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ORIGINAL ARTICLE

Morphological, molecular and phylogenetic characterization of *Leptopsylla segnis* and *Leptopsylla taschenbergi* (Siphonaptera)

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Abstract

The taxonomic status of Leptopsyllidae family has remained controversial over the years. Thus, some entomologists placed this group of fleas within Ceratophyllidae family, considering it at level of Leptopsyllinae subfamily or even appearing as a paraphyletic group within Siphonaptera phylogeny. This fact is emphasized by the lack of molecular and phylogenetic data of Leptopsyllidae taxa available in public databases. The aim of this study was to carry out a comparative morphological, phylogenetic and molecular study of two species of *Leptopsylla* genus with zoonotic relevance (*Leptopsylla segnis* and *Leptopsylla taschenbergi*) isolated from rodents collected from different geographical areas of Europe in order to molecularly characterize both taxa and to establish their taxonomic and phylogenetic status within Leptopsyllidae family. For this purpose, we have analysed and compared several morphological traits between *L. segnis* and *L. taschenbergi* and compared five different molecular markers (ITS2, *EF1*-α, *cox*1, *cox*2 and *cyt*b) among these both species and others belonging to Leptopsyllidae family. Based on the morphological results, we found a phenotypic plasticity phenomenon in one female specimen showing morphological characters of *L. segnis* but molecular sequences distinctive for *L. taschenbergi*. Furthermore, the molecular and phylogenetic analysis could easily discriminate among both species providing, by the first time, a monophyletic origin of Leptopsyllidae family. Lastly, with this work, we demonstrate one more time, the usefulness of the combination of mitochondrial and nuclear markers to solve taxonomic and phylogenetic issues within Siphonaptera field by the use of concatenated dataset.

KEYWORDS

Siphonaptera, *Leptopsylla*, Insecta, taxonomy and phylogeny

1 | INTRODUCTION

Leptopsyllidae family includes 22 genera and 331 named taxa mainly distributed in Palearctic region, although we can find leptopsyllids in Neartic, Afrotropic, Neotropic and Indo-Malayan region (Johnson, 1957; Lewis, 1993; Whiting et al., 2008). Most leptopsyllids are parasites of rodents, but a few parasitize lagomorphs (hares, rabbits and pikas), insectivores and birds (Lewis, 1993, 1999). The taxonomic status of Leptopsyllidae family has remained controversial over the years. Thus, some entomologists placed this group of fleas within Ceratophyllidae family, considering it at level of Leptopsyllinae subfamily (Beaucournu & Launay, 1990; Johnson, 1957; Smit, 1987); however, most recent phylogenetic studies placed Leptopsyllidae family as a whole family close related with Ceratophyllidae. According to this idea Lewis (1993), Medvedev (1994, 1998) recognized five major superfamilies within Siphonaptera, including Ceratophyllidae, Leptopsyllidae, Ischnopsyllidae and Xiphiopsyllidae within Ceratophylloidea superfamily.

In spite of the Palearctic distribution of Leptopsyllidae species, only two genera (*Leptopsylla* sp. and *Peromyscopsylla* sp.) are presented in Mediterranean subregion. Eleven taxa, three species (*Leptopsylla segnis*, *Leptopsylla algira* and *Leptopsylla taschenbergi*) and eight subspecies have been described so far within *Leptopsylla* genus in this geographical area (Beaucournu & Launay, 1990). All these species use to colonize murid species although *L. algira* (Jordan & Rothschild, 1912) can commonly parasite shrews of the genus *Croccidura*. *Leptopsylla segnis* (Schönherr, 1811) is considered a cosmopolitan flea labelled as the European mouse flea for some authors (Durden & Traub, 2002). They typically parasitize the house mouse (*Mus musculus*), including laboratory colonies. Rarely, large populations of *L. segnis* cause host anaemia or other problems in mouse-rearing facilities (Durden & Traub, 2002); however, flea infestations of these rodents are usually more important for its zoonotic implications by the potential transmission of pathogens rather than their discomforting bites. Therefore, *L. segnis* has appeared in some flea-borne disease studies in the last years; for example, some species of *Bartonella* sp., such as *B. elizabethae* have been detected in *L. segnis* (Loftis et al., 2006). As well as Bartonellosis, the causative agents of murine typhus (*Rickettsia typhi*) and the emerging disease known as flea-borne spotted fever (*Rickettsia felis*) have also been detected in *L. segnis* (Christou et al., 2010; Durden & Traub, 2002). In this sense, Azad and Traub (1987) reported that *L. segnis* could transmit murine typhus infection in an effective way as much as *Xenopsylla cheopis*, considered the main vector of this illness.

On the contrary, the presence of *L. taschenbergi* (Jordan & Rothschild, 1914) in rodents has been reported in some epidemiological studies, including those regarding to the detection of some pathogen's bacteria in flea populations. Three subspecies have been described for this species so far (*L. taschenbergi*, *L. taschenbergi amitina* and *L. taschenbergi calamana*). These taxa use to parasitize Muroidea from the genera *Apodemus* and *Mus* spreading out throughout Mediterranean countries including North Africa and Southwestern of Europe (Beaucournu & Launay, 1990). *Leptopsylla taschenbergi* has been recently founded in one study conducted in intensively farmed landscapes in the Castilla-y-León region (Northwest Spain) mainly parasitizing mice (*M. spretus* and *A. sylvaticus*) (Herrero-Cófreces et al., 2021). Previously, Cevidanes et al. (2016) also assessed the prevalence of *L. t. amitina* in wood mice (*A. sylvaticus*) among seasons in natural and residential habitats in Barcelona, Northwest Spain.

From a zoonotic point of view, Zurita et al. (2021) recently detected the presence of three different *Bartonella* species (*B. doshiae*, *B. elizabethae* and *B. taylorii*) in *L. taschenbergi* specimens collected from Southwestern Europe including this rodent flea species in the list of fleas which could potentially play a role in the transmission of these pathogens in Europe.

Based on all these studies mentioned above, we can confirm the common presence of *Leptopsylla* specimens parasitizing rodents in urban and rural areas and their closeness to human populations in some cases. In spite of that, there is no taxonomic and phylogenetic studies of *Leptopsylla* species based on molecular data or combining it with morphological traits. In fact, the most completed phylogenetic study done so far (Whiting et al., 2008) did not use any sequence of *Leptopsylla* specimens for its analysis, highlighting the necessity to provide new molecular data from this genus and update its phylogenetic and taxonomical status within Leptopsyllidae family.

Within this context, the aim of this study was to carry out a comparative morphological, phylogenetic and molecular study of two species of *Leptopsylla* genus (*L. segnis* and *L. taschenbergi*) isolated from rodents collected from different geographical areas of Europe to molecularly characterize both taxa and to establish their taxonomic and phylogenetic status within Leptopsyllidae family. For this purpose, we have analysed and compared several morphological traits between *L. segnis* and *L. taschenbergi* and compared five different molecular markers among these both species and others belonging to Leptopsyllidae family. We amplified and sequenced the nuclear *Elongation Factor 1 alpha* (*EF1-α*), the Internal Transcribed Spacer 2 (ITS2) ribosomal DNA (rDNA) and the *cytochrome c oxidase* subunit 1 (*cox*1), *cytochrome c oxidase* subunit 2 (*cox*2)

and *cytochrome* b (*cyt*b) mitochondrial DNA (mtDNA) partial genes of both populations.

2 | MATERIAL AND METHODS

2.1 | Collection of samples

Specimens of *L. taschenbergi* were collected from wood mice *Apodemus sylvaticus* trapped from the Nature reserve of Py-Conat (France) during two sampling periods in September 2019 and September 2021, whereas *L. segnis* specimens were collected from different rodent hosts (*Mus spretus*, *Rattus rattus*, *Apodemus mystacinus* and *Heteromys* sp.) trapped from different regions of France, Israel, Belgium and Spain (see Table S1). All *L. segnis* specimens were donated from colleagues (see Acknowledgments). All rodent specimens were captured using live traps; afterwards, each rodent was exhaustively examined for fleas by combing through an inspection of head, neck, body, sides, tail and ventral regions of each animal. Fleas were collected manually and kept in Eppendorf tubes with 96% ethanol for subsequent identification and DNA extraction.

2.2 | Morphological identification

For morphological analysis, all whole specimens were examined and photographed under an optical microscope to carry out a first specific classification. In total, 271 flea specimens from different localities from France, Israel, Belgium and Spain were morphologically classified as *L. segnis* (39 fleas) and *L. taschenbergi* (232 fleas). Thirty-three specimens (15 males: 7 *L. segnis* and 8 *L. taschenbergi* and 18 females: 8 *L. segnis* and 10 *L. taschenbergi*) from both species 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 were studied by comparison with figures, keys and descriptions by Lewis (1993) and Beaucournu and Launay (1990). Subsequently, 37 flea specimens from both species were put away for DNA analysis.

2.3 | Molecular and phylogenetic study

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

The DNA markers sequenced in the present study (*EF1-α*, ITS2 rDNA, *cox*1, *cox*2 and *cyt*b) 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 S2). For the sequencing of *cox*1 and *EF1-α*, we had to use two different set of forward primers for each species. Thus, we use LCO1490 (Folmer et al., 1994) and M47F (Zhu et al., 2015) as forward primers for the amplification of *cox*1 and *EF1-α* sequences of *L. taschenbergi*, respectively. Nevertheless, using this set of forward primers, we could not amplify these gene fragments for *L. segnis*; therefore, it was necessary the use of Kmt6 (Zhu et al., 2015) and M46-1 (Whiting, 2002), respectively, obtaining shorter gene sequences for those two markers.

The *EF1-α*, ITS2, *cox*1, *cox*2 and *cyt*b sequences were deposited in GenBank (Table S1).

The PCR products were checked on SYBR Safe stained 2% Tris–borate–ethylenediaminetetraacetic acid (TBE) agarose gels. PCR products were purified using the QWizard SV Gel and PCR Clean-Up System Kit (Promega, Madison, WI, U.S.A.). Once purified, these products were sent to the commercial company Stab Vida (Lisbon, Portugal) for sequencing process. We separately sent purified PCR products and 20 μl of 100 μM of each pair of primers (see Table S1) for each molecular marker. Sanger sequencing was carried out using an automatic LI-COR® DNA sequencer. Sequences were aligned with the MUSCLE alignment method (Edgar, 2004) in MEGA, version 5.2 (Tamura et al., 2011). Alignment settings comprised a gap open = −400.00, a gap extend = 0.0, an UPGMA (Unweighted Pair Group Method with Arithmetic Mean) as a cluster method and a minimum Diagonal length = 24. Sequence similarity was expressed as percentage using uncorrected p-distances method as implemented in MEGA, version 5.2 (Tamura et al., 2011). In this study, we compare the sequence divergence among *L. segnis* and *L. taschenbergi* including others taxa (species and genus) from Leptopsyllidae family: *Leptopsylla nana*, *Amphipsylla* sp., *Frontopsylla* sp., *Ophthalmopsylla* sp., *Pectinoctenus* sp., *Peromyscopsylla* sp., *Paractenopsyllus* sp. and *Paradoxopsyllus* sp.

Phylogenetic trees were inferred by maximum likelihood (ML) and Bayesian inferences (BI). Maximum likelihood trees were generated using the PHYML package from Guindon and Gascuel (2003), whereas Bayesian inferences were generated using MRBAYES, version 3.2.6 (Ronquist & Huelsenbeck, 2003). JMODELTEST (Posada, 2008) was used to determinate the best-fit substitution model

for *EF1-α*, *cox*1, *cox*2 and *cyt*b. Models of evolution were chosen for subsequent analyses according to the Akaike information criterion (Huelsenbeck & Rannala, 1997; Posada & Buckley, 2004). The concatenated alignment of *EF1-α*, *cox*1, *cox*2 and *cyt*b was analysed by BI after partitioning and model selection with JMODELTEST. For ML inference, best-fit nucleotide substitution models included GTR + I + G for all markers assessed. Support for the topology was examined using bootstrapping (heuristic option) (Felsenstein, 1985) over 1000 replications. The commands used in MRBAYES, version 3.2.6 for BI, were *nst* = 6 with invgamma rates (*EF1-α*, *cox*1, *cox*2 and *cyt*b). For BI, the standard deviation of split frequencies was used to determine whether the number of generations completed was enough; the chain was sampled every 500 generations and each dataset was run for 10 million generations. Adequacy of sampling and run convergence were assessed using the effective sample size diagnostic in tracer, version 1.6 (Rambaut & Drummond, 2007). Trees from the first million generations were discarded based on an assessment of convergence. Burn-in was determined empirically by examination of the log likelihood values of the chains. Bayesian posterior probabilities (BPP) were used to assess the reliability of nodes.

The phylogenetic analyses of single gene fragments *EF1-α*, *cox*1, *cox*2 and *cyt*b were carried out using our sequences and those obtained from GenBank (Table S3). Phylogenetic trees were rooted using *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).

ITS2 sequences were exclusively used to characterize and compare *L. segnis* and *L. taschenbergi* species assessed in this study.

3 | RESULTS

3.1 | Morphological results

All the specimens collected from El Hierro (Spain), Beit Oren and Upper Galilee (Israel), Pont-à-Celles (Belgium) and only one specimen (sample ID: LS38) from the Nature reserve of Py-Conat (France) (Table S1) studied in this work showed morphological characteristics expected for the species *L. segnis*:

- Presence of pronotal and genal comb. Genal comb composed of four well-developed spines. Presence of two small spiniform setae in the head front (Figure 1a).
- Spermatheca of females showing a bulga wider than the hilla but practically with the same length. Ventral margin of sternite VII with a straight profile ending on right angle to the posterior margin (Figure 1b).
- Male with a big hamulus dilated in its apical portion (Figure 1c).
- Telomere or movable process of males width and curved. Basimere or fixed process with a straight apical margin without any lobes observed (Figure 1d).

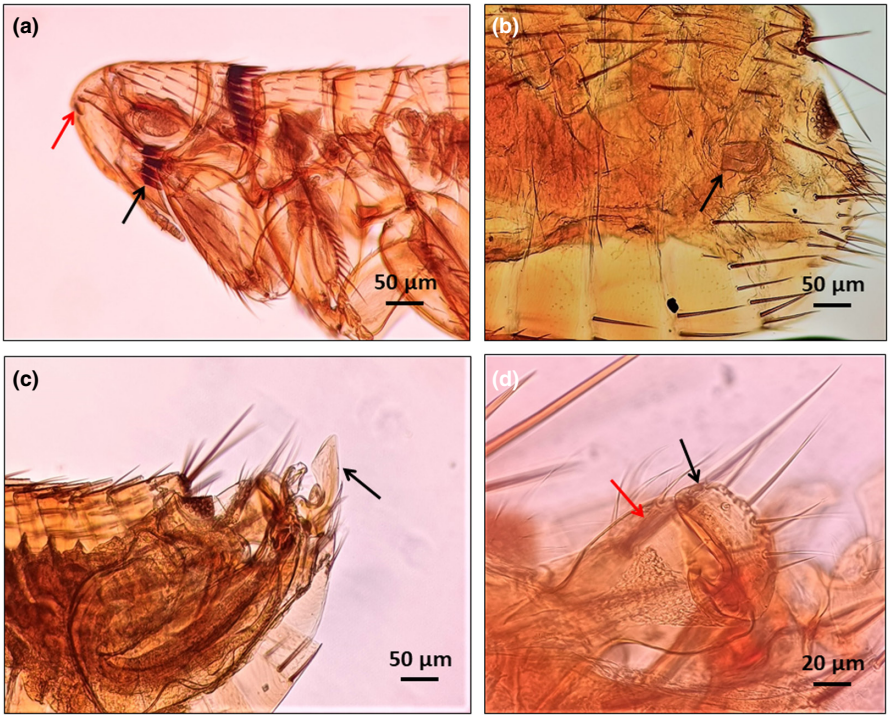

FIGURE 1 Morphological characteristics of *L. segnis* specimens assessed in this study. (a) Head and front with genal comb and two small spiniform setae arrowed. (b) Spermatheca (arrowed) of females. (c) Hamulus of males (arrowed). (d) Telomere and Basimere (both arrowed) of males.

All the remaining specimens collected from the Nature reserve of Py-Conat (France) (see Table S1) showed diagnostic morphological traits of the species *L. taschenbergi*:

- Presence of pronotal and genal comb. Genal comb composed of three well-developed spines. Presence of two small spiniform setae in the head front (Figure 2a).
- Antesensiliales setae regrouped on one pedestal of each side (Figure 2b,c).

Additionally, we classified all *L. taschenbergi* specimens at subspecies level, showing diagnostic morphological characters for the subspecies *L. t. amitina*:

- Spermatheca of females very similar to *L. segnis* specimens, showing a bulga wider than the hilla but practically with the same length. Sternite VII showing a low neckline in its ventral margin. This neckline appeared with different forms even disappearing in some female specimens (Figure 2D). Detailed drawing about these different forms observed in the neckline of the Sternite VII are available in Beaucournu and Launay (1990).
- Male with a long and thin hamulus with a membranous lobe in its apical portion (Figure 2e).
- Telomere or movable process of males thin and curved. Basimere or fixed process with a typical apical lobe (Figure 2f).

3.2 | Molecular results

ITS2 and *EF1-α* analysis:

The length of ITS2 was 464 base pairs (bp) for both species, whereas *EF1-α* showed a 975 bp length, except for *L. segnis* specimens collected from El Hierro (766 bp) (Table S1). This difference in the *EF1-α* sequence length was due to the use of different forward primers (see Material and Methods section). Due to these differences in the total base length of *EF1-α*, the molecular and phylogenetic analyses were based on the common base pair fragment for both species. We did not find any base pair differences among ITS2 sequences of specimens from the same species, while interspecific similarity observed was 98.7% with a total of 6 mutational sites between both taxa (table not shown). According to *EF1-α*, intraspecific similarity ranged from 99.3% to 100% for *L. taschenbergi* and 100% for *L. segnis*, whereas interspecific similarity ranged from 97.0% to 97.5% between both species (Table 1). When we compared the *EF1-α* sequences obtained in this work with another Leptopsyllidae taxa, we noticed that *L. nana* showed a similar percentage of similarity with the other *Leptopsylla* species ranging from 96.5% to 96.9%, while genera *Amphipsylla*, *Pectinoctenus* and *Peromyscopsylla* appeared less molecularly divergent respecting to *Leptopsylla* genus than *Ophthalmopsylla* and *Frontopsylla* genera (Table 1). It should be highlighted that the sample ID: LS38, morphologically identified as *L. segnis*, provided ITS2 and *EF1-α* sequences typical of *L. taschenbergi* with a percentage of similarity of 98.7% (table not shown) and 97.3% (Table 1), respectively, with *L. segnis*.

Partial *cox*1, *cox*2 and *cyt*b analysis:

The lengths of *cox*1, *cox*2 and *cyt*b sequences were 658 bp, 732 bp and 374 bp, respectively, for *L. taschenberg*i and *L. segnis* sample ID: LS38. For the remaining *L. segnis* specimens, the total lengths of the previous molecular markers were 453, 686 and 374 bp, respectively (Table S1). This difference in the *cox*1 sequence length was due to the use of different forward primers (see material and methods section), whereas the length difference observed in the *L. segnis cox*2 sequence was due to the poor quality obtained in the last 50 bp of the DNA chromatogram during three different sequencing attempts. Due to these differences in the total base length, the molecular and phylogenetic analyses were based on the common base pair fragment for both species.

The intraspecific similarity observed for *L. taschenbergi* was nearly to 100% for each mitochondrial primer assessed in this study, as well as, based on these three primers, molecular characterization of *L. segnis* ID: LS38, corresponded again with *L. taschenbergi*, with similarity percentage values always ranged from 99.7% to 100% (Tables 1 and 2). According to the remaining *L. segnis* specimens, intraspecific similarity observed ranged from 97.6% (*cox*2) to 100% (*cox*1, *cox*2 and *cyt*b). According to *Leptopsylla* genus, when we compared the mitochondrial partial gene sequences obtained in this work with another Leptopsyllidae taxa, we did not observe any common pattern of molecular divergence with other genera since molecular similarity data overlapped each other for each mitochondrial maker assessed in this study (Tables 1 and 2). Likewise, based on mitochondrial markers *cox*2 and *cyt*b, we again noticed that *L. nana* showed a slightly similar percentage respect to the remaining *Leptopsylla* species without any specific molecular relationship pattern with another taxa.

3.3 | Phylogenetic results

Phylogenetic trees inferred from *EF1-α*, *cox*2 and *cyt*b showed similar topologies respecting to *Leptopsylla* genus phylogeny (Figures S1, S2 and S3, respectively). Thus, specimens belonging to this genus appeared comprising a monophyletic clade for each cited marker, although these clades showed low BPP and Bootstrap values. Furthermore, within this genus, two

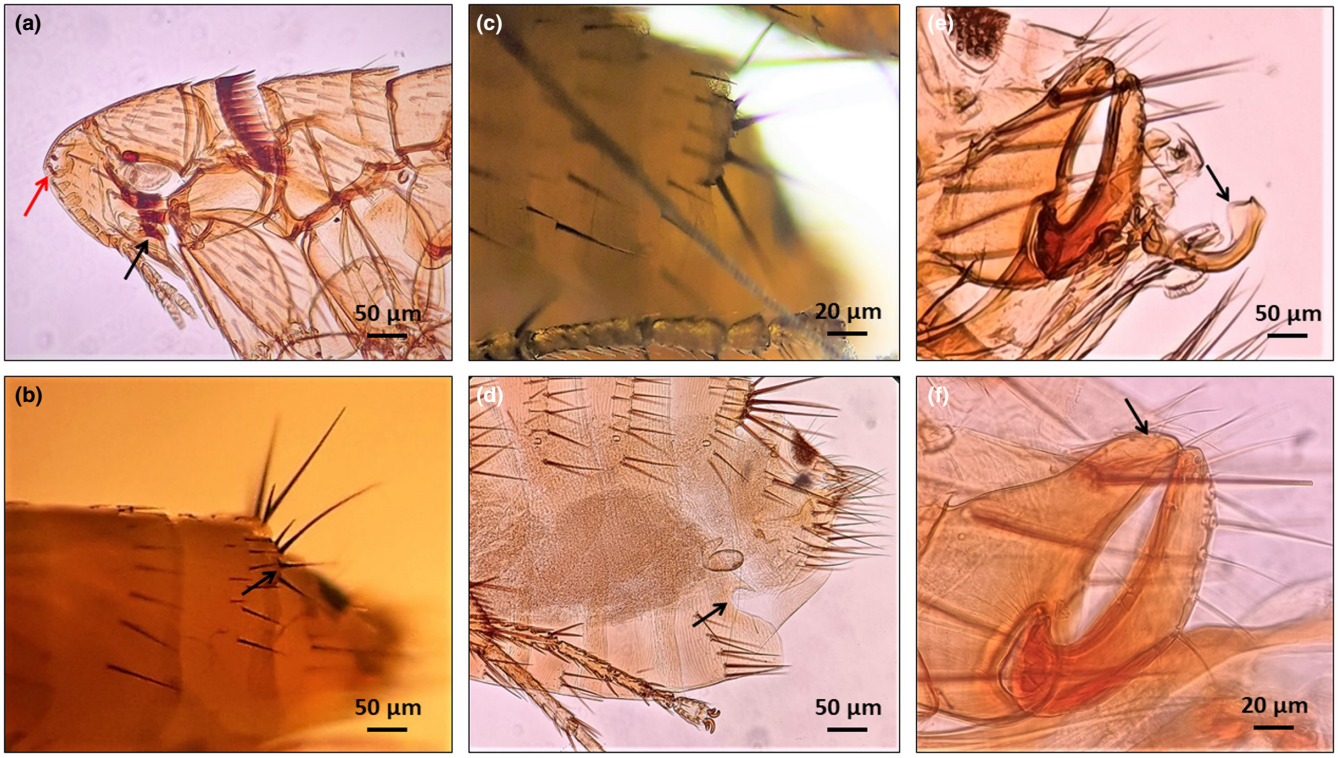

FIGURE 2 Morphological characteristics of *L. taschenbergi* specimens assessed in this study. (a) Head and front with genal comb and two small spiniform setae arrowed. (b) and (c) Antesensiliales setae (arrowed in b) regrouped on one pedestal of each side. (d) Spermatheca and ventral margin of sternum VII of females. (e) Hamulus (arrowed) of males. (f) Telomere and Basimere (both arrowed) of males.

well-supported subclades were noticed corresponding with *L. segnis* and *L. taschenbergi* specimens, respectively. In agreement with molecular analysis, *L. segnis* specimen collected from Py-Conat (ID: LS38) always appeared clustering within *L. taschenbergi* subclade (Figures S1, S2 and S3), whereas *L. nana* did not show any repetitive phylogenetic pattern since it was placed out of *Leptopsylla* clade in *EF1-α* phylogenetic tree (Figure S1) but clustering inside this group for *cox*2 and *cyt*b with low BPP and Bootstrap values (Figure S2 and S3). Additionally, based on *cyt*b phylogenetic tree, we observed two different subclades inside *L. segnis* group, corresponding with specimens from El Hierro (Spain) and those collected from Israel and Belgium (Figure S3). According to phylogenetic relationships among different Leptopsyllidae genera, we could observe *Peromyscopsylla* sp. and *Amphipsilla* sp. genera as closer groups of *Leptopsylla* sp., based on *EF1-α* phylogenetic tree topology (Figure S1). This fact could not be observed in the remaining phylogenetic trees since all Leptopsyllidae genera clustered together comprising a non-defined general polytomy (Figure S2 and S3).

On the contrary, *cox*1 phylogenetic tree (Figure S4) did not provide too much additional information since both *Leptopsylla* species assessed in this work, appeared setting up two well-supported clades but clustering in polytomy with the remaining Leptopsyllidae species and genus even with Pulicidae family. Once again, *L. segnis* (ID: LS38) clustering together with all *L. taschenbergi* specimens in the same clade.

The concatenated dataset of *EF1-α*, *cox*1, *cyt*b and *cox*2 comprised 1837 aligned sites and 48 taxa, including *L. segnis* and *L. taschenbergi* assessed in this study and outgroups (*P. meridionalis*) (Figure 3). Phylogenetic analysis of this dataset yielded a tree in which Leptopsyllidae and Ceratophyllidae (*Nosopsyllus* genus) specimens formed a well-supported clade (93/100-BPP/Bootstrap) clearly separated from the remaining flea families (Pulicidae, Ctenophthalmidae, Stephanocircidae, Hystrichopsyllidae and Stenoponiidae). Assessing this clade, we noticed a well-supported group comprising *Leptopsylla* species, in which, *L. segnis* and *L. taschenbergi* appeared clearly sorted in different subclades with high percentages of BPP and Bootstrap values (Figure 3). On the contrary, the phylogenetic position of *L. segnis* specimen ID: LS38 was in concordance with the previous molecular and phylogenetic results; therefore, this taxon clustered together with *L. taschenbergi* specimens. Lastly, *Pectinoctenus* sp. appeared as the most nearby Leptopsyllidae genus from *Leptopsylla* sp. in comparison with the remaining genera belonging to Leptopsyllidae family (Figure 3).

TABLE 1 Intraspecific (#) and interspecific similarity observed among all the partial *EF1-α* gene sequences of nuclear DNA and the partial *cyt*b mtDNA gene sequences of *L. taschenbergi* and *L. segnis* (obtained in this study) and different species and genus belonging to Leptopsyllidae family retrieved from GenBank database. Values are given in percentages. (*) *L. segnis* specimen ID: LS38 isolated from Py-Conat, France

EF1-α/Cytb	*L. taschenbergi* OU922490-98/ OU919865-67	*L. segnis* OM321875/ OU919868-72	*L. segnis** OU922489/ OU91986864	*L. nana* KM890556/ KM890726	*Amphipsylla* sp. KM890531, −33 EU336274/ KM890700 KM890702-03	*Frontopsylla* sp. EU336286 KM890539/ KM890704, −08 KM890608	*Ophthalmopsylla* sp. KM890545 KM890557/ KM890727 KM890714	*Pectinoctenus* sp. KM890481-82 KM890551/ KM890643-44 KM890721	*Peromyscopsylla* sp. KM890484 EU336278/ KM890743, −45
L. taschenbergi OU922490-98/OU919865-67	99.3–100#/99.5–100#								
L. segnis OM321875/-72	97.0–97.5/83.5–84.0	100#/98.1–100#							
*L. segnis** OU922489 OU91986864	99.3–99.7/99.7–100	97.3/83.5–83.7	-						
L. nana KM890556/KM890726	96.5–97.0/88.5–88.8	96.7/87.8–88.5	96.9/88.5	-					
Amphipsylla sp. KM890531, −33 EU336274/KM890700 KM890702-03	95.2–96.4/87.0–87.8	95.2–95.7/84.5–86.2	95.5–95.8/87.0–87.5	94.8–95.4/88.5–88.8	96.9–97.8/96.5–99.7				
Frontopsylla sp. EU336286 KM890539/KM890704, −08 KM890608	90.7–92.0/81.9–87.8	90.1–90.8/83.5–87.5	90.5–91.4/81.9–87.5	90.7–91.0/83.5–85.9	89.6–89.985.9–88.5	97.5/90.0–93.9			
Ophthalmopsylla sp. KM890545 KM890557/KM890727 KM890714	91.4–92.2/80.2–86.2	90.7–90.8/82.7–86.2	91.3–91.7/80.5–85.9	90.4–90.7/85.4–85.9	90.1–91.0/85.2–86.8	93.6–94.0/85.7–89.0	97.9/91.7		
Pectinoctenus sp. KM890481-82 KM890551/KM890643-44 KM890721	95.2–96.3/84.5–87.8	94.6–95.7/80.7–85.4	94.9–95.8/84.5–87.5	95.1–95.8/87.8–88.5	93.9–94.6/87.0–89.3	90.7–92.3/84.5–86.2	90.7–91.4/81.7–87.8	96.0–97.9/89.8–94.9	
Peromyscopsylla sp. KM890484 EU336278/KM890743, −45 KM890601	95.5–96.4/81.7–85.9	95.7–95.8/80.7–87.0	95.8–96.3/81.7–85.7	95.4–95.5/86.8–89.0	95.2–96.0/86.4–89.8	90.2–92.2/85.2–88.8	89.9–90.8/82.5–87.0	93.9–94.3/83.5–86.8	96.1/83.7–90.3

TABLE 2 Intraspecific (#) and interspecific similarity observed among all the partial *cox*1 and *cox*2 mtDNA gene sequences of *L. taschenbergi* and *L. segnis* (obtained in this study) and different species and genus belonging to Leptopsyllidae family retrieved from GenBank database

*Cox*1/*cox*2	***L. taschenbergi*** **OU903832-34/ OU943325-29**	***L. taschenbergi*** **KM891007/–**	***L. segnis*** **OU905174 OU903835/ OU943331-33**	***L. segnis**** **OU919276/ OU943330**	***L. segnis*** **MG138247/ MG637367**	***L. nana*** **-/KM890862**	***Amphipsylla*** **sp. MG138307, –281, –310, –313, –334, –276/ KM890837-39**	***Frontopsylla*** **sp. KM890973/ KM890840, KM890844**	***Paracteno psyllus*** **sp. KM890955-56/ KX982876-78 KM890828**	***Ophthalmo psylla*** **sp. MG138268, KM890979/ KM890845, KM890768**	***Paradoxopsyllus*** **sp. MF000661, MG138271/–**	***Pectinoctenus*** **sp. MF000662, KM890986, MG138249/ KM890778-79 KM890857**	***Peromysco psylla*** **sp. KM891011, KM891006/ KM890878, KM890882 KM890781**
L. taschenbergi OU903832-34/ OU943325-29	99.8–100#/ 99.7–100#												
L. taschenbergi KM891007/–	97.8–98.0/–	-											
L. segnis OU905174, OU903835/ OU943331-33	82.9–83.1/ 88.8–89.2	84.1–84.3/–	99.8–100#/ 97.6–100#										
*L. segnis** OU919276/ OU943330	99.8–100/ 99.7–100	97.8/–	82.9–83.1/ 88.9–89.1	-									
L. segnis MG138247/ MG637367	84.5–84.7/ 88.5–88.8	85.7/–	87.1–87.3/ 96.6–98.7	84.7/88.6	-								
L. nana -/KM890862	–/88.5–88.9	–/–	–/87.9–88.2	–/88.6	–/87.9	-							
Amphipsylla sp. MG138307, –281, –310, –313, –334, –276/ KM890837-39	81.1–88.0/ 85.3–86.2	82.0–88.6/–	81.0–84.7/ 84.5–85.6	81.2–88.0/ 85.6–86.1	81.8–84.7/ 84.1–85.0	–/86.5–86.6	85.7–98.2/96.6						
Frontopsylla sp. KM890973/ KM890840 KM890844	83.3–83.5/ 85.6–86.9	83.5/–	82.4–82.6/ 84.7–85.3	86.1/85.6–86.9	86.1/85.0–85.2	–/86.1–86.6	81.4–86.9/ 85.5–87.7	–/94.9					
Paractenopsyllus sp. KM890955-56/ KX982876-78 KM890828	83.3–84.7/ 83.4–85.5	83.5–85.3/–	78.7–80.6/ 82.3–84.0	83.5–84.9/ 83.7–85.3	84.9–83.5/ 82.7–84.1	–/83.8–85.6	79.5–85.1 82.7–85.3	83.9–84.5/ 83.7–87.4	95.8/96.3–99.1				

TABLE 2 (Continued)

	L. taschenbergi	*L. taschenbergi*	*L. segnis*	*L. segnis**	*L. segnis*	*L. nana*	*Amphipsylla* sp.	*Frontopsylla* sp.	*Paracteno psyllus* sp.	*Ophthalmo psylla* sp.	*Paradoxopsyllus* sp.	*Pectinoctenus* sp.	*Peromysco psylla* sp.
			OU905174				MG138307, −281, −310, −313, −334, −276/		KM890955-56/ KX982876-78	MG138268, KM890979/		MF000662, KM890986, MG138249/ KM890778-79	KM891011, KM891006/ KM890878, KM890882
Cox1*/*cox2	**OU903832-34/ OU943325-29**	**KM891007/−**	**OU903835/ OU943331-33**	**OU919276/ OU943330**	**MG138247/ MG637367**	**-/KM890862**	**KM890837-39**	**KM890973/ KM890840, KM890844**	**KM890828**	**KM890845, KM890768**	**MF000661, MG138271/−**	**KM890857**	**KM890781**
Ophthalmopsylla sp. MG138268, KM890979/ KM890845, KM890768	84.7–85.1/ 84.3–85.3	85.1/−	83.5–84.3/ 82.0–84.5	83.9–85.3/ 84.3–85.2	83.9–85.3/ 82.3–84.1	−/84.0–84.5	81.8–87.5/ 83.5–83.8	86.5–88.4/ 87.3–88.9	83.5–84.9/ 81.7–84.1	92.0/89.3			
Paradoxopsyllus sp. MF000661, MG138271/−	84.7–85.7/−	84.7–86.3/−	81.4–82.4/−	84.3–84.9/−	84.3–84.9/−	−/	80.2–86.5/−	89.7–90.4/−	84.3–86.9/−	87.5–89.8/−	91.5/−		
Pectinoctenus sp. MF000662, KM890986 MG138249/ KM890778-79 KM890857	84.9–86.5/ 86.6–87.7	84.9–87.5/−	81.6–83.5/ 86.6–87.4	85.3–86.3/ 86.9–87.6	85.3–85.9/ 87.1–87.4	−/87.1–87.6	79.7–86.1/ 84.7–86.8	83.9–85.7/ 84.1–86.8	82.0–83.3/ 82.7–85.8	84.9–87.9/ 83.0–84.7	84.7–86.5/−	90.9–96.7/ 90.6–95.5	
Peromyscopsylla sp. KM891011, KM891006/ KM890878, KM890882 KM890781	85.3–87.5/ 83.8–86.2	85.5–87.7/−	83.1–85.1/ 83.2–86.2	85.3–87.5/ 84.0–86.1	87.3–87.7/ 84.3–86.6	−/84.0–87.9	81.0–88.6/ 84.7–86.8	85.9–86.1/ 85.2–87.6	85.5–87.3/ 81.7–87.6	85.3–87.9/ 82.7–86.5	84.9–87.7/−	84.7–88.4/ 83.3–86.8	89.8/84.4–86.5

Note: Values are given in percentages. (*) *L. segnis* specimen ID: LS38 isolated from Py-Conat, France.

4 | DISCUSSION

The work published by Whiting et al. (2008) is considered the first comprehensive attempt to reconstruct deep-level evolutionary relationships for fleas using a formal analysis of character data from multiple loci. These data and analyses resulted in a robust phylogenetic hypothesis for fleas, although these authors claimed for the necessity to carry out further investigation focusing on deepest nodes and taxonomic levels of flea since it showed limited phylogenetic support. In this study, the family Leptopsyllidae was grossly paraphyletic including two subfamilies Amphipsyllinae and Leptopsyllinae that appear closely related to Ceratophyllidae, and to each other. The phylogenetic analysis done by Whiting et al. (2008) did not support the monophyly of the family Leptopsyllidae nor the subfamilies Amphipsyllinae and Leptopsyllinae even not addressing the systematic position of certain taxa placed between Leptopsyllidae and Ceratophyllidae as in the case of *Dolichopsyllus* genus. Furthermore, as we mentioned in introduction section, these authors did not use any sequence of *Leptopsylla* specimens for its phylogenetic analysis.

From an epidemiology point of view, in agreement with Cevidanes et al. (2016) and Herrero-Cófreces et al. (2021), this work confirmed the status of *A. sylvaticus* as the main host of *L. taschenbergi*, at least in the Iberian Peninsula, and its distribution throughout the southwest area of the Mediterranean region. On the contrary, *L. segnis*, showed a major variety of host range parasitizing different species of mice and rats..

To differentiate both species based on morphological traits, flea's specialist have classically used the difference in the number of spines present in the genal comb as a diagnostic criterion (Beaucournu & Launay, 1990). In that study, we follow this morphological pattern of discrimination that agreed with molecular and phylogenetic results. Apart from the difference in the number of genal spines, we did not find clear morphological differences between females of both species since the shape of the spermatheca and the ventral margin of the sternite VII showed very variable in both species with any specific trait to differentiate each other. Therefore, the use of female genitalia as a diagnostic criterion for *Leptopsylla* species diagnosis, at least between *L. segnis* and *L. taschenbergi*, should be discarded, whereas the study of male genitalia must remain as a useful tool for this aim. This fact has just been observed in the differentiation of female and male of genus *Ctenophthalmus* (Zurita et al., 2020; Zurita & Cutillas, 2021).

In spite of that, in our study, one female specimen (ID: LS38) collected from Py-Conat showed morphological characters typical from *L. segnis* (genal comb with four spines), but molecular and phylogenetic diagnostic features from *L. taschenbergi* based on both, nuclear and mitochondrial markers. Divergence process between molecular and morphological data in fleas is not a new issue for entomologists; thus, some recent studies have reported this fact in some cosmopolitan fleas as *Ctenocephalides felis*, *Nosopsyllus fasciatus*, *Pulex irritans* or *Ctenophthalmus* sp. (Marrugal et al., 2013; Zurita et al., 2018, 2019, 2020). In these publications, authors noticed a certain degree of phenotypic plasticity which did not correspond with molecular differences, or even Zurita et al. (2018) found that some morphological diagnostic characters historically used to discriminate between two congeneric species (*Nosopsyllus fasciatus* and *Nosopsyllus barbarus*) should be revised considering both species as synonymous taxa. Phenotypic plasticity is defined as the ability of an individual organism to change its phenotype in response to stimuli or inputs from the environment (West-Eberhard, 2003). Thus, many organisms can modify their phenotype in response to several environmental factors, such as temperature, nutrition, light, pressure or the presence of predators (Gilbert & Epel, 2009). Phenotypic plasticity involves a change in some aspect of the phenotype, including morphology, without a change in the individual's genes, or the genetic underpinnings of a particular trait (West-Eberhard, 2003). Other authors have also defined this biological phenomenon as the environmental sensitivity of a genotype; however, in our study, all 233 *Leptopsylla* specimens collected from Py-Conat shared same host (*A. sylvaticus*) and environmental conditions. In this sense, and based on its molecular and phylogenetical characterization, we could consider the possibility that specimen ID: LS38 could really represent a *L. taschenbergi* female showing a morphological variation in the genal comb derived from some mutational process not detected in the five molecular loci assessed in our work. We could also consider a mitochondrial introgression phenomenon, which have been observed between close related fleas as *C. felis* and *C. canis* (Zurita et al., 2016), but *EF1-α* also provided a *L. taschenbergi* identification, discarding this possibility. We cannot consider the hypothesis of hybridization between both species since this option in fleas have been rejected by Beaucournu and Guiller (2006). Within this context, we must take into consideration this morphological variability observed in the genal comb of the complex *L. taschenbergi—L. segnis* for further morphological identification, especially when we deal with female specimens.

Using molecular and phylogenetic data, we could easily discriminate between both taxa comparing both nuclear and mitochondrial markers. In this sense, the most molecular divergence between both species was observed

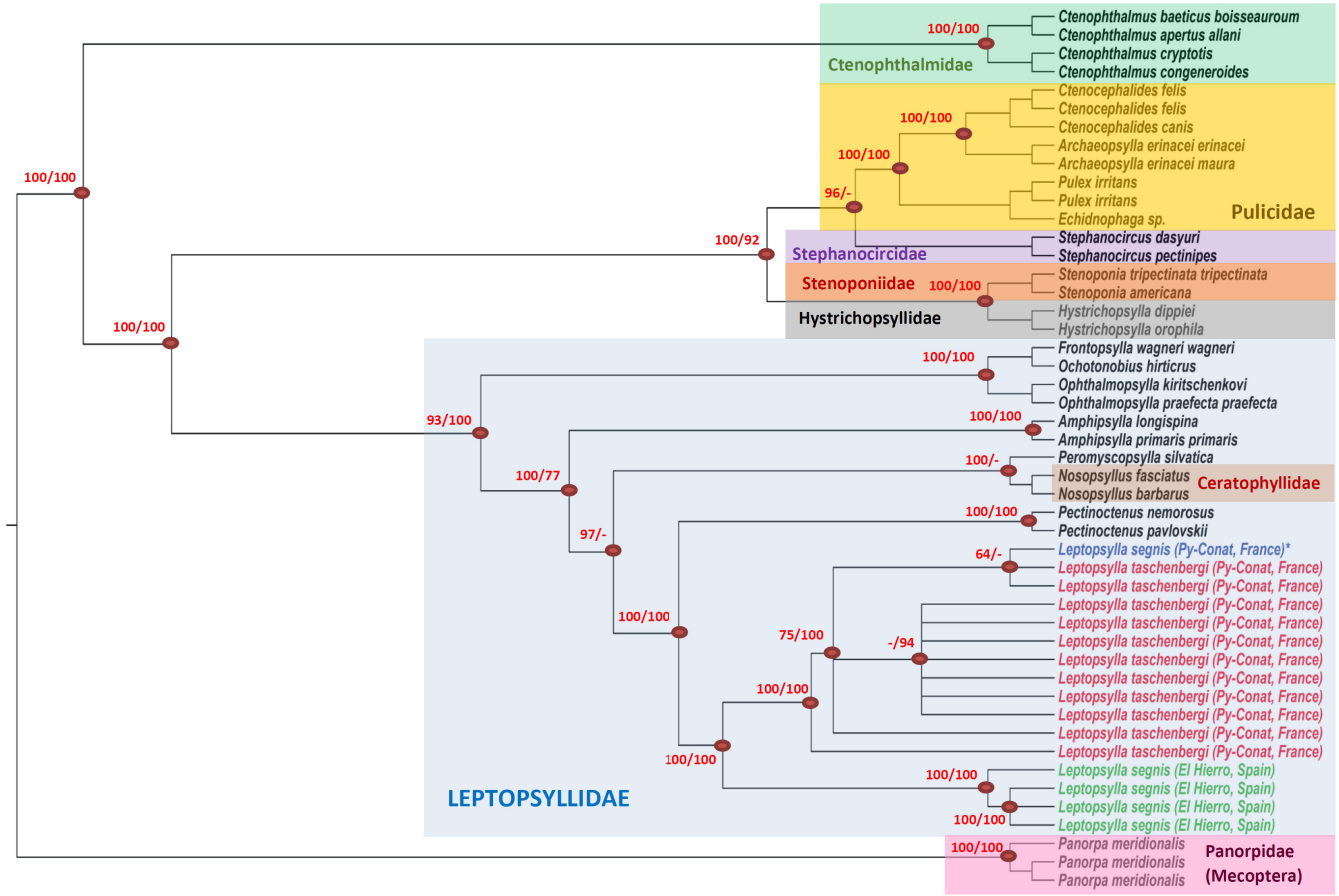

FIGURE 3 Phylogenetic tree of *L. taschenbergi* and *L. segnis* specimens assessed in this study (see Table S1). This analysis was based on concatenated sequences of partial *elongation factor 1 alpha* (*EF1-α*) of nuclear DNA, partial *cytochrome c oxidase* subunit 1 (*cox*1), *cytochrome c oxidase* subunit 2 (*cox*2) 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 (BPP/bootstrap). The Bayesian posterior probabilities (BPP) are percentage converted. BPP and bootstrap values lower than 60% are not shown. (*) *L. segnis* specimen ID: LS38 isolated from Py-Conat, France.

when we assess mitochondrial markers, while ITS2 and *EF1-α* appeared less variable. This molecular pattern was expected since ITS2 and *EF1-α* are known to have a high conserved sequence (Friedlander et al., 1994; Zurita et al., 2019) as well as some authors have expressed that the inheritance properties of mtDNA make it more likely than any single nuclear marker to accurately reflect recent divergence, so they use to show higher degrees of variability (Toews & Brelsford, 2012). For that reason, if we want to infer a robust and feasible phylogeny, we should include multiple independent loci combining nuclear and mitochondrial data (Edwards & Bensch, 2009). This fact has been largely proved in flea's field as for genus and species level (Lawrence et al., 2019; Zurita & Cutillas, 2021) as family and higher stratum (Whiting et al., 2008; Zhu et al., 2015).

The only phylogenetic attempt to evaluate the taxonomic status of *Leptopsylla* genus so far was carried out by Guernier et al. (2014) who sequenced the nuclear 28S and mitochondrial *cox*2 partial gene of *L. segnis* isolated from rodents from Reunion Island. The *cox*2 sequence provided in this work has not been included in our study due to a considerable divergence observed in the total sequence length, which could have limited our phylogenetic analysis and sequence comparison process among Leptopsyllidae taxa. Anyways, these authors observed incongruent results between phylogenetic analysis of 28S and *cox*2 since *L. segnis* unexpectedly appeared clustering together with Pulicidae family in *cox*2 phylogenetic tree. With these results and the lack of molecular data available in GenBank database for *Leptopsylla* taxa, these authors claimed about the necessity to investigate additional and more informative markers in order to address *L. segnis* taxonomic status together with other more basic questions such as a previously reported paraphyly of Leptopsyllidae (Guernier et al., 2014).

Our phylogenetic and molecular analysis based on each single marker and concatenated dataset reached to discriminate between both *Leptopsylla* species, proving the usefulness of ITS2, *EF1-α*, *cox*1, *cyt*b and *cox*2 in

order to identify *Leptopsylla* taxa. Additionally, based on *cyt*b phylogenetic analysis, we could observe a geographical signal in *L. segnis* between specimens collected from mainland of Europe and those collected from Canary Islands, Spain. This fact again supports the molecular divergence observed in flea's populations isolated from island, contributing to the appearance of cryptic species (Zurita et al., 2015; Zurita & Cutillas, 2021). In addition, the phylogenetic position of *L. nana* remains incongruent in this work due to it seems to cluster within *Leptopsylla* species in *cyt*b and *cox*2 analysis, but it was placed out this group based on *EF1-α* phylogenetic results.

On the contrary, based on phylogenetic analysis of concatenated dataset together with the percentage of molecular similarity observed among different Leptopsyllidae genera, we could conclude that *Pectinoctenus* sp. placed as a sister genus of *Leptopsylla* sp. We also observed by the first time, a possible monophyletic origin of Leptopsyllidae family, which had been reported in previous studies as a paraphyletic group, pending to be studied in a deeper way (Guernier et al., 2014; Whiting et al., 2008). This fact highlights the need to provide new molecular and phylogenetic approaches in order to resolve flea systematic issues. Nevertheless, phylogenetic relationships between Ceratophyllidae and Leptopsyllidae families should be considered for further analysis since *Nosopsyllus* sp. (Ceratophyllidae) clustered inside Leptopsyllidae clade. This fact was reported by Whiting et al. (2008) and Zurita et al. (2018); therefore, to clear this subject, further taxonomic studies are needed in flea's field, including and providing more Ceratophyllids taxa and additional molecular markers.

5 | CONCLUSIONS

In conclusion, the main findings of this work can be sorted as follow:

(i) We molecularly and phylogenetically characterize, by the first time, two *Leptopsylla* species with zoonotic importance: *L. segnis* and *L. taschenbergi*; (ii) although these species can have discriminated each other by the number of spines presented in the genal comb, we found one female specimen showing morphological characters of *L. segnis* but molecular sequences distinctive for *L. taschenbergi*. This incongruence between molecular and morphological results emphasizes, one more time, the necessity to combine morphological, phylogenetic and molecular data in order to assess and elucidate taxonomic issues regarding to Siphonaptera Order. At this point, entomologist should take in consideration this possible phenotypic plasticity observed in the genal comb of both species, especially in females, which genitalia appear very similar each other; (iii) furthermore, we observed a monophyletic origin of Leptopsyllidae family; however, the phylogenetic relationships between Leptopsyllidae and Ceratophyllidae taxa or the phylogenetic position of *L. nana* within Leptopsyllidae remain problematic and should be take into consideration in further studies; (iv) once again within Siphonaptera field, the combination of mitochondrial and nuclear markers resulted in a useful tool to solve taxonomic and phylogenetic issues by the use of concatenated data.

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SUPPORTING INFORMATION

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