Molecular study of *Stenoponia tripectinata tripectinata* (Siphonaptera: Ctenophthalmidae: Stenoponiinae) from the Canary Islands: taxonomy and phylogeny.

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RUNNING HEAD: *Stenoponia tripectinata tripectinata***:** a molecular study

ABSTRACT

In the present work, we carried out a comparative molecular study of *Stenoponia tripectinata tripectinata* isolated from *Mus musculus* from the Canary Islands, Spain. The Internal Transcribed Spacers 1 and 2 (ITS1, ITS2) and 18S ribosomal RNA partial gene and cytochrome c-oxidase 1 (*cox1*) mitochondrial DNA partial gene sequences of this subspecies were determined to clarify the taxonomic status of this subspecies and to assess inter-population variation and inter-specific sequence differences. In addition, we have carried out a comparative phylogenetic study with other species of fleas using Bayesian, Maximum Parsimony, Maximum Likelihood and Neighbor-Joining analysis. A geographical signal was detected between the *cox1* partial gene sequences of *S. t. tripectinata* isolated from *M. musculus* from different islands and those isolated from *Apodemus sylvaticus* from the Iberian Peninsula. Our results assess the monophyletic origin of Stenoponiinae and a different genetic lineage from Ctenophthalmidae. Thus, the elevation of subfamily Stenoponiinae to family level (Stenoponiidae) is suggested.

Keywords: *Stenoponia tripectinata tripectinata*, ribosomal RNA, cytochrome c-oxidase 1, Canary Islands, Siphonaptera.

Introduction

Fleas (Insecta, Siphonaptera) form a distinct group of wingless bloodsucking insects with complete metamorphosis. About 2574 species belonging to 16 families and 238 genera were described (Bitam *et al*., 2010). Lewis (1998) recognized 15 families considering Ctenophthalmidae, while Medvedev (1998) treated Hystrichopsyllidae as a large family that includes Hystrichopsyllinae and the subfamilies that were traditionally placed within Ctenophthalmidae. This family (*sensu* Lewis, 1993a) consists of nine subfamilies and 17 described tribes, with 42 genera and 664 species. Roughly one quarter of flea species are placed within this group, and Ctenophthalmidae has been traditionally the ‘‘catchall’’ family for fleas that have been difficult to assign to other families (Whiting *et al*., 2008). These authors reconstructed deep level evolutionary relationships for fleas (Insecta: Siphonaptera) based on 28S, 18S, COII and EFI-α sequences and found, in their analysis, that this family was paraphyletic. The current arrangement of Ctenophthalmidae is clearly in a state of disarray; however, if one assesses the phylogeny based on the subfamily, five natural groupings may be observed: Ctenophthalminae, Doratopsyllinae, Neopsyllinae, Stenoponiinae and the Rhadinopsyllinae (Whiting *et al*., 2008). These authors concluded that the catchall group Ctenophthalmidae is clearly an unnatural grouping of fleas, and elevating each of its constituent subfamilies to family level would be a closer reflection of their phylogeny. Furthermore, Ctenophthalmidae has been generally associated with insectivorous hosts (Soricidae) as the main hosts, but members of this family have been reported parasitizing rodents (Muridae) (Acosta, 2005).

The Holarctic subfamily Stenoponiinae are all very large and darkly pigmented fleas with a striking genal comb spanning most of the lateral portion of the head. Species from the Nearctic (*Stenoponia americana*), Palearctic (*Stenoponia tripectinata medialis*), and the Oriental (*Stenoponia sidimi*) regions parasitize murid rodents.

The genus *Stenoponia* (Ctenophthalmidae) Hopkins & Rothschild, 1962, is a Holarctic genus of 16 species and 14 subspecies which includes *Stenoponia tripectinata tripectinata,* the vector of plague in Asia Minor and European Russia (Lewis, 1993b). To date, *S. tripectinata* has been documented in Turkey, Greece, Romania, Italy, France and the Iberian Peninsula (Sánchez & Gómez, 2012). Furthermore, Sánchez & Gómez (2012) reported, for the first time the geographical and host distribution of *S. t. tripectinata* parasitizing *Mus musculus* on the Canary Islands, Spain.

The specific differentiation of fleas has been carried out according to morphological characteristics based on the shape and structure of their complex genitalia and the presence and the distribution of setae, spines and ctenidia on the body (Dunnet & Mardon, 1999; Whiting, 2002). Nevertheless, the phenotype is conditioned by different factors: host, ambient conditions, feeding, etc., and many species and subspecies of fleas were reported based on a new host or on the presence or absence of putative "specific" morphological and biometrical characters. All these difficulties and this incertitude, in discriminating among flea species, claims for the need of adding molecular data to the observation of morphological characters to study the taxonomy of the group.

Among the different molecular markers used in systematics, the Internal Transcribed Spacer regions 1 and 2 (ITS1 and ITS2) ribosomal DNA (rDNA) remains a valuable marker, in particular arthropods to discriminate between species (Marrugal *et al*., 2013; Monje *et al*., 2014) or also within species (Essig *et al*., 1999; Marcilla *et al*., 2002) and it has been revealed to be informative to establish phylogenetic relationships at the genus level (Zagoskin *et al*., 2014). Vobis *et al*. (2004) carried out a molecular phylogeny of isolates of *Ctenocephalides felis* based on analysis of the ITS1 and ITS2. These regions have also been used to differentiate populations within mite species (de Rojas *et al*., 2007).

Furthermore, mitochondrial DNA (mtDNA) has remained as evaluable marker for population, biogeographic and phylogenetic studies. It is also used for taxonomic purposes, where determinate fragments are used as mtDNA sequence tags or bar-code for species diagnostics (Hebert *et al*., 2003). It remains, however, that while mtDNA sequences are very useful markers, their use is not without complication. Ballard & Whitlock (2004) argued that mtDNA evolution is non-neutral with sufficient regularity to question its utility as a marker for genomic history. Direct selection (selection on mtDNA itself) and indirect selection (selection arising from disequilibrium with other maternally transmitted genes) is sufficiently common to impose caution when making phylogenetic inferences based on mtDNA data alone. Thus, Hurst & Jiggins (2005) concluded that mtDNA is inappropriate as a sole marker in studies of the recent history of arthropods and, potentially, other invertebrates.

In the present work, we carried out a comparative molecular study of *S. t. tripectinata* isolated from *M. musculus* from different islands from the Canary Islands, Spain. To this end, the ITS1, ITS2 and 18S of the rDNA and a fragment of the cytochrome c-oxidase 1 (*cox1*) gene of the mitochondrial DNA of this subspecies were sequenced in order to clarify the taxonomic status of this subspecies and to assess inter-population variation and inter-specific sequence differences. Based on the sequences produced here, together with data of additional flea species retrieved from public databases, we also carried out a comparative phylogeographic analysis Bayesian, Maximum Parsimony, Maximum Likelihood and Neighbor-Joining inference.

Material and Methods

Collection of samples

Rodents were captured using live traps on all the islands. Fleas were collected from mice (*M. musculus*) from different islands of the Canary Islands (Gran Canaria, La Palma, El Hierro, La Gomera and Tenerife) (Spain) (Table 1). Fleas were collected manually and kept in an Eppendorf tube with 70 % ethanol until required for subsequent identification and sequencing. Specific identification was based on morphological characteristics (Jordan 1958; Hopkins & Rothschild, 1962; Beaucournu & Launay, 1990). For details concerning host distribution on each island of the Canarian Archipelago and distribution of *S. t. tripectinata* in different biotopes (Laurisilva, Pine forest, etc) see Sánchez & Gómez (2012).

Molecular study

Single fleas were frozen in liquid nitrogen and pulverized in a mortar. Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. The ITS1 region was amplified by PCR using a thermocycler (Perkin Elmer) and the PCR mix and PCR conditions were applied as previously described by Marrugal *et al*. (2013). Primers were NC5 (Gasser *et al*., 1996) and ITS1rev (Marrugal *et al*., 2013). For the ITS2 region the PCR mix used was the same as for the ITS1 region and the conditions were: 94 °C at 5 min (denaturing), 35 cycles at 94 °C at 60 s (denaturing), 55 °C at 60 s (annealing), 72 °C at 60 s (primer extension), followed by 10 min at 72 °C. Forward and reverse primers for ITS2 region were senITS2 (Vobis *et al*., 2004) and ITS2R, respectively (Vobis *et al*., 2004). In the case of 18S partial gene region, the PCR mix was: 5µl 10× PCR buffer, 1µl 10 Mm dNTP mixture (0.2 mM each), 2µl 50 mM MgCl2, 5µl primer mix (1 mM each), 5µl template DNA, 0.5µl *Taq* DNA polymerase (2.5 units) and

135 autoclaved distilled water to 50 µl. The PCR conditions and primers (18SF and 18SR)
136 were defined by Kaewmongkol *et al*. (2011). *Cox1* mtDNA partial gene was amplified
137 using PCR conditions designed for amplification of *cox1* from fleas' isolates by
138 Kaewmongkol *et al*. (2011). The cycling conditions consisted of a pre-PCR step of 96 °C
139 for 2 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 30 s and an extension of
140 72 °C for 60 s with a final extension of 72 °C for 7 min. Forward and reverse primers for
141 the *cox1* were: LCO1490 and HCO2198 (Folmer *et al*., 1994), respectively.

142 The rDNA intra-individual variation was determined by sequencing four to seven clones
143 of one individual per geographical population of *S. t. tripectinata.* The PCR products were
144 eluted from the agarose by using the WIZARD® SV Gel and PCR Clean-Up System
145 (Promega) and transformation was carried out as cited by Cutillas *et al.* (2009). Plasmids
146 were purified using a Wizard Plus SV (Promega) and sequenced by Stab Vida (Portugal)
147 with a universal primer (M13).

148 All the phylogenetic analyses were performed on the rDNA and mtDNA datasets, and
149 sequences were aligned using the Clustal W program version 2.0 (Larkin *et al*., 2007).
150 The intra-population variation was determined for the rDNA and mtDNA by sequencing
151 three individuals from each island. Furthermore, all the sequences were aligned and
152 compared with each other using the CLUSTAL W program. Alignments were manually
153 adjusted.

154 Phylogenetic relationships were analyzed using four different methods: Neighbor-Joining
155 (NJ) and Maximum Parsimony (MP) trees were generated from methods using the MEGA
156 5 program from Tamura *et al*. (2011), Maximum Likelihood (ML) using the PHYML
157 package from Guindon & Gascuel (2003) and Bayesian inferences (B) were performing
158 from Mr Bayes-3.1.2. For the Bayesian analysis, we ran three independent runs of four

159 Markov chains for 10 million generations, sampling every 500 generations. The Bayesian
160 posterior probabilities are percentage converted. For ML inference, the JMODELTEST
161 (Posada, 2008) program was also used to determine the best fit substitution model for the
162 parasite data (18S, ITS1, ITS2 and *cox1*). Models of evolution were chosen for
163 subsequent analysis according to the Akaike Information Criterion (Huelsenbek and
164 Rannala, 1997; Posada and Buckley, 2004). Best-fit nucleotide substitution models
165 included general time-reversible model with gamma-distributed rate variation and a
166 proportion of invariable sites, GTR+I+G (18S), Hasegawa-Kishino-Yano, HKY85+I+G
167 (ITS1), GT+G (ITS2) and GTR+G (*cox1*). Support for the topology was examined using
168 bootstrapping (heuristic option) (Felsenstein, 1985) over 1,000 replications.

169 The phylogenetic and phylogeographic analysis, based on ITS1, ITS2, 18S and *cox1*
170 sequences was carried out using sequences obtained from GenBank (appendix 1).
171 Phylogenetic trees based on 18S rRNA and *cox1* mtDNA were rooted including two
172 outgroup species representing members of the Order Mecoptera: *Microchorista philpotti*
173 and *Boreus elegans* (*cox1*) and *Nannochorista dipteroides* and *Boreus coloradensis* (18S)
174 (appendix 1), whereas phylogenetic trees based on ITS1 and ITS2 sequences were
175 constructed using different outgroup species representing members of Order Diptera
176 (*Anopheles farauti*, *Anopheles lesteri*, *Anopheles anthropophagus*, *Muscina stabulans*
177 and *Philornis seguyi*). No ITS sequences of Order Mecoptera were found in public
178 database.

Results

No morphological differences were observed between individuals of *S. t. tripectinata* isolated from *Mus musculus* from different islands. ITS1 sequences of the ribosomal DNA (rDNA) of different populations of *S. t. tripectinata* were 1204-1209 base pairs (bp) in length (Table 1), while the ITS2 sequences of *S. t. tripectinata* were 332 bp in length (Table 1). Furthermore, the *cox1* and 18S partial gene sequences of *S. t. tripectinata* were 677 bp and 1095-1098 bp in length, respectively (Table 1). All the sequences (ITS1, ITS2, 18S and *cox1* partial gene) of *S. t. tripectinata* isolated from *M. musculus* from different islands were deposited in GenBank database (Table 1).

ITS1 and ITS2

The intra-individual, intra-population and inter-population similarities of *S. t. tripectinata* isolated from *M. musculus* are shown in Table 2 (ITS1) and Table 3 (ITS2). No ITS1 sequences of others species of family Ctenophthalmidae were found in GenBank. Thus, no molecular comparative analysis between them could be performed.

The phylogenetic analysis based on ITS1 and ITS2 sequences showed a substantial length variation in the alignment which compromised inferences of positional homology. Furthermore, *Anopheles* spp. seemed to be a poor outgroup due to long-branch problems affecting root-placement.

18S rRNA partial gene

The intra-population and inter-population similarities were of 100 %. Furthermore, the inter-specific similarity was of 99.6 % (*S. t. tripectinata*-*S. t. medialis*), 99.8 % (*S. t. tripectinata*-*S. americana*) and 99.9 % (*S. t. tripectinata*-*S. sidimi*). The Bayesian, Maximum Parsimony, Neighbor-Joining and Maximum Likelihood analysis

reconstructed a similar topology. The phylogenetic tree (Fig. 1) constructed for the 18S rRNA partial gene sequences of *S. t. tripectinata* with those sequences from GenBank of species belonging to the family Ctenophthalmidae, Leptopsyllidae and Ceratophyllidae revealed the individuals of *S. t. tripectinata* clustering together with *S. t. medialis, S. americana* and *S. sidimi* (Fig. 1). Subfamily Stenoponiinae appeared related with family Ceratophyllidae and Leptopsyllidae and separated, in polytomy, from Ctenophthalmidae (Fig. 1).

Cox1 mtDNA partial gene

The intra-population and inter-population similarities are shown in Table 4. When sequences of this *cox1* mtDNA partial gene of *S. t. tripectinata* isolated from different islands were compared with those obtained in GenBank from the Iberian Peninsula (see appendix 1) we noticed that both populations displayed slight differences (98.9 % to 99.7 %) (Table 4). Based on the *cox1* mtDNA partial gene sequences, a restriction map was constructed. Three endonucleases located at position 200 (*Mse*1, *Ase*1 and *Vsp*1) differentiated, clearly, both geographical regions (the Canary Islands and the Iberian Peninsula).

The phylogenetic tree topology of *S. t. tripectinata* from different geographical origins showed all the individuals from the Canary Islands clustered together, and separated from those individuals from the Iberian Peninsula (Fig. 2). Furthermore, all the individuals of *S. t. tripectinata* appeared as a compact group and separated, in polytomy, with the remaining species belonging to different families of Siphonaptera: Ctenophthalmidae, Pygiopsillidae, and Pulicidae (Fig. 2).

Discussion

Fleas are holometabolous insects with an uncertain taxonomic classification. This is due to the extreme morphological specialization and the use of the quetotaxy, and the complex genitalia as the main differential diagnostic criteria. Nevertheless, phenotypic characters are influenced by different external factors and there might be synonymies among the described Siphonaptera species reflecting an accepted species being found in a different host and determined as a new species when host species and external factors influence results in a flea with different morphological characteristics. Thus, Marrugal *et al*. (2013) found in *C. felis*, collected from dogs from different geographical locations, four populations with different morphological characteristics which did not correspond with molecular differences. These authors concluded that ITS1 region is a useful tool to approach different taxonomic and phylogenetic questions in *Ctenocephalides* species and they found clear molecular differences between *C. felis* and *C. canis*. In addition, they detected some specific recognition sites for endonucleases in order to differentiate both species.

In the present work, *S. t. tripectinata* isolated from *M. musculus* from different islands from the Canary Islands was studied by amplification and sequencing of ribosomal (ITS1 and ITS2, and 18S rRNA partial gene) and mitochondrial (*cox1* partial gene) DNA markers.

The differences in length in the ITS1 sequences of *S. t. tripectinata* were due to the presence or absence of nucleotides not only among different populations from different islands but also among different clones of the same individual (intra-individual variation). Nevertheless, the range of percentages of variation observed between different populations was higher than those observed intra-individually (Tables 1 and 2).

The ITS2 sequences were markedly shorter than ITS1. This difference in the length of ITS1 and ITS2 sequences was also observed in triatomines by Bargues *et al*. (2006). The intra-population and inter-population similarity was nearly 100 % and the highest differences were observed between individuals from La Palma (99.4 %). At the inter-population level, it is to be noted that in all sequences analysis (ITS1, ITS2, 18S and *cox1* partial gene), *S. t. tripectinata* populations from the Canary Islands appeared without any particular geographical pattern. ITS2 sequences evolve following the so-called concerted evolution (Smith, 1976) through a process known as molecular drive (Dover, 2002). Molecular drive, involving genomic turnover mechanisms and population dynamic processes, make it possible to homogenize and fix a particular repeat variant within each single reproductive unit. This leads to a lower degree of divergence within than between populations and/or species. This phenomenon clearly explains the lack of nucleotide variation within analyzed populations of *S. t. tripectinata* from different islands of the Canary Islands (see Tables 2, 3 and 4). This result seems to be consistent with other studies of Dipteran species that suggested that ITS2 cannot be utilized in differentiation of geographical populations of some blowfly species (Zaidi et al., 2011).

ITS1 and ITS2 sequences of different species of genus *Stenoponia* were not available in GenBank, thus, we could not confirm that the approach employed here is useful to distinguish species within this genus as cited by other authors for the genus *Ctenocephalides* (Marrugal et al., 2013; Vobis et al., 2004).

The phylogenetic analysis was carried out considering different outgroups (Diptera) but we had problems in performing a multiple alignment correctly. Thus, to address this problem and the absence of other ITS sequences that affects *Stenoponia* genus, 18S rRNA partial gene was sequenced and compared.

Whiting *et al*. (2008) based on 28S, 18S, COII and EF1-α markers reported the monophily of Stenoponiinae and Rhadinopsyllinae and placed both subfamilies as sister groups but with limited support. In our results, the 18S partial gene tree topology showed Rhadinopsyllinae clustered together with all the subfamilies and tribes included in family Ctenophthalmidae while Stenoponiinae clustered with Ceratophyllidae and Leptopsyllidae.

Furthermore, *cox1* mtDNA partial gene sequences clustered all the populations from the Canary Islands and from the Iberian Peninsula with high support. Nevertheless, island populations showed a lower polymorphism than those from the Iberian Peninsula population. Island populations have shown to have lower levels of genetic variation than those populations from mainland (Dietzen *et al*., 2006). These two geographical lineages (Iberian Peninsula and Canary Islands) could have arisen due to the existence of geographical barriers.

The *cox1* partial gene phylogenetic tree showed subfamily Stenoponiinae clustering all the species of *Stenoponia* from different geographical origins and in polytomy with Pygiopsyllidae, Ctenophthalmidae and Pulicidae.

This seems to suggest a new status for subfamily Stenoponiinae that was not related with family Ctenophthalmidae, and the suggestion of a new family: Stenoponiidae including species of the genus *Stenoponia*. Unfortunately, 18S partial gene and *cox1* partial gene phylogenetic trees did not resolve at higher taxonomic levels. Furthermore, no other ITS1 sequences of *Stenoponia*, and related genera molecular data are available in GenBank for intra-generic comparisons.

In conclusion, ITS1 and ITS2 sequences were used as molecular markers to characterize *S. t. tripectinata*, while 18S rRNA partial gene and *cox1* mtDNA partial gene assess the

296 monophyletic origin of Stenoponiinae and a different genetic lineage from
297 Ctenophthalmidae. Thus, the elevation of subfamily Stenoponiinae to family level
298 (Stenoponiidae) would be considered. Nevertheless, we must be expecting since the
299 molecular studies in Siphonaptera are scarce and the number of sequences of
300 Siphonaptera in GenBank is low. Thus, the lack of knowledge of mitochondrial and
301 ribosomal genomics for this group is a major limitation for phylogenetic studies.
302 Furthermore, *cox1* sequences revealed two different genetic lineages: the Canary Islands
303 and the Iberian Peninsula, both being separated by specific restriction endonucleases.

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References

Acosta, R. (2005) Relationship host-parasite in fleas (Insecta: Siphonaptera) and rodents (Mammalia: Rodentia) from Querétaro state, México. *Folia Entomologica Mexicana* **44**, 37-47.

Ballard, J.W.O. & Whitlock, M.C. (2004) The incomplete natural history of mitochondria. *Molecular Ecology* **13**, 729-744.

Bargues, M.D., Klisiowicz, D.R., Panzera, F., Noireau, F., Marcilla, A., Perez, R., Rojas, M.G., O´Connor, J.E., Gonzalez-Candelas, F., Galvão, C., Jurberg, J., Carcavallo, R.U., Dujardin, J.P. & Mas-Coma, S. (2006) Origin and phylogeography of the Chagas disease main vector *Triatoma infestans* based on nuclear rDNA sequences and genome size. *Infection, Genetics and Evolution* **6**, 46-62.

Beaucournu, J.C. & Launay, H. (1990) Les Puces (Siphonaptera) de France et du Bassin méditerranéen occidental. Faune de France, 76, Paris. *Fédération Française des Sociétés de Sciences Naturelles* pp. 548.

Bitam, I., Dittmar, K., Parola, P., Whiting, M.F. & Raoult, D. (2010) Fleas and flea-borne diseases. *International Journal of Infectious Diseases* **14**, 667-676.

Cutillas, C., Callejón, R., de Rojas, M., Tewes, B., Ubeda, J.M., Ariza, C. & Guevara, D.C. (2009) *Trichuris suis* and *Trichuris trichiura* are different nematode species. *Acta Tropica* **111**, 299-307.

De Rojas, M., Úbeda, J.M., Cutillas, C., Mora, D., Ariza, C. & Guevara, D.C. (2007) Utility of ITS1-5.8S-ITS2 and 16S mitochondrial DNA sequences for species identification and phylogenetic inference within the genus *Rhinonyssus*: the *Rhinonyssus coniventris* complex. *Parasitology Research* **100**, 1041-1046.

Dietzen, C., Voigt, C., Wink, M., Gahr, M. & Leitner, S. (2006) Phylogeography of island Canary (*Serinus canaria*) populations. *Journal of Ornithology* **147**, 485-494.

Dover, G.A. (2002) Molecular drive. *Trends Genetics* **18**, 587-589.

Dunnet, G.M. & Mardon, D.K. (1999) Siphonaptera, the insects of Australia: a textbook for students and research workers, 2nd ed. Csiro and Melbourne University Press, Melbourne pp. 125-140.

Essig, A., Rinder, H., Gothe, R. & Zahler, M. (1999) Genetic differentiation of mites of the genus *Chorioptes* (Acari: Psoroptidae). *Experimental and Applied Acarology* **23**, 309-18.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783-791.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R., (1994) DNA primers for amplification of mitochondrial *cytochrome c oxidase* subunit I from diverse metazoan invertebrates. *Molecular Marine Biology Biotechnology* **3**, 294-299.

Gasser, R.B., Nansen, P. & Guldberg, P. (1996) Fingerprinting sequence variation in ribosomal DNA of parasites by DGGE. *Molecular and Cellular Probes* **10**, 99-105.

Guindon, S. & Gascuel, O. (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**, 696-704.

349 Hebert, P.D.N., Cywinska, A., Ball, S.L. & DeWaard, J.R. (2003) Biological identifica-
350 tions of birds through DNA barcodes. *Proceedings of the Royal Society B* **270**, 313-321.

351 Hopkins, G.H.E. & Rothschild, M. (1962) An illustrated catalogue of the Rothschild col-
352 lection of fleas (Siphonaptera) in the British Museum (Natural History). Vol. 3. Hystri-
353 chopsyllidae. *Trustees of the British Museum (Natural History)*, London.

354 Huelsenbeck, J.P. & Rannala, B. (1997) Phylogenetic methods come of age: testing hy-
355 potheses in an evolutionary context. *Science* **276**, 227-232.

356 Hurst, G.D.D. & Jiggins, F.M. (2005) Problems with mitochondrial DNA as a marker in
357 population, phylogeographic and phylogenetic studies: the effects of inherited symbionts.
358 *Proceedings of the Royal Society* B **272**, 1525-1534.

359 Jordan, K. (1958) A contribution to the taxonomy of *Stenoponia* J. & R. (1911), a genus
360 of Palaeartic and Neartic fleas. *British Museum (Natural History)* **6**, 169-202.

361 Kaewmongkol, G., Kaewmongkol, S., Mclnnes, L.M., Burmej, H., Bennet, M.D., Adams,
362 P.J., Ryan, U., Irwin, P.J. & Fenwick, S.G. (2011) Genetic characterization of flea derived
363 *Bartonella* species from native animals in Australia suggest host-parasite co evolution.
364 *Infection, Genetics and Evolution* **11**, 1868-1872.

365 Larkin, M.A., Blackshields, G. & Brown, N.P. (2007) Clustal W and Clustal X version
366 2.0. *Bioinformatics* **23**, 2947-2948.

367 Lewis, R.E. (1993a) Notes on the geographical distribution and host preferences in the
368 order Siphonaptera. Part 8. New taxa described between 1984 and 1990, with a current
369 classification of the order. *Entomological Society of America* **30**, 239-256.

Lewis, R.E. (1993b) Fleas (Siphonaptera). In Medical Insects and Arachnids (Lane, R.P. & Crosskey, R.W., eds), pp. 529-575. Chapman and Hall, London.

Lewis, R.E. (1998) Résumé of the Siphonaptera (Insecta) of the World. *Journal of Medical Entomology* **35**, 377-389.

Marcilla, A., Bargues, M.D., Abad-Franch, F., Panzera, F., Carcavallo, R.U., Noireau, F., Galvão, C., Jurberg, J., Miles, M.A., Dujardin, J.P. & Mas-Coma, S. (2002) Nuclear rDNA ITS-2 sequences reveal polyphyly of *Panstrongylus* species (Hemiptera: Reduviidae: Triatominae), vectors of *Trypanosoma cruzi*. *Infection, Genetics and Evolution* **1**, 225-35.

Marrugal, A., Callejón, R., de Rojas, M., Halajian, A. & Cutillas, C., (2013) Morphological, biometrical and molecular characterization of *Ctenocephalides felis* and *Ctenocephalides canis* isolated from dogs from different geographical regions. *Parasitology Research* **112**, 2289-2298.

Medvedev, S.G. (1998) Classification on fleas (Order Siphonaptera) and its theoretical foundations. *Entomological Review* **78**, 1080-1093.

Monje, L.D., Quiroga, M., Manzoli, D., Couri, M.S., Silvestri, L., Venzal, J.M., Cuervo, P. & Beldomenico, P.M. (2013) Sequence analysis of the internal transcribed spacer 2 (ITS2) from *Philornis seguyi* (García, 1952) and *Philornis torquans* (Nielsen, 1913) (Diptera: Muscidae). *Systematic Parasitology* **86**, 41-53.

Posada, D. & Buckley, T.R. (2004) Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Systematic Biology* **53,** 793-808.

392 Posada, D. (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and*
393 *Evolution* **25**, 1253-1256.

394 Sánchez, S. & Gómez, M.S. (2012) Presence of *Stenoponia tripectinata* (Tiraboschi,
395 1902) (Siphonaptera, Ctenophtalmidae) in murine (Rodentia) from the Canary Islands.
396 *Acta Parasitologica* **57**, 190-193.

397 Smith, G.P. (1976) Evolution of repeated DNA sequences by unequal crossover. *Science*
398 **191**, 528-535.

399 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011) MEGA5:
400 Molecular evolutionary genetics analysis using maximum likelihood, evolutionary dis-
401 tance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**, 2731-
402 2739.

403 Vobis, M., D´Haese, J., Mehlhorn, H., Mencke, N., Blagburn, B.L., Bond, R., Denholm,
404 I., Dryden, M.W., Payne, P., Rust, M.K., Schroeder, I., Vaughn, M.B. & Bledsoe, D.
405 (2004) Molecular phylogeny of isolates of *Ctenocephalides felis* and related species based
406 on analysis of ITS1, ITS2 and mitochondrial 16S rDNA sequences and random binding
407 primers. *Parasitology Research* **94**, 219-226.

408 Whiting, M.F. (2002) Mecoptera is paraphyletic: multiple genes and phylogeny of
409 Mecoptera and Siphonaptera. *Zoologica scripta* **31**, 93-104.

410 Whiting, M.F., Whiting, A.S., Hastriter, M.W. & Dittmar, K. (2008) A molecular phy-
411 logeny of fleas (Insecta: Siphonaptera): origins and host associations. *Cladistics* **24**, 677-
412 707.

413 Zagoskin, M.V., Lazareva, V.I., Grishanin, A.K. & Mukha, D.V. (2014) Phylogenetic
414 information content of Copepoda ribosomal DNA repeat units: ITS1 and ITS2 impact.
415 *BioMed Research International* **2014**, ID: 926342.

416 Zaidi, F., Wei, S.J., Shi, M. & Chen, X.X. (2011) Utility of multi-gene loci for forensic
417 species diagnosis of blowflies. *Journal of insect science* **11**, 59.

418 **Figure captions**

419 Figure 1. Phylogenetic tree of *Stenoponia tripectinata tripectinata* from different
420 geographical origins (see Table 1) based on 18S partial gene of ribosomal RNA inferred
421 using the Bayesian (B), Maximum Likelihood (ML), Maximum Parsimony (MP) and
422 Neighbor-Joining (NJ) methods and Bayesian topology. The percentage of replicate trees
423 in which the associated taxa clustered together in the bootstrap test (1.000 replicates) is
424 shown onto the branches (B/ML/MP/NJ). Bootstrap values lower than 60 % are not
425 shown. The Bayesian Posterior Probabilities (BPP) are percentage converted.

426 Figure 2. Phylogenetic tree of *Stenoponia tripectinata tripectinata* from different
427 geographical origins (see Table 1) based on cytochrome c-oxidase 1 (*cox1*) partial gene
428 of mitochondrial DNA inferred using the Bayesian (B), Maximum Likelihood (ML),
429 Maximum Parsimony (MP) and Neighbor-Joining (NJ) methods and Bayesian topology.
430 The percentage of replicate trees in which the associated taxa clustered together in the
431 bootstrap test (1.000 replicates) is shown onto the branches (B/ML/MP/NJ). Bootstrap
432 values lower than 60 % are not shown. The Bayesian Posterior Probabilities (BPP) are
433 percentage converted.

434 Figure 3. Specific restriction endonucleases observed in the *cox1* sequences of *Stenoponia*
435 *tripectinata tripectinata* from the Canary Archipelago and the Iberian Peninsula.

Figure 1


61/75/75/65
Conorhinopsylla stanfordi EU336127
Megarthroglossus divisus AF286276
Corypsylla ornata EU336092
Jordanopsylla becki EU336071
Carteretta clavata EU336072
Chiliopsylla allophyla allophyla EU336094
Neotyphloceras crassispina chilensis EU336079
Dinopsyllus ellobius EU336070
Catallagia borealis EU336100
Catallagia sp. AF423890
Catallagia decipiens EU336058
Epitedia wenmanni EU336033
Neopsylla bidentatiformis EU336074
Meringis hubbardi AF423891
Meringis dipodomys EU336032
Rhadinopsylla difficilis EU336044
Rhadinopsylla masculana EU336056
Corrodopsylla birulai EU336063
Corrodopsylla curvata curvata EU336064
Nearctopsylla traubi EU336093
Ctenophthalmidae
Neopsyllinae
Rhadinopsyllinae
Doratopsyllinae
63/83/-/-
100/97/-/80
98/90/62/60
100/99/99/100
100/92/-/-
Stenoponia americana AF423893
Stenoponia sidimi EU336078
Stenoponia tripectinata medialis EU336050
Stenoponia tripectinata tripectinata (Gran Canaria) LK937066
Stenoponia tripectinata tripectinata (El Hierro) LK937067
Stenoponia tripectinata tripectinata (La Palma) LK937068
Stenoponia tripectinata tripectinata (La Gomera) LK937069
Stenoponia tripectinata tripectinata (Tenerife) LK937070
Stenoponiinae
80/77/-/62
87/84/-/-
Aetheca wagneri EU336040
Ceratophyllus gallinae EU336041
Ceratophyllus petrochelidoni AF423888
Citellophilus tesquorum mongolicus EU336103
Dactylopsylla bluei bluei EU336124
Foxella ignota EU336030
Ceratophyllidae
74/78/-/61
100/93/-/-
Ophthalmopsylla jettmari EU336101
Ophthalmopsylla volgensis palestinica AF423895
Amphipsylla sibirica sibirica EU336061
Leptopsylla segnis DQ298442
Peromyscopsylla hamifer viaens EU336104
Peromyscopsylla selensis EU336043
Leptopsyllidae
100/97/84/83
100/96/68/81
Jellisonia guerrerensis EU336107
Ceratophyllidae
Ctenophthalmus pseudagyrtes AF423892
Ctenophthalmus sanborni EU336067
Ctenophthalmus formosanus EU336131
Anomiopsyllus amphibolus EU336036
Stenistomera alpina EU336037
Ctenophthalminae
Ctenophthalmidae
100/100/99/100
70/74/-/-
Nannochorista dipteroides AF334796
Nannochoristidae
Boreus coloradensis AF286285
Boreidae
0.2

Figure 2

FIGURE 2

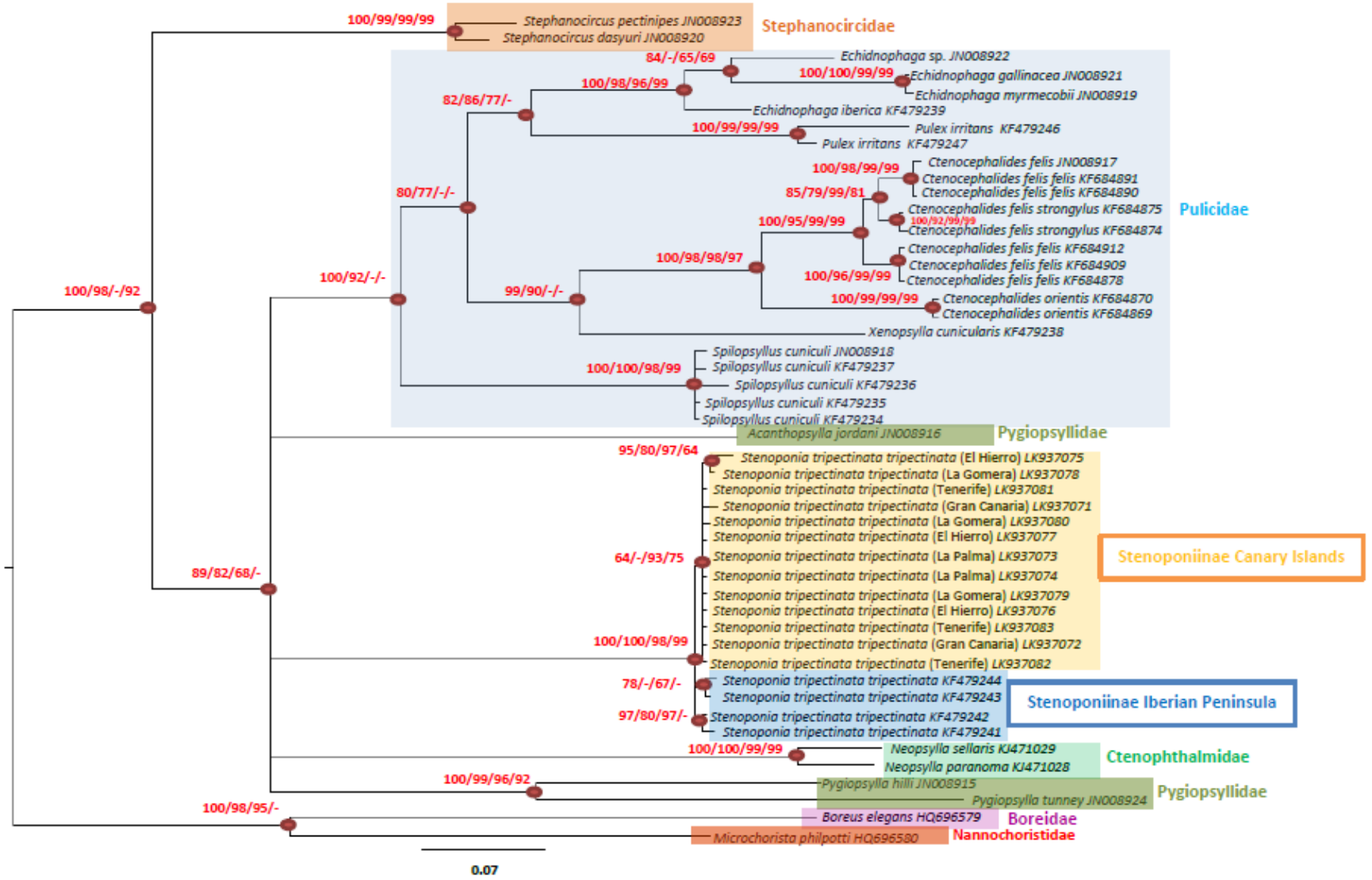

Figure 3

FIGURE 3

Stenoponia tripectinata tripectinata (the Canarian archipelago)

AseI **at|taat** 209
MseI **t|taa** 209
VspI **at|taat** 209

181 ATTTTAATTGGAGGATTTGGAAATTG**ATTAAT**TCCTTTAATACTTGGAGCTCCTGATATA
181 190 200 210 220 230
181 TAAAATTAACCTCCTAAACCTTTAAC**TAATTA**AGGAAATTATGAACCTCGAGGACTATA

S. t. tripectinata (the Iberian Peninsula)

181 ATTTTAATTGGAGGATTTGGAAATTG**ATTAGT**TCCTTTAATACTTGGAGCTCCTGATATA
181 190 200 210 220 230
181 TAAAATTAACCTCCTAAACCTTTAAC**TAATCA**AGGAAATTATGAACCTCGAGGACTATA

Table 1. GenBank accession numbers of ITS1, ITS2, *cox1* partial gene and *18S* partial gene sequences of individuals of *Stenoponia tripectinata tripectinata* isolated of *Mus musculus* from the Canary Islands (Spain).

ITS1 *S. t. tripectinata*			
Island	Number of base pairs (bp)	G+C %	Accession number
Gran Canaria	1205	52.4	LK937051
Gran Canaria	1205	52.5	LK937052
Gran Canaria	1205	52.6	LK937053
Gran Canaria (Clone 1)	1205	52.5	LN847260
Gran Canaria (Clone 2)	1205	52.5	
Gran Canaria (Clone 3)	1205	52.6	LN847261
Gran Canaria (Clone 4)	1205	52.3	LN847262
La Palma	1204	52.4	LK937054
La Palma	1205	52.7	LK937055
La Palma	1205	52.5	LK937056
El Hierro	1207	52.3	LK937057
El Hierro	1209	52.5	LK937058
El Hierro	1205	52.5	LK937059
La Gomera	1205	52.5	LK937060
La Gomera	1205	52.6	LK937061
La Gomera	1205	52.6	LK937062
Tenerife	1205	52.5	LK937063
Tenerife	1205	52.5	LK937064
Tenerife	1207	52.5	LK937065
ITS2 *S. t. tripectinata*			
Island	Number of base pairs (bp)	G+C %	Accession number
Gran Canaria	332	48.2	LK937035
Gran Canaria	332	48.2	LK937036
Gran Canaria	332	48.2	LK937037
Gran Canaria	332	48.2	LK937038
Gran Canaria (Clone 1)	332	48.2	LN847258
Gran Canaria (Clone 2)	332	48.2	
Gran Canaria (Clone 3)	332	48.2	
Gran Canaria (Clone 4)	332	48.2	
Gran Canaria (Clone 5)	332	48.2	
Gran Canaria (Clone 6)	332	48.2	
Gran Canaria (Clone 7)	332	47.9	LN847259
La Palma	332	48.2	LK937039
La Palma	332	47.9	LK937040
La Palma	332	48.2	LK937041
El Hierro	332	48.2	LK937042
El Hierro	332	48.2	LK937043
El Hierro	332	48.3	LK937044
La Gomera	332	48.2	LK937045
La Gomera	332	48.2	LK937046
La Gomera	332	48.2	LK937047
Tenerife	332	48.2	LK937048
Tenerife	332	48.2	LK937049
Tenerife	332	48.2	LK937050
cox1 S. t. tripectinata			
Island	Number of base pairs (bp)	G+C %	Accession number
Gran Canaria	677	28.8	LK937071
Gran Canaria	677	28.6	LK937072
La Palma	677	29	LK937073
La Palma	677	28.8	LK937074
El Hierro	677	28.8	LK937075
El Hierro	677	28.8	LK937076
El Hierro	677	28.8	LK937077
La Gomera	677	28.6	LK937078
La Gomera	677	28.9	LK937079
La Gomera	677	28.8	LK937080
Tenerife	677	28.8	LK937081
Tenerife	677	28.8	LK937082
Tenerife	677	28.8	LK937083
18S *S. t. tripectinata*			
Island	Number of base pairs (bp)	G+C %	Accession number
Gran Canaria	1095	50.4	LK937066
La Palma	1098	50.5	LK937068
El Hierro	1096	50.5	LK937067
La Gomera	1096	50.5	LK937069
Tenerife	1096	50.5	LK937070

Table 2. Intra-individual, intra-population (*) and inter-population similarity observed in ITS1 sequences in *Stenoponia tripectinata tripectinata* populations isolated from different islands from the Canary Islands (Spain).

ITS1	GRAN CANARIA	EL HIERRO	LA GOMERA	LA PALMA	TENERIFE
GRAN CANARIA	Intra-individual 99.6-100% Intra-population (*) 99.2-99.8				
EL HIERRO	99-100%	99.2-99.8%*			
LA GOMERA	98.9-99.9%	99-99.9%	99.2-99.8%*		
LA PALMA	99.9-100%	99.2-99.8%	99-99.8%	99.2-99.6%*	
TENERIFE	99.6-100%	99.4-100%	99.3-99.9%	99.4-99.6%	100%*

Table 3

Table 3. Intra-individual, intra-population (*) and inter-population similarity observed in ITS2 sequences in *Stenoponia tripectinata tripectinata* populations isolated from different islands from the Canary Islands (Spain).

ITS2	GRAN CANARIA	EL HIERRO	LA GOMERA	LA PALMA	TENERIFE
GRAN CANARIA	Intra-individual 99.7-100% Intra-population (*) 100%				
EL HIERRO	100%	100% (*)			
LA GOMERA	100%	100%	100% (*)		
LA PALMA	99.7-100%	99.7-100%	99.7-100%	99.4-99.7% (*)	
TENERIFE	100%	100%	100%	99.7-100%	100% (*)

Table 4

Table 4. Intra-population (*) and inter-population similarity observed in *cox1* mtDNA partial gene sequences in *Stenoponia tripectinata tripectinata* populations isolated from different islands from the Canary Islands (Spain) and the Iberian Peninsula.

COX1	GRAN CANARIA	EL HIERRO	LA GOMERA	LA PALMA	TENERIFE	*Stenoponia tripectinata tripectinata* KF479241	*Stenoponia tripectinata tripectinata* KF479242	*Stenoponia tripectinata tripectinata* KF479243	*Stenoponia tripectinata tripectinata* KF479244
GRAN CANARIA	99.7% (*)								
EL HIERRO	99.7-100%	100% (*)							
LA GOMERA	99.6-100%	99.9-100%	99.7-99.9% (*)						
LA PALMA	99.6-100%	99.9-100%	99.7-100%	99.9% (*)					
TENERIFE	99.7-100%	100%	99.9-100%	99.9-100%	100% (*)				
Stenoponia tripectinata tripectinata KF479241	99.1-99.4%	99.4%	99.2-99.4%	99.2-99.4%	99.4%	-			
Stenoponia tripectinata tripectinata KF479242	99.4-99.7%	99.4%	99.2-99.4%	99.6-99.7%	99.7%	99.7%	-		
Stenoponia tripectinata tripectinata KF479243	99.2-99.6%	99.6%	99.4-99.6%	99.4-99.6%	99.6%	99.2%	99.6%	-	
Stenoponia tripectinata tripectinata KF479244	98.9-99.2%	99.2%	99.1-99.2%	99.1-99.2%	99.2%	99.2%	99.2%	99.7%	-

Appendix

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

Specie	Family	Host	Accession number	Gen Region
Aetheca wagneri	Ceratophyllidae	*Peromyscus maniculatus*	EU336040	18S
Ceratophyllus gallinae	Ceratophyllidae	*Woodpecker nest*	EU336041	18S
Ceratophyllus petrochelidoni	Ceratophyllidae	*Petrochelidon pyrrhonota*	AF423888	18S
Citellophilus tesquorum mongolicus	Ceratophyllidae	*Citellus dauricus dauricus*	EU336103	18S
Dactylopsylla bluei bluei	Ceratophyllidae	*Thomomys bottae*	EU336124	18S
Foxella ignota	Ceratophyllidae	*Thomomys* sp.	EU336030	18S
Jellisonia guerrerensis	Ceratophyllidae	*Oryzomys alfaroi*	EU336107	18S
Amphipsylla sibirica sibirica	Leptopsyllidae	*Clethrionomys rutilus*	EU336061	18S
Peromyscopsylla hamifer vigens	Leptopsyllidae	*Microtus* sp.	EU336104	18S
Peromyscopsylla selensis	Leptopsyllidae	*Peromyscus maniculatus*	EU336043	18S
Leptopsylla segnis	Leptopsyllidae	Unknown	DQ298442	18S
Ophthalmopsylla jettmari	Leptopsyllidae	*Citellus dauricus dauricus*	EU336101	18S
Ophthalmopsylla volgensis palestinica	Leptopsyllidae	*Jaculus jaculus*	AF423895	18S
Stenoponia americana	Ctenophthalmidae	*Peromyscus leucopus*	AF423893	18S
Stenoponia sidimi	Ctenophthalmidae	*Clethrionomys rufocans*	EU336078	18S
Stenoponia tripectinata medialis	Ctenophthalmidae	*Gerbillus dasyurus*	EU336050	18S
Catallagia borealis	Ctenophthalmidae	*Clethrionomys gapperi*	EU336100	18S
Catallagia decipiens	Ctenophthalmidae	*Microtus* sp.	EU336058	18S
Neopsylla bidentatiformis	Ctenophthalmidae	*Cricetulus triton*	EU336074	18S
Ctenophthalmus pseudagyrtes	Ctenophthalmidae	*Condylura cristata*	AF423892	18S
Ctenophthalmus sanborni	Ctenophthalmidae	*Habromys lophurus*	EU336067	18S
Megarthroglossus divisus	Ctenophthalmidae	*Tamiasciurus hudsonicus*	AF286276	18S
Conorhinopsylla stanfordi	Ctenophthalmidae	*Glaucomys volans*	EU336127	18S
Rhadinopsylla difficilis	Ctenophthalmidae	*Vole nest*	EU336044	18S
Rhadinopsylla masculana	Ctenophthalmidae	*Gerbillus dasyurus*	EU336056	18S
Jordanopsylla becki	Ctenophthalmidae	*Neotoma lepida*	EU336071	18S
Carteretta clavata	Ctenophthalmidae	*Peromyscus eremiens*	EU336072	18S
Corypsylla ornata	Ctenophthalmidae	*Scapanus orarius*	EU336092	18S
Anomiopsyllus amphibolus	Ctenophthalmidae	*Peromyscus eremicus*	EU336036	18S
Stenistomera alpina	Ctenophthalmidae	*Neotoma lepida*	EU336037	18S
Chiliopsylla allophyla allophyla	Ctenophthalmidae	Unknown	EU336094	18S
Ctenophthalmus formosanus	Ctenophthalmidae	*Eothenomys melanogaster*	EU336131	18S
Neotyphloceras crassispina chilensis	Ctenophthalmidae	*Abrocoma bennetti*	EU336079	18S
Dinopsyllus ellobius	Ctenophthalmidae	*Mastomys natalensis*	EU336070	18S
Corrodopsylla birulai	Ctenophthalmidae	*Sorex araneus*	EU336063	18S
Corrodopsylla curvata curvata	Ctenophthalmidae	*Sorex* sp.	EU336064	18S
Catallagia sp.	Ctenophthalmidae	*Scapanus orarius*	AF423890	18S
Epitedia wenmanni	Ctenophthalmidae	*Thomomys bottae*	EU336033	18S

Meringis dipodomys	Ctenophthalmidae	*Dipodomys merriami*	EU336032	18S
Meringis hubbardi	Ctenophthalmidae	*Perognathus parvus*	AF423891	18S
Nearctopsylla traubi	Ctenophthalmidae	*Scapanus townsendii*	EU336093	18S
Nannochorista dipteroides	Nannochoristidae	Unknown	AF334796	18S
Boreus coloradensis	Boreidae	Unknown	AF286285	18S
Echidnophaga sp.	Pulicidae	*Oryctolagus cuniculus*	JN008922	*Cox1*
Echidnophaga gallinacea	Pulicidae	*Oryctolagus cuniculus*	JN008921	*Cox1*
Echidnophaga myrmecobii	Pulicidae	*Oryctolagus cuniculus*	JN008919	*Cox1*
Echidnophaga iberica	Pulicidae	*Oryctolagus cuniculus*	KF479239	*Cox1*
Pulex irritans	Pulicidae	*Meles meles*	KF479246	*Cox1*
Pulex irritans	Pulicidae	*Meles meles*	KF479247	*Cox1*
Spilopsyllus cuniculi	Pulicidae	*Felis silvestris catus*	JN008918	*Cox1*
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	KF479234	*Cox1*
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	KF479235	*Cox1*
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	KF479236	*Cox1*
Spilopsyllus cuniculi	Pulicidae	*Oryctolagus cuniculus*	KF479237	*Cox1*
Ctenocephalides felis	Pulicidae	*Vulpes vulpes*	JN008917	*Cox1*
Xenopsylla cunicularis	Pulicidae	*Oryctolagus cuniculus*	KF479238	*Cox1*
Ctenocephalides felis felis	Pulicidae	*Canis lupus familiaris*	KF684891	*Cox1*
Ctenocephalides felis felis	Pulicidae	*Canis lupus familiaris*	KF684890	*Cox1*
Ctenocephalides felis felis	Pulicidae	*Canis lupus familiaris*	KF684912	*Cox1*
Ctenocephalides felis felis	Pulicidae	*Canis lupus familiaris*	KF684909	*Cox1*
Ctenocephalides felis felis	Pulicidae	*Canis lupus familiaris*	KF684878	*Cox1*
Ctenocephalides felis strongylus	Pulicidae	*Canis lupus familiaris*	KF684875	*Cox1*
Ctenocephalides felis strongylus	Pulicidae	*Canis lupus familiaris*	KF684874	*Cox1*
Ctenocephalides orientis	Pulicidae	*Canis lupus familiaris*	KF684870	*Cox1*
Ctenocephalides orientis	Pulicidae	*Canis lupus familiaris*	KF684869	*Cox1*
Acanthopsylla jordani	Pygiopsyllidae	*Antechinus flavipes*	JN008916	*Cox1*
Pygiopsylla tunney	Pygiopsyllidae	*Perameles bougainville*	JN008924	*Cox1*
Pygiopsylla hilli	Pygiopsyllidae	*Bettongia penicillata*	JN008915	*Cox1*
Stephanocircus pectinipes	Stephanocircidae	*Rattus fuscipes*	JN008923	*Cox1*
Stephanocircus dasyuri	Stephanocircidae	*Rattus fuscipes*	JN008920	*Cox1*
Neopsylla sellaris	Ctenophtalmidae	*Canis lupus familiaris*	KJ471029	*Cox1*
Neopsylla paranoma	Ctenophtalmidae	*Canis lupus familiaris*	KJ471028	*Cox1*
Stenoponia tripectinata tripectinata	Ctenophthalmidae	*Apodemus sylvaticus*	KF479241	*Cox1*
Stenoponia tripectinata tripectinata	Ctenophthalmidae	*Apodemus sylvaticus*	KF479242	*Cox1*
Stenoponia tripectinata tripectinata	Ctenophthalmidae	*Apodemus sylvaticus*	KF479243	*Cox1*
Stenoponia tripectinata tripectinata	Ctenophthalmidae	*Apodemus sylvaticus*	KF479244	*Cox1*
Boreus elegans	Boreidae	Unkonwn	HQ696579	*Cox1*
Microchorista philpotti	Nannochoristidae	Unknown	HQ696580	*Cox1*

Appendix 2. Alignment (by CLUSTALW) of the different ITS1 clones nucleotide sequences of *Stenoponia tripectinata tripectinata* from *Mus musculus* from Gran Canaria. Polymorphism is remarked by bold letter. The ITS1 clones sequence data have been deposited in GenBank database under the following Accession Numbers: LN847260, LN847261 and LN847262.

```
CLONE1          TGTTGTCTGACGACGACATTGAAATTGAACGACGTATTACGATGGACGGCGTTCTCATTG 60
CLONE2          TGTTGTCTGACGACGACATTGAAATTGAACGACGTATTACGATGGACGGCGTTCTCATTG 60
CLONE3          TGTTGTCTGACGACGACATTGAAATTGAACGACGTATTACGATGGACGGCGTTCTCATTG 60
CLONE4          TGTTGTCTGACGACGACATTGAAATTGAACGACGTATTACGATGGACGGCGTTCTCATTG 60
                ************************************************************

CLONE1          ATCGCCGACCGGAGTACGTCGATGGAACGGGACTCTTCTAGAGGACCGAGCGCCTCCCGC 120
CLONE2          ATCGCCGACCGGAGTACGTCGATGGAACGGGACTCTTCTAGAGGACCGAGCGCCTCCCGC 120
CLONE3          ATCGCCGACCGGAGTACGTCGATGGAACGGGACTCTTCTAGAGGACCGAGCGCCTCCCGC 120
CLONE4          ATCGCCGACCGGAGTACGTCGATGGAACGGGACTCTTCTAGAGGACCGAGCGCCTCCCGC 120
                ************************************************************

CLONE1          CGTGTACGGCTTCGTGCTCTACCGGCGGGAAAATATGGAACGGGACTCTTCTCGAGGACC 180
CLONE2          CGTGTACGGCTTCGTGCTCTACCGGCGGGAAAATATGGAACGGGACTCTTCTCGAGGACC 180
CLONE3          CGTGTACGGCTTCGTGCTCTACCGGCGGGAAAATATGGAACGGGACTCTTCTCGAGGACC 180
CLONE4          CGTGTACGGCTTCGTGCTCTACCGGCGGGAAAATATGGAACGGGACTCTTCTCGAGGACC 180
                ************************************************************

CLONE1          GAGCGCCTCCCGCCGCGTACGGCTTCGTGCTGTATCGGCGGGAAAATATGGAACGGGATT 240
CLONE2          GAGCGCCTCCCGCCGCGTACGGCTTCGTGCTGTATCGGCGGGAAAATATGGAACGGGATT 240
CLONE3          GAGCGCCTCCCGCCGCGTACGGCTTCGTGCTGTATCGGCGGGAAAATATGGAACGGGATT 240
CLONE4          GAGCGCCTCCCGCCGCGTACGGCTTCGTGCTGTATCGGCGGGAAAATATGGAACGGGATT 240
                ************************************************************

CLONE1          CATACGGGCACATAGCCCCGAAGACCGAGCGCTTCGACGGAGAGATTTTATTATTTCTCT 300
CLONE2          CATACGGGCACATAGCCCCGAAGACCGAGCGCTTCGACGGAGAGATTTTATTATTTCTCT 300
CLONE3          CATACGGGCACATAGCCCCGAAGACCGAGCGCTTCGACGGAGAGATTTTATTATTTCTCT 300
CLONE4          CATACGGGCACATAGCCCCGAAGACCGAGCGCTTCGACGGAGAGATTTTATTATTTCTCT 300
                ************************************************************

CLONE1          GTCGCTATATGCACATCTGTTCTGTGTGGGAACGGTGTCGCACACTGCTACGTATCATCC 360
CLONE2          GTCGCTATATGCACATCTGTTCTGTGTGGGAACGGTGTCGCACACTGCTACGTATCATCC 360
CLONE3          GTCGCTATATGCACATCTGTTCTGTGTGGGAACGGTGTCGCACACTGCTACGTATCATCC 360
CLONE4          GTCGCTATATGCACATCTGTTCTGTGTGGGAACGGTGTCGCACACTGCTACGTATCATCC 360
                ************************************************************

CLONE1          GATGCGTGAGCGAATAGTGGAGCTTCGCTTAGTTGCGTTCTCTCAAATGATTCCAGACG**T** 420
CLONE2          GATGCGTGAGCGAATAGTGGAGCTTCGCTTAGTTGCGTTCTCTCAAATGATTCCAGACG**T** 420
CLONE3          GATGCGTGAGCGAATAGTGGAGCTTCGCTTAGTTGCGTTCTCTCAAATGATTCCAGACG**C** 420
CLONE4          GATGCGTGAGCGAATAGTGGAGCTTCGCTTAGTTGCGTTCTCTCAAATGATTCCAGACG**T** 420
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CLONE1          GCGGGATCGTATCCGTCTAGTC**C**TAGTACTGTTTTCGTAACGGGAAAGGTACACCGACGG 480
CLONE2          GCGGGATCGTATCCGTCTAGTC**C**TAGTACTGTTTTCGTAACGGGAAAGGTACACCGACGG 480
CLONE3          GCGGGATCGTATCCGTCTAGTC**C**TAGTACTGTTTTCGTAACGGGAAAGGTACACCGACGG 480
CLONE4          GCGGGATCGTATCCGTCTAGTC**T**TAGTACTGTTTTCGTAACGGGAAAGGTACACCGACGG 480
                ********************* ************************************

CLONE1          TACGACGCAAACAACCCCGCGTCGTTTGCTACGTATCATCCGATGCGTGAGCGAATAGTG 540
CLONE2          TACGACGCAAACAACCCCGCGTCGTTTGCTACGTATCATCCGATGCGTGAGCGAATAGTG 540
CLONE3          TACGACGCAAACAACCCCGCGTCGTTTGCTACGTATCATCCGATGCGTGAGCGAATAGTG 540
CLONE4          TACGACGCAAACAACCCCGCGTCGTTTGCTACGTATCATCCGATGCGTGAGCGAATAGTG 540
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CLONE1          GAGCTTCGCTTAGTTGCGTTCTCTTAAATGATATGAAGCGCGCGAACATCGTTCGTGTCT 600
CLONE2          GAGCTTCGCTTAGTTGCGTTCTCTTAAATGATATGAAGCGCGCGAACATCGTTCGTGTCT 600
CLONE3          GAGCTTCGCTTAGTTGCGTTCTCTTAAATGATATGAAGCGCGCGAACATCGTTCGTGTCT 600
CLONE4          GAGCTTCGCTTAGTTGCGTTCTCTTAAATGATATGAAGCGCGCGAACATCGTTCGTGTCT 600
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CLONE1          CGCCGAGTACCAGCGCTATGGGAAAAT**C**GTGACTCGCCTAGTACCGTAAAGGGAAAATCG 660
CLONE2          CGCCGAGTACCAGCGCTATGGGAAAAT**C**GTGACTCGCCTAGTACCGTAAAGGGAAAATCG 660
CLONE3          CGCCGAGTACCAGCGCTATGGGAAAAT**C**GTGACTCGCCTAGTACCGTAAAGGGAAAATCG 660
CLONE4          CGCCGAGTACCAGCGCTATGGGAAAAT**T**GTGACTCGCCTAGTACCGTAAAGGGAAAATCG 660
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CLONE1          TATCGCGGCCAGTACTGTATGTTGGGAAAATCTTGACAGTTCGCTGTTATAACCAGCGCG 720
CLONE2          TATCGCGGCCAGTACTGTATGTTGGGAAAATCTTGACAGTTCGCTGTTATAACCAGCGCG 720
CLONE3          TATCGCGGCCAGTACTGTATGTTGGGAAAATCTTGACAGTTCGCTGTTATAACCAGCGCG 720
CLONE4          TATCGCGGCCAGTACTGTATGTTGGGAAAATCTTGACAGTTCGCTGTTATAACCAGCGCG 720
                ************************************************************

CLONE1          TGATGGGCATAGCTTCCGTTGACGGTTGCGTCGCCAAAAATCTGCAAATATTCCGAGTCA 780
CLONE2          TGATGGGCATAGCTTCCGTTGACGGTTGCGTCGCCAAAAATCTGCAAATATTCCGAGTCA 780
CLONE3          TGATGGGCATAGCTTCCGTTGACGGTTGCGTCGCCAAAAATCTGCAAATATTCCGAGTCA 780
CLONE4          TGATGGGCATAGCTTCCGTTGACGGTTGCGTCGCCAAAAATCTGCAAATATTCCGAGTCA 780
                ************************************************************

CLONE1          CACGGGACTCCGCTCATTGCGGATCCCGGGCGGATGGTTCGTCTGTCGAACCTCAGCTAC 840
CLONE2          CACGGGACTCCGCTCATTGCGGATCCCGGGCGGATGGTTCGTCTGTCGAACCTCAGCTAC 840
CLONE3          CACGGGACTCCGCTCATTGCGGATCCCGGGCGGATGGTTCGTCTGTCGAACCTCAGCTAC 840
CLONE4          CACGGGACTCCGCTCATTGCGGATCCCGGGCGGATGGTTCGTCTGTCGAACCTCAGCTAC 840
                ************************************************************

CLONE1          AGGCCGTGATCGCGGGCATCCTGTTAGCGAAAGTGAACGTTCTCGCTTGATTGCGACGTT 900
CLONE2          AGGCCGTGATCGCGGGCATCCTGTTAGCGAAAGTGAACGTTCTCGCTTGATTGCGACGTT 900
CLONE3          AGGCCGTGATCGCGGGCATCCTGTTAGCGAAAGTGAACGTTCTCGCTTGATTGCGACGTT 900
CLONE4          AGGCCGTGATCGCGGGCATCCTGTTAGCGAAAGTGAACGTTCTCGCTTGATTGCGACGTT 900
                ************************************************************

CLONE1          TCAAATAAAACGGCCGTTACTCGCAACGATTAATTTCGTTCGGGTAGGCAAACATGTCCC 960
CLONE2          TCAAATAAAACGGCCGTTACTCGCAACGATTAATTTCGTTCGGGTAGGCAAACATGTCCC 960
CLONE3          TCAAATAAAACGGCCGTTACTCGCAACGATTAATTTCGTTCGGGTAGGCAAACATGTCCC 960
CLONE4          TCAAATAAAACGGCCGTTACTCGCAACGATTAATTTCGTTCGGGTAGGCAAACATGTCCC 960
                ************************************************************

CLONE1          CCAGACCTCGAAAGTCTTCGGGAAACCAGACAGCACGGCATCCGTTTCGGCTACGTGATT 1020
CLONE2          CCAGACCTCGAAAGTCTTCGGGAAACCAGACAGCACGGCATCCGTTTCGGCTACGTGATT 1020
CLONE3          CCAGACCTCGAAAGTCTTCGGGAAACCAGACAGCACGGCATCCGTTTCGGCTACGTGATT 1020
CLONE4          CCAGACCTCGAAAGTCTTCGGGAAACCAGACAGCACGGCATCCGTTTCGGCTACGTGATT 1020
                ************************************************************

CLONE1          CACGCAATCACACGGATTATTTGA**T**CCACACTATATCTCCAATGTTGGAATATACGAGAC 1080
CLONE2          CACGCAATCACACGGATTATTTGA**T**CCACACTATATCTCCAATGTTGGAATATACGAGAC 1080
CLONE3          CACGCAATCACACGGATTATTTGA**C**CCACACTATATCTCCAATGTTGGAATATACGAGAC 1080
CLONE4          CACGCAATCACACGGATTATTTGA**T**CCACACTATATCTCCAATGTTGGAATATACGAGAC 1080
                ********************** ***********************************

CLONE1          CGAGCGTCTCGAGCCTGAGGCTTCGGCCGACGGTGTACTAATTGTAACTGCGCAATCGTA 1140
CLONE2          CGAGCGTCTCGAGCCTGAGGCTTCGGCCGACGGTGTACTAATTGTAACTGCGCAATCGTA 1140
CLONE3          CGAGCGTCTCGAGCCTGAGGCTTCGGCCGACGGTGTACTAATTGTAACTGCGCAATCGTA 1140
CLONE4          CGAGCGTCTCGAGCCTGAGGCTTCGGCCGACGGTGTACTAATTGTAACTGCGCAATCGTA 1140
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CLONE1          CATATAATTCGAGCTCGGAGGGTCTCCTCGCGGGACCGTCCGACCGAGACTTGTACAAAT 1200
CLONE2          CATATAATTCGAGCTCGGAGGGTCTCCTCGCGGGACCGTCCGACCGAGACTTGTACAAAT 1200
CLONE3          CATATAATTCGAGCTCGGAGGGTCTCCTCGCGGGACCGTCCGACCGAGACTTGTACAAAT 1200
CLONE4          CATATAATTCGAGCTCGGAGGGTCTCCTCGCGGGACCGTCCGACCGAGACTTGTACAAAT 1200
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CLONE1          TATAA 1205
CLONE2          TATAA 1205
CLONE3          TATAA 1205
CLONE4          TATAA 1205
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Appendix 3. Alignment (by CLUSTALW) of the different ITS2 clones nucleotide sequences for *Stenoponia tripectinata tripectinata* from *Mus musculus* from Gran Canaria. Polymorphism is remarked by bold letter. The ITS2 clones sequence data have been deposited in GenBank database under the following Accession Numbers: LN847258 and LN847259.

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CLONE1          TATATTATTACCAGACTGCTGCTTGCCCTCGGGCTCGCCAGCGAATGATGGGACTCTTCG 60
CLONE2          TATATTATTACCAGACTGCTGCTTGCCCTCGGGCTCGCCAGCGAATGATGGGACTCTTCG 60
CLONE3          TATATTATTACCAGACTGCTGCTTGCCCTCGGGCTCGCCAGCGAATGATGGGACTCTTCG 60
CLONE4          TATATTATTACCAGACTGCTGCTTGCCCTCGGGCTCGCCAGCGAATGATGGGACTCTTCG 60
CLONE5          TATATTATTACCAGACTGCTGCTTGCCCTCGGGCTCGCCAGCGAATGATGGGACTCTTCG 60
CLONE6          TATATTATTACCAGACTGCTGCTTGCCCTCGGGCTCGCCAGCGAATGATGGGACTCTTCG 60
CLONE7          TATATTATTACCAGACTGCTGCTTGCCCTCGGGCTCGCCAGCGAATGATGGGACTCTTCG 60
                ************************************************************

CLONE1          CTTAATTGCGTCTCCCTAAATTATTCACTCAACGTGTGAGCCAGTTCCGTTTGCAACGCG 120
CLONE2          CTTAATTGCGTCTCCCTAAATTATTCACTCAACGTGTGAGCCAGTTCCGTTTGCAACGCG 120
CLONE3          CTTAATTGCGTCTCCCTAAATTATTCACTCAACGTGTGAGCCAGTTCCGTTTGCAACGCG 120
CLONE4          CTTAATTGCGTCTCCCTAAATTATTCACTCAACGTGTGAGCCAGTTCCGTTTGCAACGCG 120
CLONE5          CTTAATTGCGTCTCCCTAAATTATTCACTCAACGTGTGAGCCAGTTCCGTTTGCAACGCG 120
CLONE6          CTTAATTGCGTCTCCCTAAATTATTCACTCAACGTGTGAGCCAGTTCCGTTTGCAACGCG 120
CLONE7          CTTAATTGCGTCTCCCTAAATTATTCACTCAACGTGTGAGCCAGTTCCGTTTGCAACGTG 120
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CLONE1          ACGTGCATAACGTTCGTTGTCGGTACGCGGAAGTAGGGCGGATTGTGTGTCCTAGATTAA 180
CLONE2          ACGTGCATAACGTTCGTTGTCGGTACGCGGAAGTAGGGCGGATTGTGTGTCCTAGATTAA 180
CLONE3          ACGTGCATAACGTTCGTTGTCGGTACGCGGAAGTAGGGCGGATTGTGTGTCCTAGATTAA 180
CLONE4          ACGTGCATAACGTTCGTTGTCGGTACGCGGAAGTAGGGCGGATTGTGTGTCCTAGATTAA 180
CLONE5          ACGTGCATAACGTTCGTTGTCGGTACGCGGAAGTAGGGCGGATTGTGTGTCCTAGATTAA 180
CLONE6          ACGTGCATAACGTTCGTTGTCGGTACGCGGAAGTAGGGCGGATTGTGTGTCCTAGATTAA 180
CLONE7          ACGTGCATAACGTTCGTTGTCGGTACGCGGAAGTAGGGCGGATTGTGTGTCCTAGATTAA 180
                ************************************************************

CLONE1          TTTCTTTGACTCTACGCCGTTTTATTTGCACGAAAGTGTACCGAAACCCCGTACCACACG 240
CLONE2          TTTCTTTGACTCTACGCCGTTTTATTTGCACGAAAGTGTACCGAAACCCCGTACCACACG 240
CLONE3          TTTCTTTGACTCTACGCCGTTTTATTTGCACGAAAGTGTACCGAAACCCCGTACCACACG 240
CLONE4          TTTCTTTGACTCTACGCCGTTTTATTTGCACGAAAGTGTACCGAAACCCCGTACCACACG 240
CLONE5          TTTCTTTGACTCTACGCCGTTTTATTTGCACGAAAGTGTACCGAAACCCCGTACCACACG 240
CLONE6          TTTCTTTGACTCTACGCCGTTTTATTTGCACGAAAGTGTACCGAAACCCCGTACCACACG 240
CLONE7          TTTCTTTGACTCTACGCCGTTTTATTTGCACGAAAGTGTACCGAAACCCCGTACCACACG 240
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CLONE1          ACAGCTTGCCGTCACAGCTTACGGAACGATCTGATGGTTCCTCGTAAAATGCGCTGGTAG 300
CLONE2          ACAGCTTGCCGTCACAGCTTACGGAACGATCTGATGGTTCCTCGTAAAATGCGCTGGTAG 300
CLONE3          ACAGCTTGCCGTCACAGCTTACGGAACGATCTGATGGTTCCTCGTAAAATGCGCTGGTAG 300
CLONE4          ACAGCTTGCCGTCACAGCTTACGGAACGATCTGATGGTTCCTCGTAAAATGCGCTGGTAG 300
CLONE5          ACAGCTTGCCGTCACAGCTTACGGAACGATCTGATGGTTCCTCGTAAAATGCGCTGGTAG 300
CLONE6          ACAGCTTGCCGTCACAGCTTACGGAACGATCTGATGGTTCCTCGTAAAATGCGCTGGTAG 300
CLONE7          ACAGCTTGCCGTCACAGCTTACGGAACGATCTGATGGTTCCTCGTAAAATGCGCTGGTAG 300
                ************************************************************

CLONE1          ATATTTACAATATCGAACGCGCTCTCTCAATA 332
CLONE2          ATATTTACAATATCGAACGCGCTCTCTCAATA 332
CLONE3          ATATTTACAATATCGAACGCGCTCTCTCAATA 332
CLONE4          ATATTTACAATATCGAACGCGCTCTCTCAATA 332
CLONE5          ATATTTACAATATCGAACGCGCTCTCTCAATA 332
CLONE6          ATATTTACAATATCGAACGCGCTCTCTCAATA 332
CLONE7          ATATTTACAATATCGAACGCGCTCTCTCAATA 332
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