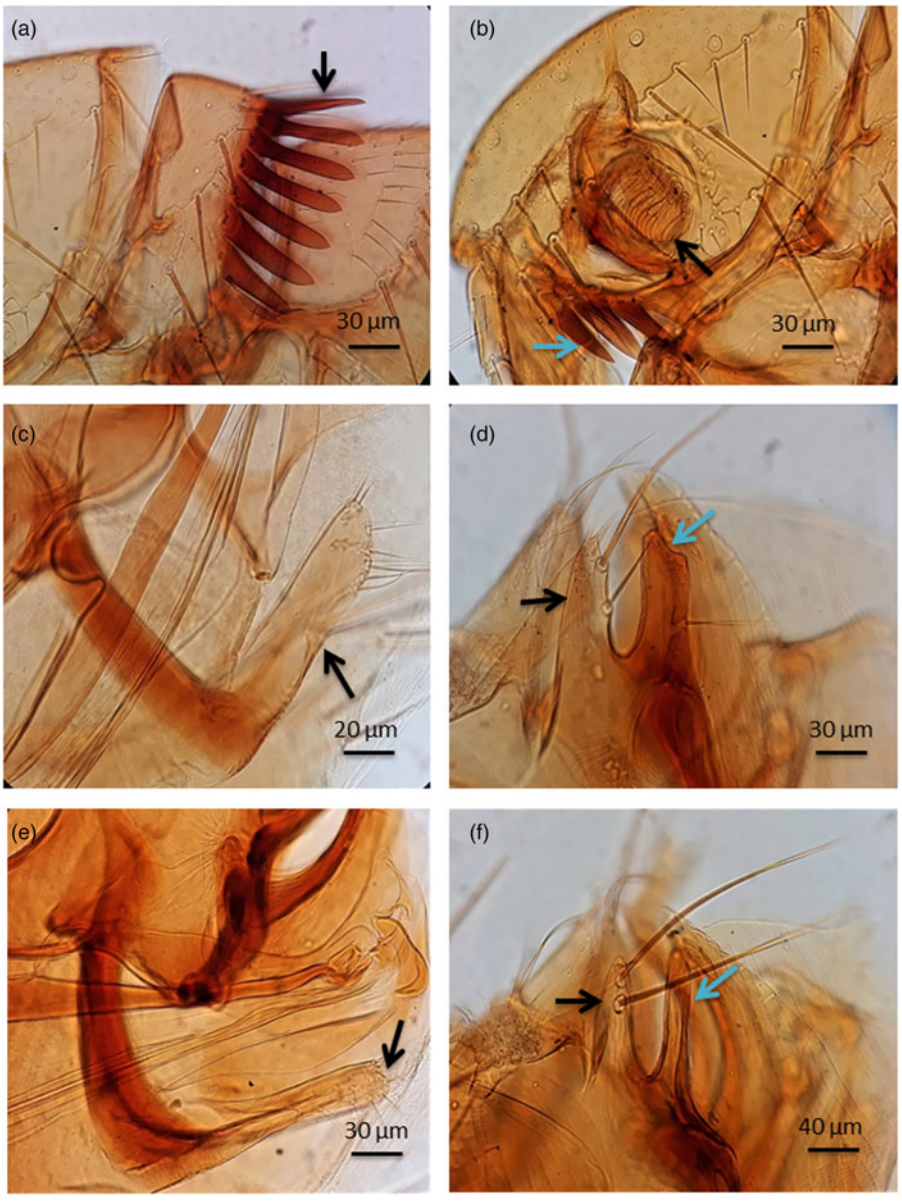

Figure 1. Morphological characteristics of *Ctenophthalmus* sp, *Ctenophthalmus baeticus boisseauorum*, and *Ctenophthalmus apertus allani*. (a) Pronotal ctenidia (black arrow) of *Ctenophthalmus* sp.; (b) Head with antennae (black arrow) and genal ctenidia of *Ctenophthalmus* sp (blue arrow); (c) Male distal branch of IX sternum (black arrow) of *C. b. boisseauorum*; (d) Dorsal processus basimere (black arrow) and ventral processus basimere (blue arrow) of males of *C. b. boisseauorum*; (e) Male distal branch of IX sternum (black arrow) of *C. a. allani*; (f) Dorsal processus basimere (black arrow) and ventral processus basimere (blue arrow) of males of *C. a. allani*.

- 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 lengths on 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 the 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 on 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

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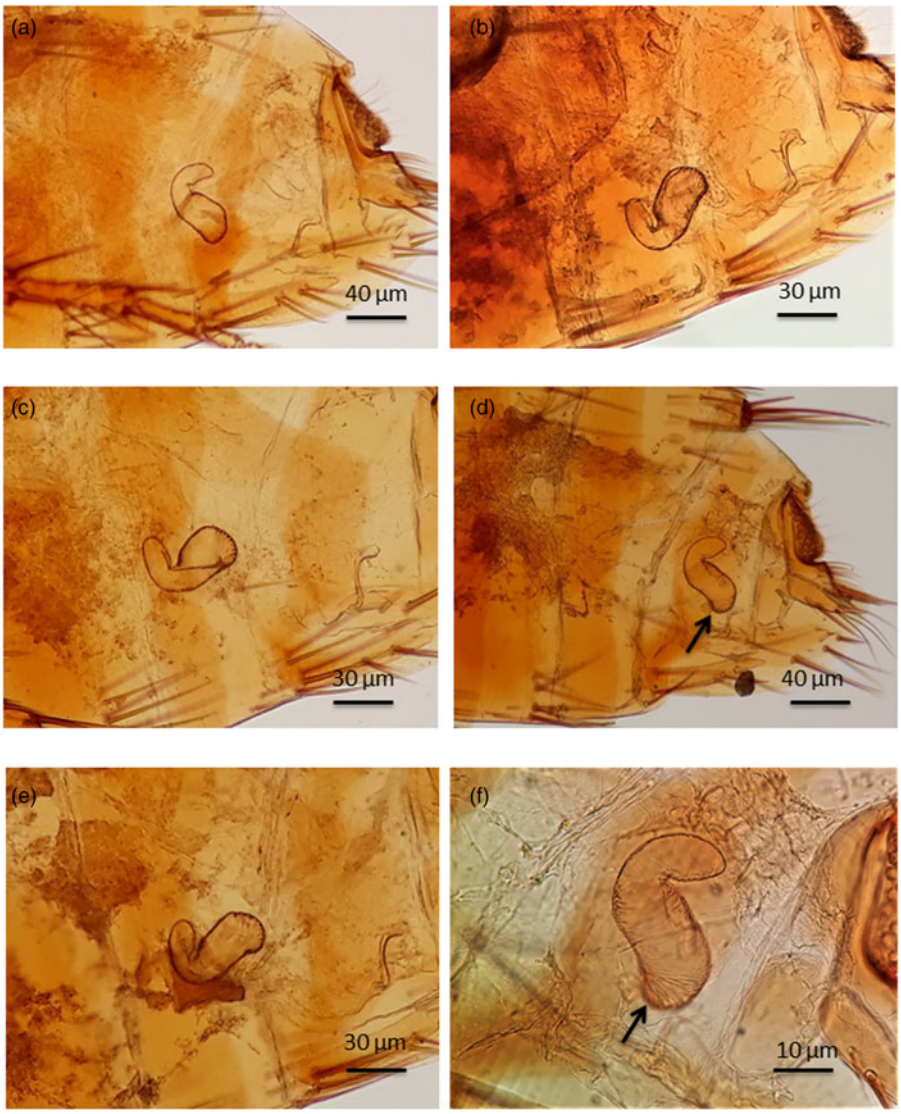

Figure 2. Spermatheca of females of *Ctenophthalmus* sp. analyzed in this study. A small prominence at the end of the bulga is arrowed in fig. 2d and f.

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, f) which sometimes could appear less prominent (fig. 2b, c). 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–g), whereas other females from both groups showed only one well-developed apical lobe (fig. 3h–k) together with a deep sinus (fig. 3i–k). According to chaetotaxy, no significant differences were observed between both females' groups. Therefore, all specimens assessed showed the presence of six setae with different degrees of development (fig. 4). The distribution of these setae changed among all the specimens analyzed; however, it was common in the presence of three strong setae, longest than the other ones, which appeared very close to each other (fig. 4a–f). 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

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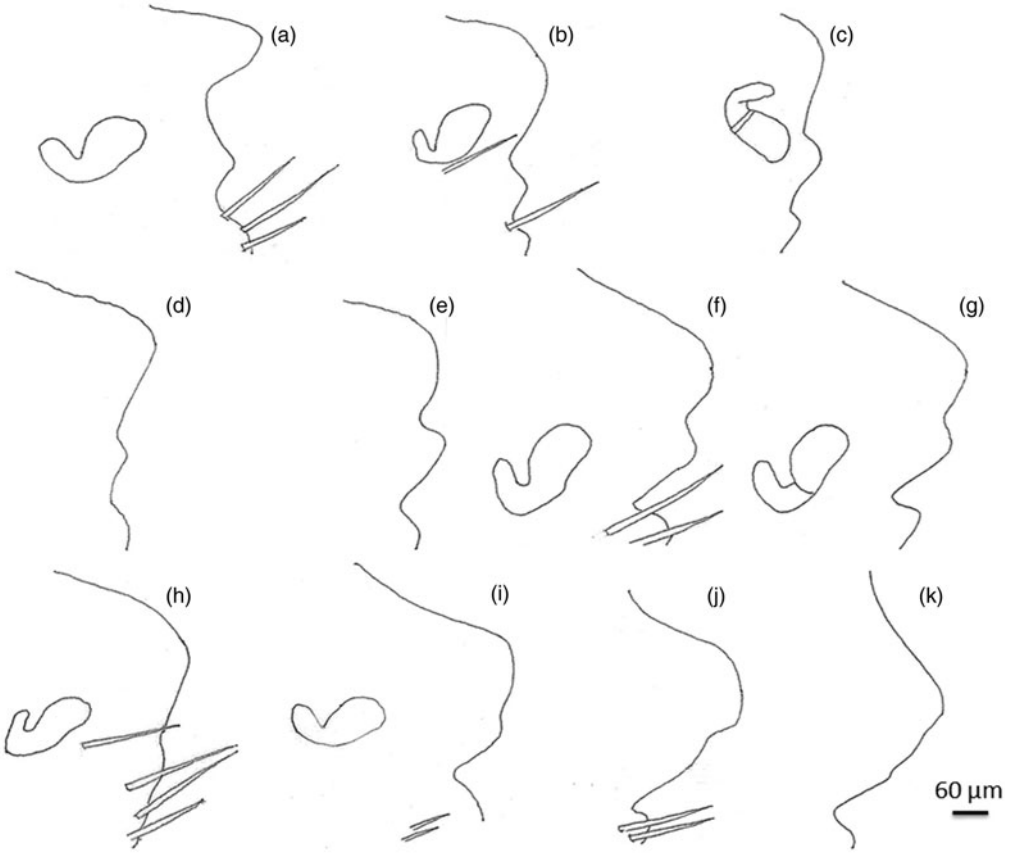

Figure 3. Variability observed in the shape of the margin of sternum VII of *Ctenophthalmus* sp. females.

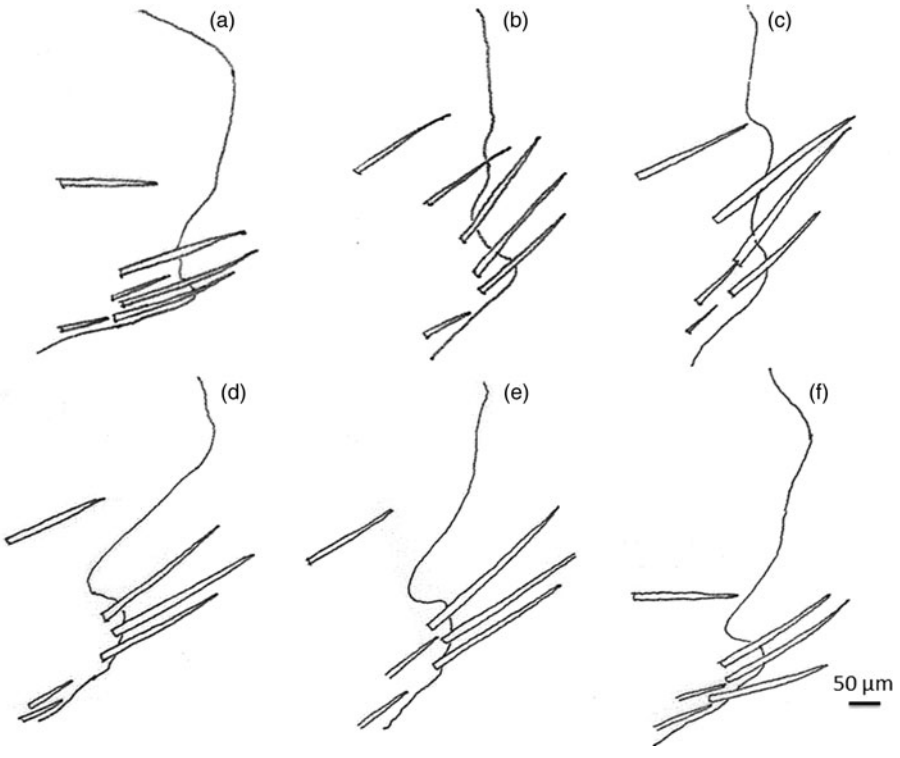

Figure 4. Variability observed in chaetotaxy of sternum VII of females belonging to *Ctenophthalmus* sp. assessed in this study.

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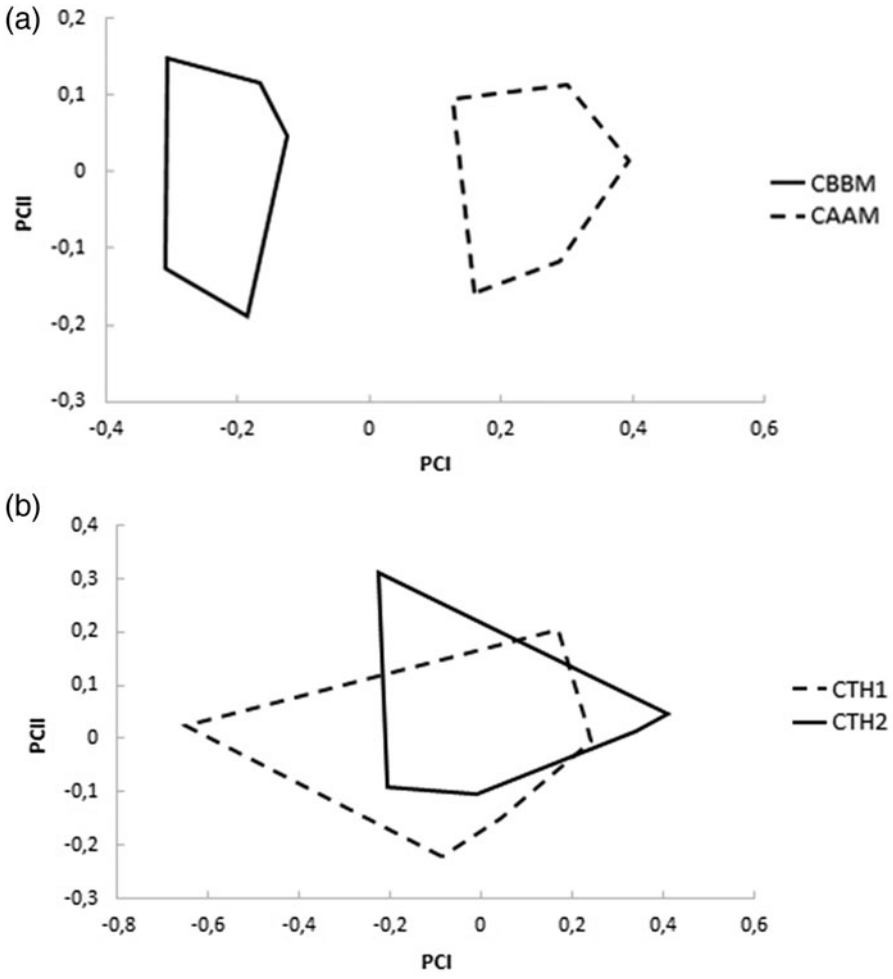

Figure 5. (a) Factor map corresponding to adult *C. b. boisseauorum* (CBBM) and *C. a. allani* (CAAM) males from Asturias (Spain). Samples are projected onto the first (PC1, 73%) and second (PC2, 9%) principal components. Each group is represented by its perimeter. (b) Factor map corresponding to adult *Ctenophthalmus* sp. females from Asturias (Spain). Samples are projected onto the first (PC1, 67%) and second (PC2, 18%) principal components. Each group is represented by its perimeter. CTH1: Females of *Ctenophthalmus* sp. isolated together with *C. b. boisseauorum* males from the same host; CTH2: Females of *Ctenophthalmus* sp. isolated together with *C. a. allani* males from the same host.

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 of 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

ITS1 and ITS2 analysis

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

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

Partial cox1 mtDNA gene analysis

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

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Table 4. Similarity observed among all the partial *cox*1 mtDNA gene sequences of different species belonging to *Ctenophthalmus* sp. obtained in this work and retrieved from Genbank database. Values are given in percentages

*COX*1	*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 among all the partial *cyt*b mtDNA gene sequences of different species belonging to *Ctenophthalmus* sp. obtained in this work and retrieved from Genbank database. Values are given in percentages

*CYT*B	*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	–

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 *cyt*b mtDNA sequences of all the *Ctenophthalmus* sp. specimens obtained in this study was 374 (table 1). The similarity observed among the partial *cyt*b 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 *cyt*b sequences of 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 *cyt*b 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*).

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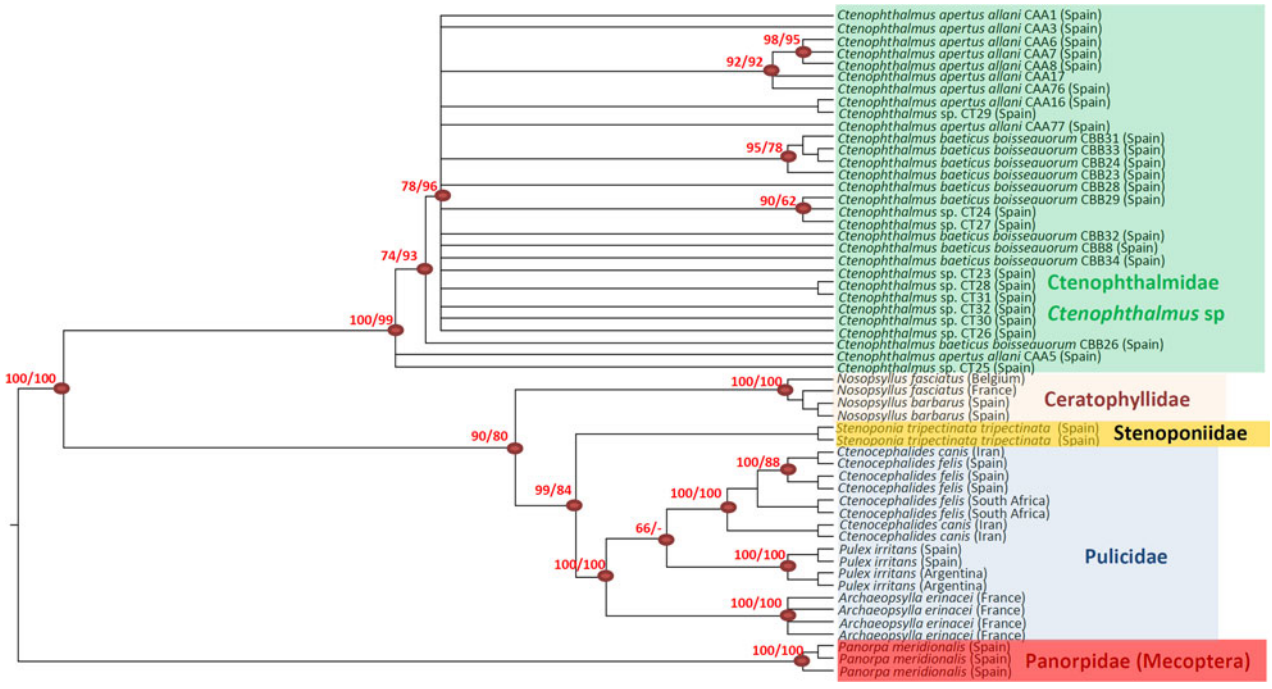

Figure 6. Phylogenetic tree of *Ctenophthalmus sp.*, *Ctenophthalmus baeticus boisseauorum*, and *Ctenophthalmus apertus allani* assessed in this study (see table 1) based on concatenated Internal Transcribed Spacer 2 (ITS2), partial *cytochrome c-oxidase* subunit 1 (*cox*1) and *cytochrome* b (*cyt*b) 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. The Bayesian posterior probabilities (BPP) are percentage converted.

The phylogenetic tree inferred from partial *cyt*b 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 *cyt*b 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 a polytomy in the same clade (Pulicidae, Ctenophthalmidae, Ceratophyllidae, Stephanocircidae, Pygiopsyllidae, Stivaliidae and Stenoponiidae) (fig. S3).

The concatenated dataset of ITS2, partial *cyt*b and *cox*1 gene sequences included 1405 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, different 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 is 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 approximately 300 valid taxa (Beaucournu and Lorvelec, 2014) representing the most abundant flea genus in Europe (Beaucournu and 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 and 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 and Launay, 1990; Beaucournu and Loverlec, 2014) have just placed *C. b. boisseauorum* in different geographical areas of the north of Spain. The morphological analysis carried out by Hopkins and Rothschild (1966) and Beaucournu and Launay (1978, 1990) reported that several specimens of each '*apertus*' subspecies evidenced great variability in male modified abdominal segments as well as in female sternum VII; however, these authors only provided taxonomical keys for males. Beaucournu and Launay (1978, 1990) speculated about the possibility that this morphological variability was possibly due to interbreeding of two subspecies

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which have sympatric distribution, but finally, they supported that this fact was just different morphotypes as a consequence of the wide morphological intraspecific '*apertus*' variations. The higher degree of morphological variation observed in males could be explained because in temporary parasites, males mostly have a shorter life period and are more active in terms of looking for new hosts. Thus, males leave earlier from their hosts (Marshall, 1981), whereas females need blood to produce their eggs, leaving their hosts later (Dryden, 1993). Attending to our morphological results, we could discriminate between the males of *C. b. boisseauorum* and *C. a. allani* generally based on the width of the ventral processus basimere and in the total distance between the two setae present on the dorsal processus basimere which showed different lengths in *C. b. boisseauorum*. Unlike males, females showed an aleatory high degree of polymorphism based on the shape of the margin of the sternum VII. These characters did not correspond with any subspecific morphological pattern between the two groups of *Ctenophthalmus* females analyzed in this study. Márquez and Soringuer (1987) observed great variability in the margin of sternum VII in the females of *C. a. meylani* noticing that some specimens showed morphological characteristics similar to the subspecies *C. a. queirozi*. These authors argued that in each population a great morphological variability could exit in females associated with different ecological traits which would be responsible for the selection of one specific morphotype. Nevertheless, in our study, the variability observed in the shape of the margin of the sternum VII was similar in both female groups isolated from the same host and from the same geographical origin.

In spite of that, Marquez and Soringuer (1987) found some differences in this region in terms of the number of setae from one population of C. *a. meylani* isolated from Granada, Córdoba, and Jaén (Spain). Nevertheless, most specimens analyzed by these authors showed six main setae in sternum VII agreeing on our results. In this sense, the chaetotaxy of sternum VII of females was assessed in our study in order to find new possible morphological variations which allow us to discriminate between females of *Ctenophthalmus* genus. Nevertheless, both characters appeared hardly identical (with slight differences in the spermatheca of some specimens) even between the two female groups of this study. These results would be in agreement with Beaucournu and Launay (1990) who did not find clear differences in this region in *Ctenophthalmus* genus. These taxonomical results were corroborated by PCA and biometrical analysis but were not in concordance with molecular and phylogenetic results, especially based on male specimens which showed a high degree of nucleotide similarity.

ITS1 and ITS2 have been reported as two useful markers in order to infer phylogenetic studies in flea taxonomy, being used with several purposes: molecular characterization of several flea species (Vobis *et al*., 2004), molecular discrimination among congeneric species (Marrugal *et al*., 2013; Zurita *et al*., 2016), molecular characterization of different geographical lineages from the same species (Luchetti *et al*., 2007; Ghavami *et al*., 2018), or even molecular discrimination among possible cryptic species (Zurita *et al*., 2019).

In our study, we observed a high similarity (99.6–100%) between *C. b. boisseauorum* and *C. a. allani* based on ITS sequence analysis. These results did not correspond with the morphological differences observed between both subspecies agreeing with Zurita *et al.* (2018*a*) who did not observe substantial nucleotide differences when they compared ITS1 and ITS2 sequences of *N. barbarus* and *N. fasciatus* supporting the idea that *N. barbarus* should be considered a junior synonym of *N. fasciatus*.

Even in a long way to ITS sequences, mitochondrial markers have been widely used for estimating molecular phylogenies in fleas in the last years (Lawrence *et al*., 2014; Hornok *et al*., 2018; Zurita *et al*., 2018*a*, 2018*b*). The *cox*1 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 studies 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). *Cyt*b partial gene has also been widely used in order to infer phylogenetic relationships among different closed flea taxa (Dittmar and 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 *cyt*b and *cox*1 (likewise ITS1 and ITS2) are easily able to discriminate themselves between two closely 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 *cyt*b and *cox*1 were able to discriminate between these 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 *cox*1 and *cyt*b 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 reinforces the results reported by the 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 and 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

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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 and Beaucournu, 2019*b*). 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 and 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.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485320000127.

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