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Article *in* Zootaxa · March 2018
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Article

ISSN 1175-5326 (print edition)
ZOOTAXA
ISSN 1175-5334 (online edition)

https://doi.org/10.11646/zootaxa.4399.2.3
http://zoobank.org/urn:lsid:zoobank.org:pub:A6236F80-3190-48B3-9D6F-AB426F37B0A6

Species diversity and phylogeny of fleas of small terrestrial mammals in the forests of the Central Highlands of Madagascar

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Abstract

Fleas are holometabolous insects forming the order of Siphonaptera. Some studies have been carried out on biology and systematic of Malagasy fleas, but little is known about their phylogenetic relationships. In this study, we focused on flea species occurring in the forests of the Central Highlands and also, on the determination of their phylogenetic relationships. Three families, five genera and thirteen species were identified. The family Pulicidae includes four species (*Centetipsylla madagascariensis* Rothschild, *Synopsyllus fonquerniei* Wagner & Roubaud*, S. estradei* Klein and *S. robici* Klein); Leptopsyllidae has eight species (*Paractenopsyllus vauceli* Klein*, P. petiti* Klein*, P. viettei* Klein*, P. grandidieri* Klein*, P. goodmani* Duchemin, *P. rouxi* Duchemin, *P. raxworthyi* Duchemin & Ratovonjato and *Tsaractenus rodhaini* Duchemin), and Ctenophtalmidae one species (*Dinopsyllus brachypecten* Smit). All are endemic to Madagascar and each differs geographically. Flea phylogenetic relationships were inferred using four molecular markers (ITS2, mtCOII, 16SrRNA and 12S rRNA) and using Neighbor-Joining, Maximum Parsimony and Bayesian methods with addition of Genbank sequences of exotic species. The Family Pulicidae was monophyletic while the families Leptopsyllidae and Ctenophtalmidae were paraphyletic. Malagasy fleas are homogeneous and all species adhere to current classification schemes.

Key words: Forest fleas, Siphonaptera, taxonomy, flea systematics

Introduction

Fleas (Siphonaptera) have been studied because of their medical importance but also as models for the relation between ectoparasites and their hosts (Eads *et al.* 2013; Krasnov *et al.* 2002; St. Juliana *et al.* 2014). Some species are vectors of pathogenic agents such as bubonic plague, murine typhus and tularaemia (Dunnet & Mardon 1991). Plague is a major concern in African countries such as Democratic Republic of the Congo and Madagascar: the number of cases in these two countries corresponds to 92.3% of the reported African cases (WHO 2013). Plague epidemics occur primarily in rural areas with a high fatality rate in case of absence of or delay in treatment. Plague was introduced into Madagascar in 1898 (Brygoo 1966) and its current endemicity provides emphasis to study the local flea fauna.

Siphonaptera contains approximately 2,575 described species belonging to 15 families and 246 genera (Lewis & Lewis 1985; Whiting *et al.* 2008).The identification of these species is carried out using morphological criteria such as the shape and structure of the complex genitalia, or the presence and distribution of setae and spines (Dunnet & Mardon 1991). Malagasy fleas comprise four families: Pulicidae, Leptopsyllidae, Ctenophtalmidae (small terrestrial mammal fleas) and Ischnopsyllidae (bat fleas). Species diversity and systematics of Malagasy fleas have been studied by many authors (Beaucournu & Fontenille 1993; Duchemin 2003a, 2004; Duchemin & Ratovonjato 2004; Girard 1942; Hastriter 2016; Hastriter & Dick 2009; Lumaret 1962; Wagner & Roubaud 1932a,

Accepted by E. Bernard: 10 Jan. 2018; published: 21 Mar. 2018

1932b). These studies strongly contributed to the knowledge of Malagasy fleas and their taxonomy. Various endemic species have been discovered, such as *Tsaractenus rodhaini* and *Paractenopsyllus goodmani* (Leptopsyllidae) (Duchemin 2003b). Five other species of *Paractenopsyllus* were thereafter described: *P. rouxi*, *P. ratovonjatoi*, *P. duplantieri*, *P. juliamarinus* and *P. gemelli* (Duchemin 2004). In recent years, *P. madagascariensis* and the female of *P. raxworthyi* (Hastriter & Dick 2009), a new bat flea, *Lagaropsylla makay* (Laudisoit *et al.* 2012) and the female of *Araeopsylla goodmani* (Hastriter 2016) were described. More than 40 flea species occur in Madagascar, of which about 30 of them are endemic.

Wildlife areas contain an important flea diversity compared to human settlements and surroundings. The forests of the Central Highlands of Madagascar are known to host various flea species. The most represented genera are *Paractenopsyllus* and *Synopsyllus*, which parasitize different small mammals (Beaucournu *et al.* 2015; Goodman *et al.* 2015). In addition, plague has been shown to be circulating in some Malagasy forests (Duplantier *et al.* 2005), extending the known range of putative plague vectors and mammal hosts. Therefore, these areas are important for systematic and diversity studies. Identification of fleas by standard morphological methods are adequate; however, molecular analysis specifies genetic relatedness of species, definitively highlighting evolutionary processes of speciation. Phylogenetic relationships within fleas of the world have been highlighted by few authors (Whiting *et al.* 2008). No study has been conducted regarding the molecular phylogeny or the evolutionary history of fleas in Madagascar with the exception of the biogeography of fleas in Madagascar (Duchemin 2003a).

With this study we aimed to identify the different forest flea species that occur in Madagascar by implementing both classic morphological and DNA analytical methods to associate natural relative flea phylogenies.

Material and methods

Flea specimens were collected and identified to species level using morphological identification keys. Total DNA was extracted in a non-destructive manner and molecular analyses were performed with different molecular markers. PCR amplicons were sequenced and nucleotide sequences obtained were used to carry out phylogenetic analyses.

Study sites. Three sites (Ankazomivady, Anorana, Lakato) located in montane forests of the Central Highland regions were chosen for flea samplings (Fig. 1). These sites are representative of three different elevations in the Central Highlands. Ankazomivady is located in the southwestern part of Ambositra, a district of the southern part of the Central Highlands. The site is included in the montane rainforest of Ankazomivady (47°09.7'E; 20°46.8'S; 1680 m). Anorana is located in the district of Anjozorobe, at the edge of the northeastern part of the Central Highlands. The site is included in the tropical rainforest corridor of Anjozorobe-Angavo (48°00'54.38"E; 18°18'15.35"S; 1300m). Lakato belongs to the district of Moramanga, which is situated on a plateau between the Central Highlands and the east coast. The site is located at the eastern limit of the montane forest (48°20'55.04"E; 19°02'38.95"S; 1007m).

These three sites are the main sampling sites; however, some nucleotide sequences obtained from specimens collected at Ambohitantely were added to construct the phylogenetic trees. This site is located in the Central Highlands in the district of Ankazobe in the northwest region of Antananarivo. The site is in the montane humid forest (18°11'45.5"S; 47°17'14.0"E; 1600m).

Flea collection and conservation. Fleas were collected from 2010 to 2012 during studies on forest small mammal diversity and their roles as potential reservoirs of pathogens. They were preserved at ambient temperature in 70% ethanol.

DNA extraction, specimen mounting and species identification. Each specimen was placed on filter paper to remove ethanol. It was transferred to a glass slide on which 10µl PBS 1X (Phosphate Buffer Saline) was added beforehand. Under a binocular magnifier, an incision was performed on the abdomen with a syringe needle. The specimen with PBS solution was removed from the glass slide and transferred into an Eppendorf tube, to which was added 100µl of Instagene™ Matrix kit (Bio-Rad Laboratories, Hercules, California) for non-destructive DNA extraction (Whiting *et al.* 2008). Once DNA extracts were obtained, specimens were permanently mounted. Morphological identification keys (Duchemin 2003a, 2003b, 2004) were used to identify fleas at species level. Specimens are stored in the flea collection of the Institut Pasteur of Madagascar.

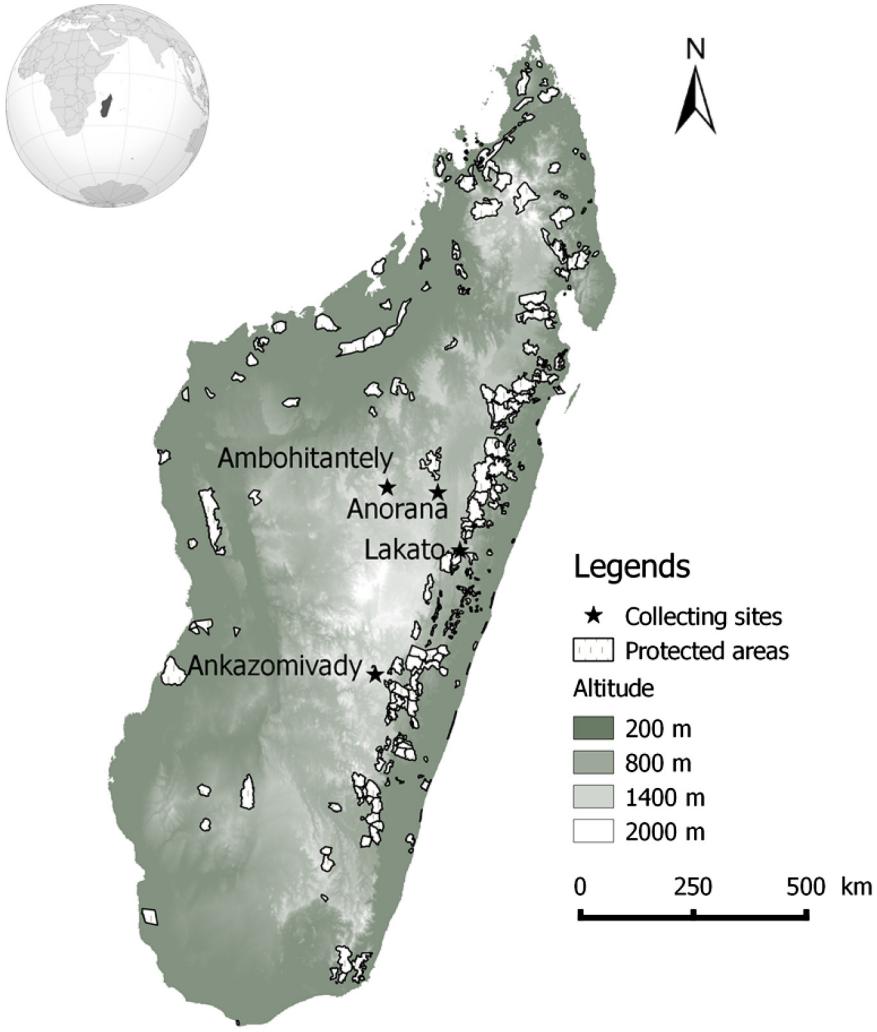

FIGURE 1. Map of Madagascar showing the three sampling sites in the Central Highlands.

PCR amplification and sequencing. PCR analyses were performed to amplify DNA fragment from DNA extracts. Different pairs of primers (see Table 1) were used to amplify ITS2 (~460bp), mtCOII (~740bp), 16S rRNA (~510bp) and 12S rRNA (~400bp) sequences (Kambhampati & Smith 1995; Luchetti *et al.* 2005). PCR amplifications were performed in 50µl of mixture using Thermoscientific Dream Taq PCR Master mix (2x) (InqabaBiotec, Pretoria, South Africa) and following the protocol as described by the manufacturers. Thermal amplification was done as following:

ITS2 sequence: 35 cycles of 94°C for 30 sec., 45°C for 30 sec., 72°C for 45 sec.
mtCOII sequence: 35 cycles of 94°C for 30 sec., 52°C for 45 sec., 72°C for 1 min.
16SrRNA sequence: 35 cycles of 94°C for 30 sec., 55°C for 45 sec., 72°C for 45 sec.
12SrRNA sequence: 35 cycles of 94°C for 30 sec., 45°C for 30 sec., 72°C for 45 sec.

For all temperature profiles, an initial denaturation step of 94°C for 5 min and a final extension step of 72°C for 8 min were added. After visualization under UV light, the amplified products were purified and sequenced at Macrogen (Seoul, South Korea).

Phylogenetic analyses. Forward and reverse sequences were assembled using GENEIOUS v7.1.3 (Biomatters; http://www.geneious.com/). For each of the four loci, sequence data were created by combining consensus sequences obtained from this assembly. Sequences of representative flea species corresponding to the regions amplified and available on Genbank were added in the data (Table 3). Most of these species are exotic to Madagascar and they are representative of different taxa of the world. Because Tungidae is considered as the sister

group of the remaining Siphonaptera (Whiting *et al.* 2008), we chose nucleotide sequences of this family as outgroups. Sequence alignments were performed using MUSCLE algorithm (Edgar 2004) implemented on MEGA 5.2.2 software (Tamura *et al.* 2011). Best-fit models of nucleotide substitution were chosen using jModelTest (Darriba *et al.* 2012; Guindon & Gascuel 2003). Neighbor-Joining (NJ), Maximum Parsimony (MP) and Bayesian inference using Markov chain Monte Carlo (MCMC) methods were used to estimate evolutionary relationships between the different Malagasy flea species. NJ analyses were carried out on MEGA 5.2.2. Genetic distances were corrected according to Kimura's two-parameter model. Bootstrap values at nodes were assessed with 1000 replications. MP analyses were also carried out on MEGA 5.2.2. MP search was performed using heuristic option and nodal support was assessed with 100 bootstrap replicates. Bayesian analyses were carried out with MrBAYES 3.2 software (Ronquist *et al.* 2012). Default priors as described by the authors were chosen when setting parameters. To run the analysis, the MCMC parameters were set as follows: sample and print frequency = 500, diagnostic frequency = 5,000 and run length = 1,000,000. Two independent runs starting from different random trees were conducted. Each run consisted to three heated chains and a single cold chain. At the end of the run, the average standard deviation of split frequencies was examined. The stationarity was attempted when its value approached zero. This means that the two runs converge onto the stationary distribution and the two trees become similar. Moreover, the Potential Scale Reduction Factor (PSRF) should approach 1.0 as runs converge (Ronquist *et al.* 2011). For each run, the first 25% of sampled trees were discarded as burn-in. Trees obtained for both methods were visualized using FIGTREE v1.4.0 software available at http://tree.bio.ed.ac.uk/software/figtree/.

TABLE 1. Pairs of primers used in this study.

Primer names	Primer sequences (5'-3')	Target sequences	References
ITS2D	**F:** CAC TGC GCT CGT GGA TCT AT	ITS2 sequence	Luchetti *et al.* 2005
ITS2R	**R:** TTT AGG GGG TAG TCT CAC CTG		
mtD-13	**F:** AAT ATG GCA GAT TAG TGC	COII gene	Luchetti *et al.* 2005
mtD-20	**R:** GTT TAA GAG ACC AGT ACT TG		
mtD-32	**F:** CCG GTC TGA ACT CAG ATC ACG	16S rRNA gene	Luchetti *et al.* 2005
mtD-34	**R:** CGC CTG TTT AAC AAA AAC AT		
SR-J-14199	**F:** TAC TAT GTT ACG ACT TAT	12S rRNA gene	Kambhampati & Smith 1995
SR-N-14594	**R:** AAA CTA GGA TTA GAT ACC C		

Results

Morphological identification. During six nights of capture per site, 382 small mammals were collected yielding 491 flea specimens. Thirteen flea species comprising five genera and three families were identified (Fig. 2). The family Pulicidae included four species (*Centetipsylla madagascariensis* Rothschild, *Synopsyllus fonquerniei* Wagner and Roubaud, *S. estradei* Klein and *S. robici* Klein), Leptopsyllidae was represented by eight species (*Paractenopsyllus vauceli* Klein, *P. petiti* Klein, *P. viettei* Klein, *P.grandidieri* Klein, *P. goodmani* Duchemin, *P. rouxi* Duchemin, *P. raxworthyi* Duchemin & Ratovonjato and *Tsaractenus rodhaini* Duchemin), and Ctenophtalmidae by one species (*Dinopsyllus brachypecten* Smit).

The geographic distribution of each of these species was different (Table 2). *Centetipsylla madagascariensis* was found in Ankazomivady and Lakato. *Paractenopsyllus goodmani* and *T. rodhaini* were only found in the forest of Anorana. *Synopsyllus robici*, *P. rouxi, P. viettei, P. raxworthyi* and *D. flacourti* were only found in the forest of Ankazomivady. All except *P. raxworthyi* were previously known to be distributed in the southern part of the Central Highlands, while *P. raxworthyi* is distributed both in the Central and in the Northern Highlands (in the Montagne d'Ambre). *Paractenopsyllus* and *Synopsyllus* species were the most abundant species at Ankazomivady and Anorana; at Lakato, the most abundant was *S. fonquerniei*.

TABLE 2. List of flea species identified in this study and their hosts.

Family and species	Distribution	Species inventory[a]			Host species
		Ank.	Ano.	Lak.	
Pulicidae, Archaeopsyllinae					
Centetipsylla madagascariensis Rothschild, 1900	endemic	+	-	+	*Setifer setosus, Tenrec ecaudatus*
Pulicidae, Xenopsyllinae					
Synopsyllus fonquerniei Wagner & Roubaud, 1932	endemic	+	+	+	*Rattus rattus, Eliurus majori, E. tanala, Hemicentetes hemispinosus, Microgale dobsoni, M. longicaudata, M. principula, M. soricoides, M. talazaci, M.* sp., *Nesomys rufus, Oryzorictes hova, S. Setosus*
Synopsyllus estradei Klein, 1964	endemic	+	+	-	*E. majori, E. minor, H. nigriceps, M. dobsoni, M. soricoides, O. hova, R. rattus, S. Setosus*
Synopsyllus robici Klein, 1966	endemic	+	-	-	*H. nigriceps*
Leptopsyllidae, Leptopsyllinae					
Paractenopsyllus vauceli Klein, 1965	endemic	+	+	-	*E. minor, H. hemispinosus, H. nigriceps, M. cowani, M. dobsoni, M. soricoides, M. thomasi, R. Rattus*
Paractenopsyllus petiti Klein, 1965	endemic	+	+	+	*E. minor, E. tanala, M. cowani, M. dobsoni, R. rattus*
Paractenopsyllus viettei Klein, 1965	endemic	+	-	-	*R. rattus*
Paractenopsyllus grandidieri Klein, 1965	endemic	+	+	-	*E. majori, E. tanala, M. dobsoni, N. rufus, O. hova, R. rattus, S. setosus*
Paractenopsyllus goodmani Duchemin, 2003	endemic	-	+	-	*M. dobsoni, M. soricoides, O. hova, R. rattus*
Paractenopsyllus rouxi Duchemin, 2004	endemic	+	-	-	*E. minor, H. nigriceps, M. cowani, M. dobsoni, O. hova, R. rattus*
Paractenopsyllus raxworthyi Duchemin & Ratovonjato, 2004	endemic	+	-	-	*M. parvula*
Tsaractenus rodhaini Duchemin, 2003	endemic	-	+	-	*E. tanala, M. dobsoni, R. rattus*
Ctenophtalmidae, Dinopsyllinae					
Dinopsyllus brachypecten Smit, 1951	endemic	+	+	-	*H. nigriceps, M. cowani, M. parvula, R. rattus*

[a] Ank.: Ankazomivady; Ano.: Anorana; Lak.: Lakato. +/-: presence/absence of species at the site.

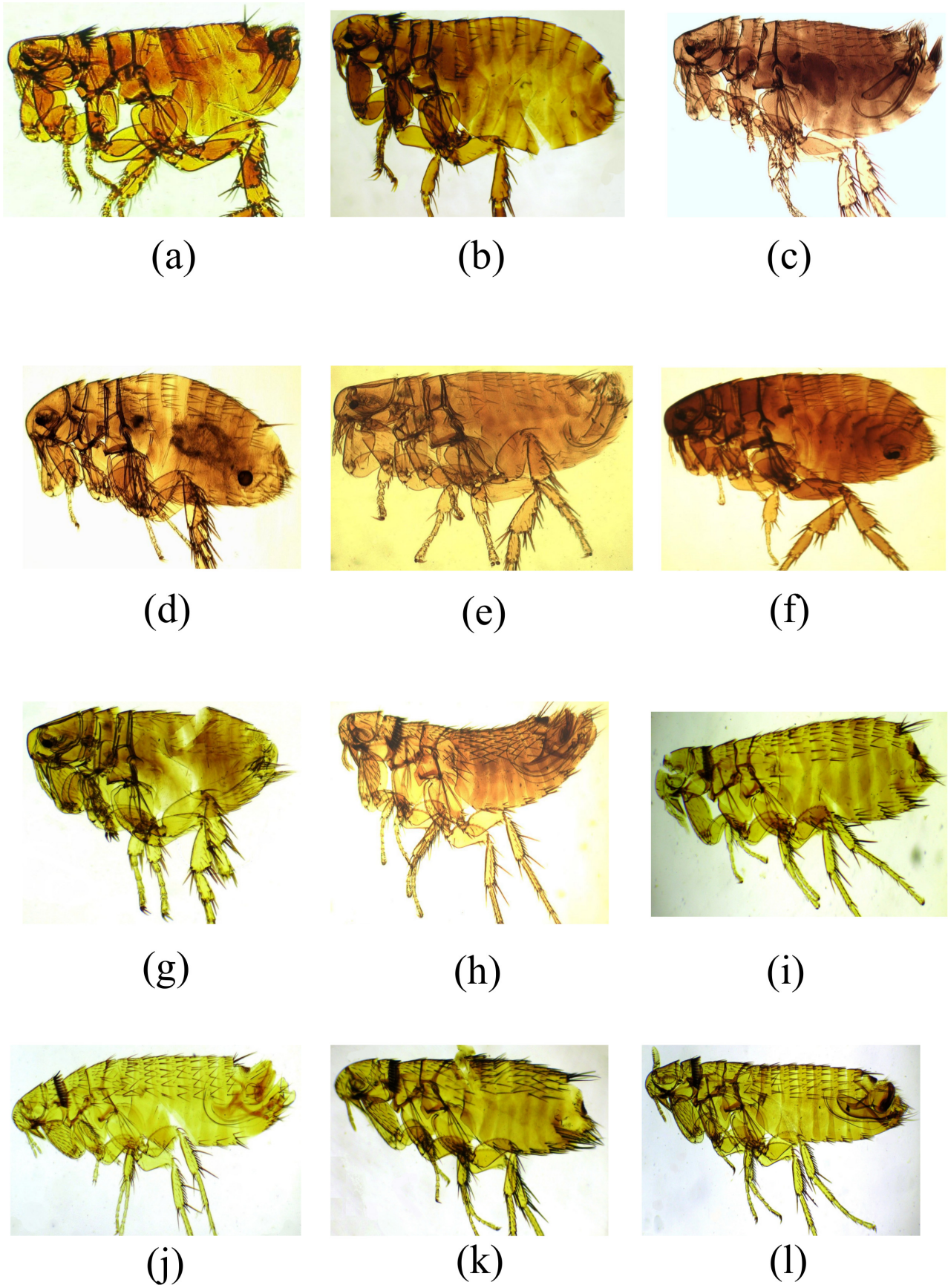

FIGURE 2. Flea species isolated in this study. (a) *Centetipsylla madagascariensis*, male. (b) *C. madagascariensis*, female. (c) *Synopsyllus fonquerniei*, male. (d) *S. fonquerniei*, female. (e) *S. estradei*, male. (f) *S. estradei*, female. (g) *S. robici*, male. (h) *Paractenopsyllus vauceli*, male. (i) *P. vauceli*, female. (j) *P. petiti*, male. (k) *P. petiti*, female. (l) *P. viettei*, male.

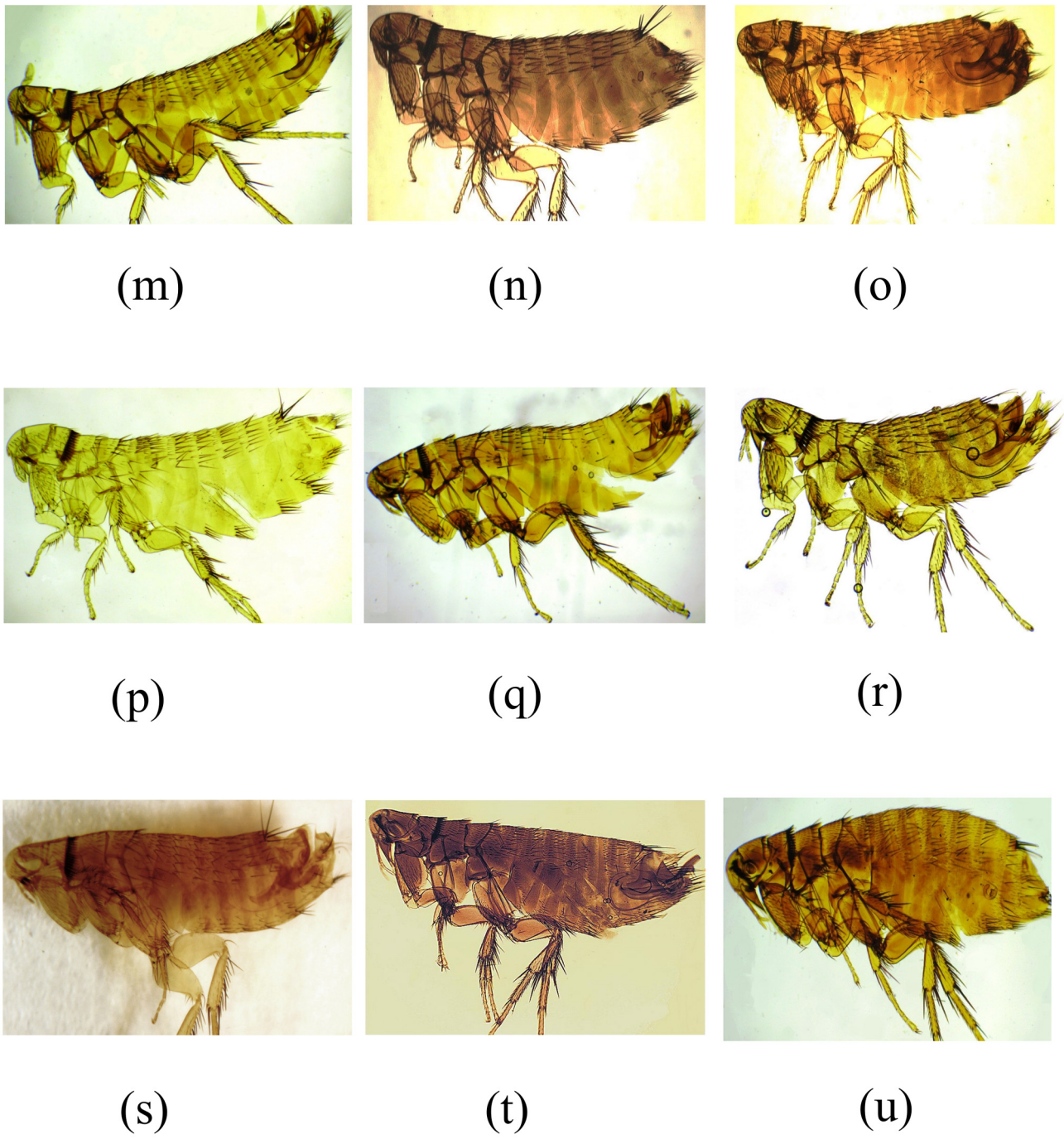

FIGURE 2 (continued). Flea species isolated in this study. (m) *Paractenopsyllus grandidieri*, male. (n) *P. grandidieri*, female. (o) *P. goodmani*, male. (p) *P. goodmani*, female. (q) *P. rouxi*, male. (r) *P. raxworthyi*, male. (s) *Tsaractenus rodhaini*, male. (t) *Dinopsyllus brachypecten*, male. (u) *D. brachypecten*, female.

Sequencing results. Among the 491 flea specimens identified, 239 individuals chosen to represent each species were used to obtain nucleotide sequences. By using ITS2, COII, 16S and 12S primers respectively, 137, 73, 62 and 73 exploitable nucleotide sequences were obtained. The sequences amplified with the different pairs of primers showed variation in their sizes. Sequences ranged from 432 to 483 bps for ITS2, 652 to 653 bps for COII, 506 to 513 bps for 16S and 335 to 337 bps for 12S.

Phylogenetic analysis. All of the four trees constructed (corresponding to the four loci) show the phylogenetic relationships within Malagasy forest fleas and their relationship with the other flea species. Each taxon belonging to a particular species is included in its cluster. In other words, no "abnormal" position of taxa was identified. This

result corresponds to a correct morphological identification of species. The trees obtained with the NJ method have the same topologies as the Bayesian trees, therefore results from the Bayesian analysis (Figs. 3, 4) are presented. Results of the MP analysis are given in Fig. 5.

All of the phylogenetic trees constructed using the different markers showed that species belonging to Pulicidae form a distinct group and evolved from a common ancestor , confirming the monophyly of the family. High posterior probability (P) was obtained at nodes for all the trees: P = 0.85–1 (Figs. 3, 4) while bootstrap values (B) were high for COII and ITS2 trees, respectively B = 89 and B = 86 (Figure 5). All of the phylogenetic trees showed that within the subfamily Xenopsyllinae, *Synopsyllus fonquerniei* is closely allied to *S. girardi* as reported in previous studies (Cheetham 1988; Duchemin 2003a) while *S. estradei* is branched outside this group.

The cluster of all the Malagasy members of the family Leptopsyllidae form two branches: one includes all *Paractenopsyllus* species and the other includes *Tsaractenus rodhaini*. This topology can be observed in all trees. Strong probability values support this relationship: P ~ 1 (Figs. 3, 4). *Paractenopsyllus* species and *T. rodhaini* are sister groups and Malagasy Leptopsyllidae is monophyletic. The other non-Malagasy species belonging to this family were found outside this Malagasy group, in sometimes distant clusters, confirming the paraphyly of the family (Whiting *et al.* 2008).

The family Ctenophtalmidae is represented by the species *Dinopsyllus brachypecten.* The position of this species in the phylogenetic trees is variable according to the markers used. Thus, its phylogenetic relationship with the remaining fleas was not resolved. In summary, tree topologies suggest the paraphyly of Ctenophtalmidae family as suggested previously (Whiting 2002; Whiting *et al.* 2008).

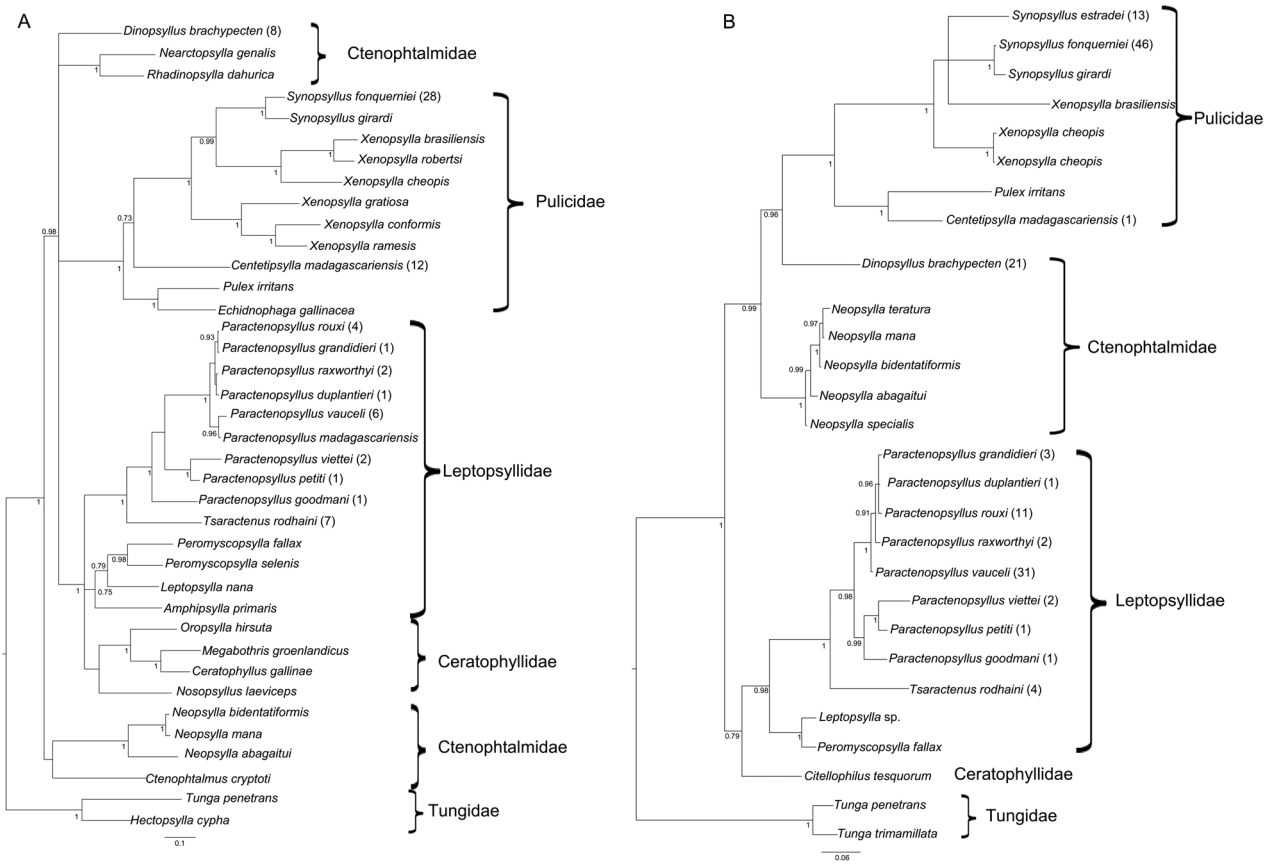

FIGURE 3. Bayesian trees constructed using COII (A) and ITS2 (B) sequences. Values at nodes correspond to the posterior probability values (P) obtained after 100 replicates. P values under 0.7 are not shown. Trees were rooted using sequences of the species *Tunga penetrans*, *T. trimamillata* and *Hectopsylla cypha*. The number of sequences we obtained for each species was given in brackets. Sequences of exotic species (available in our data) such as *Xenopsylla cheopis*, *X. brasiliensis*, *Pulex irritans*, *Peromyscopsylla fallax* and one endemic species isolated in other site *Synopsyllus girardi* were added.

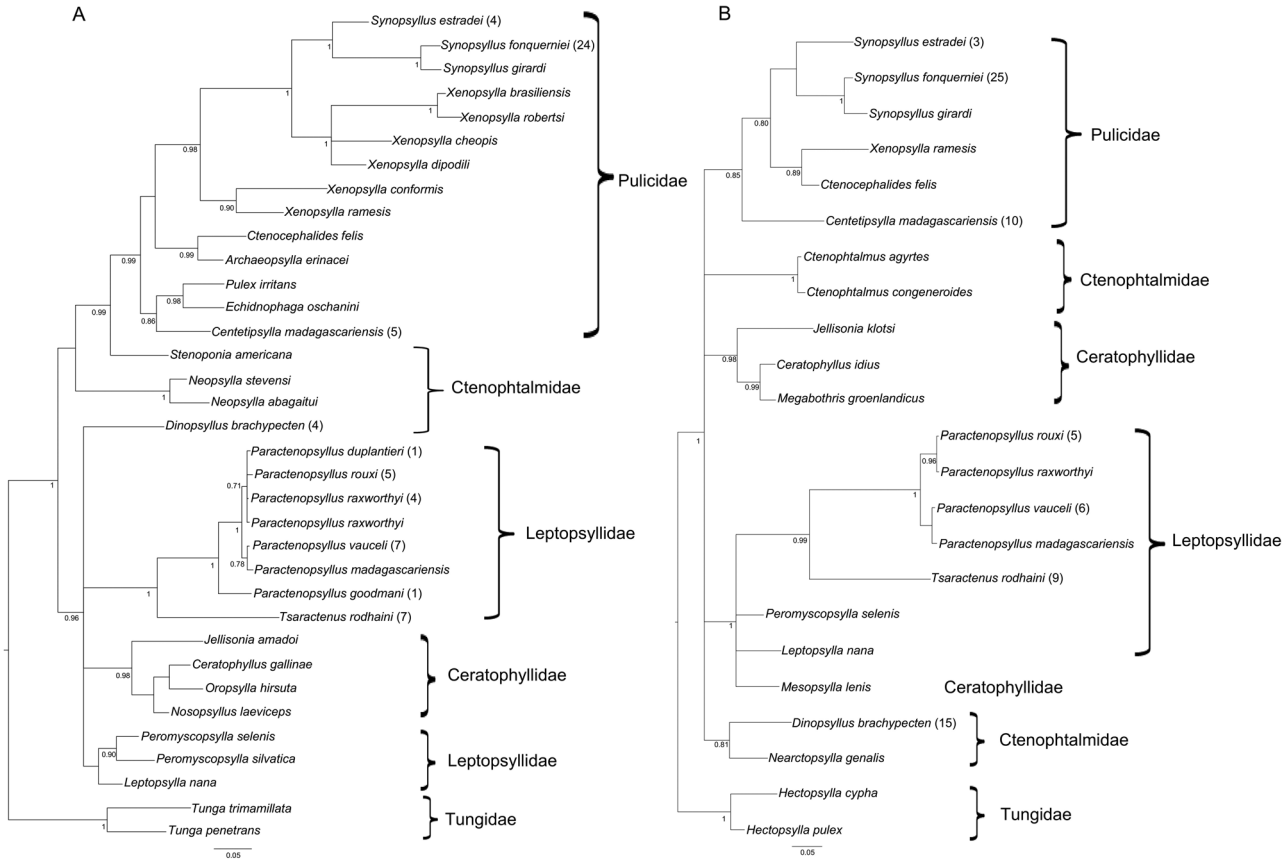

FIGURE 4. Bayesian trees constructed using 16S (A) and 12S (B) sequences. Values at nodes correspond to the posterior probability values (P) obtained after 100 replicates. P values under 0.7 are not shown. Trees were rooted using sequences of the species *Tunga trimamillata*, *T. penetrans*, *Hectopsylla cypha* and *H. pulex*. The number of sequences we obtained for each species was given in brackets. Sequences of exotic species (available in our data) such as *Xenopsylla brasiliensis* and *X. cheopis* were added.

Discussion

This study reports the diversity of fleas parasitizing small mammals in different forest sites of the Central Highlands of Madagascar. Morphological identification allowed the determination of thirteen species belonging to three flea families, while nucleotide sequences of each species allowed determination of phylogenetic relationships using different markers.

The main finding of this study is the strong homogeneity of the Malagasy flea species gathered into each of the flea families. Morphology and phylogenetic-based classifications are consistent. More importantly, this strong homogeneity reflects the evolutionary history of these fleas, which so far remains little known. What can be assumed is that they evolved from infrequent introduction events of their ancestors. As fleas are ectoparasites, their introduction would be associated with that of their mammal hosts, which probably was infrequent as well. Moreover, it is assumed that small mammals of Madagascar, especially rodents, are derived from a single colonization of rodent ancestors from the eastern African region (Goodman *et al.* 2003). Small mammals of the island are mostly comprised of endemic species. The high level of endemism of fauna and flora is the result of the past geological history of Madagascar. The evolution of Malagasy fauna is thought to have occurred after the breakup of the Gondwana landmass (Goodman *et al.* 2003), which separated Madagascar from Africa 160 million years ago, then from India 80 million years ago. These events contributed to the isolation of the island that favored the insular radiation of its fauna and flora resulting in species found nowhere else.

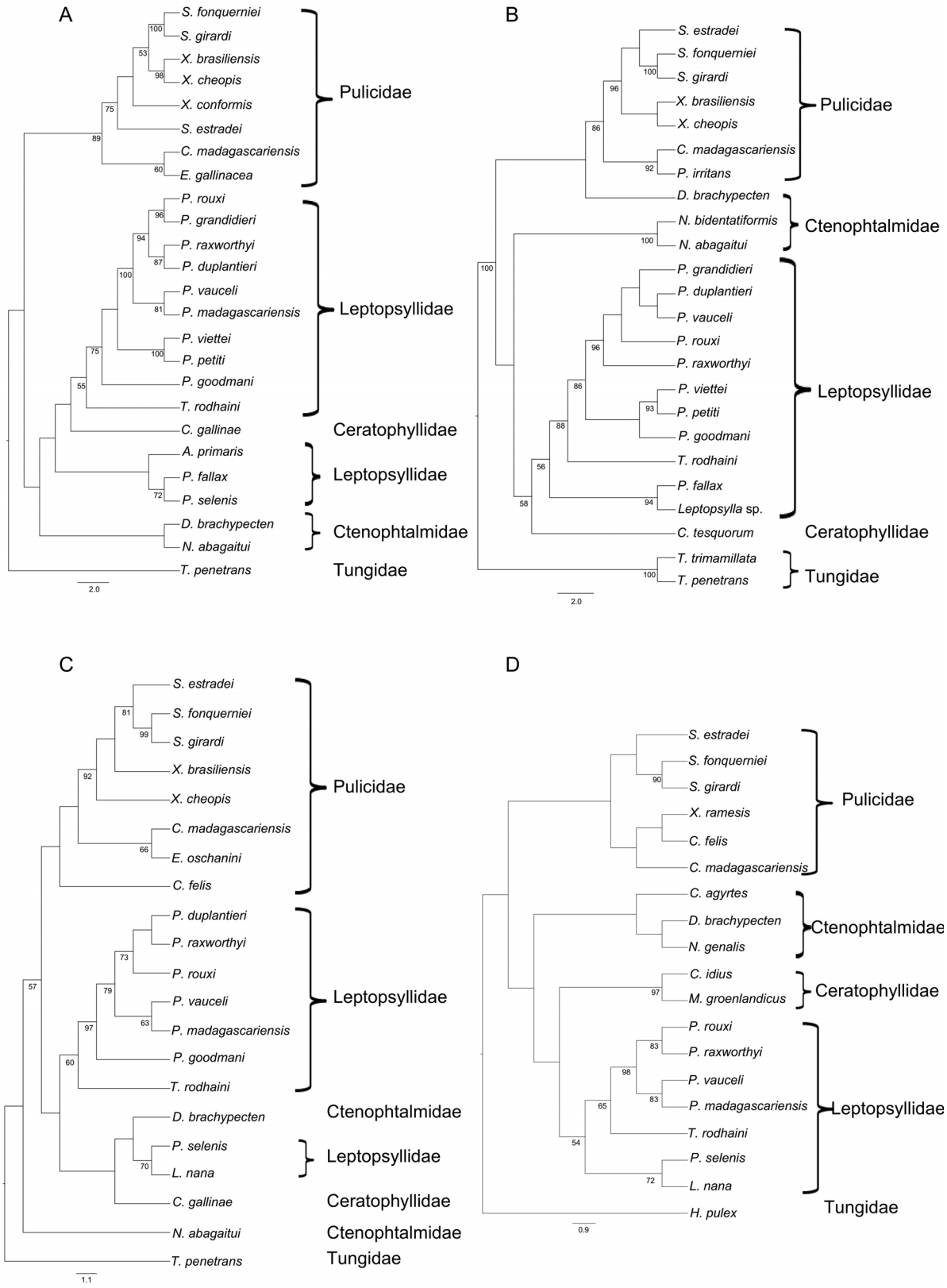

FIGURE 5. Maximum parsimonious trees using COII (A), ITS2 (B), 16S (C) and 12S (D) sequences. Values at nodes correspond to the bootstrap values (B) obtained after 100 replicates. B values under 50 are not shown. Trees were rooted using sequences of the species *Tunga penetrans*, *T. trimamillata* and *Hectopsylla pulex*.

TABLE 3. List of species used in this study with accession numbers. Accession numbers in bold correspond to Malagasy endemic taxa isolated in this study; the remaining taxa are representative flea species from Genbank and from our personal data.

Family	Subfamily	Species names	Accession numbers			
			ITS2	COII	16S	12S
Pulicidae	Xenopsyllinae	*Xenopsylla cheopis*	DQ295061	-	KY007023	-
	Xenopsyllinae	*Xenopsylla cheopis*	KX982860	KY073318	-	-
	Xenopsyllinae	*Xenopsylla robertsi*	-	KM890773	KM891279	-
	Xenopsyllinae	*Synopsyllus girardi*	KX982858	KM890823	KM891329	KM891464
	Xenopsyllinae	*Synopsyllus fonquerniei*	**KX982857**	**KX982873**	**KY007025**	**KY007036**
	Xenopsyllinae	*Synopsyllus estradei*	**KX982856**	**-**	**KY007024**	**KY007035**
	Xenopsyllinae	*Xenopsylla gratiosa*	-	EU088172	-	-
	Xenopsyllinae	*Xenopsylla conformis*	-	KM890859	KM891276	-
	Xenopsyllinae	*Xenopsylla ramesis*	-	KM890772	KM891278	KM891413
	Xenopsyllinae	*Xenopsylla dipodilli*	-	-	KM891277	-
	Xenopsyllinae	*Xenopsylla brasiliensis*	KX982859	KY073317	KY007026	-
	Archeopsyllinae	*Ctenocephalides felis*	-	-	GQ387498	JF523340
	Archeopsyllinae	*Archaeopsylla erinacei*	-	-	KM891368	-
	Archeopsyllinae	*Centetipsylla madagascariensis*	**KX982862**	**KX982874**	**KY007027**	**KY007037**
	Pulicinae	*Echidnophaga gallinacea*	-	KT376420	-	-
	Pulicinae	*Echidnophaga oschanini*	-	-	KM891362	-
	Pulicinae	*Pulex irritans*	KX982861	KY073316	GQ387497	-
Hystrichopsyllidae	-	*Doratopsylla dascynema*	-	KM890804	-	-
	Hystrichopsyllinae	*Hystrichopsylla dippiei*	-	KM890881	-	-
	Hystrichopsyllinae	*Hystrichopsylla* sp.	-	-	KM891382	-
	-	*Stenischia* sp.	-	-	-	KM891426
Ctenophtalmidae	Rhadinopsyllinae	*Nearctopsylla genalis*	-	KM890866	-	KM891508
	Rhadinopsyllinae	*Rhadinopsylla dahurica*	-	KM890851	-	-
	Neopsyllinae	*Neopsylla bidentatiformis*	AF353114	AF251152	-	-
	Neopsyllinae	*Neopsylla mana*	AF353110	KM890849	-	-

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TABLE 3. (Continued)

Family	Subfamily	Species names	Accession numbers			
			ITS2	COII	16S	12S
	Neopsyllinae	*Neopsylla abagaitui*	AF353118	KM890847	AF269121	-
	Neopsyllinae	*Neopsylla stevensi*	-	-	AY337027	-
	Neopsyllinae	*Neopsylla specialis*	AF353120	-	-	-
	Neopsyllinae	*Neopsylla teratura*	AF353122	-	-	-
	Ctenophtalminae	*Ctenophtalmus cryptoti*	-	KM890809	-	-
	Ctenophtalminae	*Ctenophtalmus agyrtes*	-	-	-	KM891515
	Ctenophtalminae	*Ctenophtalmus congeneroides*	-	-	-	KM891429
	Stenoponiinae	*Stenoponia americana*	-	-	KM891401	-
	Dinopsyllinae	*Dinopsyllus brachypecten*	**KX982863**	**KY007022**	**KY007028**	-
Ceratophyllidae	Ceratophyllinae	*Jellisonia amadoi*	-	-	KF322091	-
	Ceratophyllinae	*Jellisonia klotsi*	-	-	-	KM891531
	Ceratophyllinae	*Nosopsyllus laeviceps*	-	KM890858	KM891359	-
	Ceratophyllinae	*Citellophilus tesquorum*	EU770316	-	-	-
	Ceratophyllinae	*Megabothris groenlandicus*	-	KM890796	-	KM891437
	Ceratophyllinae	*Ceratophyllus gallinae*	-	KM890832	KM891338	-
	Ceratophyllinae	*Ceratophyllus idius*	-	-	-	KM891476
	Dactylopsyllinae	*Oropsylla hirsuta*	-	KM890877	KM891384	-
	-	*Mesopsylla lenis*	-	-	-	KM891438
Leptopsyllidae	Amphipsyllinae	*Amphipsylla primaris*	-	KM890839	-	-
	Amphipsyllinae	*Ophtalmopsylla kiritschenkoi*	GQ161961	-	-	-
	Leptopsyllinae	*Peromyscopsylla selenis*	-	KM890882	KM891269	KM891525
	Leptopsyllinae	*Peromyscopsylla silvatica*	-	-	KM891386	-
	Leptopsyllinae	*Peromyscopsylla fallax*	KX982872	KY007021	-	-
	Leptopsyllinae	*Leptopsylla nana*	-	KM890862	KM891369	KM891504
	Leptopsyllinae	*Leptopsylla* sp.	EF504226	-	-	-
	Leptopsyllinae	*Paractenopsyllus madagascariensis*	-	KM890828	KM891333	KM891468

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TABLE 3. (Continued)

Family	Subfamily	Species names	Accession numbers			
			ITS2	COII	16S	12S
	Leptopsyllinae	*Paractenopsyllus raxworthyi*	-	-	KM891332	KM891467
	Leptopsyllinae	*Paractenopsyllus raxworthyi*	**KX982867**	**KX982877**	**KY007031**	-
	Leptopsyllinae	*Paractenopsyllus rouxi*	**KX982866**	**KX982875**	**KY007030**	**KY007038**
	Leptopsyllinae	*Paractenopsyllus grandidieri*	**KX982864**	**KX982876**	-	-
	Leptopsyllinae	*Paractenopsyllus duplantieri*	**KX982865**	**KX982878**	**KY007029**	-
	Leptopsyllinae	*Paractenopsyllus vauceli*	**KX982868**	**KX982879**	**KY007032**	**KY007039**
	Leptopsyllinae	*Paractenopsyllus viettei*	**KX982869**	**KX982880**	-	-
	Leptopsyllinae	*Paractenopsyllus petiti*	**KX982870**	**KX982881**	-	-
	Leptopsyllinae	*Paractenopsyllus goodmani*	**KX982871**	**KX982882**	**KY007033**	-
	Leptopsyllinae	*Tsaractenus rodhaini*	**KX982855**	**KY007020**	**KY007034**	**KY007040**
Ischnopsyllidae	Ischnopsyllinae	*Lagaropsylla idae*	-	KM890824	KM891330	KM891465
	Ischnopsyllinae	*Lagaropsylla consularis*	-	-	KM891331	-
	Ischnopsyllinae	*Nycteridopsylla iae*	-	KM890829	KM891292	FJ422789
	Ischnopsyllinae	*Ischnopsyllus indicus*	-	KM890860	-	-
	Thaumapsyllinae	*Thaumapsylla*	-	KM890896	KM891404	-
Rhopalopsyllidae	-	*Tetrapsyllus maulinus*	-	KM890868	-	KM891449
	Parapsyllinae	*Ectinorus onychius*	-	KM890816	-	-
	Rhopalopsyllinae	*Rhopalopsyllus australis*	-	KM890865	-	-
	Rhopalopsyllinae	*Polygenis* sp.	-	-	-	KM891415
Tungidae	Tunginae	*Tunga penetrans*	DQ844725	DQ844706	AF551756	-
	Tunginae	*Tunga trimamillata*	AY425820	-	AY425834	-
	Hectopsyllinae	*Hectopsylla cypha*	-	KM890818	-	KM891459
	Hectopsyllinae	*Hectopsylla pulex*	-	-	-	KM891526
Stephanocircidae	Stephanocircinae	*Stephanocircus dasyuri*	-	-	-	KM891436
	Craneopsyllinae	*Barreropsylla excelsa*	-	-	-	KM891408
Malacopsyllidae	-	*Malacopsylla grossiventris*	-	-	-	KM891406

Madagascar does not have endemic flea families but some genera are endemic, such as *Paractenopsyllus*, *Tsaractenus*, *Synopsyllus* and *Centetipsylla*. The family Pulicidae is distributed throughout the world and has 167 species in 21 genera (Whiting *et al.* 2008). In Madagascar, it is represented by three subfamilies: Pulicinae, Archeopsyllinae and Xenopsyllinae. We collect species belonging only to the two latter subfamilies but trees suggest that Pulicidae is monophyletic and is phylogenetically distant from Tungidae, a finding that was previously reported by Whiting and co-authors (Whiting *et al.* 2008).

The family Leptopsyllidae is composed of two subfamilies: Amphipsyllinae and Leptopsyllinae, with 6 tribes, 29 genera and 260 species (Whiting *et al.* 2008). Only the subfamily Leptopsyllinae is present in Madagascar. This family is the most speciose, with seven species in *Paractenopsyllus* and one in *Tsaractenus*. Our investigation, along with previous studies (Beaucournu *et al.* 2015; Goodman *et al.* 2015) show that *Paractenopsyllus* is the most prevalent in the forest sites of the Central Highlands. Tree topologies suggest that *Paractenopsyllus* spp. and *Tsaractenus rodhaini* are sister groups. Moreover, *Paractenopsyllus* and *Tsaractenus* share some morphological similarities such as large body size and the absence of *trabecula centralis* (Beaucournu & Fontenille 1993). Malagasy species belonging to the family Leptopsyllidae are monophyletic but the family itself is paraphyletic when other representative species were added to perform the analysis.

The family Ctenophtalmidae has 9 subfamilies, 17 tribes, 42 genera and 664 species (Whiting *et al.* 2008). In Madagascar, it is represented by one subfamily, Dinopsyllinae, containing 3 endemic species of the genus *Dinopsyllus*. We collected only one species, *D. brachypecten.* The two other endemic species of the family, *D. flacourti* and *D. tsaratananae*, are rare and were not collected. *Dinopsyllus flacourti* has been isolated in the southern part of the Central Highlands at Vinanintelo (Fianarantsoa) and Andringitra massif around elevations of 1000 – 1600m, while *D. tsaratananae* has been isolated only in Tsaratanana, situated in the extreme north of Madagascar at an elevation above 2000m (Duchemin 2003a).

Forest sites in the Central Highlands are known to have significant small mammal species richness and constitute micro-endemism areas (Goodman *et al.* 2007). Small mammals hosting these different endemic flea species are diversified. Except for *Centetipsylla madagascariensis* and *Synopsyllus robici,* flea specimens were trapped not only from the introduced rodent *Rattus rattus* (Muridae), but also from different endemic species of Tenrecidae such as *Microgale cowani*, *M. soricoides*, *M. dobsoni*, *M. thomasi*, *Hemicentetes nigriceps*, *H. hemispinosus*, *Oryzorictes hova, Tenrec ecaudatus* and *Setifer setosus* (see Table 2). Some specimens were trapped on other rodents such as *Eliurus minor*, *E. majori*, *E. tanala*, and *Nesomys rufus* (Nesomyidae). *Centetipsylla madagascariensis* was trapped only on *T. ecaudatus* and *S. setosus*. Globally, there is no flea–host specificity and a flea species can be hosted by various small terrestrial mammals.

Although we sampled only in three representative sites of the Central Highlands, these sites define the occurrence of flea species diversity in high altitude in Madagascar. We observed that diversity decreases in the Lowlands. This fact coincides with the absence of plague from Lowlands to 800m. This study focuses mainly on identification of forest fleas that are important not only for taxonomic and evolutionary studies, but also to identify flea species that may play a role on the dynamics of plague in forest areas. This disease is a major health threat in the country, but there is little information on the flea vectors and their role in epizootics and transmission to humans. For instance, *Synopsyllus fonquerniei* and *Paractenopsyllus pauliani* were identified during a resurgence of plague at Antanambao-Vohidroa (Ikongo District) in 1998 (Duplantier *et al.* 2001). Also, *S. fonquerniei* is identified as a major vector on the island. Other forest species such as *Dinopsyllus brachypecten* or *Synopsyllus estradei* are suspected to be implicated in plague epidemic in Madagascar (Duchemin 2003a). To confirm their role as vectors, the diagnosis of the pathogen agent *Yersinia pestis* combined with vector competence tests are necessary. Some of these flea species were collected on small mammals seropositive for *Y. pestis* (N. Elissa, pers. comm.) and forest areas such as Ankazomivady are located inside the plague "hot spot" area of the Central Highlands (Duplantier *et al.* 2005).

This study highlights the occurrence of an important diversity of fleas in different types of forests in the Central Highlands of Madagascar and shows their phylogenetic relationships with some representative flea species external to Madagascar. Most of the fleas were trapped from *Rattus rattus* (Muridae) but also from other small mammals belonging to the families Nesomyidae and Tenrecidae. Most of these hosts are endemic except *Tenrec ecaudatus* and *R. rattus*. The knowledge of flea species occurring in Madagascar is necessary not only for systematic and biodiversity fields but also to assess their potential implication on human diseases such as plague. It is worth noting that even though several factors may be implicated in plague epidemic (e.g., virulence of *Y. pestis*,

social conditions, human behavior) the vectors have an important role especially in the spread of the disease. Forest fleas may have a role in the epidemiology of plague in Madagascar, thus future studies are urgently warranted.

Acknowledgments

We thank the *Centre de Recherche et de Veille des Maladies Emergentes dans l'Ocean Indien* (CRVOI) and the project 'Faune Sauvage' for the funding of the field research. The authors want also to thank Vahatra Association for supplying some supplementary field data.

Author contributions. Flea samplings: TR. Morphological identification: TR. DNA extraction and PCR analysis: AM and TR. Data analysis: MH. Writing paper: MH. Proofreading paper: MH, AM, JBD and SB.

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