Morphological, biometrical and molecular characterization of *Archaeopsylla erinacei* (Bouché, 1835).

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Abstract

In the present work, we carried out a morphological, biometrical and molecular study of the species *Archaeopsylla erinacei* (Bouché, 1835) and their subspecies: *Archaeopsylla erinacei erinacei* (Bouché, 1835) and *Archaeopsylla erinacei maura* (Jordan & Rothschild, 1912) isolated from hedgehogs (*Erinaceus europaeus*) from different geographical regions (Seville and Corse). We have found morphological differences in females of *A. erinacei* from the same geographical origin that did not correspond with molecular differences. We suggest that some morphological characters traditionally used to discriminate females of both subspecies should be revised as well as we set the total length of the spermatheca as a valid criterion in order to discriminate between both subspecies. The Internal Transcribed Spacers 1 and 2 (ITS1, ITS2) and partial 18S rRNA gene, and partial cytochrome c-oxidase 1 (*cox1*) and cytochrome b (*cytb*) mtDNA gene sequences were determined to clarify the taxonomic status of these taxa and to assess intra-specific and intra-population similarity. In addition, a phylogenetic analysis with other species of fleas using Bayesian and Maximum Likelihood analysis was performed. All molecular markers used, except 18S, showed molecular differences between populations corresponding with geographical origins. Thus, based on the phylogenetic and molecular study of two nuclear markers (ITS1, ITS2) and two mitochondrial markers (*cox1* and *cytb*), as well as concatenated sequences of both subspecies, we reported the existence of two geographical genetic lineages in *A. erinacei* corresponding with two different subspecies: *A. e. erinacei* (Corse, France) and *A. e. maura* (Seville, Spain), that could be discriminated by PCR-linked RFLP.

Running head: *Archaeopsylla erinacei*

Keywords: *Archaeopsylla erinacei*, morphology, molecular study, ribosomal DNA, mitochondrial DNA.

Introduction

Siphonaptera is a relative small order of secondarily wingless holometabolous insects. According to Beaucournu & Gómez (2015) the order includes 2500 species ``of fleas´´. In addition, 409 specific, 147 subspecific, 65 generic, and 7 subgenera names are considered to be synonymous (Krasnov, 2008). The Siphonaptera fauna of the Palearctic region is the richest, including 96 genera and 892 speciesconstituting a 38 % of the total number of species known, and 38 % of the known genera (Krasnov, 2008).

Within this order, the Pulicidae is the most studied family since most fleas of medical or veterinary importance (*Ctenocephalides felis*, *Ctenocephalides canis*, *Pulex irritans* or *Xenopsylla cheopis*) are members of this family. Pulicidae consists of four tribes, 21 genera, and 167 species. Some workers have treated Pulicidae as including Tungidae (Lewis, 1998); however, Whiting *et al*. (2008) placed this family as a monophyletic group and phylogenetically distant from Tungidae. Pulicidae exhibit an interesting diversity of host specificity patterns and ecological habits (Whiting *et al*., 2008). Certain species such as *Archaeopsylla erinacei* and *Spilopsyllus cuniculi* are monoxenous on hedgehog and rabbits respectively, while other Pulicidae species such as *C. felis* or *P. irritans*, are highly promiscuous, and occurs on a wide variety of Carnivora (Whiting *et al*., 2008).

Although during the last fifteen years molecular data has made a significant contribution (Dittmar & Whiting, 2003; Vobis *et al*., 2004; Gamerschlag *et al*., 2008; Whiting *et al*., 2008; Marrugal *et al*., 2013; Zurita *et al*., 2016), for decades, the genus and species differentiation of fleas has been based on morphological criteria (the shape and structure of their complex genitalia, distribution of setae, spines and ctenidia, etc) (Lane & Crosskey, 1993; Kramer & Mencke, 2001; Mehlhorn, 2001; Linardi & Santos, 2012). However, a few studies have been carried out on molecular differentiation of fleas (Lawrence *et al*., 2014; Zurita *et al*., 2015). Thus, the scientific community has a great knowledge of flea taxonomy at the species and subspecies level, and enough information to assess their biology and role in disease

transmission in recent years (Kaewmongkol *et al*., 2011; Lawrence *et al*., 2015). In contrast, a rigorous exploration of the phylogenetic relationships among fleas is needed in order to clarify their complex systematics (Whiting *et al.*, 2008). In this way, the few taxonomic and phylogenetic studies of fleas based on molecular data carried out in the last years have revealed that not all flea species previously described only by morphological methods, have always remained as defined species. Recently, Zurita *et al*. (2017) based on a comparative morphological, phylogenetic and molecular study of *Nosopsyllus fasciatus* and *Nosopsyllus barbarus*, concluded that there were no solid arguments to consider these two "morphospecies" as two different species and proposed *N. barbarus* as a junior synonym of *N. fasciatus*. These authors used two nuclear markers: Internal Transcribed Spacers 1 and 2 (ITS1 and ITS2) and two mitochondrial markers: cytochrome c-oxidase subunit 1 (*cox1*) and cytochrome b (*cytb*), in order to determine the taxonomic status of both species.

Previous studies showed that fleas have a high level of genetic intraspecific variation (Dittmar & Whiting, 2003; Brinkerhoff *et al*., 2011). Thus, several authors in the last ten years (Kaewmongkol *et al*., 2011; Lawrence *et al*., 2014; Zurita *et al*., 2015; Zhu *et al*., 2015; Zurita *et al*., 2016) have used mitochondrial DNA markers such as *cox1*, *coxII* or *cytb* as reference molecular markers in order to investigate the phylogenetic and taxonomic relationships in fleas at family, genus and species level.

Genus *Archaeopsylla* Dampf, 1908 (Pulicidae) is a great example of the shortage of molecular and phylogenetic data in fleas' taxonomy. Based on morphological criteria, two species have been described within the *Archaeopsylla* genus (Pulicidae): *Archaeopsylla sinensis*, and *A. erinacei* with two subspecies: *Archaeopsylla erinacei erinacei* (Bouché, 1835) and *Archaeopsylla erinacei maura* (Jordan & Rothschild, 1911). Both species have a Palearctic distribution; however, *A. sinensis* occurs at East-Asian subregion, Siberian province; China, Russia (Medvedev et al., 2005) whereas *A. erinacei* is distributed from European region to

Mediterranean and North Africa area (Hopkins & Rothschild, 1953). Furthermore, *A. e. erinacei* is distributed from European and Mediterranean subregions, while the distribution of *A. e. maura* is possibly partly accounted by the artificial introduction of its host (the North African hedgehog, *Atelerix algirus*), primarily a North African form, which is stated to have, probably, been introduced into southern Spain (Domínguez, 2004), the Balearic Islands and south-eastern France within historic times (Hopkins & Rothschild, 1953). Thus, we can say that both subspecies are sympatric along certain geographical areas where they coexist and particularly also in the Iberian Peninsula and south-eastern France. Furthermore, Hopkins & Rothschild (1953) and Beaucournu & Launay (1990) noticed that these two subspecies cohabit the same host (*Erinaceus europaeus*). These authors provided taxonomic keys based on morphological criteria in order to discriminate between the two subspecies; however, the close likeness of female specimens of *A. e. erinacei* and *A. e. maura* makes the differential diagnosis very difficult, especially when there are few males (easily differentiated), and when the specimens come from areas where the two subspecies coexist (Beaucournu & Launay, 1990).

The aim of this study was to carry out a comparative morphological, biometrical and molecular study of *A. erinacei* and their subspecies: *A. e. erinacei* and *A. e. maura* isolated from *Erinaceus europaeus* from Seville (southwestern of Spain) and Corse Island (France). Thus, the partial 18S rRNA gene, ITS1, ITS2 of the rDNA and partial *cox1* and *cytb* mtDNA gene of these taxa were sequenced in order to clarify their taxonomic status and to assess intra-specific and intra-population similarity. Furthermore, based on the sequences obtained and those of additional flea species retrieved from public databases, we also carried out a comparative phylogenetic analysis.

Materials and methods

Collection of samples

Hedgehog (*Erinaceus europaeus*) trapping was conducted in Dos Hermanas (37°17′01″N-5°55′20″W) and Aznalcázar (37°18′14″N-6°15′03″W), Seville (Spain). Early morning hedgehogs killed on roads at night were located and collected by hand. Collected hedgehogs were taken to the laboratory and then placed on a white sheet of paper in order to be visually examined for ectoparasites. Fleas were collected by adding 70 % ethanol and then were removed from the hedgehogs by gently shaking the animal over the white sheet of paper. Fleas from hedgehog from Corse were obtained through the assistance of colleagues (see Acknowledgements). Fleas obtained were kept in Eppendorf tubes with 70 % ethanol until required for subsequent identification and sequencing; for details on locality, host, flea species and gender, see Table 1.

Morphological identification and Biometrical study

Flea specimens collected from Spain were classified by us whereas those fleas from Corse provided by our colleagues were classified firstly by them (see Acknowledgements) and then compared morphologically with our specimens in our laboratory. For morphological analysis, all specimens were examined and photographed under optical microscope. Posteriorly, flea legs were cut off in order to carry out the DNA extraction, while the rest of the flea was used to confirm *A. erinacei* species/subspecies morphological identity. Thus, they were cleared with 10 % KOH, prepared and mounted on glass slides using conventional procedures (Lewis, 1993). Once mounted, they were examined and photographed again for a deeper morphological analysis using a Nikon microscope equipped with a camera lucid system and a photomicroscope. Generic, specific and subspecific identification was carried out according to

Jordan & Rothschild (1912, 1953) and Beaucournu & Launay (1990). Thus, the morphological characteristics considered for the specific determination include:

- Presence of a well noticed sclerotized falx of head.
- Asymmetrical antenna with partially welded basal segments.
- Presence of a pleural rod of mesothorax.
- Vestigial genal and pronotal comb. Genal comb composed of one to three spines, these being the small posterior ones. Pronotal comb composed of at most six spines on the two sides together, and sometimes only one each side. Very rarely some of these combs are entirely absent, but it can occur.
- Hind tibia with six seta-bearing notches along dorsal margin with a row of six to eleven little setae near to dorsal margin.

For the subspecific differentiation, we considered morphological characteristics reported by Jordan & Rothschild (1912, 1953) and Beaucournu & Launay (1990):

- Male specimens of *A. e. erinacei* showed the greatest length of basimere same as distance from base of spine on genal process to anterior edge of eye while, male individuals of *A. e. maura* showed the greatest length of basimere same as distance from base of spine at tip of genal process to front margin on head.
- Females of *A. e. erinacei* showed eighth abdominal tergum bearing two lateral bristles towards base and seventh sternum usually with five lateral bristles on the two sides together, whereas *A. e. maura* females presented only one bristle in eighth abdominal tergum and seventh sternum usually bore four lateral bristles on the two sides together.

Furthermore, twenty different parameters were measured of forty-eight (23 females and 25 males) *A. erinacei* specimens (Table 2). Descriptive univariate statistics (arithmetic means, standard deviations, and variation coefficients) for all parameters were determined for two

166 populations (*A. erinacei* from Seville and *A. erinacei* from Corse) using IBM® SPSS®
167 Statistics program version 24.0.0.0 (Pardo & Ruiz, 2002). Furthermore, ANOVA statistical
168 test was performed and significative values were calculated. A two-way Analysis of Variance
169 (ANOVA), with factorial design, was used to test the significance of the differences between
170 geographic origin and sex. Means were compared using the Fisher's Least Significant
171 Difference (LSD). Effect geographic origin (G.O), sex (S) and the interaction (G.O.x S) was
172 calculated as the fraction of the total variability explained. All data analysis was performed
173 with the software "Statistix 9.0". Statistically significant effects were assumed for $p < 0.05$
174 (*).

175 **Molecular study**

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

179 All molecular markers sequenced in this study were amplified by polymerase chain reaction
180 (PCR) using a thermal cycler (Eppendorf AG). PCR mix, PCR conditions and PCR primers
181 are summarized in Table S1. The 18S, ITS1, ITS2, partial *cox1* and *cytb* gene sequences
182 obtained from *A. erinacei* from the two geographical areas were deposited in GenBank
183 database (Table 1). Furthermore, we sequenced and provided ITS2 and *cytb* sequences of
184 *Xenopsylla cheopis* isolated from *Rattus* sp. from El Hierro Island (Spain) (see Table 1).

185 The PCR products were checked on ethidium bromide stained 2 % Tris–Borate–EDTA (TBE)
186 agarose gels. Bands were eluted and purified from the agarose gel by using the QWizard SV
187 Gel and PCR Clean-Up System Kit (Promega). Once purified, the products were sequenced
188 by Stab Vida (Portugal). To obtain a nucleotide sequence alignment file, we used MUSCLE
189 alignment method (Edgar, 2004) by the MEGA program version 5.2 (Tamura *et al.*, 2011).
190 The rDNA intra-individual variation was determined by sequencing 7-8 clones of one

191 individual. The PCR products were eluted from the agarose gel using the WIZARD® SV Gel
192 and PCR Clean-Up System (Promega) and transformation was carried out as cited by Cutillas
193 *et al*. (2009). Plasmids were purified using a Wizard Plus SV (Promega) and sequenced by
194 Stab Vida (Portugal) with an universal primer (M13).

195 A restriction map of the ITS1 and ITS2 sequences of *A. erinacei* from Seville and Corse was
196 constructed using The Sequence Manipulation Suite (Stothard, 2000; available at
197 http://www.bioinformatics.org/sms2/rest_map.html). For determination of PCR-linked
198 random-fragment-length polymorphism (RFLP), ITS1 and ITS2 PCR products from *A.*
199 *erinacei* were restricted directly with 2.5 endonuclease units and were incubated three hours
200 at 37º C. Digests were separated on 2 % agarose-TBE gels.

201 In order to assess the similarity among all sequences of *A. erinacei* obtained in this study we
202 analyzed the number of base differences per sequence among all of them using no. of
203 differences method of MEGA 5 program version 5.2 (Tamura *et al*., 2011). Furthermore, we
204 complemented these analyses with other Pulicidae species sequences obtained from GenBank.
205 On the other hand, similarity sequence divergence of *cox1* sequences were calculated using
206 the Kimura 2 parameter (K2P) distance model in order to apply the 10X rule (Hebert *et al*.,
207 2003) and to figure out the threshold level of nucleotide divergence to represent different
208 categories of 'species' used by Hebert *et al*. (2003). This method was included in MEGA
209 program version 5.2 (Tamura *et al*., 2011).

210 Phylogenetic trees were inferred using nucleotide data and performed using two methods:
211 Maximum Likelihood (ML) trees were generated using the PHYML package from Guindon
212 & Gascuel (2003) whereas Bayesian inferences (B) were generated using MrBayes-3.2.6
213 (Ronquist & Huelsenbeck 2003). JMODELTEST (Posada, 2008) program was used to
214 determinate the best-fit substitution model for the parasite data (18S, ITS1, ITS2, *cox1* and
215 *cytb*). Models of evolution were chosen for subsequent analyses according to the Akaike

Information Criterion (Huelsenbeck & Rannala, 1997; Posada & Buckley, 2004). For the study of the dataset containing the concatenation of four markers (18S, ITS2, *cox1*, *cytb*), analyses based on BI were partitioned by gene and models for individual genes within partitions were those selected by jModeltest. For ML inference, best-fit nucleotide substitution models included general time-reversible model with gamma-distributed rate variation and a proportion of invariable sites, GTR+I+G (ITS2, *cox1*), transition model with gamma-distributed rate variation, TIM+G (*cytb*) and general time-reversible model with gamma-distributed rate variation GTR+G (18S and ITS1). Support for the topology was examined using bootstrapping (heuristic option) (Felsenstein, 1985) over 1000 replications to assess the relative reliability of clades. The commands used in MrBayes-3.2.6 for BI were nst=6 with invgamma rates (ITS2 and *cox1*), nst=2 with gamma rates (*cytb*) and nst=6 with gamma rates (18S and ITS1). For BI, the standard deviation of split frequencies was used to assess if the number of generations completed was sufficient; the chain was sampled every 500 generations and each dataset was run for 10 million generations. Adequacy of sampling and run convergence was assessed using the effective sample size diagnostic in TRACER program version 1.6 (Rambaut & Drummond, 2007). Trees from the first million generations were discarded based on an assessment of convergence. Burn-in was determined empirically by examination of the log likelihood values of the chains. The Bayesian Posterior Probabilities (BPP) was percentage converted.

The phylogenetic analyses, based on18S rRNA, ITS1, ITS2, *cox1* and *cytb* mtDNA sequences were carried out using our sequences and those obtained from GenBank database (appendix 1). Phylogenetic trees based on 18S rRNA, ITS2, *cox1*, *cytb* mtDNA and concatenated (18S, ITS2, *cox1* and *cytb*) sequences were rooted including outgroup species representing members of the Order Mecoptera: *Panorpa meridionalis*. This choice was based on the the combination of morphological and molecular data obtained in former studies which provided compelling

evidences for a sister group relationship between Mecoptera and Siphonaptera (Whiting, 2002; Whiting *et al*., 2008). ITS1 sequence of *Panorpa meridionalis* or other species of Mecoptera was not available neither by amplification of different individuals nor in any public database. Thus, phylogenetic tree with other Siphonaptera species based on ITS1 sequences were constructed using different outgroup species representing members of Order Diptera: *Anopheles moucheti nigerensis* and *Anopheles moucheti bervoetsi*. Thus,, ITS1 was discarded for the concatenated dataset. The selection of flea taxa for the concatenated phylogenetic tree was limited to flea species whose 18S, ITS2, *cox1* and *cytb* sequences were available on GenBank database.

Results

Morphological and biometrical results

Forty-eight fleas: 13 fleas from two hedgehogs (*E. europaeus*) and 35 fleas from three hedgehogs (*E. europaeus*) were collected from Corse and Seville, respectively.

Specific morphological identification done by ourselves was in agreement with that made by our colleagues. Thus, all specimens isolated in this work showed specific morphological characteristics of *A. erinacei* (Figure 1a-f). Within this species, males of *A. erinacei* from Corse presented typical morphological characteristics of *A. e. erinacei* (See material and methods) (Figure 1g), while those from Seville presented typical morphological characteristics of *A. e. maura* (See material and methods) (Figure 1h) (Table S2). Furthermore, total length and total width of the basimere appeared as a significative value to differentiate males from both geographical regions.

On the other hand, according to previous morphological descriptions of different authors (See material and methods), we found three different operational taxonomic units (OTUs) of *A. erinacei* females:

- Population A: *A. erinacei* females which showed morphological characteristics of *A. e. erinacei* (See material and methods) (Figure 1i). This population was observed on hedgehogs from Corse and Seville (Table S2).
- Population B: *A. erinacei* females which showed eighth abdominal tergum bearing only one lateral bristle towards base (Figure 1j) and seventh sternum bore five lateral bristles on the two sides together (two and three bristles each side). This population coud not be classified neither *A. e. erinacei* nor *A. e. maura* since it showed ambiguous morphological characteristics. This population was observed on hedgehogs from Corse and Seville (Table S2).

- Population C: *A. erinacei* females which showed eighth abdominal tergum bearing only one lateral bristle towards base (Figure 1j) and seventh sternum bore six lateral bristles on the two sides together (three bristles each side) (Figure 1k). This population coud not be classified neither *A. e. erinacei* nor *A. e. maura* since it showed ambiguous morphological characteristics. This population was only observed on hedgehogs from Seville (Table S2).

Biometrical data (Table 2) showed that total width, total length of the head, total width of the head and the total length of spermatheca (Figure 1l) in females were significate values to differentiate females from both geographical regions, being the length of spermatheca considerably higher in females from Seville than that in females collected from Corse, regardless which OTU they belong. Furthermore, we also observed that total length and the length and width of the head of the females tended to be higher in *A. erinacei* specimens from Seville than those collected from Corse (Table 2).

Molecular results

Partial 18S rRNA gene analysis

Partial 18S rRNA gene sequences of different populations of *A. erinacei* were 1,160 base pairs (bp) in length (Table 1). No differences were observed between partial 18S rRNA gene sequences from both geographical origins. Partial 18S gene phylogenetic tree showed species belonging to Pulicidae family clustered together, with high bootstrap and Bayesian Posterior Probabilities (BPP) values, but phylogenetically distant from Stenoponiidae, Ctenophthamidae and Ceratophyllidae (tree not shown). Nevertheless, this tree was unable to differentiate at species and subspecies level.

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

297 The length of the ITS1 sequences of *A. erinacei* ranged from 949-950 (Seville) to 951 (Corse)
298 (Table 1). On the other hand, ITS2 sequence length ranged from 360 (Corse) to 361 (Seville).
299 This length difference was also observed in clones from individuals from two different
300 geographical origins and was due to the existence of one extra basis pair in position 258 in the
301 ITS2 sequence of the individuals from Seville.

302 ITS2 intra-individual similarity studied in seven clones of one individual of *A. erinacei* from
303 Corse ranged from 99.4 % to 100 %, whereas this value ranged from 99.2 % to 100 % when
304 eight clones of one individual of *A. erinacei* from Seville were compared. Specimens obtained
305 from the same geographical area showed the same ITS2 sequence (Intra-population similarity
306 = 100 %), indistinctly if they belong to different morphological populations (females). Unlike
307 this value, when the ITS2 sequences of individuals from both geographical origins (Corse and
308 Seville) were compared, the similarity observed was 96.9 % (Intra-specific similarity = 96.9
309 %).

310 ITS1 sequences of specimens from the same geographical origin were identical (Intra-
311 population similarity = 100 %). On the other hand, when the ITS1 sequences from both
312 geographical origins were compared, the similarity observed was 99.1 % (Intra-specific
313 similarity = 99.1 %).

314 Based on ITS1 and ITS2 sequences, restriction mapping identified endonucleases delineating
315 the two different geographical areas (Corse and Seville) (Figure 2). Thus, *EcoRV*, *HaeIII* and
316 *PhoI* presented one restriction site in ITS1 sequences of *A. e. erinacei* (male) from Seville but
317 none in *A. e. maura* (male) from Corse (Figure 2). Restriction mapping for ITS2 sequences
318 showed *AseI*, *MseI* (Position 78) and *VspI* presented one restriction site in *A. e. erinacei*
319 (male) from Corse but none in *A. e. maura* (male) from Seville, whereas, *AsuII*, *BbuI*, *DraI*,
320 *NIaIII*, *PsiI*, *MseI* (Position 179) and *SphI* presented one restriction site in ITS2 sequences of
321 *A. e. maura* from Seville but none in *A. e. erinacei* from Corse (Figure 2). The endonuclease

HaeIII was chosen for the use in the PCR-linked RFLP analysis of ITS1. As predicted by the sequence data, restriction of ITS1 PCR products of *A. erinacei* from two geographical origins with *HaeIII* produced two restriction fragments (194 bp and 755 bp) for individuals from Seville and an undigested product (951 bp) for individuals from Corse (Figure 3).

The phylogenetic tree inferred from ITS2 sequences of *A. erinacei* and other ITS2 sequences retrieved from GenBank (see appendix 1) showed all Pulicidae species clustered together with high bootstrap and BPP values and phylogenetically close to Stenoponiidae family (Figure S1). Within Pulicidae clade, *A. erinacei* specimens comprised a well-supported subclade phylogenetically related with the remaining Pulicidae species. This subclade showed individuals separated according to geographical origin with high bootstrap and BPP values, indistinctly these individuals belong to different morphological populations (Figure S1).

ITS1 phylogenetic tree revealed a subclade clustering all *A. erinacei* specimens related with *Ctenocephalides* within Pulicidae family clade. Furthermore, likewise in ITS2 phylogenetic tree, *A. erinacei* individuals clustered separated according to geographical origin with high bootstrap and BPP values (Figure S2).

Partial cox1 and cytb mtDNA gene analysis

The partial *cox1* mtDNA gene sequences of *A. erinacei* from the two geographical areas were 658 bp in length (Table 1). Intra-population similarity observed ranged from 99.8 % to 100 % in both geographical origins, while intra-specific similarity ranged from 97.7 % to 98.1 % (Table 3). Furthermore, the conspecific divergence ranged from 0 to 0.2. If we consider that the average of conspecific divergence was 0.09, we can applied the 10X rule; thus, the threshold level of nucleotide divergence between two *Archaeopsylla* species would be 0.9 %. Nevertheless, any value of conspecific divergence among all individuals analyzed in this study overcame this threshold.

On the other hand, the length of the partial *cytb* mtDNA gene sequences of *A. erinacei* from Corse and Seville was 374 bp (Table 1). Intra-population similarity of *A. erinacei* specimens from Seville ranged from 98.1 % to 100 %, while this value was 100 % for specimens collected from Corse. Intra-specific similarity ranged from 98.1 % to 98.9 % (Table 4). Furthermore, inter-specific *cytb* similarity observed between others congeneric species belonging to Pulicidae family showed quite lowest percentage values than those observed between *A. erinacei* specimens from the two different geographical origins analyzed in this work (Table 4).

Phylogenetic tree topology of both mitochondrial markers revealed a highly supported clade clustering all Pulicidae species (Figure S3 and S4). In addition, *A. erinacei* individuals from Seville clustered together with high bootstrap and BPP values and separated from *A. erinacei* specimens collected from Corse indistinctly if these individuals belong to different morphological populations (Figure S3 and S4). Particularly, in *cox1* phylogenetic tree, *Ctenocephalides* species appeared clustering near to *Archaeopsylla* with high bootstrap and BPP values (96/82), whereas in *cytb* phylogenetic tree, *Ctenocephalides* species and the others Pulicidae species clustered in polytomy in relation to *Archaeopsylla*.

The concatenated dataset of partial 18S gene, ITS2, partial *cytb* and *cox1* gene sequences included 2,558 aligned sites and 30 taxa, including outgroups. Phylogenetic analyses of the concatenated dataset yielded a tree with branches strongly supported (Figure 4). The analysis based on the concatenated dataset is concordant with all trees constructed on the basis of the single markers. Thus, all species belonging to Pulicidae family clustered together in two main subclades with high bootstrap and BPP support. The first one clustered all *Ctenocephalides* species, while in the second one all *Archaeopsylla* species clustered separated according to two different geographical origins: Corse and Seville (Figure 4).

Discussion

It has been widely reported the idea that majority of characters used for flea species and subspecies diagnoses are based on the shape and structure of their extraordinarily complex genitalia, or the presence and distribution of setae and spines (Traub & Starcke, 1980; Dunnet & Mardon, 1991). While these characters are adequate for species diagnoses, they are mostly autapomorphic at the species and subspecies level and of limited utility for phylogenetic reconstruction. Thus, Siphonaptera appears to have many instances of parallel reductions and modifications, probably associated with multiple invasions of similar hosts, which may obscure homology. In addition, from a phylogenetic standpoint, Siphonaptera has remained as the most neglected of the holometabolous insect orders (Whiting *et al*., 2008).

The present work represents the first study that provides morphological, biometrical, molecular and phylogenetic comparative data of *A. erinacei* and their subspecies: *A. e. erinacei* and *A. e. maura*, in order to assess taxonomic and phylogenetic relationships between both subspecies and to shed light on the systematics of *A. erinacei*, representing a new tool to elucidate identifications within the genus.

From a morphological standpoint, Jordan & Rothschild (1953) were the first authors who provided some morphological features in order to identify and discriminate between both subspecies. They based the male morphological identification on the length of basimere, whereas female morphological subspecies discrimination was based on the presence of one or two lateral bristles in eighth abdominal tergum and the presence of four or five lateral bristles in seventh abdominal sternum on the two sides together. Beaucournu & Launay (1990) accepted these morphological criteria in order to discriminate both subspecies, excluding the setae number observed in seventh abdominal sternum. Nevertheless, these authors pointed out the high taxonomic similarity between these two subspecies and they observed that only male specimens could be identified easily each other. Our results reinforce the idea of the use of the

length of basimere as a useful morphological criterion in order to discriminate between males of *A. e. maura* and *A. e. erinacei*. Thus, based on these criteria we conclude that males collected from Corse belong to *A. e. erinacei*, while male specimens collected from Seville belong to *A. e. maura*.

Unlike male individuals, our results showed that previous criteria used for morphological subspecific differentiation in females of *A. erinacei* were not useful to discriminate between both subspecies. Thus, we observed different morphological populations of females showing overlapped morphological characters that not corresponded with any previous subspecific morphological characterization cited by different authors. Furthermore, a geographical pattern of distribution was not observed in female specimens, appearing *A. e. erinacei* (population A) and population B in both geographical areas. With these results, two different hypotheses could be suggested. The first one would be consider that *A. e. erinacei* occurs in both geographical areas and the appearance of population B and C just mean morphological variants belonging to a polymorphic taxon. The other one, could be considering that the morphological classification of females does not support the male one, therefore, it could be suggested to discriminate between both subspecies based exclusively on the morphological characteristics of e males specimens unless new discriminative morphological characters were revealed for female subspecific classification. In this sense, we observed, by the first time, that the total length of the spermatheca could be a useful criterion in order to discriminate between both females' subspecies since this criterion display a geographical pattern of distribution corroborated by molecular and phylogenetic data. Thus, we could conclude that individuals from Seville showing a total length of spermatheca higher than 120 µm corresponded with *A. e. maura* while those from Corse showing a total length of spermatheca lower than 120 µm corresponded with *A. e. erinacei*. Furthermore, length of spermatheca appeared as a significate value calculated by ANOVA test to differentiate both subspecies.

421 The analysis of external morphological characters presents some weaknesses when are used
422 as the unique criterion to distinguish female specimens of this species. Thus, the use of
423 molecular biology is considered as an essential tool in order to clarify morphological data.

424 These facts, lead us to suggest that *A. erinacei* subspecies might have been morphologically
425 misidentified for many years in Mediterranean area. This observation could be the
426 consequence of a wrong identification practice of females based on morphological differences
427 of male specimens or the geographic origin as a valid criterion for the identification between
428 both subspecies. Lewis (1967) and Beaucournu & Launay (1990) argued that certain flea
429 subspecies admitted by some authors, could just be a morphological variant belonging to a
430 polymorphic taxon. This fact is corroborated by phylogenetic analyses in our study, in which
431 we did not find correspondence between female morphological differences analyzed and the
432 18S, ITS1, ITS2, *cox1* and *cytb* sequences.

433 According to ITS´s analyses, ITS2 sequences of both subspecies were markedly shorter than
434 ITS1 sequences. Vobis *et al*. (2004) and Zurita *et al*. (2015, 2016, 2017) have previously
435 reported this fact in other species of fleas such as *C. felis*, *Stenoponia tripectinata*
436 *tripectinata*, *C. canis*, *N. barbarus* and *N. fasciatus*.

437 Both markers (ITS1 and ITS2) did not show sequence differences among individuals from the
438 same geographical area regardless they belong to different morphological populations
439 (females). Nevertheless, they showed different percentage of similarity ranged from 96.9 %
440 (ITS2) to 99.1 % (ITS1) between specimens from two geographical regions each other. Thus,
441 these nuclear markers were useful to differentiate *A. erinacei* from Seville and Corse. Similar
442 values of similarity were reported by Marrugal *et al*. (2013) and Zurita *et al*. (2016), who
443 reported an inter-specific similarity ranged from 91.8 % to 96 % between ITS sequences of *C.*
444 *felis* and *C. canis* isolated from dogs from different geographical areas. These geographical
445 signals in fleas have previously been reported by Luchetti *et al*. (2007), who noticed the

presence of two genotypic groups (Pacific and Atlantic) based on the analysis of ITS2 sequences of *Tunga penetrans* from Ecuador, Brazil and different geographical areas of Africa. In addition, several specific recognition sites for endonucleases were detected in ITS1 and ITS2 sequences in order to differentiate two geographical lineages. Thus, *EcoRV*, *HaeIII*, *PhoI*, *AseI*, *VspI*, *AsuII*, *BbuI*, *DraI*, *NIaIII*, *MseI*, *PsiI* and *SphI* sites have diagnostic value for specific determination of subspecific discrimination in *A. erinacei*.

The partial *cox1* and *cytb* mtDNA gene sequences showed the same geographical pattern than ITS sequences analyses (Tables 3 and 4) regardless which morphological population they belong to. On the other hand, *cox1*, *cytb* and concatenated phylogenetic trees reinforce the idea of the existence of two geographical genetic lineages in *A. erinacei* (Iberian Peninsula and Corse Island). Furthermore, *cox1* phylogenetic tree showed specimens belong to *Ctenocephalides* and *Archaeopsylla* genera clustered together. This close phylogenetic relation between *Ctenocephalides* and *Archaeopsylla* genera was reported by Zhu et al. (2015) who included both genera in Archaeopsyllini subfamily.

Previous studies showed that fleas have a high level of intraspecific genetic variation (Dittmar & Whiting, 2003; Brinkerhoff *et al*., 2011). Furthermore, it has been suggested that host specificity may influence the level of intraspecific genetic divergences since more generalist parasite species will show a higher level of intraspecific genetic variation enabling them to infest a broader host range (Van der Mescht *et al*., 2015). DNA barcoding studies on insects and invertebrates have shown maximum intra-specific variation ranging from 3 % to 3.9 % (Carew *et al*., 2007), out of which are markedly higher when specimens of study come from distant geographical regions, especially islands or archipelagos. In this way, Lawrence *et al*. (2014), Zurita *et al*. (2015) and Zurita *et al*. (2017) found a high degree of intra-specific variation in some flea species when populations from islands and mainland were compared,

suggesting the existence of different geographical lineages, which could have arisen due to the existence of geographical barriers.

The *cox1* similarity values observed between both geographical genetic lineages (97.7 % - 98.1 %) in *A. erinacei* were similar with those observed among different flea species such as *C. felis* and *C. canis* (97.7) (Table 3). This fact, could suggest that individuals from Spain and Corse could be treated as different species. Nevertheless, based on K2P analysis and 10X rule reported by Hebert *et al*. (2003) we cannot assume that both geographical genetic lineages correspond with two different species within *Archaeopsylla* genus.

Our results are in agreement with Losos & Ricklefs (2009) who suggest that detailed population-level studies can chart the course of evolution over short time periods. This approach can be broadened to incorporate intra-specific level studies with geographically explicit sampling of individuals for the reconstruction of gene genealogies to reveal the extent to which natural selection, or alternative mechanisms may explain evolutionary change. In this sense, island radiations are ideal systems for such an approach, because it is frequently apparent that the arena within which inter-specific diversification has occurred is similar to the arena within which intra-specific diversification is occurring (Ricklefs & Bermingham, 2001).

In conclusion, the present study provides for the first time, comparative morphological, biometrical and molecular data of *A. erinacei* and their subspecies: *A. e. erinacei* and *A. e. maura*. On the basis on morphological results, we conclude that the number of bristles bearing in eighth abdominal tergum and seventh abdominal sternum of female specimens are not valid criteria as diagnostic characters in order to differentiate *A. e. erinacei* and *A. e. maura*. However, the total length of the spermatheca in females and the different length of basimere in males should be taking into account as characters of reference in order to discriminate between both subspecies.

On the other hand, based on phylogenetic and molecular comparative study of two nuclear markers (ITS1 and ITS2), two mitochondrial markers (*cox1* and *cytb*) and concatenated sequences, we reported the existence of two geographical genetic lineages in *A. erinacei* corresponding with two different subspecies (*A. e. erinacei* and *A. e. maura*), that could be discriminated by PCR-linked RFLP.

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631 **Figure captions**

632 Figure 1. Morphological specific and subspecific characteristics of *Archaeopsylla erinacei*
633 and their subspecies: *A. e. erinacei* (Bouché, 1835) and *A. e. maura*. a- Falx of head
634 (arrowed); b- Asymmetrical antenna with partially welded basal segments; c- Pleural rod of
635 mesothorax (arrowed) d- Vestigial genal (arrowed) and pronotal (asterisk) combs; e- *A.*
636 *erinacei* without pronotal comb, GHL: Distance from base of spine at tip of genal process to
637 front margin on head, GEL: Distance from base of spine on genal process to anterior edge of
638 eye; f- Hind tibia of *A. erinacei*; g- Male basimere of *A. e. erinacei*; h- Male basimere of *A. e.*
639 *maura*; i- Female of *A. erinacei* eighth tergum bearing two lateral bristles (arrowed); j-
640 Female of *A. erinacei* eighth tergum bearing only one lateral bristle (arrowed); k- Female of
641 *A. erinacei* seventh sternum with three lateral bristles (each side) (arrowed); l- Spermatheca of
642 *A. erinacei*.

643 Figure 2. A- Schematic representation of restriction maps of the ITS1 sequence of *A. e. maura*
644 collected from Seville. B- Schematic representation of restriction maps of the ITS2 sequence
645 of *A. e. maura* collected from Seville. C- Schematic representation of restriction maps of the
646 ITS2 sequence of *A. e. erinacei* collected from Corse.

647 Figure 3. PCR-RFLP analysis of the ITS1 of *A. erinacei* collected from different geographical
648 origins using *HaeIII* endonuclease. M = DNA Molecular Weight Marker IX (72-1353 bp);
649 Line 1 = *A. e. erinacei* from Seville; Line 2 = *A. e. maura* from Corse.

650 Figure 4. Phylogenetic tree of *Archaeopsylla erinacei* from different geographical origins (see
651 Table 1) based on concatenated partial 18S ribosomal RNA gene, Internal Transcribed Spacer
652 2 (ITS2) partial cytochrome c-oxidase 1 (*cox1*) and cytochrome b (*cytb*) gene of
653 mitochondrial DNA inferred using the Bayesian (B) method. The percentage of replicate trees

in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown on the branches. The Bayesian Posterior Probabilities (BPP) are percentage converted.

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

Figure S2. Phylogenetic tree of *Archaeopsylla erinacei* from different geographical origins (see Table 1) based on the Internal Transcribed Spacer 1 (ITS1) sequences using the Bayesian (B) and Maximum Likelihood (ML) methods and Bayesian topology. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown on the branches (B/ML). Bootstrap values lower than 60 % are not shown. The Bayesian Posterior Probabilities (BPP) is percentage converted.

Figure S3. Phylogenetic tree of *Archaeopsylla erinacei* from different geographical origins (see Table 1) based on partial cytochrome c-oxidase 1 (*cox1*) gene of mitochondrial DNA inferred using the Bayesian (B) and Maximum Likelihood (ML) methods and Bayesian topology. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown on the branches (B/ML). Bootstrap values lower than 60 % are not shown. The Bayesian Posterior Probabilities (BPP) is percentage converted.

Figure S4. Phylogenetic tree of *Archaeopsylla erinacei* from different geographical origins (see Table 1) based on partial cytochrome b (*cytb*) gene of mitochondrial DNA inferred using the Bayesian (B) and Maximum Likelihood (ML) methods and Bayesian topology. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap

678 test (1,000 replicates) is shown on the branches (B/ML). Bootstrap values lower than 60 %
679 are not shown. The Bayesian Posterior Probabilities (BPP) is percentage converted.

FIGURE S4

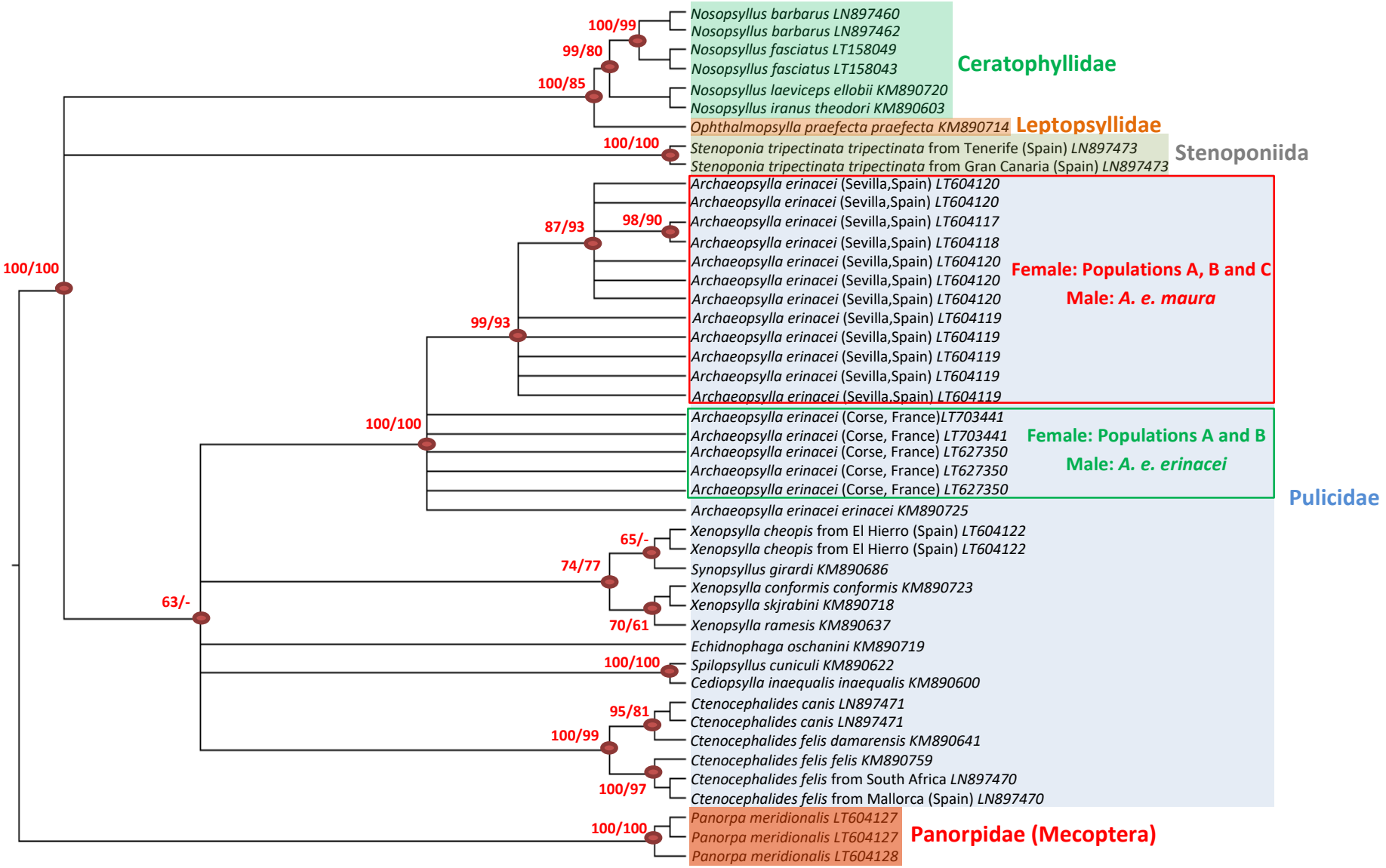

Nosopsyllus barbarus LN897460
Nosopsyllus barbarus LN897462
Nosopsyllus fasciatus LT158049
Nosopsyllus fasciatus LT158043
Nosopsyllus laeviceps ellobii KM890720
Nosopsyllus iranus theodori KM890603
Ceratophyllidae
Ophthalmopsylla praefecta praefecta KM890714
Leptopsyllidae
Stenoponia tripectinata tripectinata from Tenerife (Spain) LN897473
Stenoponia tripectinata tripectinata from Gran Canaria (Spain) LN897473
Stenoponiida
Archaeopsylla erinacei (Sevilla,Spain) LT604120
Archaeopsylla erinacei (Sevilla,Spain) LT604120
Archaeopsylla erinacei (Sevilla,Spain) LT604117
Archaeopsylla erinacei (Sevilla,Spain) LT604118
Archaeopsylla erinacei (Sevilla,Spain) LT604120
Archaeopsylla erinacei (Sevilla,Spain) LT604120
Archaeopsylla erinacei (Sevilla,Spain) LT604120
Archaeopsylla erinacei (Sevilla,Spain) LT604119
Archaeopsylla erinacei (Sevilla,Spain) LT604119
Archaeopsylla erinacei (Sevilla,Spain) LT604119
Archaeopsylla erinacei (Sevilla,Spain) LT604119
Archaeopsylla erinacei (Sevilla,Spain) LT604119
Female: Populations A, B and C
Male: A. e. maura
Archaeopsylla erinacei (Corse, France)LT703441
Archaeopsylla erinacei (Corse, France) LT703441
Archaeopsylla erinacei (Corse, France) LT627350
Archaeopsylla erinacei (Corse, France) LT627350
Archaeopsylla erinacei (Corse, France) LT627350
Female: Populations A and B
Male: A. e. erinacei
Archaeopsylla erinacei erinacei KM890725
Xenopsylla cheopis from El Hierro (Spain) LT604122
Xenopsylla cheopis from El Hierro (Spain) LT604122
Synopsyllus girardi KM890686
Xenopsylla conformis conformis KM890723
Xenopsylla skjrabini KM890718
Xenopsylla ramesis KM890637
Echidnophaga oschanini KM890719
Spilopsyllus cuniculi KM890622
Cediopsylla inaequalis inaequalis KM890600
Ctenocephalides canis LN897471
Ctenocephalides canis LN897471
Ctenocephalides felis damarensis KM890641
Ctenocephalides felis felis KM890759
Ctenocephalides felis from South Africa LN897470
Ctenocephalides felis from Mallorca (Spain) LN897470
Pulicidae
Panorpa meridionalis LT604127
Panorpa meridionalis LT604127
Panorpa meridionalis LT604128
Panorpidae (Mecoptera)
100/99
99/80
100/85
100/100
87/93
98/90
99/93
100/100
65/-
74/77
63/-
70/61
100/100
95/81
100/99
100/97
100/100

FIGURE S3

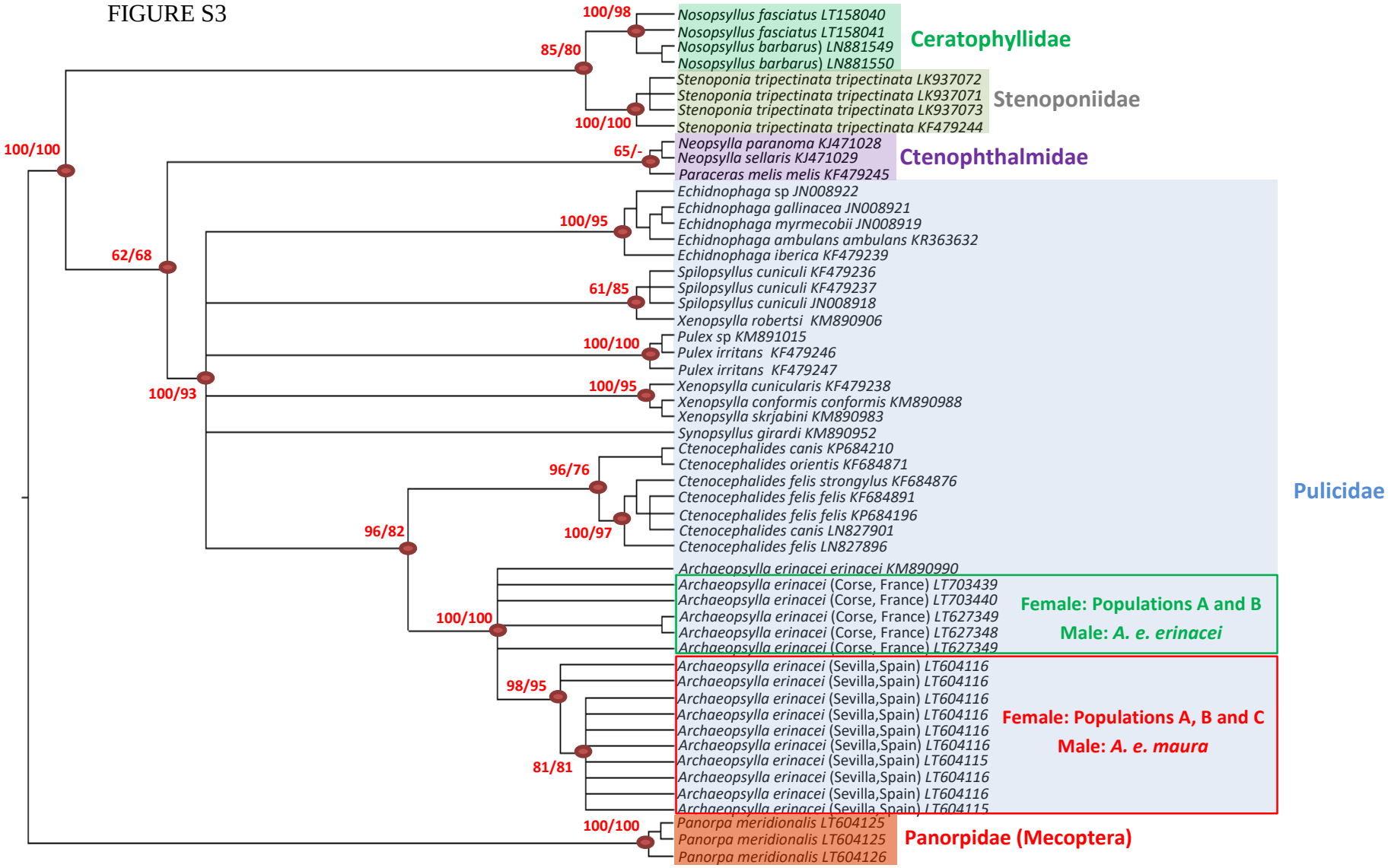

100/98
85/80
100/100
100/100
65/-
62/68
100/95
61/85
100/100
100/95
100/93
96/76
100/97
96/82
100/100
98/95
81/81
100/100
Nosopsyllus fasciatus LT158040
Nosopsyllus fasciatus LT158041
Nosopsyllus barbarus) LN881549
Nosopsyllus barbarus) LN881550
Ceratophyllidae
Stenoponia tripectinata tripectinata LK937072
Stenoponia tripectinata tripectinata LK937071
Stenoponia tripectinata tripectinata LK937073
Stenoponia tripectinata tripectinata KF479244
Stenoponiidae
Neopsylla paranoma KJ471028
Neopsylla sellaris KJ471029
Paraceras melis melis KF479245
Ctenophthalmidae
Echidnophaga sp JN008922
Echidnophaga gallinacea JN008921
Echidnophaga myrmecobii JN008919
Echidnophaga ambulans ambulans KR363632
Echidnophaga iberica KF479239
Spilopsyllus cuniculi KF479236
Spilopsyllus cuniculi KF479237
Spilopsyllus cuniculi JN008918
Xenopsylla robertsi KM890906
Pulex sp KM891015
Pulex irritans KF479246
Pulex irritans KF479247
Xenopsylla cunicularis KF479238
Xenopsylla conformis conformis KM890988
Xenopsylla skrjabini KM890983
Synopsyllus girardi KM890952
Ctenocephalides canis KP684210
Ctenocephalides orientis KF684871
Ctenocephalides felis strongylus KF684876
Ctenocephalides felis felis KF684891
Ctenocephalides felis felis KP684196
Ctenocephalides canis LN827901
Ctenocephalides felis LN827896
Pulicidae
Archaeopsylla erinacei erinacei KM890990
Archaeopsylla erinacei (Corse, France) LT703439
Archaeopsylla erinacei (Corse, France) LT703440
Archaeopsylla erinacei (Corse, France) LT627349
Archaeopsylla erinacei (Corse, France) LT627348
Archaeopsylla erinacei (Corse, France) LT627349
Female: Populations A and B
Male: A. e. erinacei
Archaeopsylla erinacei (Sevilla,Spain) LT604116
Archaeopsylla erinacei (Sevilla,Spain) LT604116
Archaeopsylla erinacei (Sevilla,Spain) LT604116
Archaeopsylla erinacei (Sevilla,Spain) LT604116
Archaeopsylla erinacei (Sevilla,Spain) LT604116
Archaeopsylla erinacei (Sevilla,Spain) LT604116
Archaeopsylla erinacei (Sevilla,Spain) LT604115
Archaeopsylla erinacei (Sevilla,Spain) LT604116
Archaeopsylla erinacei (Sevilla,Spain) LT604116
Archaeopsylla erinacei (Sevilla,Spain) LT604115
Female: Populations A, B and C
Male: A. e. maura
Panorpa meridionalis LT604125
Panorpa meridionalis LT604125
Panorpa meridionalis LT604126
Panorpidae (Mecoptera)

FIGURE S2

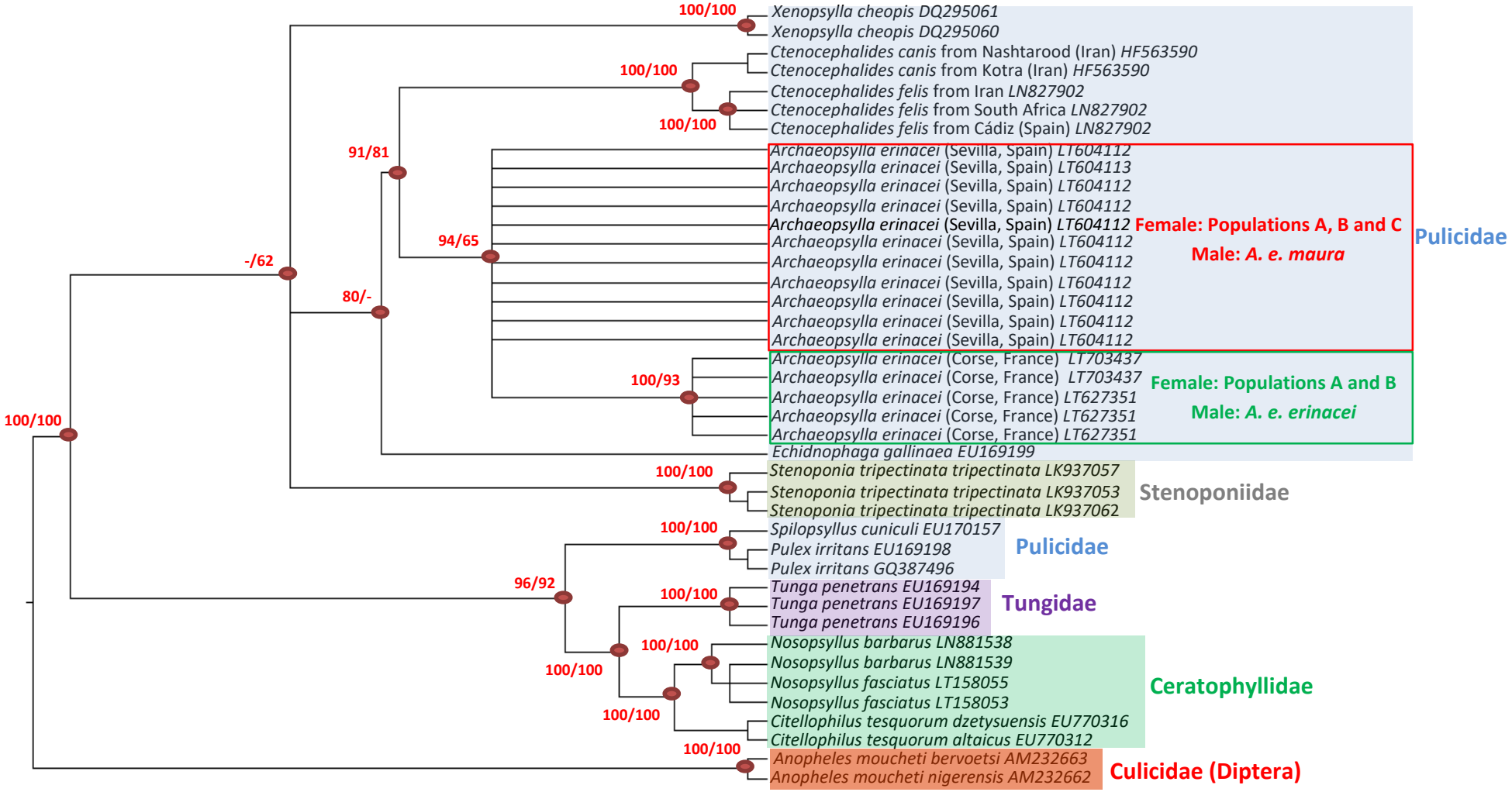
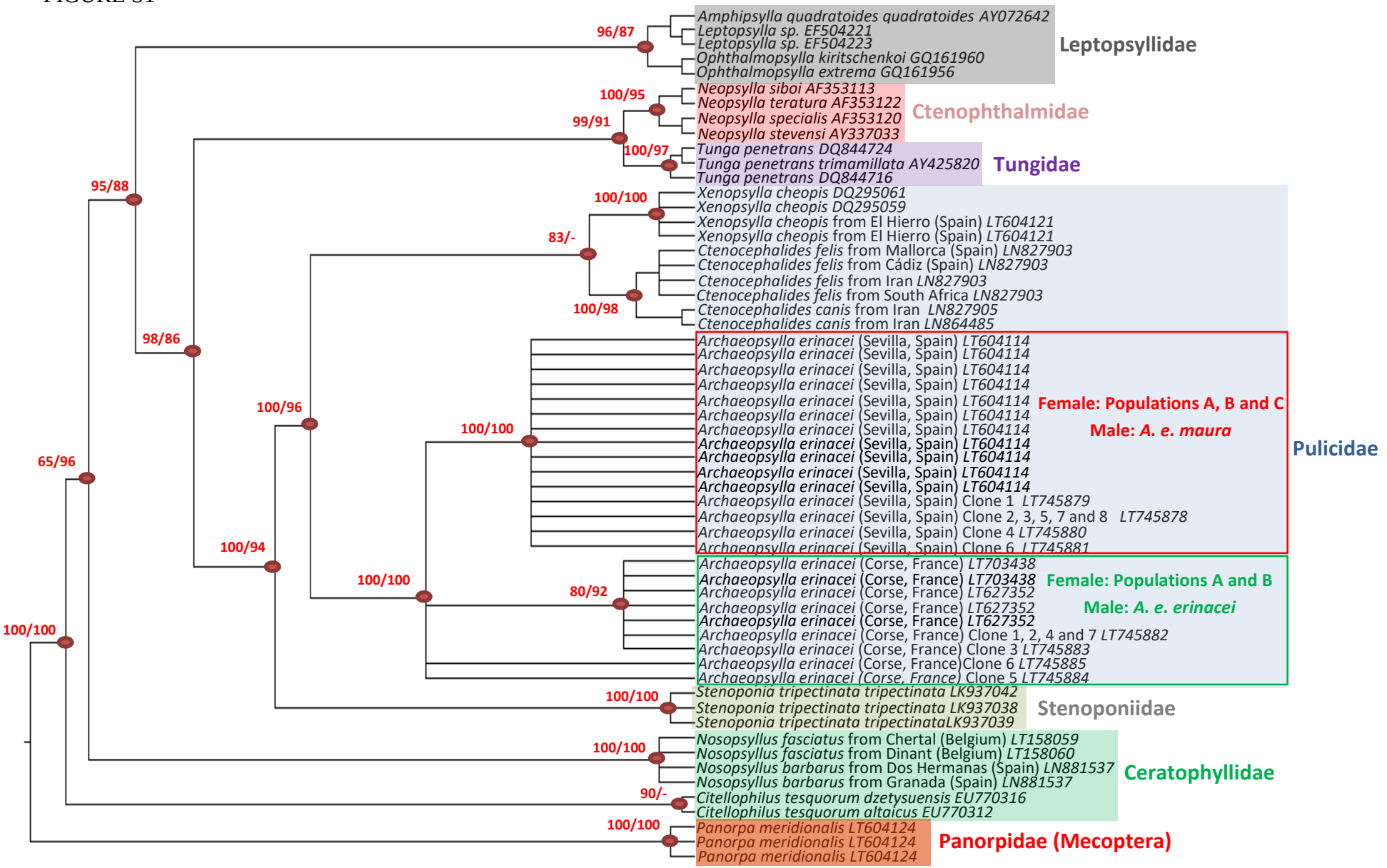

100/100
Xenopsylla cheopis DQ295061
Xenopsylla cheopis DQ295060
Ctenocephalides canis from Nashtarood (Iran) HF563590
100/100
Ctenocephalides canis from Kotra (Iran) HF563590
Ctenocephalides felis from Iran LN827902
Ctenocephalides felis from South Africa LN827902
100/100
Ctenocephalides felis from Cádiz (Spain) LN827902
91/81
Archaeopsylla erinacei (Sevilla, Spain) LT604112
Archaeopsylla erinacei (Sevilla, Spain) LT604113
Archaeopsylla erinacei (Sevilla, Spain) LT604112
Archaeopsylla erinacei (Sevilla, Spain) LT604112
Archaeopsylla erinacei (Sevilla, Spain) LT604112
Female: Populations A, B and C
Male: A. e. maura
Pulicidae
94/65
Archaeopsylla erinacei (Sevilla, Spain) LT604112
-/62
Archaeopsylla erinacei (Sevilla, Spain) LT604112
Archaeopsylla erinacei (Sevilla, Spain) LT604112
80/-
Archaeopsylla erinacei (Sevilla, Spain) LT604112
Archaeopsylla erinacei (Sevilla, Spain) LT604112
Archaeopsylla erinacei (Sevilla, Spain) LT604112
Archaeopsylla erinacei (Corse, France) LT703437
100/93
Archaeopsylla erinacei (Corse, France) LT703437
Archaeopsylla erinacei (Corse, France) LT627351
Archaeopsylla erinacei (Corse, France) LT627351
Archaeopsylla erinacei (Corse, France) LT627351
Female: Populations A and B
Male: A. e. erinacei
Echidnophaga gallinaea EU169199
100/100
Stenoponia tripectinata tripectinata LK937057
Stenoponia tripectinata tripectinata LK937053
Stenoponia tripectinata tripectinata LK937062
Stenoponiidae
100/100
Spilopsyllus cuniculi EU170157
Pulex irritans EU169198
Pulex irritans GQ387496
Pulicidae
96/92
Tunga penetrans EU169194
100/100
Tunga penetrans EU169197
Tunga penetrans EU169196
Tungidae
100/100
Nosopsyllus barbarus LN881538
100/100
Nosopsyllus barbarus LN881539
Nosopsyllus fasciatus LT158055
Nosopsyllus fasciatus LT158053
100/100
Citellophilus tesquorum dzetysuensis EU770316
Citellophilus tesquorum altaicus EU770312
Ceratophyllidae
100/100
Anopheles moucheti bervoetsi AM232663
Anopheles moucheti nigerensis AM232662
Culicidae (Diptera)

FIGURE S1


Amphipsylla quadratoides quadratoides AY072642
Leptopsylla sp. EF504221
Leptopsylla sp. EF504223
Ophthalmopsylla kiritschenkoi GQ161960
Ophthalmopsylla extrema GQ161956
Leptopsyllidae
Neopsylla siboi AF353113
Neopsylla teratura AF353122
Neopsylla specialis AF353120
Neopsylla stevensi AY337033
Ctenophthalmidae
Tunga penetrans DQ844724
Tunga penetrans trimamillata AY425820
Tunga penetrans DQ844716
Tungidae
Xenopsylla cheopis DQ295061
Xenopsylla cheopis DQ295059
Xenopsylla cheopis from El Hierro (Spain) LT604121
Xenopsylla cheopis from El Hierro (Spain) LT604121
Ctenocephalides felis from Mallorca (Spain) LN827903
Ctenocephalides felis from Cádiz (Spain) LN827903
Ctenocephalides felis from Iran LN827903
Ctenocephalides felis from South Africa LN827903
Ctenocephalides canis from Iran LN827905
Ctenocephalides canis from Iran LN864485
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) LT604114
Archaeopsylla erinacei (Sevilla, Spain) Clone 1 LT745879
Archaeopsylla erinacei (Sevilla, Spain) Clone 2, 3, 5, 7 and 8 LT745878
Archaeopsylla erinacei (Sevilla, Spain) Clone 4 LT745880
Archaeopsylla erinacei (Sevilla, Spain) Clone 6 LT745881
Female: Populations A, B and C
Male: A. e. maura
Archaeopsylla erinacei (Corse, France) LT703438
Archaeopsylla erinacei (Corse, France) LT703438
Archaeopsylla erinacei (Corse, France) LT627352
Archaeopsylla erinacei (Corse, France) LT627352
Archaeopsylla erinacei (Corse, France) LT627352
Archaeopsylla erinacei (Corse, France) Clone 1, 2, 4 and 7 LT745882
Archaeopsylla erinacei (Corse, France) Clone 3 LT745883
Archaeopsylla erinacei (Corse, France)Clone 6 LT745885
Archaeopsylla erinacei (Corse, France) Clone 5 LT745884
Female: Populations A and B
Male: A. e. erinacei
Pulicidae
Stenoponia tripectinata tripectinata LK937042
Stenoponia tripectinata tripectinata LK937038
Stenoponia tripectinata tripectinataLK937039
Stenoponiidae
Nosopsyllus fasciatus from Chertal (Belgium) LT158059
Nosopsyllus fasciatus from Dinant (Belgium) LT158060
Nosopsyllus barbarus from Dos Hermanas (Spain) LN881537
Nosopsyllus barbarus from Granada (Spain) LN881537
Citellophilus tesquorum dzetysuensis EU770316
Citellophilus tesquorum altaicus EU770312
Ceratophyllidae
Panorpa meridionalis LT604124
Panorpa meridionalis LT604124
Panorpa meridionalis LT604124
Panorpidae (Mecoptera)
96/87
100/95
99/91
100/97
95/88
100/100
83/-
100/98
98/86
100/96
100/100
65/96
100/94
100/100
80/92
100/100
100/100
90/-
100/100

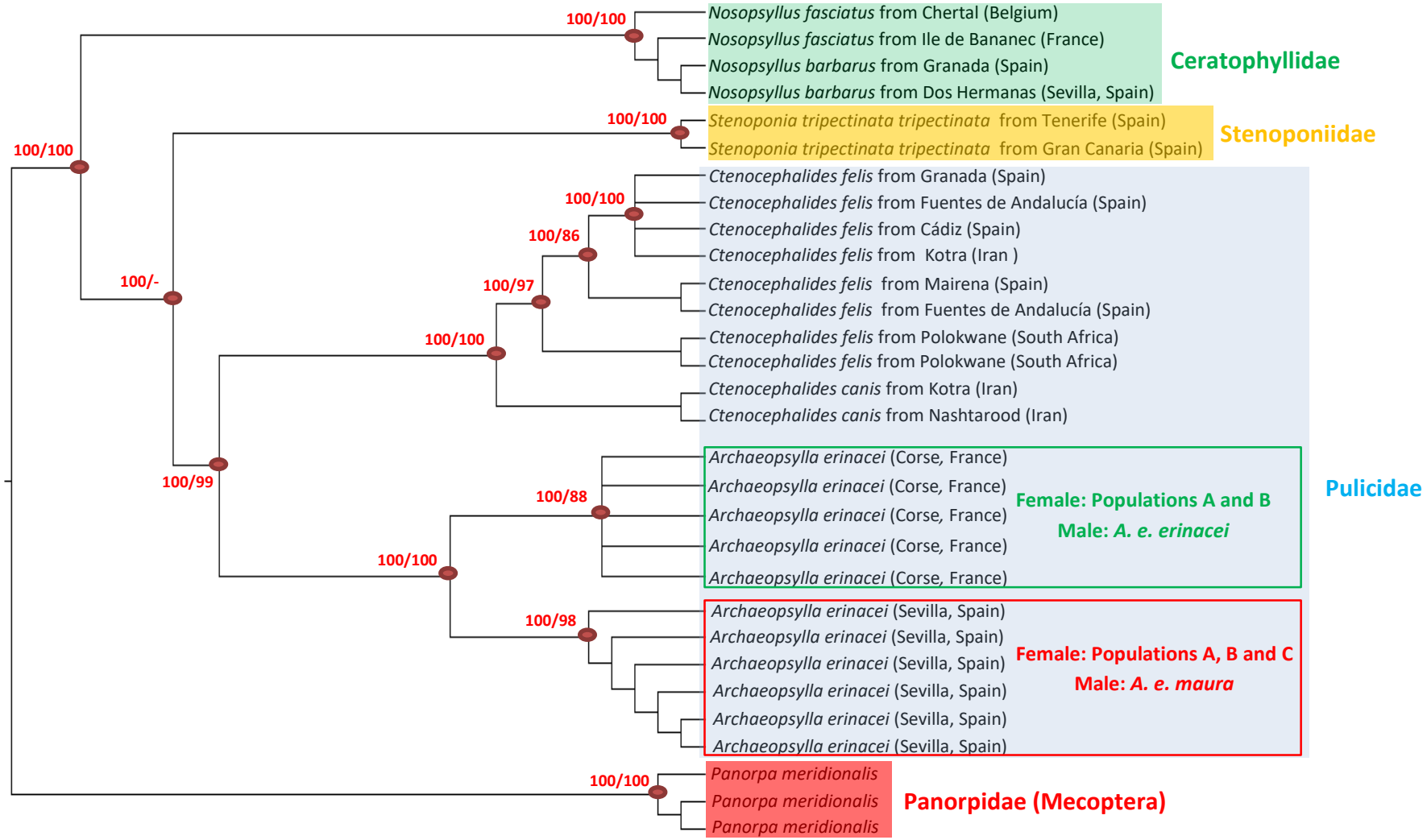

100/100
Nosopsyllus fasciatus from Chertal (Belgium)
Nosopsyllus fasciatus from Ile de Bananec (France)
Nosopsyllus barbarus from Granada (Spain)
Nosopsyllus barbarus from Dos Hermanas (Sevilla, Spain)
Ceratophyllidae
100/100
Stenoponia tripectinata tripectinata from Tenerife (Spain)
Stenoponia tripectinata tripectinata from Gran Canaria (Spain)
Stenoponiidae
100/100
Ctenocephalides felis from Granada (Spain)
Ctenocephalides felis from Fuentes de Andalucía (Spain)
Ctenocephalides felis from Cádiz (Spain)
100/86
Ctenocephalides felis from Kotra (Iran)
100/97
Ctenocephalides felis from Mairena (Spain)
Ctenocephalides felis from Fuentes de Andalucía (Spain)
100/100
Ctenocephalides felis from Polokwane (South Africa)
Ctenocephalides felis from Polokwane (South Africa)
Ctenocephalides canis from Kotra (Iran)
Ctenocephalides canis from Nashtarood (Iran)
100/-
100/99
Archaeopsylla erinacei (Corse, France)
100/88
Archaeopsylla erinacei (Corse, France)
Archaeopsylla erinacei (Corse, France)
Archaeopsylla erinacei (Corse, France)
100/100
Archaeopsylla erinacei (Corse, France)
Female: Populations A and B
Male: A. e. erinacei
Pulicidae
100/98
Archaeopsylla erinacei (Sevilla, Spain)
Archaeopsylla erinacei (Sevilla, Spain)
Archaeopsylla erinacei (Sevilla, Spain)
Archaeopsylla erinacei (Sevilla, Spain)
Archaeopsylla erinacei (Sevilla, Spain)
Archaeopsylla erinacei (Sevilla, Spain)
Female: Populations A, B and C
Male: A. e. maura
100/100
Panorpa meridionalis
Panorpa meridionalis
Panorpa meridionalis
Panorpidae (Mecoptera)

Figure 3

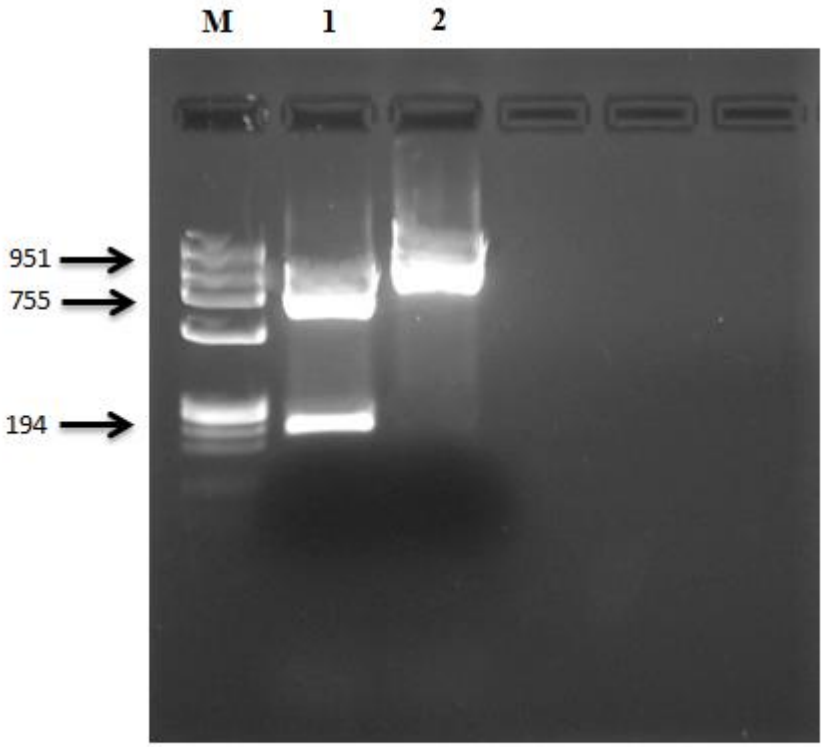
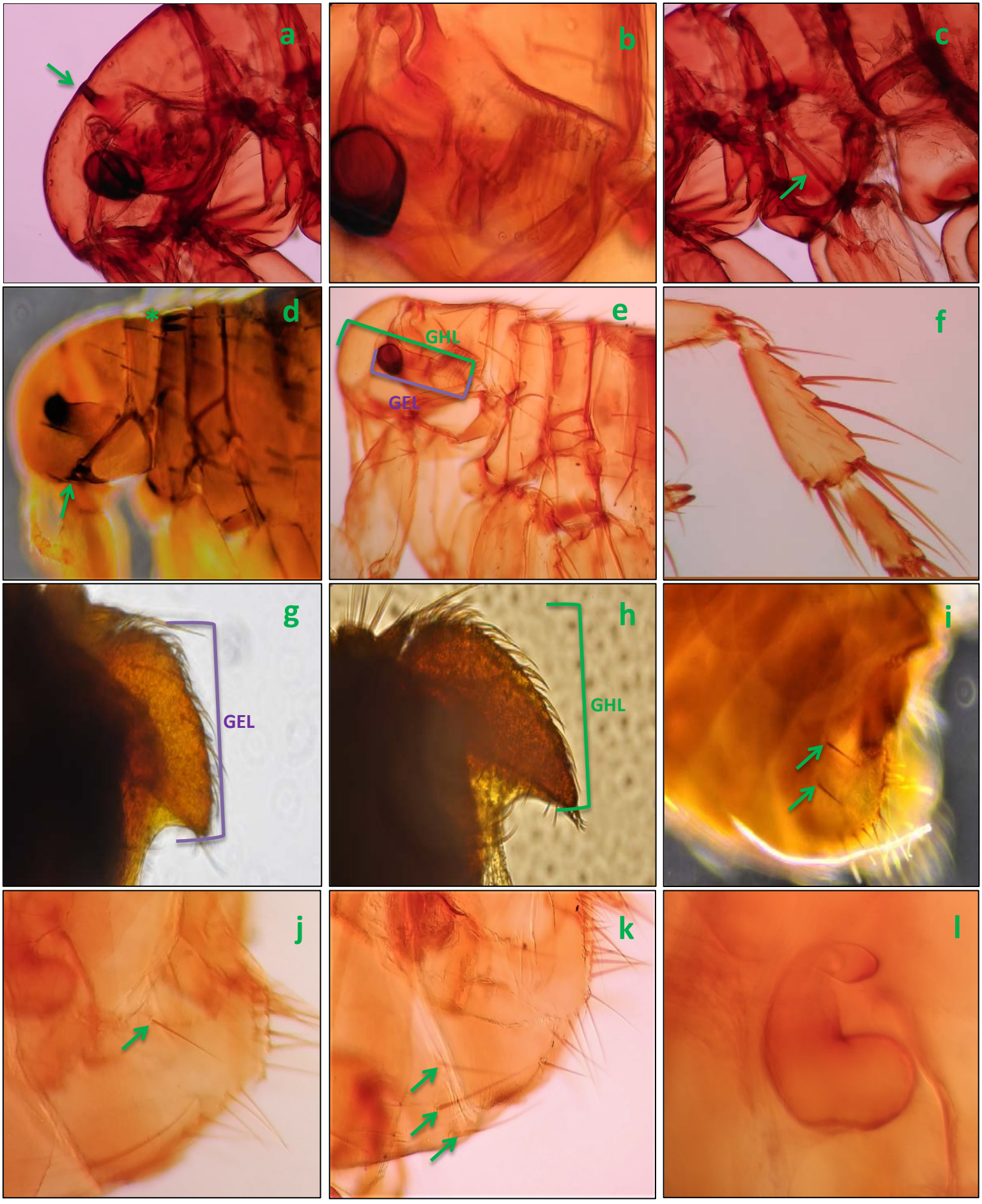
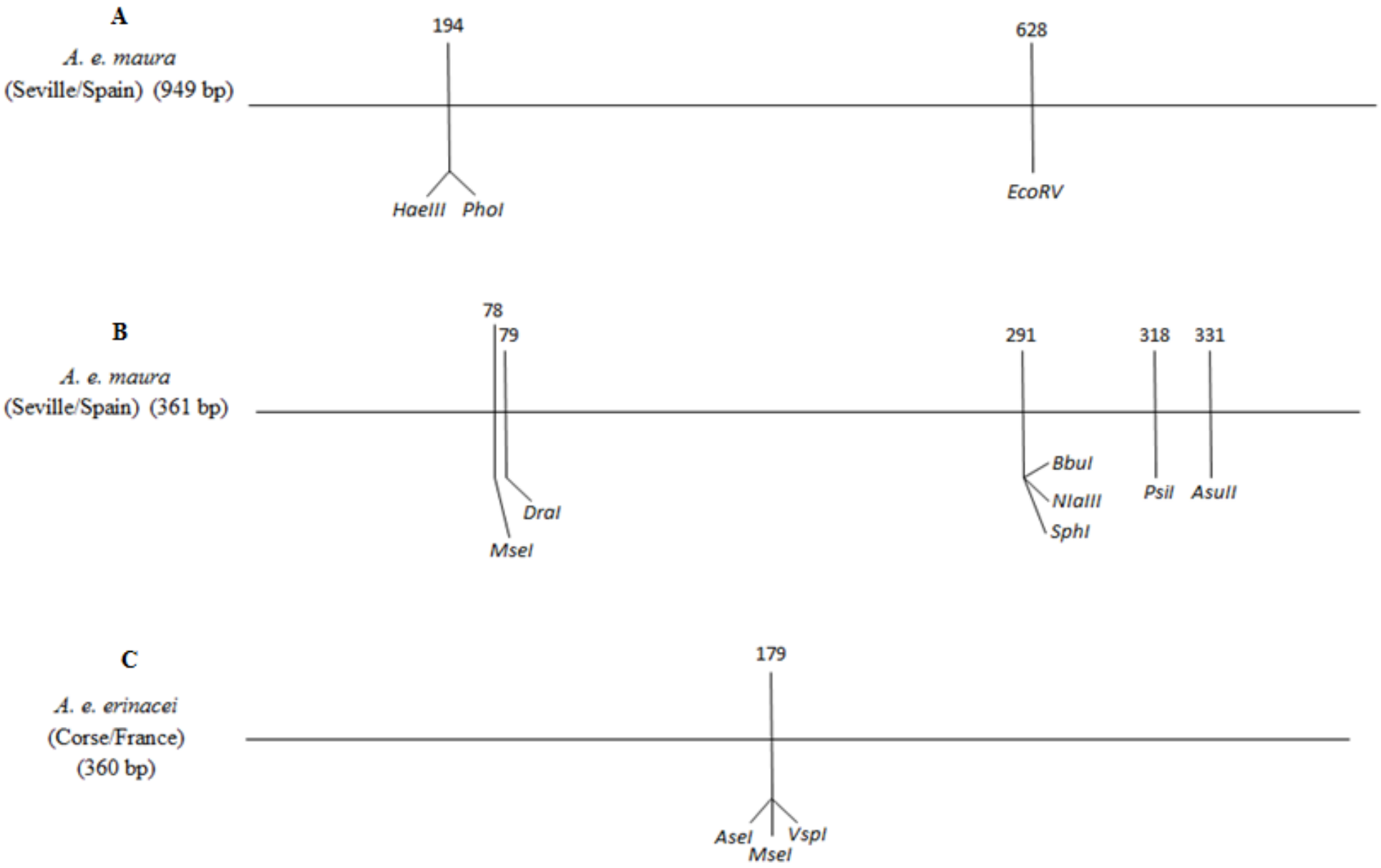


FIGURE 1




A
A. e. maura
(Seville/Spain) (949 bp)
194
628
HaeIII PhoI
EcoRV
B
A. e. maura
(Seville/Spain) (361 bp)
78
79
291
318
331
DraI
MseI
BbuI
NlaIII
SphI
PsiI
AsuII
C
A. e. erinacei
(Corse/France)
(360 bp)
179
AseI
MseI
VspI

	ITS1	**ITS2**	**18S**	***Cytb***	***cox1***
PCR Mix					
PCR Buffer (5X)	10 µl	10 µl	5 µl	10 µl	10 µl
dNTPs (10mM)	2 µl	1 µl	1 µl	1 µl	1 µl
$MgCl_2$ (25 mM)	6 µl	6 µl	4 µl	4 µl	4 µl
Forward Primer (10 µM)	5 µl	5 µl	5 µl	5 µl	5 µl
Reverse Primer (10 µM)	5 µl	5 µl	5 µl	5 µl	5 µl
Template DNA	5 µl	5 µl	5 µl	5 µl	5 µl
goTaq DNA polymerase	0,5 µl	0,5 µl	0,5 µl	0,5 µl	0,5 µl
Autoclaved distilled water to	100 µl	50 µl	50 µl	50 µl	50 µl
PCR Primers					
Forward Primer	NC5 (Gasser et al., 1996)	senITS2 (Vobis et al., 2004)	18SF (Kaewmongkol et al., 2011)	CytbF (Dittmar &Whiting, 2003)	LCO1490 (Folmer et al., 1994)
Reverse Primer	ITS1rev (Marrugal et al.., 2013)	ITS2R (Luchetti et al., 2007)	18SR (Kaewmongkol et al., 2011)	A5F (Dittmar &Whiting, 2003)	HCO2198 (Folmer et al., 1994)
PCR Conditions					
Initial Denaturing	94 ºC for 5´	94 ºC for 5´	96 ºC for 2´	95 ºC for 12´	96 ºC for 2´
Number of cycles	35	35	45	30	40
Denaturing	94 ºC for 30´´	94 ºC for 60´´	94 ºC for 50´´	95 ºC for 30´´	94 ºC for 30´´
Annealing	58 ºC for 30´´	55 ºC for 60´´	58 ºC for 60´´	40 ºC for 30´´	50 ºC for 30´´
Primer extension	72 ºC for 90´´	72 ºC for 60´´	72 ºC for 90´´	68 ºC for 2´	72 ºC for 60´´
Final extension	72 ºC for 5´	72 ºC for 10´	72 ºC for10´	68 ºC for 7´	72 ºC for 7´

CYTB	*AE*/SEVILLA/ LT604120	*AE*/SEVILLA/ LT604117	*AE*/SEVILLA/ LT604118	*AE*/SEVILLA/ LT604119	*AE*/CORSE/ LT627350, LT703441	*A. erinacei erinacei/ KM890725*	*C. felis/* LN897470	*C. canis/* LN897471	*X. cheopis/* LT604122	*X. skrjabini/* KM890718	*S. cuniculi/* KM890622	*S. girardi/* KM890686	*E. oschanini/* KM890719
AE/SEVILLA/LT604120	-												
AE/SEVILLA/LT604117	99.5	-											
AE/SEVILLA/LT604118	98.4	98.9	-										
AE/SEVILLA/ LT604119	99.3	99.2	98.1	-									
AE/CORSE/LT627350, LT703441	98.7	98.7	98.1	98.9	-								
A. erinacei erinacei/ KM890725	98.7	98.7	98.1	98.9	99.5	-							
C. felis/ LN897470	84.5	85.0	86.1	84.8	85.3	85.8	-						
C. canis/ LN897471	85.3	85.8	86.9	85.6	86.1	86.4	90.9	-					
X. cheopis/ LT604122	79.4	80.0	80.5	79.7	80.0	80.7	81.8	81.0	-				
X. skrjabini/ KM890718	81.3	81.8	82.4	81.6	82.4	82.4	84.8	83.4	81.3	-			
S. cuniculi/ KM890622	82.4	82.9	83.4	82.6	83.2	83.7	83.4	84.5	80.5	81.8	-		
S. girardi/ KM890686	83.2	83.7	84.0	83.4	84.5	84.5	85.3	84.2	80.5	82.9	78.6	-	
E. oschanini/ KM890719	84.0	84.0	84.2	84.2	84.8	85.0	83.7	84.0	78.6	83.4	82.4	79.7	-

COX1	*AE*/SEVILLA/ LT604115	*AE*/SEVILLA/ LT604116	*AE*/CORSE/ LT703439, LT627348	*AE*/CORSE/ LT703440, LT627349	*A. erinacei erinacei*/ KM890990	*C. felis*/ LN827896	*C. canis*/ LN827901	*X. cunicularis*/ KF479238	*X. skrjabini*/ KM890983	*S. cuniculi*/ KF479237	*S. girardi*/ K890952	*P. irritans*/ KF479246	*E.. gallinacea* /JN008921	*E.. iberica*/ KF479239
AE/SEVILLA/LT604115	-													
AE/SEVILLA/LT604116	99.8	-												
AE/CORSE/LT703439, LT627348	98.0	97.7	-											
AE/CORSE/ LT703440, LT627349	98.1	98.0	99.8	-										
A. erinacei erinacei/ KM890990	98.1	98.0	99.8	100	-									
C. felis/LN827896	87.3	87.0	87.5	87.7	87.7	-								
C. canis/LN827901	87.5	87.3	88.0	88.2	88.2	97.7	-							
X. cunicularis/KF479238	85.6	85.6	86.3	86.6	86.6	88.2	87.0	-						
X. skrjabini/KM890983	86,1	86.1	85.9	86.1	86.1	86.1	86.1	89.4	-					
S. cuniculi/KF479237	85.9	85.9	86.6	86.8	86.8	86.3	86.8	85.8	84.7	-				
S. girardi/K890952	85.2	85.2	85.0	85.2	85.2	85.9	86.3	85.0	83.8	84.7	-			
P. irritans/KF479246	85.0	85.0	85.4	85.4	85.4	87.5	88.2	86.8	85.0	85.6	84.7	-		
E. gallinacea/JN008921	88.4	88.4	88.9	89.1	89.1	87.5	87.7	86.6	86.3	86.6	84.7	88.2	-	
E. iberica/KF479239	88.2	88.2	88.9	88.9	88.9	88.4	88.7	87.8	87.5	87.5	85.6	88.7	92.6	-

	A. e. maura from Sevilla (Spain)					*A. e. erinacei* from Corse (France)				
	MIN	MAX	X	б	VC	MIN	MAX	X	б	VC
TLF(mm)	2.1	2.9	2.5	0.3	12	1.8	2.6	2.2	0.6	27
TLM(mm)	1.8	2.2	1.9	0.2	11	2.0	2.3	2.1	0.2	5
TWF(mm)	1.0	1.4	1.2	0.1	8	0.8	1.2	1	0.3	30
TWM(mm)	0.8	1.1	0.9	0.1	11	0.9	1	1	0.1	10
HLF(µm)	426	592	500	42	8	410	456	433	32	7
HLM(µm)	426	486	448	24	5	456	501	479	19	4
HWF(µm)	304	410	365	29	8	289	334	311	32	10
HWM(µm)	273	365	334	30	9	304	357	338	19	6
BL(µm)	328	410	362	25	7	263	275	269	5	2
BW(µm)	117	164	159	14	9	123	147	135	13	10
GHL(µm)	363	440	393	22	6	-	-	-	-	-
GEL(µm)	-	-	-	-	-	252	298	280	17	6
EL(µm)	129	199	156	20	13	100	117	109	12	11
EW(µm)	70	188	128	29	23	76	94	85	13	15
PL(µm)	47	105	73	15	20	41	76	67	12	18
DS7(µm)	23	76	39	17	44	29	35	32	4	13
DSS(µm)	234	398	285	57	20	264	270	267	4	16
PROL(µm)	100	205	150	29	28	105	188	150	29	20
MESL(µm)	70	205	141	32	23	105	193	142	26	18
METL(µm)	105	176	141	19	13	135	176	151	14	9

ITS1

Location/Country/Sample numbers	Species/Gender	Female population	Host	Number of fleas	Base pairs (bp)	Accession number
Seville/Spain/AE5-11, AE13, AE14-15	*A. erinacei*/7♂ 3♀	A, B and C	*E. europaeus*	10	949	LT604112
Seville/Spain/AE12	*A. erinacei*/1♀	A	*E. europaeus*	1	950	LT604113
Corse/France/AE17, AE19	*A. erinacei*/2♂	-	*E. europaeus*	2	951	LT703437
Corse/France/AE18, AE20-21	*A. erinacei*/3♀	A and B	*E. europaeus*	3	951	LT627351

ITS2

Location/Country/Sample numbers	Species/Gender	Female population	Host	Number of fleas	Base pairs (bp)	Accession number
Seville/Spain/AE5-7, AE9-16	*A. erinacei*/6♂ 5♀	A, B and C	*E. europaeus*	11	361	LT604114
Seville/Spain (clone 1)	*A. erinacei*	-	*E. europaeus*	-	361	LT745879
Seville/Spain (clone 2, 3, 5, 7 and 8)	*A. erinacei*	-	*E. europaeus*	-	361	LT745878
Seville/Spain (clone 4)	*A. erinacei*	-	*E. europaeus*	-	361	LT745880
Seville/Spain (clone 6)	*A. erinacei*	-	*E. europaeus*	-	361	LT745881
Corse/France/AE17, AE19	*A. erinacei*/2♂	-	*E. europaeus*	2	360	LT703438
Corse/France/AE18, AE20-21	*A. erinacei*/3♀	C and D	*E. europaeus*	3	360	LT627352
Corse/France (clone 1, 2, 4 and 7)	*A. erinacei*	-	*E. europaeus*	-	360	LT745882
Corse/France (clone 3)	*A. erinacei*	-	*E. europaeus*	-	360	LT745883
Corse/France (clone 5)	*A. erinacei*	-	*E. europaeus*	-	360	LT745884
Corse/France (clone 6)	*A. erinacei*	-	*E. europaeus*	-	360	LT745885
El Hierro/Spain/XC1, XC7	*X. cheopis*/1♂ 1♀	-	*R. rattus*	2	358	LT604121

18S

Location/Country/Sample numbers	Species/Gender	Female population	Host	Number of fleas	Base pairs (bp)	Accession number
Seville/Spain/AE5, AE7-8, AE11-12, AE14	*A. erinacei*/3♂ 3♀	A and B	*E. europaeus*	6	1,160	LT604111
Corse/France/AE17, AE19	*A. erinacei*/2♂	-	*E. europaeus*	2	1,160	LT703442
Corse/France/AE18, AE20-21	*A. erinacei*/3♀	A and B	*E. europaeus*	3	1,160	LT627347

cox1

Location/Country/Sample numbers	Species/Gender	Female population	Host	Number of fleas	Base pairs (bp)	Accession number
Seville/Spain/AE5, AE6	*A. erinacei*/2♂	-	*E. europaeus*	2	658	LT604115
Seville/Spain/AE9-13, AE14-16	*A. erinacei*/4♂ 4♀	A and C	*E. europaeus*	8	658	LT604116
Corse/France/AE17	*A. erinacei*/1♂	-	*E. europaeus*	1	658	LT703439
Corse/France/AE19	*A. erinacei*/1♂	-	*E. europaeus*	1	658	LT703440
Corse/France/AE18	*A. erinacei*/1♀	A	*E. europaeus*	1	658	LT627348
Corse/France/AE20, AE21	*A. erinacei*/2♀	B	*E. europaeus*	2	658	LT627349

cytb

Location/Country/Sample numbers	Species/Gender	Female population	Host	Number of fleas	Base pairs (bp)	Accession number
Seville/Spain/AE5, AE6, AE9, AE10, AE12	*A. erinacei*/4♂ 1♀	B	*E. europaeus*	5	374	LT604120
Seville/Spain/AE7	*A. erinacei*/1♀	B	*E. europaeus*	1	374	LT604117
Seville/Spain/AE8	*A. erinacei*/1♂	-	*E. europaeus*	1	374	LT604118
Seville/Spain/AE11, AE13-16	*A. erinacei*/2♂ 3♀	A, B and C	*E. europaeus*	5	374	LT604119
Corse/France/AE17, AE19	*A. erinacei*/2♂	-	*E. europaeus*	2	374	LT703441
Corse/France/AE18, AE20-21	*A. erinacei*/3♀	A and B	*E. europaeus*	3	374	LT627350
El Hierro/Spain/XC1, XC7	*X. cheopis*/1♂ 1♀	-	*R. rattus*	2	374	LT604122

Appendix 1

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

Species	Family	Host	Accession number	Gen Region	Sequence length
Nosopsyllus fasciatus	Ceratophyllidae	*Apodemus sylvaticus*	LT158061	18S	1,153
Nosopsyllus fasciatus	Ceratophyllidae	Muridae	LT158062	18S	1,153
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp.	LN881536	18S	1,153
Paraceras crispum	Ceratophyllidae	*Sciurotamius davidianus*	EU336075	18S	1,866
Stenoponia americana	Stenoponiidae	*Peromyscus leucopus*	AF423893	18S	1,877
Stenoponia sidimi	Stenoponiidae	*Clethrionomys rufocans*	EU336078	18S	1,869
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937066	18S	1,095
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937067	18S	1,096
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937068	18S	1,098
Neopsylla bidentatiformis	Ctenophthalmidae	*Cricetulus triton*	EU336074	18S	1,862
Pulex irritans	Pulicidae	*Speotyto cunicularia*	AF423915	18S	1,879
Xenopsylla cheopis	Pulicidae	*Rattus exulans*	EU336038	18S	1,881
Xenopsylla robertsi	Pulicidae	Unknown	KM891147	18S	1,793
Xenopsylla cunicularis	Pulicidae	*Oryctolagus cuniculus*	EU336098	18S	1,869
Cediopsylla inaequalis inaequalis	Pulicidae	*Vulpes macrotus*	EU336042	18S	1,869
Archaeopsylla erinacei	Pulicidae	Unknown	X89486	18S	1,926
Hoplopsyllus anomalus	Pulicidae	*Spermophilus variegatus*	EU336047	18S	1,881
Hoplopsyllus anomalus	Pulicidae	Unknown	AY521849	18S	1,902
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	EU336097	18S	1,881
Spilopsyllus cuniculi	Pulicidae	Mammal	JN008928	18S	1,110
Echidnophaga gallinacea	Pulicidae	*Urocyon cinereoargenteus*	EU336055	18S	1,881
Echidnophaga iberica	Pulicidae	*Oryctolagus cuniculus*	EU336099	18S	1,882
Echidnophaga myrmecobii	Pulicidae	Mammal	JN008929	18S	1,118
Ctenocephalides felis	Pulicidae	Unknown	KC177274	18S	1,884
Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LN651166	18S	989
Ctenocephalides canis	Pulicidae	Unknown	AF423914	18S	1,878
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN651167	18S	989
Echidnophaga gallinacea	Pulicidae	*Gallus gallus domesticus*	EU169199	ITS1	1,105
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	HF563590	ITS1	671
Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LN827902	ITS1	668
Xenopsylla cheopis	Pulicidae	Unknown	DQ295061	ITS1	890
Xenopsylla cheopis	Pulicidae	Unknown	DQ295060	ITS1	890
Spilopsyllus cuniculi	Pulicidae	*Felis silvestris catus*	EU170157	ITS1	760
Pulex irritans	Pulicidae	*Homo sapiens*	EU169198	ITS1	929
Pulex irritans	Pulicidae	*Homo sapiens*	GQ387496	ITS1	948
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937053	ITS1	1,205
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937057	ITS1	1,207
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937062	ITS1	1,207
Tunga penetrans	Tungidae	*Canis lupus familiaris*	EU169194	ITS1	877
Tunga penetrans	Tungidae	*Homo sapiens*	EU169197	ITS1	1,075
Tunga penetrans	Tungidae	*Homo sapiens*	EU169196	ITS1	867
Citellophilus tesquorum altaicus	Ceratophyllidae	Unknown	EU770312	ITS1	1,456
Citellophilus tesquorum dzetysuensis	Ceratophyllidae	Unknown	EU770316	ITS1	1,450
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp.	LN881538	ITS1	1,100
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp.	LN881539	ITS1	1,100
Nosopsyllus fasciatus	Ceratophyllidae	*Apodemus sylvaticus*	LT158055	ITS1	1,100
Nosopsyllus fasciatus	Ceratophyllidae	*Crocidura russula*	LT158053	ITS1	1,100
Anopheles moucheti nigerensis	Culicidae	-	AM232662	ITS1	648
Anopheles moucheti bervoetsi	Culicidae	-	AM232663	ITS1	629
Ophthalmopsylla kiritschenkoi	Leptopsyllidae	Unknown	GQ161960	ITS2	474
Ophthalmopsylla extrema	Leptopsyllidae	Unknown	GQ161956	ITS2	466

Amphipsylla quadratoides quadratoides	Leptopsyllidae	Unknown	AY072642	ITS2	497
Leptopsylla sp.	Leptopsyllidae	Unknown	EF504221	ITS2	459
Leptopsylla sp.	Leptopsyllidae	Unknown	EF504223	ITS2	449
Neopsylla siboi	Ctenophthalmidae	Unknown	AF353113	ITS2	479
Neopsylla teratura	Ctenophthalmidae	Unknown	AF353122	ITS2	479
Neopsylla stevensi	Ctenophthalmidae	Unknown	AY337033	ITS2	479
Neopsylla specialis	Ctenophthalmidae	Unknown	AF353120	ITS2	479
Xenopsylla cheopis	Pulicidae	*Rattus* sp.	DQ295061	ITS2	356
Xenopsylla cheopis	Pulicidae	*Rattus* sp.	DQ295059	ITS2	356
Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LN827903	ITS2	327
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN827905	ITS2	327
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN864485	ITS2	327
Tunga penetrans	Tungidae	*Homo sapiens*	DQ844716	ITS2	471
Tunga penetrans	Tungidae	*Homo sapiens*	DQ844724	ITS2	473
Tunga trimamillata	Tungidae	Unknown	AY425820	ITS2	470
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937042	ITS2	332
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937039	ITS2	332
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937038	ITS2	332
Citellophilus tesquorum dzetysuensis	Ceratophyllidae	Unknown	EU770316	ITS2	332
Citellophilus tesquorum altaicus	Ceratophyllidae	Unknown	EU770312	ITS2	332
Nospsyllus fasciatus	Ceratophyllidae	*Apodemus sylvaticus*	LT158059	ITS2	318
Nosopsyllus fasciatus	Ceratophyllidae	Muridae	LT158060	ITS2	318
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp.	LN881537	ITS2	318
Echidnophaga gallinacea	Pulicidae	*Oryctolagus cuniculus*	JN008921	*Cox1*	650
Echidnophaga myrmecobii	Pulicidae	*Oryctolagus cuniculus*	JN008919	*Cox1*	649
Echidnophaga iberica	Pulicidae	*Oryctolagus cuniculus*	KF479239	*Cox1*	658
Echidnophaga sp.	Pulicidae	Mammal	JN008922	*Cox1*	654
Echidnophaga ambulans ambulans	Pulicidae	*Tachyglossus aculeatus*	KR363632	*Cox1*	601
Xenopsylla cunicularis	Pulicidae	*Oryctolagus cuniculus*	KF479238	*Cox1*	658
Xenopsylla robertsi	Pulicidae	Unknown	KM890906	*Cox1*	1,179
Xenopsylla conformis conformis	Pulicidae	Unknown	KM890988	*Cox1*	1,218
Xenopsylla skrjabini	Pulicidae	Unknown	KM890983	*Cox1*	1,218
Pulex irritans	Pulicidae	*Meles meles*	KF479246	*Cox1*	658
Pulex irritans	Pulicidae	*Meles meles*	KF479247	*Cox1*	658
Pulex sp.	Pulicidae	Unknown	KM891015	*Cox1*	1,251
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	KF479236	*Cox1*	658
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	KF479237	*Cox1*	658
Spilopsyllus cuniculi	Pulicidae	Mammal	JN008918	*Cox1*	652
Synopsyllus girardi	Pulicidae	Unknown	KM890952	*Cox1*	1,251
Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LN827896	*Cox1*	600
Ctenocephalides felis	Pulicidae	Mammal	JN008917	*Cox1*	652
Ctenocephalides felis felis	Pulicidae	*Felis catus*	KF684891	*Cox1*	601
Ctenocephalides felis felis	Pulicidae	*Canis lupus familiaris*	KP684196	*Cox1*	601
Ctenocephalides felis strongylus	Pulicidae	*Canis lupus familiaris*	KF684876	*Cox1*	601
Ctenocephalides orientis	Pulicidae	*Canis lupus familiaris*	KF684871	*Cox1*	601
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	KP684210	*Cox1*	658
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN827901	*Cox1*	600
Neopsylla paranoma	Ctenophthalmidae	*Eospalax baileyi*	KJ471028	*Cox1*	1,532
Paraceras melis melis	Ctenophthalmidae	*Meles meles*	KF479245	*Cox1*	658
Neopsylla sellaris	Ctenophthalmidae	*Eospalax baileyi*	KJ471029	*Cox1*	1,532
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937072	*Cox1*	677
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937071	*Cox1*	677
Stenoponia tripectinata tripectinata	Stenoponiidae	*Mus musculus*	LK937073	*Cox1*	677
Stenoponia tripectinata tripectinata	Stenoponiidae	*Apodemus sylvaticus*	KF479244	*Cox1*	658
Nosopsyllus fasciatus	Ceratophyllidae	*Crocidura russula*	LT158040	*Cox1*	658
Nosopsyllus fasciatus	Ceratophyllidae	*Apodemus sylvaticus*	LT158041	*Cox1*	658
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp	LN881549	*Cox1*	658
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp	LN881550	*Cox1*	658
Stenoponia tripectinata	Stenoponiidae	*Mus musculus*	LN897473	*Cytb*	374

tripectinata					
Ophthalmopsylla praefecta praefecta	Leptopsyllidae	Unknown	KM890714	*Cytb*	369
Ctenocephalides felis	Pulicidae	*Canis lupus familiaris*	LN897470	*Cytb*	374
Ctenocephalides felis felis	Pulicidae	Unknown	KM890759	*Cytb*	369
Ctenocephalides canis	Pulicidae	*Canis lupus familiaris*	LN897471	*Cytb*	374
Ctenocephalides felis damarensis	Pulicidae	Unknown	KM890641	*Cytb*	369
Archaeopsylla erinacei erinacei	Pulicidae	Unknown	KM890725	*Cytb*	369
Synopsyllus girardi	Pulicidae	Unknown	KM890686	*Cytb*	369
Xenopsylla conformis conformis	Pulicidae	Unknown	KM890723	*Cytb*	369
Xenopsylla skjrabini	Pulicidae	Unknown	KM890718	*Cytb*	369
Xenopsylla ramesis	Pulicidae	Unknown	KM890637	*Cytb*	342
Echidnophaga oschanini	Pulicidae	Unknown	KM890719	*Cytb*	369
Spilopsyllus cuniculi	Pulicidae	Unknown	KM890622	*Cytb*	369
Cediopsylla inaequalis inaequalis	Pulicidae	Unknown	KM890600	*Cytb*	369
	Pulicidae	Unknown		*Cytb*	
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp	LN897460	*Cytb*	374
Nosopsyllus barbarus	Ceratophyllidae	*Rattus* sp	LN897462	*Cytb*	374
Nosopsyllus fasciatus	Ceratophyllidae	Muridae	LT158049	*Cytb*	374
Nosopsyllus fasciatus	Ceratophyllidae	*Apodemus sylvaticus*	LT158043	*Cytb*	374
Nosopsyllus iranis theodori	Ceratophyllidae	*Gerbillus dasyurus*	KM890603	*Cytb*	369
Nosopsyllus laeviceps ellobii	Ceratophyllidae	Unknown	KM890720	*Cytb*	369