1 ***Ctenocephalides felis* and *Ctenocephalides canis*: Introgressive hybridization?**

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22 **Abstract**

23 In the present work, a comparative molecular study of *Ctenocephalides felis* and
24 *Ctenocephalides canis* isolated from dogs (*Canis lupus familiaris*) from different
25 geographical regions (Spain, Iran and South Africa) has been carried out. We have
26 found morphological variations in *C. felis* which do not correspond with molecular
27 differences. The Internal Transcribed Spacers 1 and 2 (ITS1, ITS2) and 18S rRNA
28 partial gene, and cytochrome c-oxidase 1 (*cox1*) mtDNA partial gene sequences were
29 determined to clarify the taxonomic status of these two species and to assess inter-
30 population variation and inter-specific sequence differences. In addition, a comparative
31 phylogenetic study with other species of fleas using Bayesian, Maximum Parsimony,
32 Maximum Likelihood and Neighbour-Joining analysis was performed. 18S rRNA
33 partial gene fragment is not useful to discriminate *C. canis* and *C. felis* and was useless
34 to infer phylogenetic relationships at this level while ITS1 and ITS2 assessed for
35 specific determination in the genus *Ctenocephalides*. *Cox1* mtDNA sequences of *C.*
36 *felis* revealed three main haplotypes and we suggest that there has been introgression of
37 *C. canis cox1* mtDNA into *C. felis* by *Wolbachia pipientis*. Based on *cox1* sequences,
38 restriction mapping identified many endonucleases that could be used to delineate
39 different haplotypes of *C. felis* and to differentiate *C. felis* and *C. canis*.

40 **Introduction**

41 Fleas (Siphonaptera) constitute a highly distinct group of holometabolous bloodsucking
42 insects which currently involve about 2574 species-level taxa belonging to 16 families
43 and 238 genera (Bitam et al., 2010).

44 Some authors have argued that Siphonaptera is the most completely studied order of
45 insects (Medvedev, 1994), and although this is perhaps true from a morphological-level
46 classification point of view, from a phylogenetic standpoint they have been sorely
47 neglected as a group. Classically, the major obstacle in flea phylogeny has been their
48 extreme morphological specialization associated with ectoparasitism, and the inability
49 of systematics to homogenize characters adequately across flea and outgroup taxa
50 (Whiting *et al.*, 2008). In the past 30 years, there have been over 3,000 publications
51 dealing with some aspects of fleas (Lewis & Lewis, 1985), but only a few instances of
52 formal cladistics analysis (Acosta, 2010; Gao *et al.*, 2013 and Acosta & Morrone,
53 2013), so in depth and continuous studies based on molecular data are needed to clarify
54 the unknown phylogeny of this order.

55 The Order Siphonaptera has also been widely studied due to its clinical importance for
56 human health since species of this group may play a role as parasites by them causing
57 allergic dermatitis or other conditions as a result of their feeding activities. Furthermore,
58 fleas serve as intermediate hosts for parasitic worms, and transmitting important
59 pathogens such as *Rickettsia typhi, Yersinia pestis, Bartonella henselae,* or *Francisella*
60 *tularensis* (Eisen & Gage, 2012).

61 Within this order, the genus *Ctenocephalides* Stiles & Collins, 1930 is one of the most
62 studied lineages because they tend to parasitize domestic animals such as dogs, cats or
63 other pets which may play an important role as bridging hosts for fleas of different
64 animals (Dobler & Pfeffer, 2011). Thirteen species and four subspecies are recognized

65 within this genus (Beaucournu & Ménier, 1998, Lawrence *et al*., 2014) out of which
66 *Ctenocephalides felis* and *Ctenocephalides canis* have been the most studied species by
67 different authors (Gil Collado, 1949; Gil Collado, 1960; Beaucournu & Launay, 1990;
68 Lewis, 1993b; Beaucournu & Ménier, 1998; Ménier & Beaucournu, 1998; Linardi &
69 Guimarães, 2000, Durden & Traub, 2002; Linardi & Santos, 2012). From a
70 morphological point of view, four subspecies of *C. felis* have been distinguished: *C.*
71 *felis felis* Bouché, 1835, *C. felis strongylus* Jordan, 1925, *C. felis orientis* Jordan, 1925
72 and *C. felis damarensis* Jordan, 1936 (Hopkins & Rothschild, 1953). However, only a
73 few studies have been carried out based on molecular data (Vobis *et al.*, 2004; Marrugal
74 *et al.*, 2013; Lawrence *et al.*, 2014 and Lawrence *et al.*, 2015). Thus, some authors have
75 suggested that more molecular studies are needed in order to resolve and elucidate the
76 genetic diversity of *C. felis* (Lawrence *et al.*, 2014).

77 The ribosomal DNA segments Internal Transcribed Spacer 1 and 2 have been shown to
78 be two of the best molecular markers for analyzing genetic relationships at families,
79 genus and species level in arthropods (Monje *et al.*, 2013; Marcilla *et al.*, 2002; De
80 Rojas *et al.*, 2002, 2007). Recently, Marrugal *et al.* (2013) concluded that phylogenetic
81 analysis based on Internal Transcribed Spacer 1 (ITS1) region is a useful tool to
82 approach different taxonomic and phylogenetic issues in *Ctenocephalides* species and
83 they found clear molecular differences between *C. felis* and *C. canis*. In addition, 18S
84 rRNA gene has been widely used as a molecular marker in order to clarify molecular
85 relationships among families and subfamilies (Whiting *et al.*, 2008; Acosta & Morrone,
86 2013; Díaz-Nieto *et al.*, 2013).

87 Moreover, mtDNA markers have remained as the markers of choice in many
88 populations, biogeographic and phylogenetic studies. Though many species are still
89 described based on morphology, or morphometrics only, mtDNA markers has also been

90 used in taxonomic studies since all described species are given as mtDNA sequence tag
91 or bar code (Hebert *et al.*, 2003). Recently, Lawrence *et al.* (2014) carried out a study
92 based on cytochrome c-oxidase subunits 1 and 2, to investigate evolutionary
93 relationships and the genetic diversity of *C. felis* and other flea species from the genus
94 *Ctenocephalides* from different geographical areas (Australia, Fiji, Thailand and
95 Seychelles), concluding that both markers can be used to identify species of the genus
96 *Ctenocephalides*. Nevertheless, some authors cited that it is not safe to assume a priori
97 that mtDNA evolves as a strictly neutral marker because both direct and indirect
98 selection influences mitochondria. Thus, they questioned its utility as a marker for
99 genomic history (Ballard & Whitlock, 2004). On the other hand, the presence of
100 symbionts like *Wolbachia pipientis* has shown cases of reduction and increases in the
101 mtDNA genetic diversity. Thus, some authors have concluded that mtDNA on its own
102 is an unsuitable marker for the study of recent historical events in arthropods,
103 suggesting the development and use of microsatellites for intraspecific study, and
104 nuclear coding genes for phylogenetic study as a requirement to reveal the history of
105 nuclear DNA (Hurst & Jiggins, 2005; Dean *et al.*, 2003; Shoemaker *et al.*, 2003;
106 Kodandaramaiah *et al*., 2013).

107 Here we present a comparative molecular study of *C. felis* and *C. canis*, from different
108 geographical regions (Spain, Iran and South Africa). The ITS1 and ITS2, 18S rRNA
109 partial gene and cytochrome c-oxidase 1 mtDNA partial gene were sequenced in order
110 to assess inter-population and inter-specific variations to clarify the taxonomic status of
111 these species. Furthermore, comparative phylogenetic and phylogeographic analyses
112 with other species of fleas by phylogenetic methods (Bayesian, Maximum Parsimony,
113 Maximum Likelihood and Neighbor-Joining inference) were done. Finally, the presence

114 of *Wolbachia pipientis* in *C. canis* and *C. felis* has been checked to assess their

115 influence in both species´ evolutionary histories.

116 **Materials and methods**

117 Collection of samples

118 Fleas were collected from dogs (*C. l. familiaris*) from different geographical regions of
119 Spain, South Africa and Iran (Tables 1 and 2). Single individuals were preserved in 70
120 % ethanol until required for subsequent identification and sequencing.

121 Morphological identification

122 For morphological studies fleas were cleared with 10 % KOH (Lewis, 1993) and
123 examined under stereomicroscope. Morphological differentiation between *C. felis* and
124 *C. canis* individuals was carried out according to the original descriptions (Gil Collado,
125 1949, 1960; Beaucournu & Launay, 1990; Lewis, 1993b; Beaucournu & Ménier, 1998;
126 Ménier & Beaucournu, 1998; Marrugal *et al.*, 2013).

127 Criteria cited by different authors have been used for the specific determination of the
128 genus *Ctenocephalides* including:

129 • Genal ctenidium formed of eight or nine spines oriented horizontally (Lewis,
130 1993b) and relative size of the first and second genal spines (Gil Collado, 1949;
131 Beaucournu & Ménier, 1998; Durden & Traub, 2002; Marrugal *et al.*, 2013).

132 • Shape of the front of head (Gil Collado, 1949; Lewis, 1993b; Beaucournu &
133 Launay, 1990; Linardi & Santos, 2012; Marrugal *et al.*, 2013). Length/width ratio of the
134 head (Durden & Traub, 2002; Marrugal *et al.*, 2013).

135 • Male genitalia: manubrium and apex (degree of dilation) (Gil Collado 1949;
136 Lewis 1993b; Ménier & Beaucournu, 1998; Marrugal *et al.*, 2013) and aedeagus
137 (Ménier & Beaucournu, 1998; Marrugal *et al.*, 2013).

138 • Female genitalia: spermatheca and hilla (degree of elongation of the apical part
139 of the spermatheca) (Gil Collado, 1949; Lewis, 1993b; Marrugal *et al.*, 2013).

140 • Presence of two to three bristles on the lateral metanotal area (LMA) (Gil
141 Collado, 1949; Beaucournu & Launay, 1990; Beaucournu & Ménier, 1998; Linardi &
142 Santos, 2012; Marrugal *et al.*, 2013).

143 • Hind tibia with a number of seta-bearing notches along dorsal margin (Lewis,
144 1993b; Beaucournu & Launay, 1990; Beaucournu & Ménier, 1998; Durden & Traub,
145 2002; Linardi & Santos, 2012; Marrugal *et al.*, 2013).

146 Molecular study

147 All specimens were photographed and then were frozen in liquid nitrogen and
148 pulverized in a mortar. Genomic DNA was extracted using the DNeasy Blood and
149 Tissue Kit (Qiagen) following the manufacturer's protocol and was checked in a 0.8 %
150 agarose gel electrophoresis using ethidium bromide.

151 All molecular markers sequenced in this study were amplified by PCR using a
152 thermocycler (Eppendorf AG). PCR mix, PCR conditions and PCR primers used to
153 sequence each marker have been summarized in Table S1.

154 All sequenced fleas were screened for *Wolbachia* sp. using specific 16S rRNA gene
155 primers (see Table S1). As positive control, DNA from *Wolbachia pipientis* (AN:
156 LN864488) derived from *Ctenocephalides felis* from Spain was available, whereas for
157 negative controls, we used DNA from *Stenoponia tripectinata tripectinata* negative for
158 *Wolbachia pipientis*. Negative and positive controls were tested after every PCR
159 reaction sets. Sequences obtained were compared with those in the GenBank DNA
160 database by using the program BLAST (version 2.0, National Center for Biotechnology
161 Information; available from: URL: http://www.ncbi.nlm.nih.gov). (Altschul *et al.*,
162 1990). The ITS1, ITS2, 18S rRNA partial gene and *cox1* partial gene sequences
163 obtained from *C. felis* and *C. canis* and 16S rRNA of *Wolbachia pipientis* gene were
164 deposited in GenBank database (Table 1).

165 The PCR products were checked on ethidium bromide stained 2 % Tris–Borate–EDTA
166 (TBE) agarose gels. Bands were eluted and purified from the agarose gel by using the
167 QWizard SV Gel and PCR Clean-Up System Kit (Promega). Once purified, the
168 products were sequenced by Stab Vida (Portugal). All the phylogenetic analyses were
169 performed on the rDNA and mtDNA datasets, and sequences were aligned using the
170 Clustal X program version 2.0 (Larkin *et al.*, 2007).
171 The rDNA intra-individual variation was determined by sequencing four to five clones
172 of one individual per geographical population of *C. felis* and *C. canis.* The PCR
173 products were eluted from the agarose by using the WIZARD® SV Gel and PCR Clean-
174 Up System (Promega) and transformation was carried out as cited by Cutillas *et al.*
175 (2009). Plasmids were purified using a Wizard Plus SV (Promega) and sequenced by
176 Stab Vida (Portugal) with a universal primer (M13).

177 Restriction map of the *cox1* sequences of *C. felis* and *C. canis* was performed using The
178 Sequence Manipulation Suite (Stothard, 2000; available at
179 http://www.bioinformatics.org/sms2/rest_map.html).

180 Phylogenetic trees were inferred using nucleotide data and produced using three
181 methods: Maximum Parsimony (MP) trees were generated using the MEGA 5 program
182 from Tamura *et al.* (2011), Maximum Likelihood (ML) using the PHYML package
183 from Guindon & Gascuel (2003) and Bayesian inferences (B) were performing from Mr
184 Bayes-3.2.6 (Ronquist & Huelsenbeck, 2003). JMODELTEST (Posada, 2008) program
185 was used to determinate the best fit substitution model for the parasite data (18S, ITS1,
186 ITS2 and *cox1*). Models of evolution were chosen for subsequent analyses according to
187 the Akaike Information Criterion (Huelsenbeck & Rannala, 1997; Posada & Buckley,
188 2004). For the study of the four concatenated datasets (*cox1*, ITS1, ITS2 and 18S), a
189 combined partitioned analysis was performed using the model-jumping option in Mr

190 Bayes-3.2.6 (Ronquist & Huelsenbeck, 2003). For ML inference, best-fit nucleotide
191 substitution models included general time-reversible model with gamma-distributed rate
192 variation and a proportion of invariable sites, GTR+I+G (18S), Hasegawa-Kishino-
193 Yano, HKY85+I+G (ITS1) and general time-reversible model with gamma-distributed
194 rate variation GTR+G (ITS2 and *cox1*). Support for the topology was examined using
195 bootstrapping (heuristic option) (Felsenstein, 1985) over 1000 replications to assess the
196 relative reliability of clades. Models selected by jModeltest for BI nst=6 with invgamma
197 rates (18S), nst=2 with invgamma rates (ITS1) and nst=6 with gamma rates (ITS2 and
198 *cox1*). For BI, the standard deviation of split frequencies was used to assess if the
199 number of generations completed was sufficient; the chain was sampled every 500
200 generations and each dataset was run for 10 million generations. Burn-in was
201 determined empirically by examination of the log likelihood values of the chains. The
202 Bayesian Posterior Probabilities (BPP) is percentage converted.

203 NETWORK (version 4.6.1.3) was used to create inter-population and inter-specific
204 median-joining networks (Bandelt *et al*., 1999; available at www.fluxus-
205 engineering.com), to visualize evolutionary relationships between *cox1* haplotypes. This
206 approach has been shown to yield the best resolved genealogies relative to other rooting
207 and network procedures (Cassens *et al*., 2003).

208 The phylogenetic and phylogeographic analysis, based on ITS1, ITS2, 18S rRNA and
209 *cox1* mtDNA sequences was carried out using our sequences and those obtained from
210 GenBank database (appendix 1). Phylogenetic trees based on 18S rRNA and *cox1*
211 mtDNA were rooted on outgroup species representing the Order Mecoptera:
212 *Microchorista philpotti* and *Boreus elegans* (*cox1*), *Panorpa striata* and *Neopanorpa*
213 *harmandi* (18S rRNA). No ITS sequences of Order Mecoptera were found in any public
214 database, thus, phylogenetic trees with other Siphonaptera species based on ITS1 and

215 ITS2 sequences were constructed using different outgroup species representing
216 members of Order Diptera: *Anopheles farauti* and *Anopheles arabiensis* (concatenated
217 18S, ITS1, ITS2 and *cox1*).

Results

Morphological results

Two species of fleas were identified from *Canis lupus familiaris*: *Ctenocephalides felis* and *Ctenocephalides canis* (Tables 1 and 2). *C. canis* was only collected on dogs from Iran (Table 1). Some individuals characterized as *C. felis* (Table 2) showed some characters typical of *C. canis* such as the presence of two single, short and strong spines located between the post-medial and apical spines and the presence of 2 and/or 3 bristles on the LMA. These morphological variations could be found on both sides or/and on one side of the thorax, indistinctly.

18S rRNA partial gene analysis

18S rRNA partial gene sequences of all individuals from different geographical areas were 989 base pairs (bp) in length. A total of 16 sequences corresponding to 15 individuals of *C. felis* and one individual of *C. canis* were obtained (Table 1). No differences were observed between 18S rRNA partial gene sequences from both species.

Phylogenetic tree topology obtained using 18S rRNA partial gene sequences of *C. felis* and *C. canis* and other sequences from different species of fleas retrieved from GenBank (see appendix 1) showed that *C. felis* and *C. canis* clustered together, with high bootstrap and BPP values (Fig. S1), and in polytomy with *Echidnophaga iberica*, *Echinodphaga gallinacea*, *Pulex irritans*, *Spilopsyllus cuniculi*, *Parapulex cheprensis*, *Xenopsylla cunicularis* and *Xenopsylla cheopis* (Pulicidae).

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

The length of the ITS1 region of *C. felis* isolated from dogs from different localities was 668 (bp) (Table 1). A total of 25 fleas were sequenced to carry out the phylogenetic analysis. No inter-population variations in *C. felis* from different geographical areas

242 were detected. However, when the ITS1 sequences of *C. felis* were compared with *C.*
243 *canis* sequences, a total of 44 different base pairs and 11 gaps were obtained (91.9 %
244 inter-specific similarity).

245 A total of 47 *C. felis* and 3 *C. canis* ITS2 fragments were sequenced from fleas from
246 different localities (Table 1). They were 327 (bp) in length (Table 1). When the ITS2
247 sequences of *C. felis* and *C. canis* were compared, a total of 12 different base pairs were
248 detected with 96.3 % inter-specific similarity (Fig. S2). Intra-population variation was
249 not detected when *C. canis* sequences of different individuals were compared, whereas
250 only one mutation was showed in one specimen of *C. felis* (no. 113 from Sanlúcar de
251 Barrameda, Cádiz, Spain) when those sequences were compared. The intra-individual
252 variability was studied in five clones of one individual of *C. canis* isolated from *C. l.*
253 *familiaris* from Iran. The intra-individual similarity ranged from 99.1 % to 100 %
254 (Appendix 2). In relation to *C. felis,* the intra-individual similarity was studied in four
255 clones of one individual isolated from *C. l. familiaris* from Sanlúcar de Barrameda,
256 Cádiz (Spain). However, no differences among them were observed (intra-individual
257 similarity 100 %) (Data not shown).

258 The phylogenetic analysis of the ITS1 and ITS2 sequences of *C. canis* and *C. felis* with
259 different species of Siphonaptera showed a substantial length variation in the alignment
260 which compromised inferences of positional homology. In addition, species of the
261 Order Diptera were useless as outgroups.

262 *Cox1* mtDNA partial gene analysis

263 The *cox1* mtDNA partial gene sequences of *C. felis* and *C. canis* were 600 (bp) in
264 length. A total of 49 sequences were obtained from individuals from different localities
265 and countries (Table 2). Intra-specific variation was not detected when *cox1* sequences
266 of *C. canis* were compared. However, when *C. felis* sequences were analyzed, five

267 different haplotypes were obtained (A, A1, A2, B and C) (Table 2). Haplotype A was
268 the most common and included individuals from all geographical areas analyzed except
269 from South Africa (Haplotype C). On the other hand, haplotype B was only observed in
270 two individuals from Fuentes de Andalucía (Sevilla, Spain) and Mairena (Sevilla,
271 Spain) (216 and 645 sequences, respectively) (Table 2).

272 A comparative study among all the *cox1* mtDNA partial gene sequences of *C. felis* and
273 *C. canis* from different geographical areas obtained in this work was carried out (Table
274 3). Only one different base pair was noticed between haplotype A and A1 and between
275 haplotype A and A2 (99.8 % similarity). Nevertheless, a maximum of 16 differences
276 were observed between haplotype A and haplotype B and C (97.3 % similarity) (Table
277 3). Surprisingly, haplotype B and C only displayed a slight difference in respect to *C.*
278 *canis* from Iran (99.3 % and 99.7 % similarity, respectively) whereas a 97.7 %
279 similarity was observed between haplotype A and *C. canis* from Iran. Furthermore, our
280 sequences were analyzed and compared with other *cox1* mtDNA partial gene sequences
281 of *C. f. felis*, *C. f. strongylus*, *C. orientis* and *C. canis* isolated from different
282 geographical areas obtained from GenBank database. Lowest values of similarity were
283 observed when *C. canis* from Czech Republic and *C. orientis* were compared with the
284 rest of *Ctenocephalides* species (Table 3).

285 The phylogenetic tree inferred from *cox1* partial gene sequences of *C. felis* and *C. canis*
286 showed that *Ctenocephalides* species clustered together with high bootstrap and BPP
287 values. Three different subclades could be observed within the genus *Ctenocephalides.*
288 The first clade clustered all individuals corresponding to *C. canis* from the Czech
289 Republic and *C. orientis*. The second clade clustered *C. felis* (haplotypes B and C)
290 together with *C. canis* from Iran and *C. f. felis* from Queensland, Cairns (Australia)
291 (Fig. S3). The third clade included *C. felis* haplotypes A, A1 and A2, together with *C. f.*

292 *felis* from Fiji, Thailand, Mumbai (India), Pardubice and Jablonec Nad Nisou (Czech
293 Republic), Sikkim (India) and New South Wales (Australia). *Ctenocephalides felis*
294 *strongylus* appeared in polytomy between clades 2 and 3. Other species of the family
295 Pulicidae (*P. irritans*, *Echidnophaga gallinacea*, *E. myrmecobii* and *S. cuniculi*)
296 appeared separated from *Ctenocephalides* species.

297 Based on *cox1* sequences, restriction mapping identified many endonucleases that could
298 be used to delineate the haplotypes found in this study. Thus, *SacI*, *SstI* and *TaqI* sites
299 were present in the sequences of *C. felis* haplotypes A, A1 and A2 but not in *C. felis*
300 haplotypes B and C nor *C. canis*. *HpaII* and *MspI* presented one restriction site in *C.*
301 *canis* and in *C. felis* haplotypes B and C but none in *C. felis* haplotype A, A1 and A2.
302 Furthermore, *DraI* sites were present only in the sequences of *C. canis* and *C. felis*
303 haplotype C, whereas, *BclI* presented two restriction sites only in *C. felis* haplotype A,
304 A1, A2 and C.

305 The concatenated dataset of 18S partial gene, ITS1, ITS2 and *cox1* partial gene
306 sequences included 3,093 aligned sites and 25 taxa, including outgroups. Phylogenetic
307 analyses of the concatenated dataset yielded a strongly supported tree (Fig. 1). The
308 analyses of concatenated sequences are concordant with the *cox1* tree topology. Thus,
309 haplotypes A, B and C clustered in three different clades, showing haplotypes B and C
310 related with *C. canis* (Fig. 1).

311 The network of the 42 sequences of *Ctenocephalides* populations showed a general
312 congruence with the phylogenetic reconstruction. The minimum spanning network
313 showed the three main groups defined above and separated from each other by a genetic
314 distance of 9 to 41 mutational steps (Fig. 2). Clade 1 clustered *C. orientis* and *C. canis*
315 from the Czech Republic; both species seem to be more closely related to each other
316 than to any other haplotype (26 mutational steps). The second clade grouped *C. felis*

317 (haplotypes B and C) together with *C. canis* (Iran) and *C. f. felis* (Australia).
318 Nevertheless, *C. canis* (Iran) seems to be close to *C. f. felis* (Haplotype C) with 1
319 mutational step. Clade 3 clustered *C. felis* (Haplotypes A, A1 and A2) together with *C.*
320 *f. felis* fron India, the Czech Republic and Australia. A majority haplotype (H1,
321 haplotype A) was observed including 14 individuals (*C. felis* from Spain and *C. f. felis*
322 from Australia and Czech Republic). Haplotype A1 (H2) and A2 (H3) appeared related
323 to the majority haplotype of *C. felis* (H1) separated with 1 mutational step in both cases
324 (Fig. 2).

325 In order to assess the influence of *Wolbachia* sp. on the relationship inferred from flea
326 *cox1*, the presence of *Wolbachia pipientis* was tested in all fleas studied. All the PCR
327 products obtained were sequenced and the length of the 16S rRNA gene region was 334
328 base pairs. BLAST analysis showed 99 – 100 % homology to *Wolbachia pipientis* 16S
329 rRNA (Genbank accession number AY026912).

330 **4. Discussion**

331 Genus and species determination of fleas is generally based on a variety of
332 morphological criteria (Lane & Crosskey, 1993; Kramer & Mencke 2001; Mehlhorn,
333 2001 and Linardi & Santos, 2012). However, a few studies have been carried out on
334 molecular differentiation of fleas. That means we have a great knowledge of flea
335 taxonomy at the species and subspecies level, and enough information to assess their
336 biology and role in diseases transmission, which has been studied worldwide in recent
337 years. In contrast, a rigorous exploration of phylogenetic relationships among fleas is
338 needed in order to clarify their complex systematics (Whiting *et al*., 2008).

339 The morphological study of fleas from dogs from different geographical regions
340 revealed the existence of two species: *C. felis* and *C. canis*. *C. felis* showed
341 morphological variations. This fact has been cited by different authors. Thus, in
342 *Ctenocephalides* spp. the most frequent morphological variations are observed in combs
343 and chaetotaxies of LMA and in hind tibia (Amin *et al*., 1974; Amin, 1976, Linardi &
344 Santos, 2012). These alterations in chaetotaxy on the LMA and metatibia have been
345 justified by different authors (Holland, 1949, Fox, 1952, Amin *et al*., 1974 and Amin,
346 1976) as the existence of hybridization between *C. felis* and *C. canis*. However, the
347 hypothesis of hybridization between both species must be rejected because commonly
348 species do not cross each other, as reinforced by Beaucournu & Guiller (2006).
349 Sometimes, specimens exhibiting variations have been improperly treated as hybrids, in
350 spite of the nonexistence of the two species in the same municipality or region. In our
351 case, the individuals exhibiting these "abnormalities" were collected from South Spain
352 and Mallorca, where only *C. felis* has been detected (Table 2). Furthermore, these
353 alterations in chaetotaxy on the LMA did not correspond with a determined *cox1*
354 haplotype (see asterisk, Table 2).

355 Our studies are in agreement with those of Linardi & Santos (2012), who concluded that
356 the separation of the two species of *Ctenocephalides* must be based on all
357 characteristics. Data on hosts, geographical distribution and prevalence of infestation
358 may support the identification of the species. In *C. felis* four subspecies have been
359 proposed on the basis of rather minor morphological differences (Hopkins &
360 Rothschild, 1953; Haeselbarth, 1966). Analysis of some sequences of the genome of
361 these species which show high variation can be helpful to assess the validity and
362 significance of such infraspecific taxa and in investigating evolutionary relationships
363 within and between species (Vobis *et al*., 2004).

364 In the present work, *C. felis* and *C. canis* isolated from *C. l. familiaris* from different
365 geographical areas were also analyzed by amplification and sequencing of ribosomal
366 (ITS1, ITS2, and 18S rRNA partial gene) and mitochondrial (*cox1* mtDNA partial gene)
367 markers.

368 The comparative molecular study based on ITS1 sequences of *C. felis* within a
369 geographical region and from different geographical origins showed a 100 % similarity.
370 These results are in agreement with those of Vobis *et al*. (2004) and Marrugal *et al*.
371 (2013), who found that the ITS1 nucleotide sequences of different *C. felis* populations
372 from different geographical areas are practically identical. Nevertheless, other authors
373 such as Gamerschlag *et al*. (2008) reported different lengths in the ITS1 nucleotide
374 sequences in other species of fleas (*Tunga penetrans* from Africa and South America).

375 On the other hand, the comparison between *C. canis* and *C. felis* ITS1 sequences
376 revealed a clear inter-specific variability. These results are in agreement with Marrugal
377 *et al*. (2013), who concluded that the ITS1 region is a useful marker to approach
378 different taxonomic statements in the genus *Ctenocephalides.*

379 The ITS2 sequences of *C. felis* and *C. canis* were markedly shorter than ITS1. This fact
380 was reported by Vobis *et al*. (2004) in *C. felis*; however, these authors did not sequence
381 the ITS2 region of *C. canis* and we could not compare our data with theirs. The ITS2
382 sequence of *C. canis* has been reported for the first time in the present study. With
383 regard to our results, we can conclude that the analysis of ITS2 fragment constitutes a
384 useful tool for the differentiation of both *Ctenocephalides* species. There was no inter-
385 population variation and intra-population variation ranged from 99.6 % to 100 % in
386 both species. This result disagrees with that of Luchetti *et al*. (2007) who found two
387 genotypic groups (Atlantic and Pacific) in *Tunga penetrans*. Nevertheless, the peculiar
388 evolutionary dynamics of ITS2, which is a repeated sequence embodied of rDNA, is
389 well known. Therefore, this molecular marker evolves in a concerted way (Smith,
390 1976). This pattern is carried out through population dynamics processes and intra-
391 genomic unequal DNA exchanges (molecular drive; Dover, 2002). Our results confirm
392 this evolutionary process in fleas, where no marked differences were observed even
393 between different geographical populations. This phenomenon was recently reported by
394 Zurita *et al*. (2015) in *Stenoponia tripectinata tripectinata* (Siphonaptera) from the
395 Canary Islands and in Diptera species by Monje *et al*. (2013). In conclusion, ITS1 and
396 ITS2 sequences do not determine different populations within one species of the genus
397 *Ctenocephalides*; however, the analyses of both rDNA markers are a helpful tool for
398 differentiation at the species level.

399 The 18S rRNA partial gene sequences confirmed the low value of this marker for
400 phylogenetic studies at the species level. Thus, we could observe the existence of
401 politomy within the genus *Ctenocephalides* and other species of the family Pulicidae.
402 This fact is in agreement with Whiting *et al*. (2008). No differences were observed
403 between *C. felis* and *C. canis*.

404 *Cox1* mtDNA sequences of *C. felis* revealed five different haplotypes: A, A1, A2, B
405 (individuals from Spain and Iran), and C (individuals from South Africa). On the other
406 hand, no different haplotypes were observed in *cox1* mtDNA sequences of *C. canis*
407 from Iran. In addition, some specific recognition sites for endonucleases were detected
408 in order to differentiate both species (*C. felis* and *C. canis*) and to differentiate the *C.*
409 *felis* haplotypes. Thus, *SacI*, *SstI*, *TaqI*, *DraI*, *BclI*, *HpaII* and *MspI* sites have
410 diagnostic value for specific determination in *C. felis* and *C. canis.*

411 The phylogenetic tree of the *cox1* sequences revealed three different clades within the
412 genus *Ctenocephalides*. The diversity showed even within individuals of *C. felis* from
413 the same geographical origins. For example, Fuentes de Andalucía (Haplotypes B, A
414 and A2) or Mairena (Haplotypes A, A1 and B) (Table 2) is in agreement with Lawrence
415 *et al*. (2014), who found different haplotypes in *C. f. felis* from Australia. In our case,
416 we found a dominant *C. felis* group including sequences from the Czech Republic,
417 Australia, India, Iran, Thailand, Fiji and Spain (haplotypes A, A1 and A2) clustering in
418 the same clade, while haplotypes B and C clustered with *C. canis* in another clade (Fig.
419 S3). Thus, we found individuals of *Ctenocephalides* defined as *C. felis* by ITS1 and
420 ITS2 sequences but showing *cox1* partial gene sequences of *C. canis*. The phylogenetic
421 tree of the concatenated 18S, ITS1, ITS2 and *cox1* sequences corroborated these results
422 (Fig. 1). This diversity could be explained due to the presence of the symbiont
423 *Wolbachia pipientis*. This bacterium is very common in arthropods, and in recent years
424 has been thoroughly studied because of its influence on the host´s mtDNA variability.
425 Some authors (Schulenburg *et al*., 2000; Shoemaker *et al*., 2003; Behura *et al*., 2001)
426 have reported the presence of two or more haplotypes associated with this bacterium in
427 some species, such as *Adalia bipunctata* (Coleoptera), *Drosophila mauritania* (Diptera)
428 or *Solenopis invicta* (Hymenoptera). If a population becomes infected with a symbiont

429 such as *Wolbachia pipientis* that has sufficient drive to spread, the mtDNA type
430 associated with the initial infection will hitchhike through the population and further the
431 ability of symbionts to spread between populations by occasional movements of hosts
432 setting up a process named ``indirect selection on the mtDNA´´ (Hurst & Jiggins, 2005).
433 In agreement with former studies in the Order Diptera (Monnerot *et al*., 1990 and
434 Rousset & Solignac, 1995), we suggest that there has been introgression of *C. canis*
435 *cox1* mtDNA into *C. felis* by *Wolbachia pipientis*. According to Shaw (2002) it is
436 possible that symbiont-driven introgression may explain cases where mtDNA
437 phylogenetic conflicts with those obtained from nuclear DNA. The spread of the
438 introgressed symbiont would be associated with the spread of introgressed mtDNA,
439 homogenizing mtDNA variation across the species (Hurst & Jiggins, 2005).

440 Furthermore, *C. orientis*, often regarded as a subspecies of *C. felis,* should gain full
441 species status because it forms a sister clade to *cox1* sequences of *C. canis* from the
442 Czech Republic. This is in agreement with Lawrence *et al*. (2015). Nevertheless, the
443 *cox1* sequences of *C. canis* from Iran appeared separated from those sequences but
444 clustering with haplotypes B and C of *C. felis*. The network obtained for all the
445 haplotypes corroborated the phylogenetic results (Fig. S3 and 1).

446 In conclusion, we have found morphological variations in *C. felis* which do not
447 correspond with molecular differences. The analysis of 18S rRNA partial gene is not a
448 useful tool to discriminate *C. canis* and *C. felis* while ITS1 and ITS2 assessed for
449 specific determination in the genus *Ctenocephalides*. *Cox1* mtDNA sequences of *C.*
450 *felis* revealed five different haplotypes and we suggest a possible introgression of *C.*
451 *canis cox1* mtDNA into *C. felis* by *Wolbachia pipientis.* The presence of symbionts,
452 such as *Wolbachia pipientis* should be checked in species of arthropods showing

453 reduced or increased mtDNA diversity because these symbionts confound the inference

454 of an organism´s evolutionary history from mtDNA data (Hurst & Jiggins, 2005).

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664 **Figure captions**

665 Figure S1. Phylogenetic tree of *Ctenocephalides felis* and *Ctenocephalides canis* from
666 different geographical origins (see Table 1) based on 18S ribosomal RNA partial gene
667 of inferred using the Bayesian (B), Maximum Likelihood (ML) and Maximum
668 Parsimony (MP) methods and Bayesian topology. The percentage of replicate trees in
669 which the associated taxa clustered together in the bootstrap test (1,000 replicates) is
670 shown onto the branches (B/ML/MP). Bootstrap values lower than 60 % are not shown.
671 The Bayesian Posterior Probabilities (BPP) is percentage converted.

672 Figure S2. Alignment of the consensus nucleotide sequences of the Internal
673 Transcribed Spacer (ITS2) of *Ctenocephalides felis* and *Ctenocephalides canis* isolated
674 from *Canis lupus familiaris* (Gaps generated using Clustal W alignment program).

675 Figure S3. Phylogenetic tree of *Ctenocephalides felis* and *Ctenocephalides canis* from
676 different geographical origins (see Table 2) based on cytochrome c-oxidase 1 (*cox1*)
677 partial gene of mitochondrial DNA inferred using the Bayesian (B), Maximum
678 Likelihood (ML) and Maximum Parsimony (MP) methods and Bayesian topology. The
679 percentage of replicate trees in which the associated taxa clustered together in the
680 bootstrap test (1,000 replicates) is shown onto the branches (B/ML/MP). Bootstrap
681 values lower than 60 % are not shown. The Bayesian Posterior Probabilities (BPP) is
682 percentage converted.

683 Figure 1. Phylogenetic tree of *Ctenocephalides felis* and *Ctenocephalides canis* from
684 different geographical origins (see Table 1 and 2) based on concatenated 18S ribosomal
685 RNA partial gene, Internal Transcribed Spacers 1 and 2 (ITS1, ITS2) and cytochrome c-
686 oxidase 1 (*cox1*) partial gene of mitochondrial DNA inferred using the Bayesian (B) and
687 Maximum Likelihood (ML) methods and Bayesian topology. The percentage of
688 replicate trees in which the associated taxa clustered together in the bootstrap test (1,000

689 replicates) is shown onto the branches (B/ML). The Bayesian Posterior Probabilities
690 (BPP) is percentage converted.

691 Figure 2. A minimum spanning network constructed using 42 haplotypes of
692 mitochondrial *cox1* partial gene sequences of *Ctenocephalides* spp. The geographical
693 origin for each haplotype is shown in Table 2. The size of the circle is proportional to
694 the number of haplotypes represented and the numbers correspond to the mutational
695 steps observed between haplotypes. H1 (14): *C. felis* from Spain and Iran (Haplo A), *C.*
696 *felis felis* from Australia and the Czech Republic; H2 (1): *C. felis* from Spain (Haplo
697 A1); H3 (1): *C. felis* from Spain (Haplo A2); H4 (2): *C. felis* from Spain (Haplo B); H5
698 (3): *C. felis* from South Africa (Haplo C); H6 (4): *C. canis* from Iran; H7 (2): *C. felis*
699 *strongylus*; H8 (2): *C. orientis*; H9 (2): *C. felis felis* from Australia; H10 (4): *C. felis*
700 *felis* from India, Thailand and Fiji; H11 (2): *C. felis felis* from the Czech Republic; H12
701 (2): *C. felis felis* from India; H13 (3): *C. canis* from the Czech Republic.

Table 1. GenBank accession numbers of ITS1, ITS2 and 18S rRNA partial gene sequences of individuals of *Ctenocephalides felis* and *Ctenocephalides canis* isolated from *Canis lupus familiaris* from different geographical regions.

<table>
<tr><th colspan="5">ITS1</th></tr>
<tr><th>Location/Country</th><th>Number of fleas</th><th>Number of base pairs (bp)</th><th>Species</th><th>Accession number</th></tr>
<tr><td>Nashtarood, Mazandaran/Iran</td><td>1</td><td>668</td><td>C. felis</td><td rowspan="8">LN827902</td></tr>
<tr><td>Pilas, Sevilla/Spain</td><td>4</td><td>668</td><td>C. felis</td></tr>
<tr><td>Villamanrique de la Condesa, Sevilla/Spain</td><td>4</td><td>668</td><td>C. felis</td></tr>
<tr><td>Lebrija, Sevilla/Spain</td><td>4</td><td>668</td><td>C. felis</td></tr>
<tr><td>Dílar, Granada/Spain</td><td>3</td><td>668</td><td>C. felis</td></tr>
<tr><td>Fuentes de Andalucia, Sevilla/Spain</td><td>4</td><td>668</td><td>C. felis</td></tr>
<tr><td>La Luisiana, Sevilla/Spain</td><td>4</td><td>668</td><td>C. felis</td></tr>
<tr><td>Dos Hermanas, Sevilla/Spain</td><td>1</td><td>668</td><td>C. felis</td></tr>
<tr><th colspan="5">ITS2</th></tr>
<tr><th>Location/Country</th><th>Number of fleas</th><th>Number of base pairs (bp)</th><th>Species</th><th>Accession number</th></tr>
<tr><td>Sanlúcar de Barrameda, Cádiz/Spain</td><td>1 (No. 113)</td><td>327</td><td>C. felis</td><td>LN827904</td></tr>
<tr><td>Sanlúcar de Barrameda, Cádiz/Spain (Clone 1)</td><td>-</td><td>327</td><td>C. felis</td><td rowspan="4">LN864484</td></tr>
<tr><td>Sanlúcar de Barrameda, Cádiz/Spain (Clone 2)</td><td>-</td><td>327</td><td>C. felis</td></tr>
<tr><td>Sanlúcar de Barrameda, Cádiz/Spain (Clone 3)</td><td>-</td><td>327</td><td>C. felis</td></tr>
<tr><td>Sanlúcar de Barrameda, Cádiz/Spain (Clone 4)</td><td>-</td><td>327</td><td>C. felis</td></tr>
<tr><td>Sanlúcar de Barrameda, Cádiz/Spain</td><td>2</td><td>327</td><td>C. felis</td><td rowspan="12">LN827903</td></tr>
<tr><td>Mallorca/Spain</td><td>4</td><td>327</td><td>C. felis</td></tr>
<tr><td>Pilas, Sevilla/Spain</td><td>3</td><td>327</td><td>C. felis</td></tr>
<tr><td>Mairena, Sevilla/Spain</td><td>5</td><td>327</td><td>C. felis</td></tr>
<tr><td>Villamanrique de la Condesa, Sevilla/Spain</td><td>3</td><td>327</td><td>C. felis</td></tr>
<tr><td>Lebrija, Sevilla/Spain</td><td>4</td><td>327</td><td>C. felis</td></tr>
<tr><td>Dílar, Granada/Spain</td><td>4</td><td>327</td><td>C. felis</td></tr>
<tr><td>Fuentes de Andalucía, Sevilla/Spain</td><td>3</td><td>327</td><td>C. felis</td></tr>
<tr><td>La Luisiana, Sevilla/Spain</td><td>3</td><td>327</td><td>C. felis</td></tr>
<tr><td>Dos Hermanas, Sevilla/Spain</td><td>5</td><td>327</td><td>C. felis</td></tr>
<tr><td>Nashtarood, Mazandaran/Iran</td><td>2</td><td>327</td><td>C. felis</td></tr>
<tr><td>Polokwane, Limpopo/South Africa</td><td>8</td><td>327</td><td>C. felis</td></tr>
<tr><td>Tonekabon, Mazandaran/Iran</td><td>1</td><td>327</td><td>C. canis</td><td rowspan="2">LN827905</td></tr>
<tr><td>Kotra, Mazandaran/Iran</td><td>2</td><td>327</td><td>C. canis</td></tr>
<tr><td>Kotra, Mazandaran/Iran (Clone 1)</td><td>-</td><td>327</td><td>C. canis</td><td rowspan="3">LN864485</td></tr>
<tr><td>Kotra, Mazandaran/Iran (Clone 2)</td><td>-</td><td>327</td><td>C. canis</td></tr>
<tr><td>Kotra, Mazandaran/Iran (Clone 5)</td><td>-</td><td>327</td><td>C. canis</td></tr>
<tr><td>Kotra, Mazandaran/Iran (Clone 3)</td><td>-</td><td>327</td><td>C. canis</td><td>LN864486</td></tr>
<tr><td>Kotra, Mazandaran/Iran (Clone 4)</td><td>-</td><td>327</td><td>C. canis</td><td>LN864487</td></tr>
<tr><th colspan="5">18S rRNA gene</th></tr>
<tr><th>Location/Country</th><th>Number of fleas</th><th>Number of base pairs (bp)</th><th>Species</th><th>Accession number</th></tr>
<tr><td>Sanlúcar de Barrameda, Cádiz/Spain</td><td>2</td><td>989</td><td>C. felis</td><td rowspan="10">LN651166</td></tr>
<tr><td>Mallorca/Spain</td><td>2</td><td>989</td><td>C. felis</td></tr>
<tr><td>Pilas, Sevilla/Spain</td><td>2</td><td>989</td><td>C. felis</td></tr>
<tr><td>Mairena, Sevilla/Spain</td><td>2</td><td>989</td><td>C. felis</td></tr>
<tr><td>Villamanrique de la Condesa, Sevilla/Spain</td><td>1</td><td>989</td><td>C. felis</td></tr>
<tr><td>Dílar, Granada/Spain</td><td>1</td><td>989</td><td>C. felis</td></tr>
<tr><td>Fuentes de Andalucía, Sevilla/Spain</td><td>1</td><td>989</td><td>C. felis</td></tr>
<tr><td>La Luisiana, Sevilla/Spain</td><td>1</td><td>989</td><td>C. felis</td></tr>
<tr><td>Kotra, Mazandaran/Iran</td><td>1</td><td>989</td><td>C. felis</td></tr>
<tr><td>Polokwane, Limpopo/South Africa</td><td>2</td><td>989</td><td>C. felis</td></tr>
<tr><td>Kotra, Mazandaran/Iran</td><td>1</td><td>989</td><td>C. canis</td><td>LN651167</td></tr>
<tr><th colspan="5">16S rRNA gene</th></tr>
<tr><th colspan="2">Species</th><th>Number of base pairs (bp)</th><th colspan="2">Accession number</th></tr>
<tr><td colspan="2">Wolbachia pipientis</td><td>334</td><td colspan="2">LN864488</td></tr>
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702

Table 2. GenBank accession numbers of *cox1* partial gene sequences (600 base pairs) of individuals of *Ctenocephalides felis* and *Ctenocephalides canis* isolated from *Canis lupus familiaris* from different geographical regions. Asterisk represents individuals which showed two and three bristles in each side on the LMA of one individual.

Cox1 Ctenocephalides felis

Number identifier	Location/Country	*Wolbachia pipientis*	Sex	Haplotype	Accession Number
102	Sanlúcar de Barrameda, Cádiz/Spain	-	M	A	LN827896
31	Sanlúcar de Barrameda, Cádiz/Spain	-	M	A	
28	Sanlúcar de Barrameda, Cádiz/Spain	+	H	A	
223	Mairena, Sevilla/Spain	+	H	A	
228*	Mairena, Sevilla/Spain	-	M	A	
221	Mairena, Sevilla/Spain	+	H	A	
219	Mairena, Sevilla/Spain	+	H	A	
218	Mairena, Sevilla/Spain	-	H	A	
338*	Mallorca/Spain	+	H	A	
332	Mallorca/Spain	+	H	A	
330	Mallorca/Spain	+	H	A	
199	Kotra, Mazandaran/Iran	+	H	A	
200	Kotra, Mazandaran/Iran	+	H	A	
269	Nashtarood, Mazandaran/Iran	-	M	A	
271	Nashtarood, Mazandaran/Iran	-	M	A	
609	Villamanrique de la Condesa, Sevilla/Spain	+	H	A	
615	Villamanrique de la Condesa, Sevilla/Spain	+	H	A	
601	Villamanrique de la Condesa, Sevilla/Spain	+	H	A	
612	Villamanrique de la Condesa, Sevilla/Spain	+	H	A	
481	Pilas, Sevilla/Spain	+	M	A	
478	Pilas, Sevilla/Spain	+	H	A	
479	Pilas, Sevilla/Spain	+	H	A	
477	Pilas, Sevilla/Spain	-	M	A	
3	Dílar, Granada/Spain	-	M	A	
4	Dílar, Granada/Spain	+	H	A	
5	Dílar, Granada/Spain	+	H	A	
120	Lebrija, Sevilla/Spain	-	M	A	
118*	Lebrija, Sevilla/Spain	+	H	A	
643	Fuentes de Andalucía, Sevilla/Spain	+	H	A	
647	Fuentes de Andalucía, Sevilla/Spain	+	H	A	
644	Fuentes de Andalucía, Sevilla/Spain	+	H	A	
617	La Luisiana, Sevilla/Spain	-	H	A	
619*	La Luisiana, Sevilla/Spain	+	H	A	
620*	La Luisiana, Sevilla/Spain	-	H	A	
222	Mairena, Sevilla/Spain	+	H	A1	LN827897
642	Fuentes de Andalucía, Sevilla/Spain	+	H	A2	LN827898
216*	Mairena, Sevilla/Spain	+	H	B	LN827899
645*	Fuentes de Andalucía, Sevilla/Spain	+	H	B	
595	Polokwane, Limpopo/South Africa	-	M	C	LN827900
591	Polokwane, Limpopo/South Africa	+	H	C	
589	Polokwane, Limpopo/South Africa	+	H	C	
586	Polokwane, Limpopo/South Africa	+	H	C	
548	Polokwane, Limpopo/South Africa	+	H	C	
491	Polokwane, Limpopo/South Africa	+	H	C	
487	Polokwane, Limpopo/South Africa	+	H	C	

Cox1 Ctenocephalides canis

Number identifier	Location/Country	*Wolbachia pipientis*	Sex	Haplotype	Accession Number
197	Kotra, Mazandaran/Iran	+	H	-	LN827901
204	Nashtarood, Mazandaran/Iran	+	H	-	
214	Kotra, Mazandaran/Iran	+	H	-	
198	Kotra, Mazandaran/Iran	+	H	-	

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Table 3. Percentage of similarity among all the *cox1* mtDNA partial gene sequences of *C. felis* and *C. canis* from different geographical areas obtained in this work and from GenBank database.

Cox1	***C. felis* haplotype A and *C. felis* from New South Wales (Australia)**	***C. felis* haplotype A1**	***C. felis* haplotype A2**	***C. felis* haplotype B**	***C. felis* haplotype C (South Africa)**	***C. canis* from Iran**	***C. felis strongylus***	***C. orientis***	***C. felis felis* from Queensland, Cairns (Australia)**	***C. felis felis* from Fiji, Thailand and Mumbai (India)**	***C. felis felis* from Sikkim (India)**	***C. felis felis* from Jablonec nad Nisou (Czech Republic)**	***C. felis felis* from Pardubice (Czech Republic)**	***C. canis* from Czech Republic**
***C. felis* haplotype A and *C. felis* from New South Wales (Australia)**	-													
***C. felis* haplotype A1**	99.8 %	-												
***C. felis* haplotype A2**	99.8 %	99.7 %	-											
***C. felis* haplotype B**	97.3 %	97.5 %	97.2 %	-										
***C. felis* haplotype C (South Africa)**	97.3 %	97.5 %	97.2 %	99 %	-									
***C. canis* from Iran**	97.7 %	97.8 %	97.5 %	99.3 %	99.7 %	-								
C. felis strongylus	98 %	98.2 %	97.8 %	98 %	97.7 %	98 %	-							
C. orientis	91.3 %	91.5 %	91.2 %	91.8 %	91.5 %	91.8 %	91.7 %	-						
***C. felis felis* from Queensland, Cairns (Australia)**	97.3 %	97.5 %	97.2 %	98.7 %	98.3 %	98.7 %	97.7 %	92.3 %	-					
***C. felis felis from* Fiji, Thailand and Mumbai (India)**	98.3 %	99 %	98.7 %	97.8 %	97.8 %	98.2 %	98.5 %	92 %	98.2 %	-				
***C. felis felis* from Sikkim (India)**	99.7 %	99.5 %	99.5 %	97.3 %	97.5 %	97.7 %	98 %	92.3 %	97.5 %	98.8 %	-			
***C. felis felis* from Jablonec nad Nisou (Czech Republic)**	100 %	99.8 %	99.8 %	97.3 %	97.3 %	97.7 %	98 %	91.3 %	97.3 %	98.8 %	99.7 %	-		

***C. felis felis* from Pardubice (Czech Republic)**	98.8 %	98.7 %	98.7 %	97.5 %	97.5 %	97.8 %	98.2 %	92.2 %	97.8 %	99 %	98.7 %	98.8 %	-	
***C. canis* from Czech Republic**	91.5 %	91.7 %	91.5 %	91.2 %	90.8 %	91.2 %	91.2 %	95.5 %	91.7 %	91.5 %	92.7 %	91.5 %	91.7 %	-

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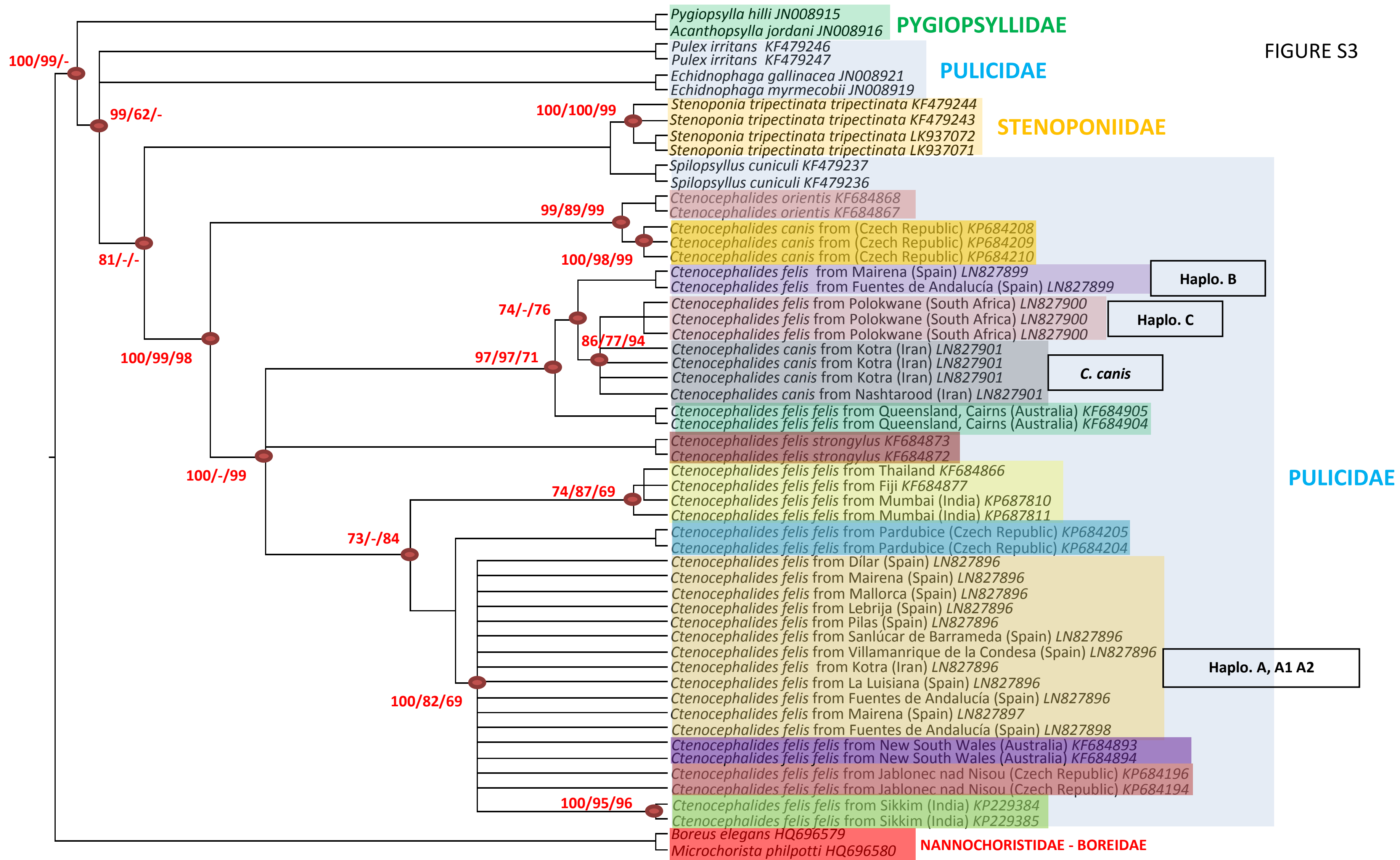
FIGURE S3
100/99/-
99/62/-
81/-/-
100/99/98
100/-/99
73/-/84
100/82/69
100/100/99
99/89/99
100/98/99
74/-/76
86/77/94
97/97/71
74/87/69
100/95/96
Pygiopsylla hilli JN008915
Acanthopsylla jordani JN008916
PYGIOPSYLLIDAE
Pulex irritans KF479246
Pulex irritans KF479247
Echidnophaga gallinacea JN008921
Echidnophaga myrmecobii JN008919
PULICIDAE
Stenoponia tripectinata tripectinata KF479244
Stenoponia tripectinata tripectinata KF479243
Stenoponia tripectinata tripectinata LK937072
Stenoponia tripectinata tripectinata LK937071
STENOPONIIDAE
Spilopsyllus cuniculi KF479237
Spilopsyllus cuniculi KF479236
Ctenocephalides orientis KF684868
Ctenocephalides orientis KF684867
Ctenocephalides canis from (Czech Republic) KP684208
Ctenocephalides canis from (Czech Republic) KP684209
Ctenocephalides canis from (Czech Republic) KP684210
Ctenocephalides felis from Mairena (Spain) LN827899
Ctenocephalides felis from Fuentes de Andalucía (Spain) LN827899
Haplo. B
Ctenocephalides felis from Polokwane (South Africa) LN827900
Ctenocephalides felis from Polokwane (South Africa) LN827900
Ctenocephalides felis from Polokwane (South Africa) LN827900
Haplo. C
Ctenocephalides canis from Kotra (Iran) LN827901
Ctenocephalides canis from Kotra (Iran) LN827901
Ctenocephalides canis from Kotra (Iran) LN827901
Ctenocephalides canis from Nashtarood (Iran) LN827901
C. canis
Ctenocephalides felis felis from Queensland, Cairns (Australia) KF684905
Ctenocephalides felis felis from Queensland, Cairns (Australia) KF684904
Ctenocephalides felis strongylus KF684873
Ctenocephalides felis strongylus KF684872
Ctenocephalides felis felis from Thailand KF684866
Ctenocephalides felis felis from Fiji KF684877
Ctenocephalides felis felis from Mumbai (India) KP687810
Ctenocephalides felis felis from Mumbai (India) KP687811
Ctenocephalides felis felis from Pardubice (Czech Republic) KP684205
Ctenocephalides felis felis from Pardubice (Czech Republic) KP684204
Ctenocephalides felis from Dílar (Spain) LN827896
Ctenocephalides felis from Mairena (Spain) LN827896
Ctenocephalides felis from Mallorca (Spain) LN827896
Ctenocephalides felis from Lebrija (Spain) LN827896
Ctenocephalides felis from Pilas (Spain) LN827896
Ctenocephalides felis from Sanlúcar de Barrameda (Spain) LN827896
Ctenocephalides felis from Villamanrique de la Condesa (Spain) LN827896
Ctenocephalides felis from Kotra (Iran) LN827896
Ctenocephalides felis from La Luisiana (Spain) LN827896
Ctenocephalides felis from Fuentes de Andalucía (Spain) LN827896
Ctenocephalides felis from Mairena (Spain) LN827897
Ctenocephalides felis from Fuentes de Andalucía (Spain) LN827898
Haplo. A, A1 A2
Ctenocephalides felis felis from New South Wales (Australia) KF684893
Ctenocephalides felis felis from New South Wales (Australia) KF684894
Ctenocephalides felis felis from Jablonec nad Nisou (Czech Republic) KP684196
Ctenocephalides felis felis from Jablonec nad Nisou (Czech Republic) KP684194
Ctenocephalides felis felis from Sikkim (India) KP229384
Ctenocephalides felis felis from Sikkim (India) KP229385
PULICIDAE
Boreus elegans HQ696579
Microchorista philpotti HQ696580
NANNOCHORISTIDAE - BOREIDAE

FIGURE S2

```
C. felis        TATAACATTACCAGACTGCCGCTTGCTTTAAACGGCTTGCCGGCGAATGATGGAGTTTCG
C. canis        TATAACATTACCAGACTGCCGCTTGCTTTAACCGGCTTGCCGGCGAATGATGGAGTTTCG
                ******************************* ****************************

C. felis        CGTAAATGCGTGCTCTTAAATAATTCACTCAACGTGTGAGCCAGTCCATTTTGCAACATC
C. canis        CGTATACGCGTGCTCTTAAATAATTCACTCAACGTGTGAGCCAGCCCAGTTTGCAACATC
                **** * ************************************* *** ***********

C. felis        GGACATTACCGTTCGTTGACGTTCTGTGGGATTACGGCGGGTTGTGTGTCCTAGAGATTT
C. canis        GGACATTACCGTTCGTTGACGTTCTGTGGGATTACGGCGGGTTGTGTGTCCTAGAGATTT
                ************************************************************

C. felis        TATATTCTTGCGACCCTCCCGATAACCGGAAACCCGAACTCTTCGAAATGCGGTTTCGTT
C. canis        TATATTCTTGCGACCCTCCCGATCACCGGAAACCCGAACTCTTCGTAATGCGGTATCGTT
                *********************** ********************* ******** *****

C. felis        CCGGCAAATTTGGACGATTCAAAGGTTCCTCGTCGATTTTGTGTGCGCTTGCTTACTAAC
C. canis        CCGGCAAATTTGGACGATTCAAAGGTTCCTCGTCGATATTGAGTGCGCTTGCTTACTATC
                ************************************* *** **************** *

C. felis        GTTTGCGAGCACATCATAATCATAATA
C. canis        GTTTGCGAGCACACCATAATCATAATA
                ************* *************
```

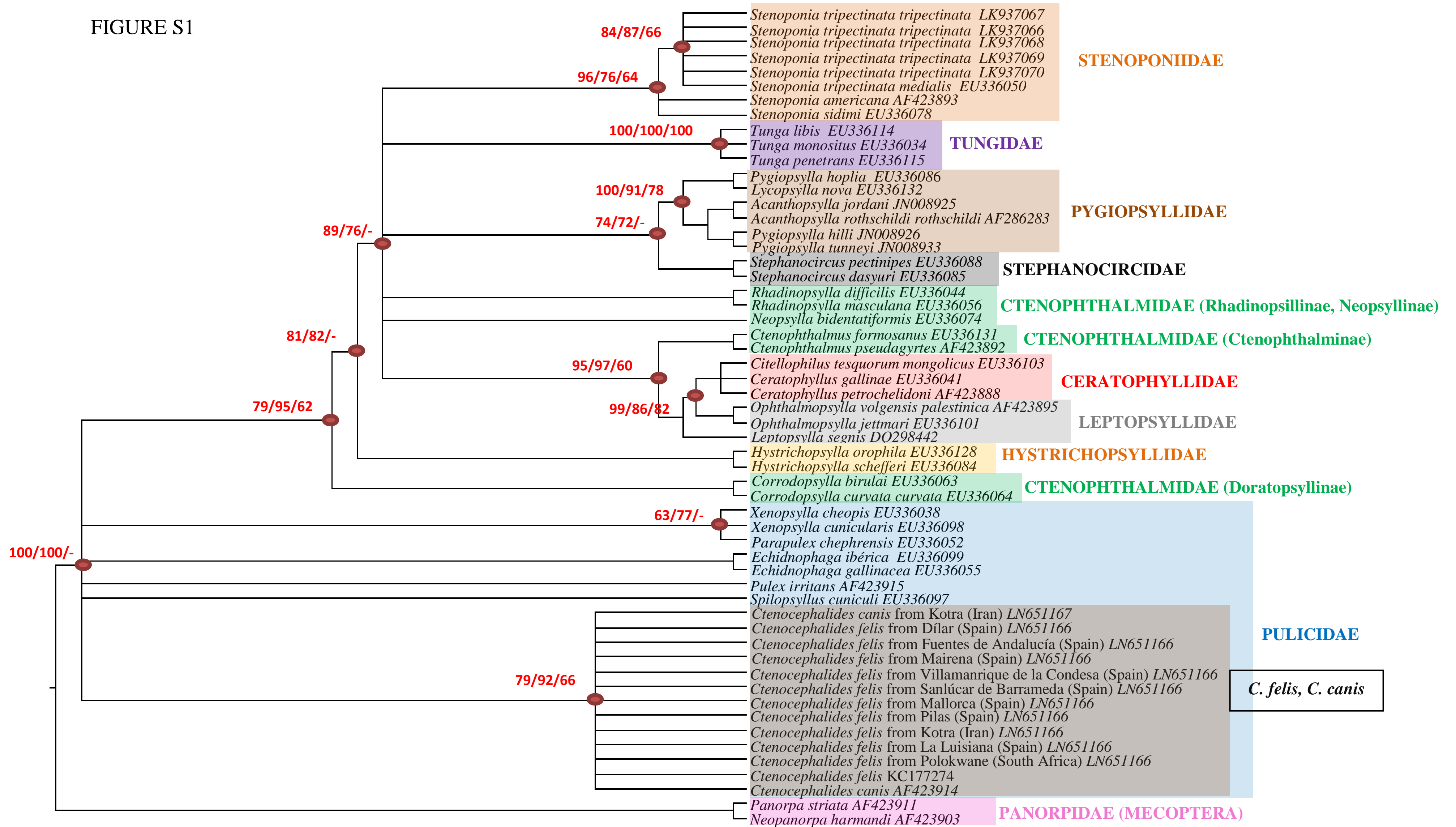
FIGURE S1
84/87/66
96/76/64
Stenoponia tripectinata tripectinata LK937067
Stenoponia tripectinata tripectinata LK937066
Stenoponia tripectinata tripectinata LK937068
Stenoponia tripectinata tripectinata LK937069
Stenoponia tripectinata tripectinata LK937070
Stenoponia tripectinata medialis EU336050
Stenoponia americana AF423893
Stenoponia sidimi EU336078
STENOPONIIDAE
100/100/100
Tunga libis EU336114
Tunga monositus EU336034
Tunga penetrans EU336115
TUNGIDAE
100/91/78
74/72/-
89/76/-
Pygiopsylla hoplia EU336086
Lycopsylla nova EU336132
Acanthopsylla jordani JN008925
Acanthopsylla rothschildi rothschildi AF286283
Pygiopsylla hilli JN008926
Pygiopsylla tunneyi JN008933
PYGIOPSYLLIDAE
Stephanocircus pectinipes EU336088
Stephanocircus dasyuri EU336085
STEPHANOCIRCIDAE
Rhadinopsylla difficilis EU336044
Rhadinopsylla masculana EU336056
Neopsylla bidentatiformis EU336074
CTENOPHTHALMIDAE (Rhadinopsillinae, Neopsyllinae)
81/82/-
Ctenophthalmus formosanus EU336131
Ctenophthalmus pseudagyrtes AF423892
CTENOPHTHALMIDAE (Ctenophthalminae)
95/97/60
Citellophilus tesquorum mongolicus EU336103
Ceratophyllus gallinae EU336041
Ceratophyllus petrochelidoni AF423888
CERATOPHYLLIDAE
79/95/62
99/86/82
Ophthalmopsylla volgensis palestinica AF423895
Ophthalmopsylla jettmari EU336101
Leptopsylla segnis DQ298442
LEPTOPSYLLIDAE
Hystrichopsylla orophila EU336128
Hystrichopsylla schefferi EU336084
HYSTRICHOPSYLLIDAE
Corrodopsylla birulai EU336063
Corrodopsylla curvata curvata EU336064
CTENOPHTHALMIDAE (Doratopsyllinae)
63/77/-
Xenopsylla cheopis EU336038
Xenopsylla cunicularis EU336098
Parapulex chephrensis EU336052
100/100/-
Echidnophaga ibérica EU336099
Echidnophaga gallinacea EU336055
Pulex irritans AF423915
Spilopsyllus cuniculi EU336097
Ctenocephalides canis from Kotra (Iran) LN651167
Ctenocephalides felis from Dílar (Spain) LN651166
Ctenocephalides felis from Fuentes de Andalucía (Spain) LN651166
Ctenocephalides felis from Mairena (Spain) LN651166
Ctenocephalides felis from Villamanrique de la Condesa (Spain) LN651166
79/92/66
Ctenocephalides felis from Sanlúcar de Barrameda (Spain) LN651166
Ctenocephalides felis from Mallorca (Spain) LN651166
Ctenocephalides felis from Pilas (Spain) LN651166
Ctenocephalides felis from Kotra (Iran) LN651166
Ctenocephalides felis from La Luisiana (Spain) LN651166
Ctenocephalides felis from Polokwane (South Africa) LN651166
Ctenocephalides felis KC177274
Ctenocephalides canis AF423914
PULICIDAE
C. felis, C. canis
Panorpa striata AF423911
Neopanorpa harmandi AF423903
PANORPIDAE (MECOPTERA)

Figure 2

- *Ctenocephalides felis felis* (Australia)
- *Ctenocephalides felis felis* (Fiji)
- *Ctenocephalides canis* and *C. felis* (Iran)
- *Ctenocephalides felis strongylus*
- *Ctenocephalides felis* (South Africa)
- *Ctenocephalides felis felis* (India)
- *Ctenocephalides felis* (Spain)
- *Ctenocephalides orientis*
- *Ctenocephalides felis felis* and *C. canis* (Czech Republic)
- *Ctenocephalides felis felis* (Thailand)

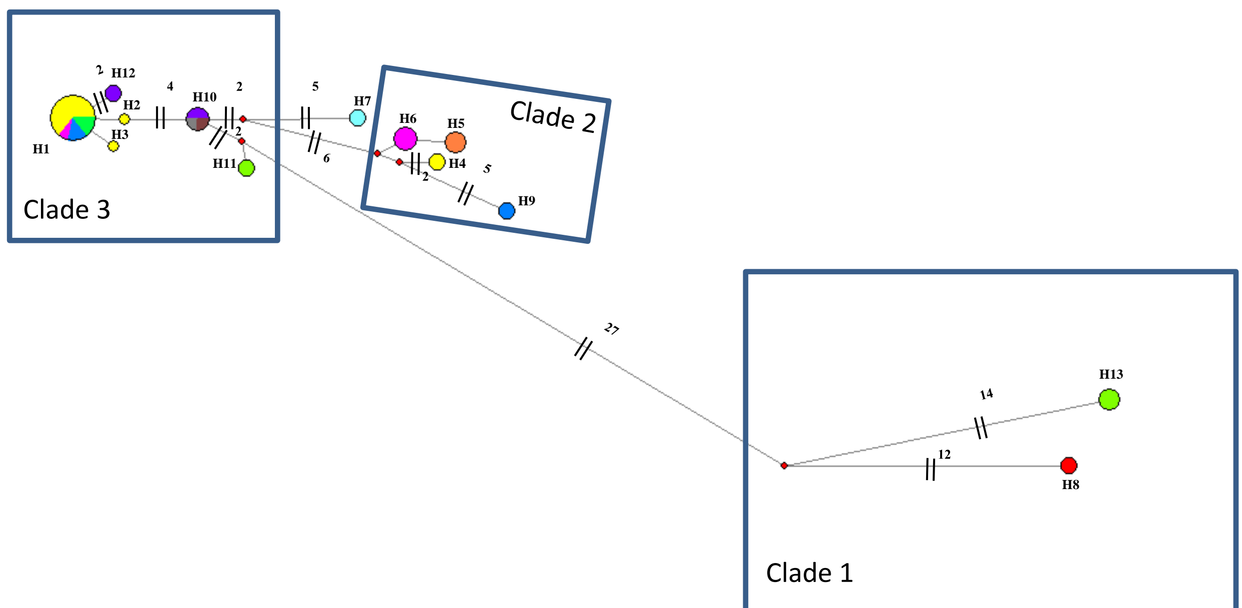

Figure 1