Co-evolution of *Leptospira* and Tenrecidae (Afrotheria) on Madagascar

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Abstract

Leptospirosis is one of the most prevalent bacterial zoonoses in the world. The disease is caused by pathogenic *Leptospira* that are maintained in the kidney lumen of infected animals, mostly mammals, acting as reservoirs and contaminating the environment via infected urine. The investigation of leptospirosis through a *One Health* framework has been stimulated by notable genetic diversity of pathogenic *Leptospira* together with a high infection prevalence in certain animal reservoirs. Extensive sampling and associated microbiological and molecular studies of Madagascar's native mammal fauna have revealed a diversity of pathogenic *Leptospira* with high levels of host-specificity. Native rodents, tenrecids, and bats shelter a number of distinct lineages and species of *Leptospira*, some of which have also been detected in acute human cases. Specifically, *L. mayottensis*, first discovered in humans on Mayotte, an island neighboring Madagascar, was subsequently identified in a few species of Malagasy tenrecids, an endemic family. Distinct *L. mayottensis* lineages were identified in shrew tenrecs (*Microgale cowani* and *Nesogale dobsoni*) on Madagascar, and later in spiny tenrecs (*Tenrec ecaudatus*) on Mayotte. These findings suggest that *L. mayottensis* (i) has experienced co-evolutionary processes during the radiation of tenrecids on Madagascar, and (ii) has recently emerged in human populations on Mayotte following the introduction of *T. ecaudatus* from Madagascar. Hitherto, *L. mayottensis* has not been detected in spiny tenrecs on Madagascar. In the present study, we broaden the investigation of Malagasy tenrecids and describe the presence of *L. mayottensis* in Malagasy populations of *T. ecaudatus*, as well as in *M. thomasi*. These results confirm the hypothesis that *L. mayottensis* was introduced to Mayotte, presumably via *T. ecaudatus,* and provide additional data on the co-evolution of *Leptospira* and Tenrecidae.

Keywords: microbial endemism, *Leptospira mayottensis*, tenrecids, co-evolution, Madagascar, Mayotte, zoonoses

Introduction

Leptospirosis is a zoonotic disease that results annually in around 100 000 human cases and 58 000 deaths (Costa et al., 2015). Pathogenic *Leptospira* are maintained in the lumen of the kidney tubules of animal reservoirs (Ristow et al., 2008), which can chronically shed viable bacteria in their urine and contaminate the environment (Adler, 2015). Although humans can be infected through direct contact with infected reservoirs, indirect transmission via contact with a contaminated environment during outdoor activities is most frequent (Ko et al., 2009). Infection leads to a wide range of symptoms ranging from mild flu-like syndromes to multi-organ failure causing death in 5-10% of the cases.

The genus *Leptospira* is currently composed of more than 60 species including saprophytic and pathogenic species (Adler 2015; Masuzawa et al. 2018; Thibeaux et al. 2018a; Thibeaux et al. 2018b; Vincent et al. 2019). Investigations carried out in different areas of the world on this genus of bacteria through a *One Health* approach have revealed distinct transmission chains composed of species or lineages and reservoirs that vary from one environmental setting to another (Bourhy et al. 2012; Cosson et al. 2014; Guernier et al. 2016; Biscornet et al. 2017; Guernier et al. 2017). Investigations carried out in the ecosystems of Madagascar and surrounding islands, hereafter referred to as the Malagasy Region, have provided new information on transmission chains on the different islands (Gomard et al., 2021). Indeed, while leptospirosis in humans is mostly caused by broadly distributed *Leptospira* that are likely of introduced origin on La Réunion and Seychelles (Biscornet et al., 2017; Guernier et al., 2016), Madagascar and Mayotte Island shelter distinctly more diversified *Leptospira* assemblages including species and lineages that can be considered endemic (Bourhy et al., 2010, 2012; Dietrich et al., 2014, 2018; Gomard et al., 2016; Tortosa et al., 2017).

Among pathogenic *Leptospira* described and investigated in the Malagasy Region, *L. mayottensis*, the principal focus of the current study, warrants further characterization. These bacteria were first isolated from human acute cases on Mayotte and initially named *L. borgpetersenii* group B (Bourhy et al., 2010, 2012). A thorough characterization of serological and genomic features of these isolates led the French Reference Center on Spirochetes to elevate this bacterium to the rank of a new species, which was named *L. mayottensis* in reference to the geographic origin of human isolates (Bourhy et al., 2014). A comprehensive investigation of the Malagasy wild mammal fauna allowed identification of

Leptospira samples imbedded in the genetic clade of *L. mayottensis* and shed by two endemic small mammal species, namely *Microgale cowani* and *Nesogale dobsoni* (Dietrich et al., 2014). These two species belong to the endemic family Tenrecidae, composed of omnivorous small mammals known to play an important role in *Leptospira* maintenance as reservoirs of two distinct species: *L. borgpetersenii* and *L. mayottensis* (Dietrich et al. 2014; Dietrich et al. 2018; Dietrich et al. in press). The origin of the Tenrecidae, a monophyletic group, is the result of a single colonization event originating from Africa that took place 30-56 My ago, followed by an extraordinary radiation leading to the currently known nearly 40 extant species or confirmed candidate species (Everson et al., 2016; Goodman et al., 2018). These findings strongly suggest that *L. mayottensis* has co-evolved with tenrecid hosts on Madagascar.

It has been proposed that *L. mayottensis* was introduced from Madagascar to Mayotte (Lagadec et al., 2016). This was supported by an investigation of animal reservoirs on Mayotte identifying *Tenrec ecaudatus*, a spiny tenrec introduced from Madagascar for human consumption, as the local reservoir of *L. mayottensis*. However, the hypothesis that *T. ecaudatus* sheds *L. mayottensis* currently suffers from a lack of evidence for Malagasy populations of this animal. In the present investigation, we screened *T. ecaudatus* specimens together with other tenrecid species sampled on Madagascar to broaden information on the presence of *L. mayottensis* in these animals, and to test the hypothesis of *L. mayottensis* introduction to Mayotte associated with that of *T. ecaudatus*.

Materials and methods

Ethical statement

Biological sampling permits were obtained from the Ministry of Water and Forests and registered under the following numbers 20/16/MEEMF/SG/DGF/DAPT/SCBT.Re and 85/18/MEEF/SG/DGF/DSAP/SCB.Re.

Biological sample

All investigated shrew tenrecs were sampled in February 2016 in a forest neighboring the village of Anjozorobe, in the Central Highlands of Madagascar (see Figure 1). The samples included 31 specimens belonging to the following nine species: *Microgale taiva* (n=15), *M. thomasi* (n=3), *M. majori* (n=3), *M. parvula* (n=2), *M. soricoides* (n=2), *M. cowani* (n=1), *M. longicaudata* (n=1), *M. fotsifotsy* (n=1), and *Nesogale dobsoni* (n=3). All specimens were captured, manipulated, and euthanized following guidelines accepted by the scientific community for the handling of wild mammals (Sikes et al., 2011) and in strict accordance with permits issued by Malagasy national authorities (see ethical section). The spiny tenrec samples, *Tenrec ecaudatus* were composed of 24 specimens collected in villages adjacent to the Makira Natural Park in the Commune Antsirabe-Sahatany (Maroantsetra District) (Figure 1), an area with heavy human hunting pressure (Annapragada et al., 2021). All samples in this region were collected from captured animals and were provided by the hunters to the research team. All kidney samples from the collected animals from both project areas were removed and immediately stored in ethanol 70% until DNA extraction and molecular analyses.

Leptospira detection and sequencing

For DNA extraction, kidneys were first rinsed with water and subsequently immersed in 2 mL of sterile water overnight. Then a thin transversal slice (approximately 0.5 mm thick) was cut in the central part of the kidney using a sterile scalpel, chopped in small pieces, and then immersed into lysis buffer provided in the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) used for DNA extraction. All subsequent extraction steps were carried out following the manufacturer's instructions. *Leptospira* detection was then carried out on 2 µL of eluted DNA using a probe-specific Real-Time Polymerase Chain Reaction system (RT-PCR)

targeting a fragment of the 16S rRNA gene (Smythe et al., 2002). DNA templates leading to positive RT-PCR results were further subjected to an end-point PCR targeting the *secY* and *adk* loci as previously described (Dietrich et al., 2014). Amplicons were Sanger sequenced on both strands at Genoscreen (Lille, France) using the same PCR primers. The produced chromatograms were visually edited using Geneious software version 9.0.5 (Kearse et al., 2012).

Phylogeny

A phylogeny was constructed for the *secY* gene based on the bacterial sequences generated in the present study and previous *secY* sequences from other research in the Malagasy Region (Bourhy et al., 2012; Dietrich et al., 2014, 2018; Gomard et al., 2016; Lagadec et al., 2016) (Table S1). The best model of sequence evolution was determined with jModelTest v.2.1.4 (Darriba et al., 2012). Phylogenetic reconstructions were performed with MrBayes v.3.2.3 (Ronquist et al., 2012). The analyses consisted of two independent runs of four incrementally heated Metropolis Coupled Markov Chain Monte Carlo (MCMCMC) starting from a random tree. MCMCMC was run for 2 million generations with trees and associated model parameters sampled every 100 generations. The convergence level of each phylogeny was validated by an average standard deviation of split frequencies inferior to 0.05. The initial 10% of trees for each run were discarded as burn-in and the consensus phylogeny along with posterior probabilities were obtained from the remaining trees. The resulting Bayesian phylogenies were visualized, annotated, and rooted to midpoint with FigTree v.1.4.2 (Rambaut, 2014).

Results

The detection by RT-PCR indicates a global leptospiral infection rate of 8.9% (4/45) with bacteria detected in three out of the nine tested tenrecids species: *T. ecaudatus* (1 positive specimen), *M. thomasi* (2 positive specimens), and *M. taiva* (1 positive specimen). The PCR protocols allowed leptospiral sequences to be obtained from the RT-PCR positive *T. ecaudatus* (*secY*) and from one out of the two RT-PCR positive *M. thomasi* (*secY* and *adk*). No bacterial sequence was obtained from the second RT-PCR positive *M. thomasi* or from the RT-PCR positive *M. taiva*. The three produced sequences were deposited in GenBank under the accession numbers: MT442041-MT442043.

We present in Figure 1 the Bayesian phylogeny obtained from the *secY* gene, which includes four principal clades corresponding to *L. borgpetersenii*, *L. mayottensis*, *L. interrogans*, and *L. kirschneri*. Within this phylogeny, the bacterial sequences obtained from *T. ecaudatus* and *M. thomasi* fall in the *L. mayottensis* clade and form a well-supported subclade with a leptospiral sequence obtained from *N. dobsoni*. This subclade is related to one subclade of *L. mayottensis* detected in humans and tenrecs from Mayotte. All *Leptospira* sequences from *Microgale* and *Nesogale* species fall within two distinct clades: *L. borgpetersenii* and *L. mayottensis*. Interestingly, tenrecid species have been found infected by these two *Leptospira* bacteria, specifically *L. borgpetersenii* in *M. longicaudata, M. principula* and *M. majori* or *L. mayottensis* in *M. cowani* and *N. dobsoni* clades. Our results confirm this observation with the detection of *L. mayottensis* in *M. thomasi* and Malagasy populations of *T. ecaudatus*.

Discussion

The Tenrecidae are placental mammals grouped within a monophyletic and endemic Malagasy family composed of nearly 40 species, including confirmed candidate species (Olson 2013; Everson et al. 2016; Goodman et al. 2018). This highly diversified family is currently considered the result from a single colonization event originating from East Africa that took place between 30 and 56 My ago, followed by speciation that resulted in an exceptional adaptive radiation (Emerson, 2002; Krause, 2010). Some tenrecids exhibit a number of biological features unique among mammals, such as the ability of hibernating without interbout arousal, partial heterothermy or elementary echolocation (Lovegrove et al., 2014; Olson, 2013).

The long evolutionary history of the Tenrecidae also makes these mammals suitable for investigating the evolution of host-parasite interactions. Tenrecids host a diversity of Paramyxoviruses, some of which having experienced host switches with introduced murid rodents (Wilkinson et al., 2014). Tenrecidae are known to be hosts of two species of pathogenic *Leptospira*, namely *L. borgpetersenii* and *L. mayottensis* (Dietrich et al., 2014, 2018; Lagadec et al., 2016). While *L. mayottensis* has been essentially identified in tenrecids or acute human cases, a study on Madagascar reported the presence of *L. mayottensis* in introduced *Rattus rattus* but only as co-infections with other *Leptospira* species (Moseley et al., 2018). The strong host-specificity of *L. mayottensis* towards tenrecids was recently tested

through experimental infection in which *L. mayottensis* isolated from *T. ecaudatus* failed to colonize the kidneys of *R. norvegicus* (Cordonin et al., 2020). The present study was carried out to (i) further explore the diversity of *L. mayottensis* sheltered by tenrecids and (ii) confirm a previous hypothesis that proposed *L. mayottensis* arrived on Mayotte with the introduction of *T. ecaudatus* for human consumption.

Analyzed samples confirmed tenrecids as being a reservoir of *L. mayottensis* and added *M. thomasi* to the list of animal reservoirs of this pathogenic bacteria. Most interestingly, we report the first characterization of *L. mayottensis* from *T. ecaudatus* on Madagascar. Together with previous data reported on Mayotte (Lagadec et al., 2016), the present work supports the introduction of this mammal species being associated with the emergence of a zoonotic pathogen, *L. mayottensis*, on Mayotte. *Tenrec ecaudatus* has also been introduced to other islands in the Malagasy Region with the purpose of providing bush meat, most notably La Réunion, Mauritius, Mahé (Seychelles), and other islands in the Comoros archipelago, but to our knowledge *L. mayottensis* has been not isolated in these non-native tenrec populations or reported in local human inhabitants.

The data presented herein also strongly support that *L. mayottensis* is an endemic zoonotic pathogen to Madagascar. It has been hypothesized nearly a century ago that the extreme abundance and unbounded dispersal capacities of microorganisms limit endemism to some extreme environments and that biogeographical patterns result from contemporary selective pressures rather than from limited dispersal capacity. This dogma, often referred to as Bass Becking hypothesis - "*everything is everywhere but the environment selects*" (Baas Becking, 1934) - has been challenged, and microbial biogeography is still in its infancy (de Wit and Bouvier 2006; Fierer 2008). The present study supports that host-specificity needs be considered as a driver of microbial endemism: the dispersal capacities of host-specific microbes is indeed limited by that of their hosts. In other words, when considering host-parasite pairs, the dispersal capacities of hosts drive the biogeographical patterns of their associated microorganisms and may, in the case of strong host-parasite specificity, lead to microbial endemism.

Table and figure legends

Figure 1. **Geographical context and Bayesian phylogenetic tree of *Leptospira* species from Mayotte (blue) and Madagascar (green) based on *secY* gene (482 bp).** The analysis was conducted under the HKY+I+G substitution model and the tree is midpoint rooted. Black circles at the nodes indicate posterior probabilities superior or equal 0.90. The red stars indicate new sequences generated in the present study and were obtained from two regions on Madagascar: Anjozorobe and Antsirabe-Sahatany. The map was realized with the function *worldHires* from R package *mapdata* (Becker & Wilks, 2018) under the R software version 4.1.1.

Table S1. **Details on *Leptospira secY* sequences used in the present study: host families and species, geographical origins, *Leptospira* species and GenBank accession number.** **Sequences generated in the present study.*

Host families	Host species	Islands	*Leptospira* species	*SecY* GenBank accession number
Hipposideridae	*Macronycteris commersoni*	Madagascar	*Leptospira* sp.	KP822608
	Macronycteris commersoni	Madagascar	*Leptospira* sp.	KP822609
	Macronycteris commersoni	Madagascar	*Leptospira* sp.	KP822611
Hominidae	*Homo sapiens*	Mayotte	*Leptospira borgpetersenii*	JN683935
	Homo sapiens	Mayotte	*Leptospira borgpetersenii*	JN683944
	Homo sapiens	Mayotte	*Leptospira borgpetersenii*	JN683945
	Homo sapiens	Mayotte	*Leptospira interrogans*	JN683938
	Homo sapiens	Mayotte	*Leptospira kirschneri*	JN683930
	Homo sapiens	Mayotte	*Leptospira kirschneri*	JN683932
	Homo sapiens	Mayotte	*Leptospira kirschneri*	JN683936
	Homo sapiens	Mayotte	*Leptospira mayottensis*	JN683931
Miniopteridae	*Miniopterus griffithsi*	Madagascar	*Leptospira borgpetersenii*	KP822628
	Miniopterus griffithsi	Madagascar	*Leptospira kirschneri*	KP822629
	Miniopterus mahafaliensis	Madagascar	*Leptospira borgpetersenii*	KP822630
	Miniopterus mahafaliensis	Madagascar	*Leptospira borgpetersenii*	KP822642
	Miniopterus mahafaliensis	Madagascar	*Leptospira kirschneri*	KP822640
	Miniopterus majori	Madagascar	*Leptospira borgpetersenii*	KP822650
	Miniopterus manavi	Madagascar	*Leptospira borgpetersenii*	KP822623
	Miniopterus manavi	Madagascar	*Leptospira kirschneri*	KP822622
	Miniopterus sororculus	Madagascar	*Leptospira borgpetersenii*	KP822651
Molossidae	*Mormopterus jugularis*	Madagascar	*Leptospira* sp.	KP822656
	Otomops madagascariensis	Madagascar	*Leptospira* sp.	KP822664
Muridae	*Rattus rattus*	Mayotte	*Leptospira borgpetersenii*	KT338849
	Rattus rattus	Mayotte	*Leptospira interrogans*	KT338838
Nesomyidae	*Eliurus minor*	Madagascar	*Leptospira borgpetersenii*	KF928130
Pteropodidae	*Rousettus madagascariensis*	Madagascar	*Leptospira* sp.	KP822667

Rhinonycteridae	*Triaenops menamena*	Madagascar	*Leptospira* sp.	KP211750
	Triaenops menamena	Madagascar	*Leptospira* sp.	KP822613
	Triaenops menamena	Madagascar	*Leptospira sp.*	KP822616
Tenrecidae	*Hemicentetes nigriceps*	Madagascar	*Leptospira kirschneri*	KF928148
	Microgale cowani	Madagascar	*Leptospira mayottensis*	KF928128
	Microgale longicaudata	Madagascar	*Leptospira borgpetersenii*	KF928137
	Microgale majori	Madagascar	*Leptospira borgpetersenii*	KF928124
	Microgale majori	Madagascar	*Leptospira borgpetersenii*	KF928136
	Microgale principula	Madagascar	*Leptospira borgpetersenii*	KF928121
	Microgale principula	Madagascar	*Leptospira borgpetersenii*	KF928127
	Microgale thomasi	Madagascar	*Leptospira mayottensis*	MT422042*
	Nesogale dobsoni	Madagascar	*Leptospira mayottensis*	KF928135
	Nesogale dobsoni	Madagascar	*Leptospira mayottensis*	KT338848
	Nesogale dobsoni	Madagascar	*Leptospira mayottensis*	KT338850
	Nesogale dobsoni	Madagascar	*Leptospira mayottensis*	KT338851
	Tenrec ecaudatus	Madagascar	*Leptospira mayottensis*	MT422041*
	Tenrec ecaudatus	Mayotte	*Leptospira mayottensis*	KT338858
Vespertilionidae	*Myotis goudoti*	Madagascar	*Leptospira borgpetersenii*	KP822668
	Myotis goudoti	Madagascar	*Leptospira borgpetersenii*	KP822673
	Myotis goudoti	Madagascar	*Leptospira kirschneri*	KP822672
	Laephotis robertsi	Madagascar	*Leptospira* sp.	KP822680

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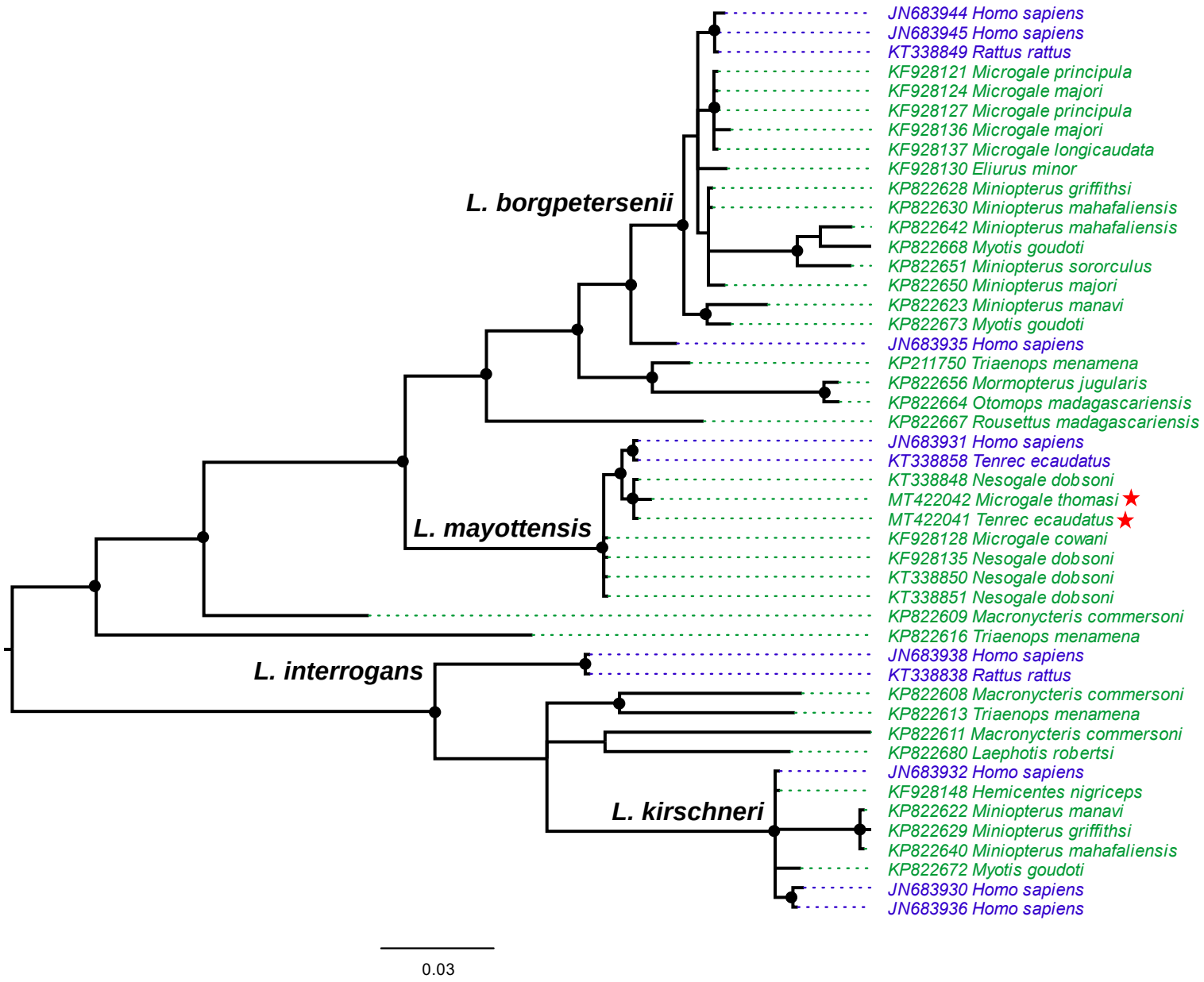
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KF928121 Microgale principula
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KF928137 Microgale longicaudata
KF928130 Eliurus minor
KP822628 Miniopterus griffithsi
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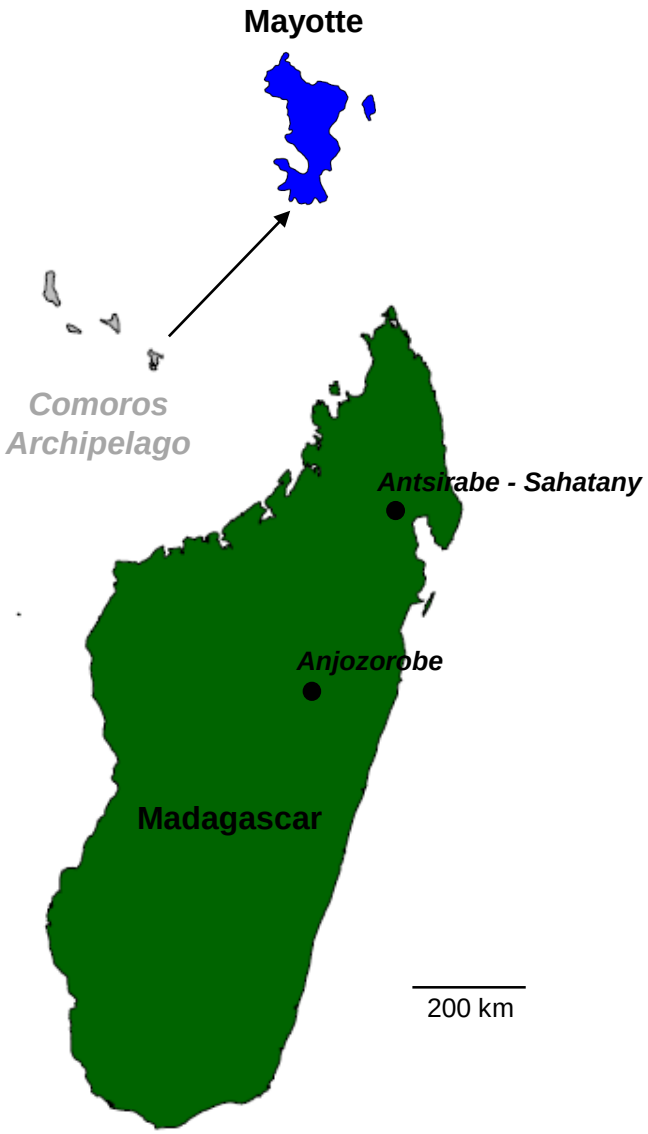
Mayotte
Comoros
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200 km