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New Insights into the Taxonomy of Malacopsylloidea Superfamily (Siphonaptera) Based on Morphological, Molecular and Phylogenetic Characterization of *Phthiropsylla agenoris* (Malacopsyllidae) and *Polygenis (Polygenis) rimatus* (Rhopalopsyllidae)

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Abstract: From a phylogenetic point of view, the Malacopsyllidae family and the Rhopalopsillidae family (comprising Parapsyllinae and Rhopalopsyllinae subfamilies) have been traditionally classified within the Malacopsylloidea superfamily, mostly restricted to South America. The phylogenetic relationships and taxonomic status of Malacopsyllidae and Rhopalopsillidae have never been assessed since no molecular loci of Malacopsyllidae have been sequenced by any authors, and the phylogeny provided so far was not based on any sort of formal quantitative analysis of flea morphology. Based on these precedents, the objective of this study was to carry out a comparative phylogenetic, molecular and morphological study of two different species belonging to each family, *Phthiropsylla agenoris* (Malacopsylla) and *Polygenis (Polygenis) rimatus* (Rhopalopsyllidae, Rhopalopsyllinae). In this study, we demonstrated the usefulness of several morphological features as diagnostic characters to differentiate between *P. (P.) rimatus* and *P. agenoris*. Using molecular and phylogenetic data, we easily discriminated between the two taxa (*P. agenoris* and *P. (P.) rimatus*) by comparing both nuclear and mitochondrial markers. This fact proves the usefulness of ITS2, *EF1−α*, *cox*1, *cyt*b and *cox*2 as molecular diagnostic markers to characterize and identify different Siphonaptera taxa. Additionally, the phylogenetic results confirm, for the first time, the monophyly of the Malacopsyllidae family and suggest a clear paraphyletic position of the Paraspsyllinae subfamily and, consequently, the Rhopalopsyllidae family.

Keywords: Malacopsyllidae; Rhopalopsyllidae; Siphonaptera; phylogeny; taxonomy

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1. Introduction

Fleas, as blood−sucking insects parasitic on wild and domestic birds and mammals, including humans, are important in public health as parasites, intermediate hosts and vectors of pathogens [1]. Phylogenetic relationships among flea taxa based on molecular data were virtually neglected until Whiting et al. [2] published the first comprehensive attempt to reconstruct deep evolutionary relationships for fleas using a formal analysis of character data from four loci: 18S ribosomal DNA, 28S ribosomal DNA, *cytochrome c oxidase* subunit 1 (*cox*1) and *elongation factor 1−alpha* (*EF1−α*). For this purpose, they used 128 different taxa representing 16 families, 25 subfamilies, 26 tribes and 83 flea genera collected from around the world. Among their findings, they confirmed the monophyly of ten families: Tungidae, Lycopsyllidae, Pygiopsyllidae, Stivaliidae, Stephanocircidae, Rhopalopsyllidae, Chimaeropsyllidae, Pulicidae, Ischnopsyllidae and Ceratophyllidae.

In spite of that, they claimed the necessity to keep providing and re−examining new molecular, phylogenetic and morphological flea data to clarify the systematics of fleas, especially those regarding the Ctenophthalmidae family, which was defined by these authors as a "catch−all group" or an unnatural grouping of fleas that are grossly paraphyletic. In this sense, few papers have been published since 2008 in order to improve and update some taxonomically doubtful points exposed by [2]. While it is true that the recent studies published by [3–5] have tried to clarify the taxonomic status of different Ctenophthalmidae taxa, other families of fleas, such as Ancistropsyllidae, Malacopsyllidae and Xiphiopsyllidae, not included in the study published by [2], remain unknown from a phylogenetic point of view.

Malacopsyllidae is a very small family endemic to Argentina, including only two genera, each one with only one species, *Malacopsylla grossiventris* (Weyenbergh, 1879) [6] and *Phthiropsylla agenoris* (Rothschild, 1904) [7,8]. These two taxa share similar morphological traits and geographical distributions, with armadillos (Xenarthra, Dasypodidae) (e.g., *Chaetophractus villosus* (Desmarest, 1904), *Dasypus* sp. and *Zaedyus pichiy* (Desmarest, 1904)) as their main hosts. In addition, Malacopsyllidae fleas have also been reported on carnivores (*Lycalopex gymnocercus* (Fischer, 1814) and *Cerdocyon thous* (Linnaeus, 1766)) and some caviid rodents (e.g., *Microcavia australis* (Geoffroy y d'Orbigny, 1833) and *Galea musteloides* (Meyen, 1832)) [9]. Malacopsyllids are large fleas; engorged females can reach a length of 6.5 mm with an abdominal diameter of 3 mm [8]. These fleas are confined to the ventral dermecos of armadillos, and as this underside is liable to brush against the substrate, malacopsyllids must therefore be able to cling very firmly to the coarse hairs of these hosts [10]. In addition, although laciniae are not heavily serrated, females were observed fixed with their mouthparts to the skin of their hosts, like ticks. Indeed, some of these specimens were observed copulating on the ventral region of their hosts [6,11].

From a phylogenetic point of view, Malacopsyllidae and Rhopalopsillidae families have been traditionally classified within Malacopsylloidea by several authors, a superfamily mostly restricted to South America, with some exceptions [8,12,13]. The Rhopalopsyllidae family comprises 14 genera and about 130 species and subspecies distributed in 2 subfamilies, Parapsyllinae and Rhopalopsyllinae, each of them of monophyletic origin [2]. These fleas mainly have a Neotropical distribution and parasitize birds and mammals, mainly cricetid rodents [2,8,9]. Rhopalopsyllinae comprises eight different genera (*Gephyropsylla*, *Hechtiella*, *Ayeshaepsylla*, *Neotropsylla*, *Polygenis*, *Rhopalopsyllus*, *Scolopsyllus* and *Tiamastus*), including species that mainly infest cricetid and octodontid rodents in the Neotropical region [1]. From these, *Polygenis* is the most important genus because of its wide geographical distribution and a high number of species and subspecies (44 in total). In addition, species of *Polygenis* were reported related to the maintenance of sylvatic plague among wild rodents [1], as well as associated with *Rickettsia felis*, the etiologic agent of flea−borne spotted fever [14–16].

In spite of that, the phylogenetic relationships and taxonomic status of Malacopsyllidae and Rhopalopsillidae have never been assessed since no molecular loci of Malacopsyllidae were sequenced and assessed by [1] or any other authors. Thus, the taxonomic relationship between these two families should be considered clearly unresolved since the intuitive phylogeny provided in [8,12,13] was not based on a formal quantitative analysis of flea morphology.

Based on these precedents, the objective of this study was to carry out a comparative phylogenetic, molecular and morphological study of two different species belonging to Malacopsyllidae and Rhopalopsyllidae families, *P. agenoris* and *Polygenis (Polygenis) rimatus* [17], in order to clarify the taxonomic status and phylogenetic relationships of these families for the first time. In addition, we examined the morphological features and performed the molecular characterization of *P. agenoris* and *P. (P.) rimatus* (Jordan, 1932).

2. Materials and Methods

2.1. Collection of Samples

Fleas were recovered from hosts (trapped alive) by using tweezers and stored in 96% ethanol. Three males and four females of Malacopsyllidae fleas were collected from an armadillo identified as *Zaedyus pichiy* (PPA 693), captured alive, 20 km S Perito Moreno and RN 40, Santa Cruz Province, Argentina, 6−II−2013, coll. Marcela Lareschi.

Four males of Rhopalopsyllidae fleas were collected individually from four different rondent hosts, all of them captured at Parque Provincial Ernesto Tornquist, Sierra de la Ventana, Buenos Aires Province, Argentina, 14−IX−2010 and 21−V−2011, coll. Marcela Lareschi. The rodents were identified as *Akodon azarae* (Fischer, 1829) (CNP 4333) and *Akodon dolores* (Thomas, 1916) (CNP3773, ROB 15, and ROB17). Voucher hosts were housed at the Mammals Collection of the Patagonic Nacional Center (CNP; Puerto Madryn, Chubut, Argentina) (CNP4333 and CNP3773). The remaining rodents and the armadillo were released in the same places where they were captured.

2.2. Morphological Study

Fleas were cleared and softened in 10% KOH, dehydrated in an increasing series of ethanol (80–100%), further diaphanized in eugenol, mounted in Canadian balsam to study them under a light microscope and photographed by using a microscope (Olympus BX51) equipped with a Photographic Camera (Olympus DP71, BX51TF, Tokyo, Japan). Morphology was studied by comparing our specimens with the male lectotype of *P. agenoris* and the male holotype of *P. (P.) rimatus*, as well as other specimens of the latter species deposited at the Natural History Museum (NHM), London, U.K. Additionally, we followed descriptions and illustrations provided in the original descriptions of the species in [7] and in [8]. The morphological terminology used by these authors was followed. The fleas studied will be deposited at the Department of Entomology of the Museum of La Plata, Argentina.

2.3. Molecular and Phylogenetic Study

We amplified and sequenced five different molecular markers of five specimens of *P. agenoris* and three specimens of *P. (P.) rimatus*: nuclear *elongation factor 1 alpha* (*EF1*−*α*), Internal Transcribed Spacer 2 (ITS2) ribosomal DNA (rDNA) and partial *cytochrome c oxidase* subunits 1 and 2 (*cox*1 and *cox*2) and *cytochrome* b (*cyt*b) mitochondrial (mt) gene fragments. Our study was completed using several sequences retrieved from GenBank, representing 12 families, 26 genera and 41 species of fleas, to set up a molecular matrix of four loci (*EF1*−*α*, *cox*1, *cox*2 and *cyt*b).

All molecular markers sequenced in the present study were amplified by polymerase chain reaction (PCR) using a thermal cycler (Eppendorf AG; Eppendorf, Hamburg, Germany). The PCR mix, PCR conditions and PCR primers are summarized in the Supporting Information (Table S1). The *EF1*−*α*, ITS2, and partial *cox*1, *cox*2 and *cyt*b gene sequences obtained from all specimens analyzed were deposited in the GenBank database (Table 1).

The PCR products were checked on SYBR Safe−stained 2% Tris–borate–ethylenediaminetetraacetic acid agarose gels. Bands were eluted and purified from the agarose gel using the QWizard SV Gel and PCR Clean−Up System Kit (Promega, Madison, WI, USA). Once purified, the products were sequenced by Stab Vida (Lisbon, Portugal). To obtain a nucleotide sequence alignment file, the MUSCLE alignment method [18] was used in MEGA, version 10.1.8 [19]. To assess the similarity among all marker sequences of all specimens analyzed in the present study and other flea taxa, the number of base differences per sequence was assessed using the number of differences method in MEGA, version 10.1.8 [19]. For this purpose, we used species and genera belonging to Rhopalopsyllidae (*Polygenis pradoi* (Wagner, 1937), *Polygenis roberti roberti* (Jordan, 1939), *Polygenis rimatus* (Jordan, 1932), *Ectinorus* sp., *Tiamastus cavicola* (Weyenbergh, 1881), *Rhopalopsyllus australis* (Rothschild, 1904), *Listronius fortis* (Jordan & Rothschild, 1923), *Parapsyllus humboldti* (Jordan,

1942), *Parapsyllus longicornis* (Enderlein, 1901) and *Tetrapsyllus* sp.) and Malacopsyllidae (*P. agenoris* and *M. grossiventris*) families available in GenBank.

Table 1. GenBank accession numbers of ITS2, *EF1−α* and partial *cyt*b, *cox*1 and *cox*2 gene sequences of individuals of *Phthiropsylla agenoris* and *Polygenis rimatus* obtained in this study.

Species	Sample ID/ Geographical Area	Host	Number of Fleas	Base Pairs (bp)	Accession Number
		ITS2			
P. agenoris	PA1−PA5/Santa Cruz, Argentina	*Zaedyus pichiy*	5	482	OU706236
P. (P.) rimatus	P1−P3/Buenos Aires, Argentina	*Akodon dolores*	3	453	OU706235
		*Cox*1			
P. agenoris	PA1−PA5/Santa Cruz, Argentina	*Zaedyus pichiy*	5	658	OU706243
P. (P.) rimatus	P1/Buenos Aires, Argentina	*Akodon dolores*	1	658	OU706244
P. (P.) rimatus	P2−P3/Buenos Aires, Argentina	*Akodon dolores*	2	658	OU706245
		*Cox*2			
P. agenoris	PA1/Santa Cruz, Argentina	*Zaedyus pichiy*	1	729	OU707013
P. agenoris	PA3/Santa Cruz, Argentina	*Zaedyus pichiy*	1	729	OU707015
P. agenoris	PA5/Santa Cruz, Argentina	*Zaedyus pichiy*	1	729	OU707016
P. agenoris	PA2, PA4/Santa Cruz, Argentina	*Zaedyus pichiy*	2	729	OU707014
P. (P.) rimatus	P1/Buenos Aires, Argentina	*Akodondolores*	1	739	OU707017
P. (P.) rimatus	P2−P3/Buenos Aires, Argentina	*Akodon dolores*	2	739	OU707018
		*Cyt*b			
P. agenoris	PA1, PA3/Santa Cruz, Argentina	*Zaedyus pichiy*	2	374	OU706744
P. agenoris	PA2, PA4−PA5/Santa Cruz, Argentina	*Zaedyus pichiy*	3	374	OU706745
P. (P.) rimatus	P1/Buenos Aires, Argentina	*Akodon dolores*	1	374	OU706746
P. (P.) rimatus	P2−P3/Buenos Aires, Argentina	*Akodon dolores*	2	374	OU706743
		EF1−α			
P. agenoris	PA1, PA3−PA5/Santa Cruz, Argentina	*Zaedyus pichiy*	4	975	OU706239
P. agenoris	PA2/Santa Cruz, Argentina	*Zaedyus pichiy*	1	975	OU706240
P. (P.) rimatus	P1, P3/Buenos Aires, Argentina	*Akodon dolores*	2	975	OU706241
P. (P.) rimatus	P2/Buenos Aires, Argentina	*Akodon dolores*	1	975	OU706242

The concatenated phylogenetic tree was inferred using nucleotide data and constructed using two methods: maximum likelihood (ML) and Bayesian inference (BI). The maximum likelihood tree was generated using the PHYML package from [20], whereas Bayesian inference was generated using MRBAYES, version 3.2.6 [21]. JMODELTEST [22] was used to determine the best−fit substitution model for the parasite data (*EF1−α*, *cox*1, *cox*2 and *cyt*b). Models of evolution were chosen for subsequent analyses according to the Akaike information criterion [23,24]. To investigate the dataset containing the concatenation of four markers (*EF1−α*, *cox*1, *cox*2 and *cyt*b), analyses based on BI were partitioned by gene, and models for individual genes within partitions were selected by JMODELTEST. For ML inference, best−fit nucleotide substitution models included Transitional Model 2 with gamma−distributed rate variation and a proportion of invariable sites equal to TIM2 + I + G (*cox*2), Transitional Model 1 with gamma−distributed rate variation and a proportion of invariable sites equal to TIM1 + I + G (*cox*1) and a general time−reversible model with gamma−distributed rate variation and a proportion of invariable sites equal to GTR + I + G (*EF1−α* and *cyt*b). Support for the topology was examined using bootstrapping (heuristic option) [25] with 1000 replications to assess the relative reliability of clades. The commands used in MRBAYES, version 3.2.6, for Bayesian inference were *nst* = 6 with invgamma rates (*EF1−α*, *cox*1, *cox*2 and *cyt*b). For BI, the standard deviation of split frequencies was used to determine whether the number of generations completed was enough; the chain was sampled every 500 generations, and each dataset was run for 10 million generations. The adequacy of sampling and run convergence was assessed using the effective sample size

(ESS) diagnostic in Tracer, version 1.6 [26]. Trees from the first million generations were discarded based on the assessment of convergence. Burn−in was determined empirically by examining the log−likelihood values of the chains. The Bayesian posterior probabilities (BPPs) comprise the significance scores for the nodes. The obtained phylogenetic tree was then visualized and edited in Figtree 1.4.4 [27].

Phylogenetic analyses, based on concatenated markers *EF1*−*α*, *cox*1, *cox*2 and *cyt*b sequences, were carried out using our sequences and those obtained from the GenBank database (see Table S2). The phylogenetic tree was rooted using *Panorpa meridionalis* (Rambur, 1842) (Mecoptera: Panorpidae) as the outgroup. This choice was based on the combination of morphological and molecular data obtained in previous studies, which provided compelling evidence for a sister−group relationship between Mecoptera and Siphonaptera [2,28].

The ITS2 sequences obtained in this work were exclusively used to molecularly characterize *P. agenoris* and *P. (P.) rimatus* fleas isolated from Argentina. Thus, no phylogenetic trees with other Siphonaptera species based on ITS2 sequences were constructed, so this marker was removed from the concatenated dataset. This decision was based on the high length and nucleotide divergence observed among Mecoptera and Siphonaptera ITS2 sequences, together with the absence of other Malacopsyllidae and Rhopalopsyllidae ITS2 sequences available in GenBank.

3. Results

3.1. Morphological Characterization of Fleas

The seven fleas collected from the armadillo (*Zaedyus pichiy*) were identified as *P. agenoris* (three males and four females), and those from *Akodon* species were identified as *P. (P.) rimatus* (four males), in accordance with morphological features observed in specimens deposited at the NHM, as well as those presented in the literature, as detailed below.

3.1.1. *Phthiropsylla agenoris*

Description (females and males): Frons rounded, without frontal tubercle; with the lower two setae of the ocular row very short, stout and spiniform; pronotum with rows of long setae and two short blunt pseudo−spines, each side widely separated from each other (Figure 1a); setae on the posterior margin of the fore tibia, not spiniform (Figure 1b); oblique break of mid coxa complete (Figure 1c); hindtarsus with distitarsomeres with five pairs of lateral plantar setae (Figure 1d). Female spermatheca with an oval bulga and a long and narrow hilla. The basis of the hilla appeared to be penetrating the lumen of the bulga (Figure 1e); male terminalia, with telomere rather small and inserted in the posterior part of the basimere. Phallosome and aedeagus as in Figure 1f.

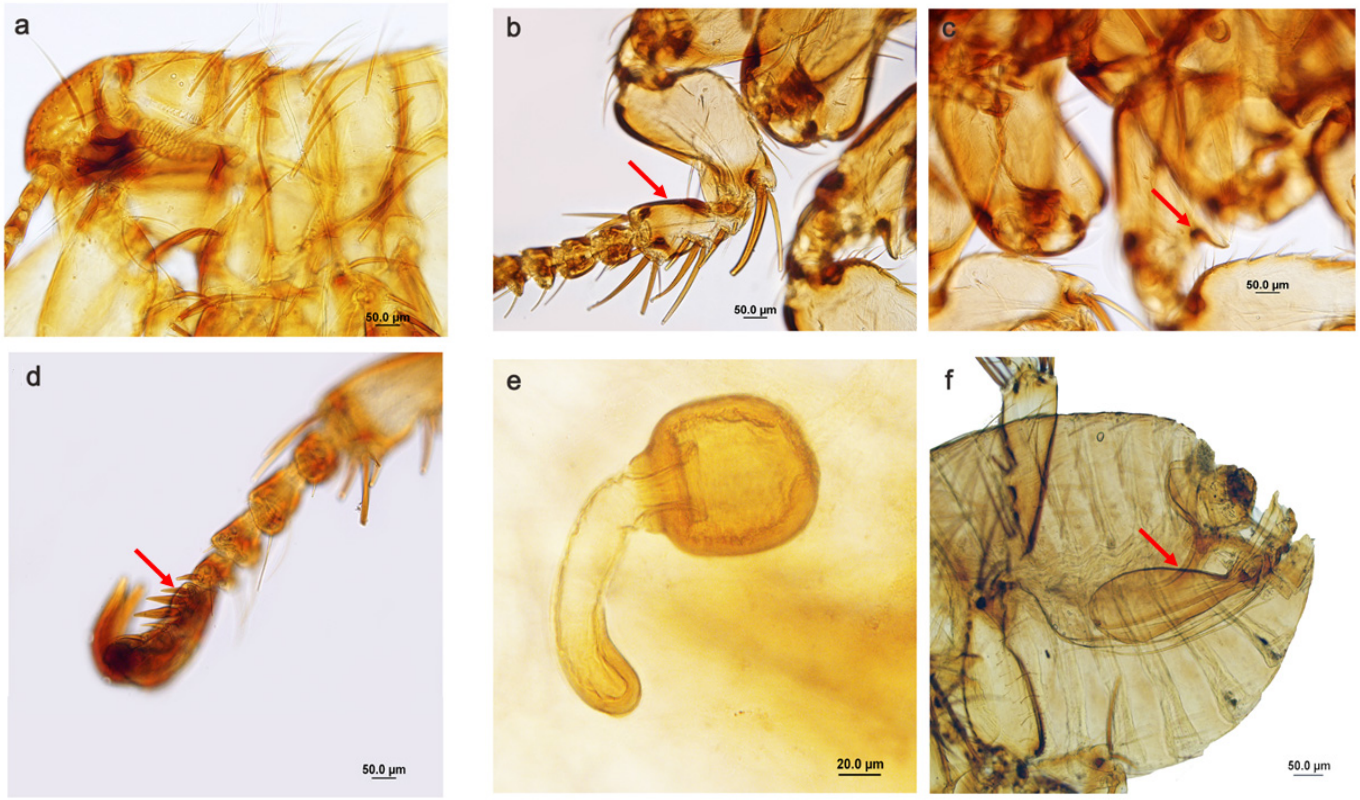

Figure 1. (**a–f**) *Phthiropsylla agenoris*: (**a**) Details of the head and thorax; (**b**) details of fore tibia (arrow); (**c**) oblique break of mid coxa (arrow); (**d**) hindtarsus with distitarsomeres with 5 pairs of lateral plantar setae (arrow); (**e**) spermatheca; (**f**) male terminalia, paramere and aedeagus (arrow).

3.1.2. *Polygenis (Polygenis) rimatus*

Description (males): Labial palp not reaching apex of fore coxa (Figure 2a); oblique break of mid coxa uncomplete (Figure 2b); acetabular seta below the level of the upper margin of acetabulum; distal arm of ninth sternum laterally with setae extending two−thirds the distance from the apex; angle between distal and proximal arms of the basal part of aedeagal tubus about 90°; coil of aedeagal inner tube making 2.5 turns (Figure 2c). Unfortunately, we could not find female specimens of *P. (P.) rimatus* in this study in order to compare the main morphological traits between the two taxa.

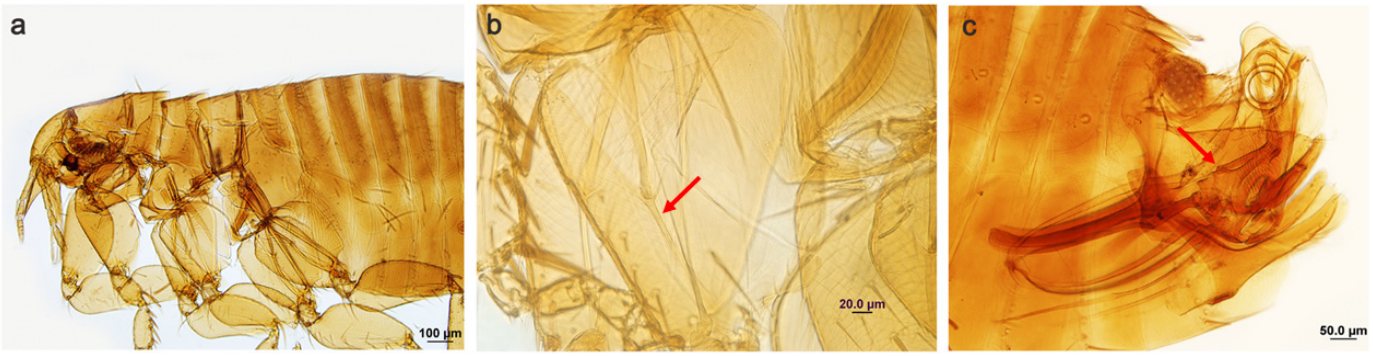

Figure 2. (**a–c**) *Polygenis (Polygenis) rimatus*: anterior general view (**a**); oblique break of mid coxa (arrow) (**b**); male terminalia, paramere and aedeagus (arrow) (**c**).

3.2. Molecular Results

3.2.1. ITS2 Fragment and *EF1−α* Partial Gene Analysis

The lengths of the ITS2 sequences of *P. agenoris* and *P. (P.) rimatus* specimens were 482 and 453 base pairs (bp), respectively, whereas the length of the partial *EF1−α* gene was 975 bp for all sequences analyzed (Table 1). There were no intraspecific nucleotide differences among any of the ITS2 sequences assessed in this study, whereas a total of 77 different base pairs were observed between *P. agenoris* and *P. (P.) rimatus* for the same molecular marker (84.9% nucleotide similarity). The *EF1−α* intraspecific similarity between *P. agenoris* and *P. (P.) rimatus* ranged from 99.8% to 100% and 99.4% to 100%, respectively, observing two different haplotypes for the two species and showing that the interspecific similarity between the two species ranged from 80.4% to 80.7% (Table 2). On the other hand, when we compared our sequences with other partial *EF1−α* gene sequences from Rhopalopsyllidae and Malacopsyllidae species retrieved from GenBank, we noted that the percentages of similarity ranged from 80 to 90%, except among congeneric *Polygenis* species, where these values always appeared higher than 90% (Table 2).

3.2.2. Partial *cox*1, *cox*2 and *cyt*b mtDNA Gene Analysis

The partial *cox*1 and *cyt*b mtDNA gene sequences of *P. agenoris* and *P. (P.) rimatus* specimens amplified in our study were 658 bp and 374 bp in length, respectively. However, the length of *cox*2 sequences differed by 10 bp between the two taxa (739 bp for *P. (P.) rimatus* and 729 bp for *P. agenoris*) (Table 1). When we compared the nucleotide similarities for these mitochondrial markers among all of the sequences obtained in this work, we noted that the intraspecific similarity ranged from 99% to 100%, whereas, the interspecific similarities between *P. agenoris* and *P. (P.) rimatus* were lower (80–85%), with the maximum nucleotide divergence between the two species observed in the *cox*2 analysis (Tables 3–5). In concordance with the nuclear marker analysis, the percentage of similarity among our sequences and other congeneric species retrieved from GenBank always appeared higher than 90%, in contrast to percentages observed among our sequences and those from other specimens belonging to Rhopalopsyllidae and Malacopsyllidae families, which had values lower than 90% (Tables 3–5).

3.3. Phylogenetic Results

The concatenated dataset of *EF1−α* and partial *cyt*b, *cox*1 and *cox*2 gene sequences included 2059 aligned sites and 56 taxa, including *P. agenoris* and *P. (P.) rimauts* specimens from Argentina and the outgroup (*P. meridionalis*). The phylogenetic analysis of the concatenated dataset yielded a tree with strongly supported nodes (Figure 3). This analysis showed a well−supported clade comprising all genera and species belonging to Rhopalopsyllidae and Malacopsyllidae families. Within this group, we note three well−defined subclades. The first one included all Malacopsyllidae taxa (*P. agenoris* and *M. grossiventris*), clustering phylogenetically close to the second one, which comprised some genera belonging to the Rhopalopsyllidae family (*Polygenis* sp., *Ectinorus* sp., *Listronius* sp. and *Parapsyllus* sp.) (Figure 3). Finally, *Tetrapsyllus thombus* and *Tetrapsyllus maulinus* (Rhopalopsyllidae) set up the third subclade within the Rhopalopsyllidae–Malacopsyllidae group, which appeared phylogenetically separated from the remaining families (Cthenophthalmidae, Pulicidae, Stenoponiidae, Ceratophyllidae, Stephanocircidae, Hystrichopsyllidae, Vermipsyllidae, Pygiopsyllidae and Stivaliidae) (Figure 3).

Table 2. Intraspecific (*) and interspecific similarities observed among all partial *EF1−α* gene sequences of nuclear DNA of *P. agenoris* and *P. (P.) rimatus* (obtained in this study) and different species and genera belonging to Rhopalopsyllidae and Malacopsyllidae families retrieved from GenBank database. Values are given in percentages.

EF1−α	***P. agenoris* (This Study) OU706239−40**	***P. (P.) rimatus* (This Study) OU706241−42**	***Polygenis rimatus* EU336290**	***Polygenis pradoi* EU336289**	***Polygenis roberti roberti* KM890524**	***Malacopsylla grossiventris* KM890469**	***Tetrapsyllus* sp. KM890506 KM890507**	***Ectinorus* sp. KM890519 KM890515 KM890512 EU336294**	***Listronius fortis* KM890511**	***Parapsyllus* sp. AF423872 EU336266**	***Tiamastus cavicola* EU336279**
P. agenoris (this study) OU706239−40	99.8–100 *										
P. (P.) rimatus (this study) OU706241−42	80.4–80.7	99.4–100 *									
Polygenis rimatus EU336290	80.7–80.9	98.0–98.1	–								
Polygenis pradoi EU336289	82.6–82.8	90.8–91.0	90.0	–							
Polygenis roberti roberti KM890524	81.0–81.1	97.6–97.7	97.4	91.4	–						
Malacopsylla_grossiventris KM890469	80.6–80.7	83.2–83.4	83.7	83.2	83.0	–					
Tetrapsyllus sp. KM890506 KM890507	81.9–82.5	84.0–84.4	84.4	82.3–85.9	84.0–84.7	84.2–84.8	96.6				
Ectinorus sp. KM890519 KM890515 KM890512 EU336294	80.0–81.4	83.0–85.9	83.1–85.8	83.2–87.4	82.5–85.4	86.6–89.7	84.4–88.7	87.1–90.4			
Listronius fortis KM890511	81.3–81.4	86.5–86.6	86.6	87.3	87.1	88.0	87.3–88.0	88.2–90.9	–		
Parapsyllus sp. AF423872 EU336266	82.0–82.6	84.5–85.4	85.2–85.4	85.0–86.4	84.9–85.4	86.0–88.5	85.5–87.1	86.4–89.7	88.7–89.4	90.8	
Tiamastus cavicola EU336279	83.0–83.1	86.3–86.5	86.8	90.0	87.4	84.4	85.0–85.9	84.5–86.7	87.1	86.186.3	–

Table 3. Intraspecific (*) and interspecific similarities observed among all partial cox1 mtDNA gene sequences of *P. agenoris* and *P. (P.) rimatus* (obtained in this study) and different species and genera belonging to Rhopalopsyllidae and Malacopsyllidae families retrieved from GenBank database. Values are given in percentages.

***Cox*1**	***P. agenoris* (This Study) OU706243**	***P. (P.) rimatus* (This Study) OU706244–45**	***P. agenoris* KM891005, KM890899**	***Polygenis roberti roberti* KM890958**	***Malacopsylla_grossiventris* KM890898**	***Ectinorus* sp. KM890943, KM890949**	***Rhopalopsyllus australis* KM890994**	***Listronius fortis* KM890945**	***Tetrapsyllus* sp. KM890937–38**	***Parapsyllus humboldti* MK104348**
P. agenoris (this study) OU706243	100 *									
P. (P.) rimatus (this study) OU706244–45	83.2–83.5	99.8–100 *								
P. agenoris KM891005, KM890899	99.2	83–83.5	99.5 *							
Polygenis roberti roberti KM890958	83.5	93.4–93.6	83.7–83.9	–						
Malacopsylla_grossiventris KM890898	88.0	86.1–86.3	88.4–88.6	84.6	–					
Ectinorus sp. KM890943, KM890949	85.6–85.9	83.7–83.9	85.1–86.1	83.0–83.5	84.6–86.1	84.0				
Rhopalopsyllus australis KM890994	86.1	83.7–83.9	85.9	83.7	86.5	80.9–83.9	–			
Listronius fortis KM890945	86.3	83.0	86.1–86.3	83.0	84.8	83.9–85.6	83.2	–		
Tetrapsyllus sp. KM890937–38	83.2–85.6	82.8–83.0	82.6–85.3	80.9–81.1	85.4–88.4	78.4–84.8	81.3–85.1	80.9–81.3	88.4	
Parapsyllus humboldti MK104348	84.8	83.7–83.9	85.1–85.3	83.7	88.6	84.6–87.7	84.6	82.6	81.9–86.7	–

Table 4. Intraspecific (*) and interspecific similarities observed among all partial *cytb* mtDNA gene sequences of *P. agenoris* and *P. (P.) rimatus* (obtained in this study) and different species and genera belonging to Rhopalopsyllidae and Malacopsyllidae families retrieved from GenBank database. Values are given in percentages.

Cytb	***P. agenoris* (This Study) OU706744−45**	***P. (P.) rimatus* (This Study) OU706746, OU706743**	***P. agenoris* KM890590, KM890742**	***Polygenis roberti roberti* KM890693**	***Malacopsylla_grossiventris* KM890589**	***Ectinorus* sp. KM890676, KM890682−83**	***Rhopalopsyllus australis* KM890729**	***Listronius fortis* KM890675**	***Tetrapsyllus* sp. KM890670−71**	***Parapsyllus longicornis* KM890604**
P. agenoris (this study) OU706744−45	99.7–100 *									
P. (P.) rimatus (this study) OU706746, OU706743	84.0–84.3	99.4–100 *								
P. agenoris KM890590, KM890742	99.4–99.7	84.0	100*							
Polygenis roberti roberti KM890693	82.8–83.1	92.3	82.8	–						
Malacopsylla_grossiventris KM890589	85.2–85.5	81.9	85.8	80.7	–					
Ectinorus sp. KM890676, KM890682−83	83.4–85.5	81.0–83.4	84.0–85.8	81.6–82.8	84.0–86.6	84.3–86.1				
Rhopalopsyllus australis KM890729	82.8–83.1	82.5	83.4	82.8	83.7	83.4–84.0	–			
Listronius fortis KM890675	83.4–83.7	85.5	84.0	84.6	83.4	83.4–87.2	86.0	–		
Tetrapsyllus sp. KM890670−71	81.9–83.7	81.3–82.8	82.5–84.0	80.1–81.3	79.2–81.6	81.9–84.3	81.9–85.5	84.3–85.8	91.7	
Parapsyllus longicornis KM890604	85.5–85.8	85.8	86.0	83.7	89.0	86.3–90.0	84.6	87.5	82.8–87.2	–

Table 5. Intraspecific (*) and interspecific similarities observed among all partial *cox*2 mtDNA gene sequences of *P. agenoris* and *P. (P.) rimatus* (obtained in this study) and different species and genera belonging to Rhopalopsyllidae and Malacopsyllidae families retrieved from GenBank database. Values are given in percentages.

***Cox*2**	***P. agenoris* (This Study) OU707013−16**	***P. (P.) rimatus* (This Study) OU707017−18**	***P. agenoris* KM890763**	***Polygenis pradoi* AF424043**	***Polygenis roberti roberti* KM890830**	***Malacopsylla grossiventris* KM890589**	***Ectinorus* sp. KM890813 KM890816 EU336012 KM890820**	***Rhopalopsyllus australis* KM890865**	***Listronius fortis* KM890815**	***Tetrapsyllus* sp. KM890807−08**	***Parapsyllus longicornis* EU335985**
P. agenoris (this study) OU707013−16	99.6–100 *										
P. (P.) rimatus (this study) OU707017−18	80.0–80.8	99.6–100 *									
P. agenoris KM890763	99.2	80.0–80.4	–								
Polygenis pradoi AF424043	79.8–80.2	91.6	80.2	–							
Polygenis roberti roberti KM890830	78.2–78.6	90.1	78.2	91.4	–						
Malacopsylla_grossiventris KM890589	85.9–86.3	81.1–81.5	86.1	82.1	80.8	–					
Ectinorus sp. KM890813 KM890816 EU336012 KM890820	82.3–85.2	80.2–84.6	83.1–84.8	80.6–84.6	78.6–83.1	81.7–85.5	85.0–89.0				
Rhopalopsyllus australis KM890865	82.3–82.7	86.5–86.9	82.3	86.3	85.2	84.4	80.0–86.5	–			
Listronius fortis KM890815	82.5–82.9	80.6	82.3	80.6	78.9	82.3	83.3–85.0	82.9	–		
Tetrapsyllus sp. KM890807−08	81.3–81.9	78.2–81.5	81.3–81.5	79.1–79.8	79.1–79.3	81.3–82.9	81.5–84.8	81.7–82.9	80.0–81.1	91.8	
Parapsyllus longicornis EU335985	84.4–84.8	84.4–84.8	84.2	83.5	84.8	84.8	89.7–91.8	85.5	84.8	84.0	–

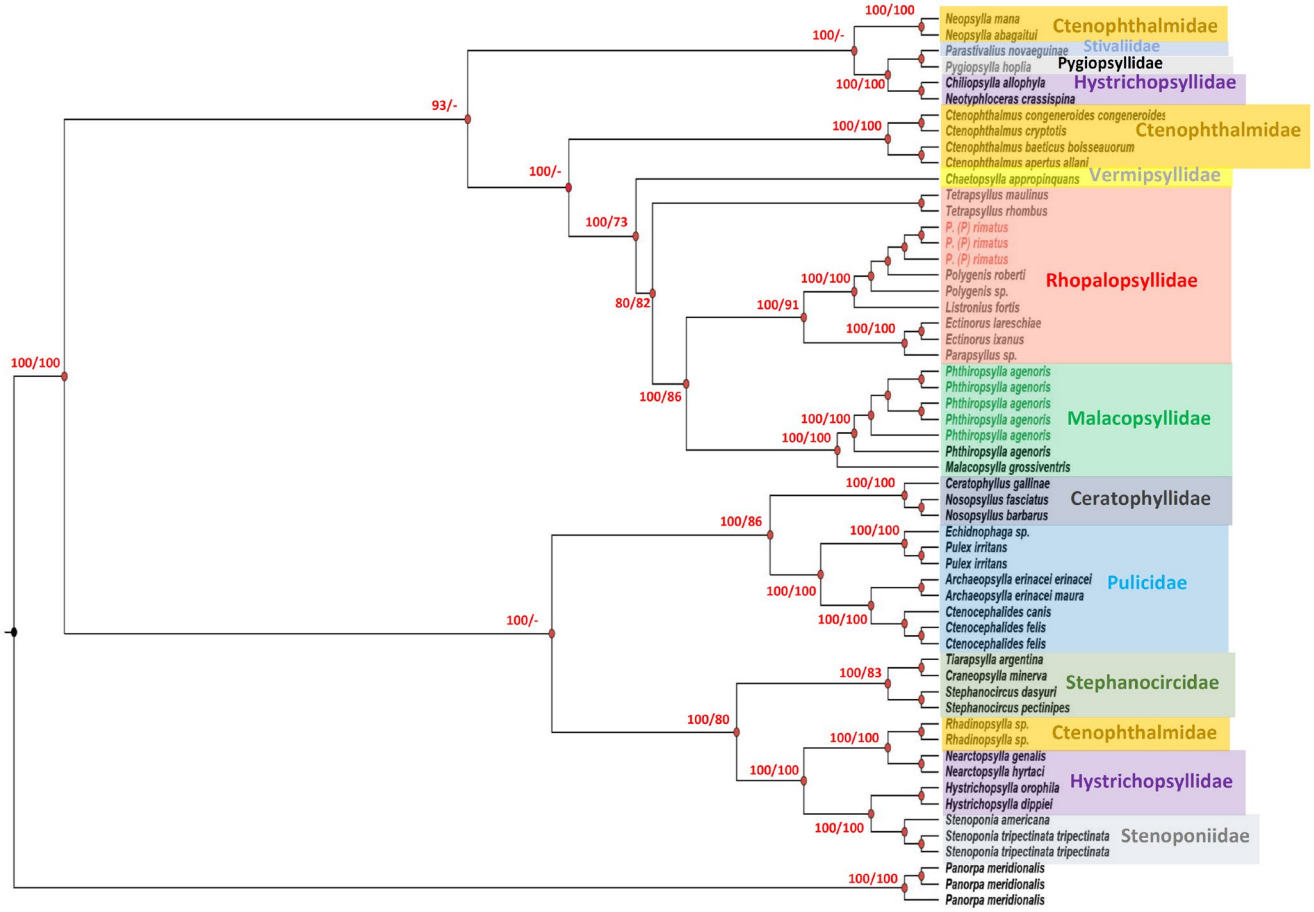

Figure 3. Phylogenetic tree of *P. agenoris* (bold and green) and *P. (P) rimatus* (bold and red) specimens assessed in this study (see Table 1). This analysis was based on concatenated sequences of partial *elongation factor 1 alpha* (*EF1−α*) from nuclear DNA and partial *cytochrome c oxidase* subunit 1 (*cox*1), *cytochrome c oxidase* subunit 2 (*cox*2) and *cytochrome* b (*cyt*b) genes from mitochondrial DNA inferred using the Bayesian inference (BI) and maximum likelihood (ML) methods and Bayesian topology. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown on the branches (BPP/bootstrap). The Bayesian posterior probabilities (BPPs) are converted to percentages. BPP and bootstrap values lower than 60% are not shown.

4. Discussion

Phthiropsylla agenoris was originally described as *Malacopsylla agenoris* on the basis of female and male specimens collected from *Dasypus* sp. in Cruz del Eje, Córdoba Province, Argentina [7]. Originally, the genus *Phthiropsylla* was described to include only this species and differentiate it from *Malacopsylla* [29]. Currently, the species, as well as the family, is considered endemic to Argentina. On the other hand, *P.* (*P.*) *rimatus* was originally described as *Pulex bohlsi* [30] from female specimens collected from the marsupial *Didelphis* sp. from Sapucay, Paraguay. Subsequently, the species was moved to *Rhopalopsyllus bohlsi* (Jordan and Rothschild, 1908) [31] based on specimens collected from Paraguay and Argentina, and later specimens previously thought to be *R. bohlsi* were described as a new species, *Rhopalopsyllus rimatus* [17]. Afterward, the species was included in the genus *Polygenis* as *P. rimata* by [32] and later renamed *P. rimatus* [33–37]. Linardi and Guimarães [38] proposed two subgenera within the *Polygenis* genus, *Neopolygenis* and *Polygenis*, and the species was renamed to its current name, *P. (P.) rimatus*. From the original description, new diagnostic features were described in order to differentiate *P. (P.) rimatus* from other sympatric congeneric species [39–41], and interspecific morphological variations in males and females were also provided that differentiate between specimens from Argentina and

those from Brazil [42]. The species was also recorded in Paraguay and Peru, but most of the records were from Brazil and Argentina [8].

The morphological analysis conducted for specimens of *P. (P.) rimatus* assessed in this study agrees with figures and descriptions given by [31], on the basis of a specimen collected from Paraguay, as well as figures and descriptions presented in [43] and the original figures and descriptions given in [8] on the basis of the male paratype collected in Los Ingleses (Buenos Aires Province, Argentina). In the same way, the morphological description of *P. agenoris* provided in this study agrees with those described in [8] based on male and female paratypes collected in Rio Negro, Argentina.

The geographical origin of the samples analyzed in this study again confirmed the South−American distribution of Malacopsylloidea. In this sense, several authors [8,9,42,44] have reported that Malacopsyllidae and Rhopallopsyllidae are mainly located in the Neotropical region, although they could reach as far north as the southernmost United States, with one notable exception, the genus *Parapsyllus*, which has adapted to penguins and sea birds (albatrosses, fulmars, petrels, shags, prions and shearwaters) and has a pan−Antarctic distribution, primarily on Antarctica, Southern Hemisphere islands and the southern coastal areas of the southern continents [2].

The phylogenetic relationship between Malacopsyllidae and Rhopallopsyllidae was first mentioned in [44] and then by several authors [8,13,45,46]. As we mention in the Introduction section, these authors included both families within the Malacopsylloidea superfamily based exclusively on morphological features and their geographical distribution. The main morphological characteristic within the Malacopsylloidea group is the absence of ctenidia; however, this character is common in other families of fleas, such as Vermipsyllidae or Coptopsyllidae [47]. Thus, other morphological traits, some of them observed and shown in our study, have acquired an important diagnostic value within the Malacopsylloidea superfamily: the presence of an anterior branch of the tentorium, often joined to a ridge arising from the anterior margin of the eye; hind coxa without spiniform setae on the inner side; tooth at the apex of the outer side of the hind tibia, pointed; distitarsomeres with four or five (exceptionally three) pairs of lateral plantar setae; terga with one or two rows of setae; and only one antesensilial seta on each side [8,44]. In this study, we demonstrate the usefulness of these morphological features, described above, as diagnostic characters in order to differentiate between *P. (P.) rimatus* and *P. agenoris*.

On the other hand, this work has tried to assess, for the first time, the phylogenetic relationship between Malacopsyllidae and Rhopallopsyllidae from a molecular point of view. The combination of mitochondrial and nuclear markers as a useful taxonomic and phylogenetic tool has been more than sufficiently proven within the Siphonaptera field. Therefore, this approach has recently been used to clarify the taxonomic status of congeneric species and subspecies [48,49], to gain evolutionary insights on the cat flea, *Ctenocephalides felis* (Bouché, 1835) [50,51], or even to reconstruct a general Siphonaptera phylogeny [2].

Using molecular data and phylogenetic approaches, we could easily discriminate between the two taxa (*P. agenoris* and *P. (P.) rimatus*) by comparing both nuclear and mitochondrial markers. This fact proves the usefulness of ITS2, *EF1*−*α*, *cox*1, *cyt*b and *cox*2 as molecular diagnostic markers to characterize and identify different Siphonaptera taxa, even when they belong to the same superfamily. In this sense, the molecular divergence observed between the two species appeared quite similar among all molecular markers assessed, always ranging from 80% to 85% nucleotide similarity. This molecular pattern was not expected since nuclear markers such as ITS2 and *EF1*−*α* are known to have higher sequence conservation than mitochondrial ones [52,53]. This fact has been widely expressed by several authors, who highlighted the fact that the inheritance properties of mtDNA make it more likely than any single nuclear marker to accurately reflect recent divergence, so it is used to show higher degrees of variability [54]. In addition, the difference observed in the length of ITS2 sequences could also be a useful strategy to discriminate between the two species, even more so when using fleas' universal and conserved primers, such as those used in this study (see Table S1). Using these primers, if we review the

large flea literature, we can note that the sequence lengths of *cox*1, *cox*2, *cyt*b and *EF1*−*α* remain fixed at 658, 727–739, 374 and 975–976 bp, respectively [2,18,49,55]; however, ITS1 and ITS2 fragments appear quite variable within the Siphonaptera Order. Thus, we can observe sequence lengths of ITS2 ranging from 318 bp in *Nosopsyllus barbarous* (Jordan and Rothschild, 1912) and *Nosopsyllus fasciatus* (Bosc, 1800) [56] to 492 bp in different congeneric taxa belonging to *Ctenophthalmus* sp. [53] for the same nuclear marker. The results of the present study also agree with [57,58], who found different ITS sequence lengths in some flea populations belonging to Tungidae and Pulicidae families. Hence, our results reinforce the idea supported by several authors about the usefulness of ITSs as markers of choice for carrying out phylogenetic studies [59].

Our phylogenetic analysis based on a concatenated dataset was able to discriminate between *P. agenoris* and *P. (P.) rimatus* species, again proving the usefulness of the combination of nuclear and mitochondrial markers in order to discriminate among flea taxa belonging to different but closely related families.

The phylogenetic results confirm, for the first time, the monophyly of the Malacopsyllidae family, taking into account that this family consists of only two taxa, *P. agenoris* (assessed in this study) and *M. grossiventris* (sequences retrieved from the GenBank database). Therefore, this family could join the remaining 10 families, whose monophyletic origin was confirmed in [2]: Tungidae, Lycopsyllidae, Pygiopsyllidae, Stivaliidae, Stephanocircidae, Rhopalopsyllidae, Chimaeropsyllidae, Pulicidae, Ischnopsyllidae and Ceratophyllidae. Regarding the Rhopalopsyllidae family, we note two well−supported subclades, one of them corresponding to the Parapsyllinae subfamily (*Ectinorus* sp. and *Parapsyllus* sp.) and a second one clustering *Listronius* sp. (Parapsyllinae) and *Polygenis* spp. (Rhopalopsyllinae). In addition, the phylogenetic position of *Tetrapsyllus* (Parapsyllinae) remains controversial since this genus clustered separately from the Malacopsyllidae and Rhopalopsyllidae clades. This fact was also observed in the phylogenetic tree provided in [60] and could suggest the clear paraphyletic position of the Paraspsyllinae subfamily and, consequently, of the Rhopalopsyllidae family. For that reason, we suggest the necessity to provide further molecular and phylogenetic analyses based on different taxa belonging to Rhopalopsyllidae, specifically focused on the *Tetrapsyllus* genus, in order to clarify the taxonomic and phylogenetic position of this family and genus in particular. Recently, Ref. [61] provided an exhaustive morphological key to identify different *Tetrapsyllus* species, as well as a phylogenetic analysis based on different morphological traits. These authors observed a monophyletic origin for this genus, closely related to *Ectinorus* sp.; however, no taxonomic studies of *Tetrapsyllus* sp. based on molecular data have been published so far.

Finally, our phylogenetic analysis confirms the close relationship between Malacopsyllidae and Rhopalopsyllidae within the taxonomy of the Siphonaptera Order, both clustering in the same clade corresponding to the Malacopsylloidea group (BPP/Bootstrap−100/86). Additionally, our phylogenetic tree also agrees with the taxonomy and systematics provided in [12] and [2] since the Vermipsyllidae family appeared as a sister group of Malacopsylloidea, and we reinforce the theory of the paraphyletic origin of Hystrichopsyllidae and Ctenophthalmidae [2].

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d15020308/s1, Table S1: PCR mix, primers and conditions used for each molecular marker sequenced in this study. Table S2: List of taxa used in the analysis, including GenBank accession numbers and taxonomic information. References [62–64] are cited in the Supplementary Materials.

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