Malagasy bats shelter a considerable genetic diversity of pathogenic *Leptospira* suggesting notable host-specificity patterns.

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Running title: *Leptospira* host-specificity in Malagasy Chiroptera

One sentence summary: This study highlights the high genetic diversity of *Leptospira* in Madagascar bats and demonstrates that this genetic diversity is structured by a notable host-specifity pattern.

ABSTRACT

Pathogenic *Leptospira* are the causative agents of leptospirosis, a disease of global concern with major impact in tropical regions. Despite the importance of this zoonosis for human health, the evolutionary and ecological drivers shaping bacterial communities in host reservoirs remain poorly investigated. Here, we describe *Leptospira* communities hosted by Malagasy bats, composed of mostly endemic species, in order to characterize host–pathogen associations and investigate their evolutionary histories. We screened 947 individual bats (representing 31 species, 18 genera and seven families) for *Leptospira* infection and subsequently genotyped positive samples using three different bacterial *loci*. Molecular identification showed that these *Leptospira* are notably diverse and include several distinct lineages mostly belonging to *L. borgpetersenii* and *L. kirschneri*. The exploration of the most probable host-pathogen evolutionary scenarios suggests that bacterial genetic diversity results from a combination of events related to the ecology and the evolutionary history of their hosts. Importantly, based on the data set presented herein, the notable host-specificity we have uncovered, together with a lack of geographical structuration of bacterial genetic diversity, indicates that the *Leptospira* community at a given site depends on the co-occurring bat species assemblage. The implications of such tight host-specificity on the epidemiology of leptospirosis are discussed.

Keywords: *Leptospira*, Chiroptera, Madagascar, host-parasite association, host-specificity, co-phylogeny.

INTRODUCTION

Bats (Order Chiroptera) represent the second most diversified group of mammals on Earth, with over 1,100 recognized species (Simmons 2005). These flying mammals play important roles in ecological processes (Kunz et al. 2011) and are known reservoirs of numerous pathogens, including zoonotic viruses that in some cases have been comprehensively studied mainly due to their medical importance (Calisher et al. 2006; Wong et al. 2007; Chomel et al. 2015). However, the ecological factors and evolutionary processes shaping this microbial genetic diversity remain poorly understood.

Among bat infecting agents, bacteria have been less explored as compared to viruses, but different investigations have reported bats as carriers of pathogenic *Leptospira* (Emanuel, Mackerras and Smith 1964; Fennestad and Borg-Petersen 1972; Bunnell et al. 2000; Smythe et al. 2002a; Cox, Smythe and Leung 2005; Matthias et al. 2005; Zetun et al. 2009; Bessa et al. 2010; Tulsiani et al. 2011; Desvars et al. 2012; Lagadec et al. 2012; Mgode et al. 2014; Ogawa et al. 2015), the etiological agent of leptospirosis, a prototypical environmental zoonosis with a nearly global distribution. The bacterial cycle is maintained in nature by infected mammals, which excrete living *Leptospira* in their urine, contaminating the environment where the bacteria remain viable for several weeks or months (Levett 2001). The survival time of *Leptospira* in the environment appears to depend on different factors such as temperature, pH, nutrients and the presence of other organisms (Chang, Buckingham and Taylor 1948; Okazaki and Ringen 1957; Levett 2001; Andre-Fontaine, Aviat and Thorin 2015; Kumar et al. 2015); it is important to note that most of these studies have been carried out under laboratory conditions. Humans are accidentally infected, either during outdoor activities through contact with contaminated water or mud, or via direct exchange with infected animal secretions (Levett 2001; Bharti et al. 2003; Adler and de la Peña Moctezuma

2010).

Leptospira are typically classified into serovars and serogroups according to their antigenic determinants (Ahmed et al. 2012). More recently, genetic classifications have been developed allowing the identification of *Leptospira* species, including pathogenic species such as *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. mayottensis* (Ahmed et al. 2006; Lehmann et al. 2013; Bourhy et al. 2014).

Studies have revealed a high genetic diversity of *Leptospira* in bats, some of which are highly divergent from known lineages (Matthias et al. 2005; Lagadec et al. 2012; Dietrich et al. 2014). Using a multilocus sequence analysis, Dietrich et al. (2014) showed tight host-parasite specificity between *Leptospira* spp. and endemic Malagasy small terrestrial mammals (rodents [Nesomyinae] and tenrecs [Tenrecidae]) and some Malagasy bats species, suggesting that long-term co-evolution has shaped the leptospiral genetic diversity in these animals. However, Lei and Olival (2014) recently proposed that *Leptospira* communities in bats are structured by geography rather than by the phylogenetic relationships of their hosts. Given that bats evolved relatively deep in geological time (Agnarsson et al. 2011) and are a highly diversified order (Simmons 2005) occupying different ecological niches, they represent an outstanding biological model to investigate the biotic and abiotic drivers shaping host–parasite associations.

Considered as one of the five most important hot spots of macro-organism biodiversity in the world (Myers et al. 2000), Madagascar shelters over 46 bat species of which nearly 80% are endemic (Goodman and Ramasindrazana 2013; Goodman et al. 2015a;b). Of interest in the present work, Malagasy bats occupy different types of day roosts, including synanthropic structures and natural sites (such as caves), often composed of distinct taxonomic assemblages with certain species in physical contact (Goodman 2011). Moreover,

some of these taxa have broad distributions on the island, while others are geographically limited (Goodman and Ramasindrazana 2013). These different aspects allow testing for geographical *vs.* taxonomical structure of bacterial genetic diversity within these organisms.

In the present work, we screened 947 bats representing 31 species sampled along a broad north-south transect encompassing distinct bioclimatic zones of the island, to analyse the effect of host specificity and geography on the structure of the genetic diversity of *Leptospira*. For this, samples were screened through RT-PCR for *Leptospira* infection and positive samples were further genotyped using three housekeeping genes as markers. The role of host species assemblages on the composition of *Leptospira* was investigated at the roost site scale and the geographical patterns of bacteria distribution was tested across sampling sites. Finally, cophylogenetic signals and evolutionary scenarios that may account for the contemporary bat-*Leptospira* associations were evaluated using both distance- and event-based approaches.

MATERIALS AND METHODS

Study design and sampling

Bats were sampled on Madagascar from February 2012 to March 2013 in the context of a multi-level research program examining the taxonomy and biogeography of the island's bat fauna (e.g. Goodman and Ramasindrazana 2013; Goodman et al. 2015a;b; Christidis et al. 2014) with the description of their associated micro- and ecto-parasites (Tortosa et al. 2013; Duron et al. 2014; Wilkinson et al. 2014). Bats were trapped using mist nets, harp traps and butterfly nets in different natural or synanthropic habitats, encompassing four of the five recognized bioclimatic zones of the island (Cornet 1974). Specimens were captured,

manipulated and euthanized following guidelines accepted by the scientific community for the handling of wild mammals (Sikes and Gannon 2011) and in strict accordance with permits issued by Malagasy national authorities (see further details in the acknowledgements section). Herein we use the designation *Miniopterus manavi* (sensu lato) for members of this genus captured at Ambohitantely, where a range of divergent forms of this species complex occur in sympatry (Goodman et al. 2015b). Standard external morphological measurements were taken from each individual and several tissue samples (kidney, spleen, lung, salivary gland, brain, liver, intestine and urine or bladder) were preserved in liquid nitrogen soon after the animals were dispatched. Once back to the laboratory, the samples were stored at -80°C until molecular analyses. Voucher specimens were deposited at the Université d'Antananarivo, Département de Biologie Animale (UADBA), Antananarivo, and at the Field Museum of Natural History (FMNH), Chicago.

DNA extraction, *Leptospira* detection and prevalence

For each specimen, total nucleic acids were extracted from a pool of kidney, spleen and lung tissues using EZ1Virus Mini Kits version 2.0 (Qiagen, Les Ulis, France) on an EZ1 Biorobot as previously described (Wilkinson et al. 2012). A Reverse Transcription step was performed on total nucleic acids with GoScript Reverse Transcriptase (Promega, Madison, WI) and molecular detection was carried out on cDNA with a Real Time – Polymerase Chain Reaction (RT-PCR) using a pathogenic *Leptospira* specific fluorescent probe targeting the 5' end of the 16S encoding gene, following a previously described procedure (Smythe et al. 2002b). We used a Fisher's exact test implemented in R software version 2.15.3 (R Core Team 2013) to test the significance of difference in prevalence of infection among bat species.

Leptospira identification

Leptospira were identified to the species level by sequencing a portion of *secY*, a highly polymorphic housekeeping gene (Ahmed et al. 2006) commonly used for the determination of members of this genus (Perez and Goarant 2010; Dietrich et al. 2014). In addition, *Leptospira* phylogenies were constructed using two additional genes, *adk* and *rrs2*, both markers used in combination with *secY* in a previously described multilocus sequence typing (MLST) scheme (Ahmed et al. 2006), which was recently optimized by Dietrich et al. (2014). In each reaction, Polymerase Chain Reaction (PCR) mixture contained 12.5 µL of GoTaq Hot Start Green Master Mix 2X (Promega, Madison, WI), 1 µL (10 mM) of each primer, 8.5 µL of nuclease free water and 2 µL of cDNA. The PCR conditions consisted of an initial denaturation step at 95°C for 5 min followed by 45 cycles at 94°C for 30 s, 52 – 56°C for 30 s and 72°C for 1 min and a final elongation step of 7 min at 72°C. PCR products were visualized under UV light after electrophoresis on a 2% agarose gel stained with 1X GelRed^{TM} (Biotium Inc.) and sequenced on both strands through direct Sanger sequencing (Genoscreen, Lille, France) using the same amplification primer set.

Phylogenetic analyses

Nucleotide sequences were verified, corrected by visual inspection and aligned under the software Geneious pro software v.5.4 (Drummond et al. 2011). Nucleotide polymorphism was evaluated for each gene by quantifying the number of polymorphic sites using DnaSP 5.10.01 (Librado and Rozas 2009). The model of sequence evolution that best fit the data was determined for each marker using jModelTest v.0.1.1 (Posada 2008) based on the Akaike Information Criterion (AIC). Phylogenies were first constructed separately for each marker using sequences generated in the present work together with those available on GenBank (see

Supplementary Table 1). Phylogenetic constructions were carried out using Bayesian Inference analyses implemented in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). 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 2,000,000 generations with trees and associated model parameters sampled every 100 generations. For each phylogeny, the convergence level was validated by an average standard deviation of split frequencies inferior to 0.05. The initial 10% of trees from each run were discarded as burn-in and the consensus phylogeny and posterior probabilities were obtained from the remaining trees. The congruence between topologies obtained using each specific marker was verified using the Incongruence Length Difference (ILD) test implemented in PAUP 4.0b10 (Swofford 2002).

Genetic diversity and structure of bat-borne *Leptospira*

Although *adk* and *secY* markers both display a relatively high level of polymorphism, the number of obtained sequences was more important for *secY* (see results). Hence, only *secY* data were used to analyze the genetic structure of *Leptospira*, in order to maximize the number of species and sampling sites integrated into the analyses. The relative role of host specificity *vs.* spatial structure in shaping the genetic diversity of *Leptospira* was examined by performing a molecular variance analysis (AMOVA) implemented in ARLEQUIN v.3.5.1.3 (Excoffier and Lischer 2010). In our analyses, a “population” corresponds to *Leptospira* sequences obtained from a single bat species at a given sampling site. Geographical structure was firstly addressed by grouping populations by bioclimatic regions (humid, sub-humid, dry and sub-arid) (Cornet 1974) and subsequently by grouping populations by sampling site. Host specificity was analyzed by grouping populations at the level of host family (Hipposideridae,

Miniopteridae, Molossidae, Pteropodidae, Rhinonycteridae and Vespertilionidae). The significance levels of the fixation indices were obtained using 1023 nonparametric permutations. Finally, we assessed the role of host species community composition in shaping the genetic diversity of *Leptospira* by analysing the correlation between host species richness at a given sampling site and the number of detected *Leptospira* haplotypes, using a Pearson's R correlation test under R software version 2.15.3 (R Core Team 2013).

Co-phylogeny

Host-parasite associations were analysed using the phylogenies of bats and associated *Leptospira*. We specifically used sequentially a pruned phylogeny of *secY*, *adk* and *rrs2*, which resulted in a tree including only one sequence by well-supported *Leptospira* group detected in the Bayesian analyses. Thus, we defined 17, 12 and 22 *Leptospira* groups for *secY*, *adk* and *rrs2*, respectively. Bat phylogenies were built using the mitochondrial cytochrome b encoding gene (*cyt b*) available from GenBank (see Supplementary Table 2). In the case of Malagasy bat species for which *cyt b* sequences were incomplete or not available (*Hipposideros commersoni*, *Triaenops menamena*, *Paratriaenops furculus*, *Mormopterus jugularis*, *Neoromicia robertsi* and *Otomops madagascariensis*), PCRs and subsequent *cyt b* sequencing were carried out using L14724 and H15915 primers (Irwin, Kocher and Wilson 1991) (see Supplementary Table 2). Finally, bat and pruned bacteria phylogenies were generated by Maximum Likelihood under GTR + G model with RAxML (v.8.0.26) (Stamatakis 2006) by using rapid bootstrapping with 1,000 repetitions using the RAxML GUI (Silvestro and Michalak 2011). Parasite-host associations were visualized using TreeMap 3b software (Charleston 2011).

In order to assess the levels of congruence between bats and *Leptospira* phylogenies,

two programs based on global-fit methods were used: ParaFit (Legendre, Desdevises and Bazin 2002) and Procrustean Approach to Cophylogeny (PACo) (Balbuena, Míguez-Lozano and Blasco-Costa 2013). These programs require two input matrixes of patristic distances obtained from bat and *Leptospira* phylogenies together with a matrix of parasite-host associations (presence/absence of a *Leptospira* lineage in a bat species). The null hypothesis tested by ParaFit was a random association between parasites and hosts, while PACo tests the dependence of parasite and host phylogenies (Balbuena, Míguez-Lozano and Blasco-Costa 2013). ParaFit and PACo were performed with 999 and 10,000 permutations, respectively. Both tests were conducted using R software version 2.15.3 (R Core Team 2013) with APE package (version 3.0-8) (Paradis, Claude and Strimmer 2004) for both ParaFit and PACo, and with VEGAN packages (version 2.0-7) (Dixon 2003) for PACo.

Investigation of evolutionary history of host–parasite association

The reconciliation tool CoRe-PA version 0.5.1 (Merkle, Middendorf and Wieseke 2010), an event-based tree reconciliation program, was used to determine the most probable evolutionary scenarios (considering co-speciation, sorting, duplication and host-switching events) leading to the contemporary structure of *Leptospira* spp. within Malagasy bats. CoRe-PA is able to incorporate timing information to restrict possible co-evolutionary scenarios. An applicable timing for the nodes of the aforementioned phylogenetic trees was computed using the branch lengths of the given tree. As a first step, the branch lengths were adjusted to obtain trees with all paths from the root to a leaf having the same length. Thereafter, an optimization problem was defined using integer linear programming (ILP) (Wolsey and Nemhauser 1999). For each branch length b_i of a branch i, a factor/multiplier f_i, ranging from 0.25 to 4.0 was sought, such that for the tree with adjusted branch lengths b_i*f_i all paths from the root to a

leave were equal. From all possible sets of factors, the set finally selected was the one minimizing the branch length adjustment \sum_i |b_i-(b_i*f_i)|. In a second step, time zones were assigned to nodes with root node designated to time zone 0 and leaves to time zone 10. The inner nodes were assigned to time zones proportionally to the length of the path from the root to the respective node in the adjusted tree. In order to lower the stringency of evolutionary scenario selection, a time zone interval of ± 5 of the previously computed time zones was assigned to each node of the *Leptospira* tree. CoRe-PA analyses were performed using the "rank" and "automatic cost evaluation" options with 5,000 random cycles. The statistical significance was assessed with 100 reconstructions of the same trees and randomly generated *Leptospira* - bat leaf-to-leaf associations.

RESULTS

Bats sampling

In total, tissue samples from 947 bat specimens representing 31 species, 18 genera and seven families (Table 1) were obtained from 52 sites on Madagascar (see Supplementary Table 3). The sampling was conducted in caves (n = 22), synanthropic sites (n = 18) and forested zones (outdoor trapping) (n = 12). Thirty-nine percent (n = 7) of synanthropic sites were composed of two or three bat species and harboured only Molossidae species: *Chaerephon* spp., *Mops* spp. and *Mormopterus jugulari*s. Seventy-three percent (n = 16) of caves contained two to seven sympatric bat species. Within these sites, the bats composition varied from two bat families (example: Miniopteridae and Vespertilionidae or Hipposideridae and Molossidae) to five families (example: Hipposideridae, Miniopteridae, Molossidae, Rhinonycteridae and Vespertilionidae). Few details are available about possible physical contact between sympatric bat taxa.

Leptospira detection and identification

Real-Time PCR revealed 203 positive samples, indicating a global prevalence of *Leptospira* infection of 21.4% and at least one infected bat species per family (Table 1). At the species level, 18 of the 31 bat taxa were infected, with significant differences in leptospiral infection between species (P-value < 0.001, Fisher's exact test). 120 out of 203 RT-PCR-positive *Leptospira* samples we successfully amplified and sequenced for at least one of the three bacterial markers (*secY, adk* and *rrs2*). Amplification failed at all three loci for 83 RT-PCR positive samples. Based on *secY*–phylogeny (see below), all identified *Leptospira* clustered into the pathogenic clade and most of them (67.0%) were typed as *L. borgpetersenii* and *L. borgpetersenii*-related. *Leptospira kirschneri* was identified in 12.3% of genotyped

Table 1. *Leptospira* detection in Malagasy bats by Real-Time PCR. Infected species are indicated in bold. I: insectivorous species, F: frugivorous species.

Bat family	Bat species	Sample size	Number positive (%)
Emballonuridae (I) (n = 9)	***Coleura kibomalandy***	3	1 (33.3)
	Paremballonura tiavato	6	0
Hipposideridae (I) (n = 27)	***Hipposideros commersoni***	27	13 (48.1)
Miniopteridae (I) (n = 289)	*Miniopterus aelleni*	7	0
	***Miniopterus manavi* sensu lato**	19	14 (73.7)
	Miniopterus gleni	22	0
	Miniopterus griffithsi	7	5 (71.4)
	Miniopterus griveaudi	116	10 (8.6)
	Miniopterus mahafaliensis	89	45 (50.6)
	Miniopterus majori	7	2 (28.6)
	Miniopterus sororculus	22	13 (59.1)
Molossidae (I) (n = 406)	*Chaerephon atsinanana*	34	0
	Chaerephon leucogaster	94	1 (1.1)
	Mops leucostigma	68	0
	Mops midas	19	0
	Mormopterus jugularis	152	27 (17.8)
	Otomops madagascariensis	39	4 (10.3)
Rhinonycteridae (I) (n=56)	***Paratriaenops furculus***	14	1 (7.1)
	Triaenops menamena	42	27 (64.3)
Pteropodidae (F) (n = 80)	*Eidolon dupreanum*	11	0
	Pteropus rufus	20	2 (10.0)
	Rousettus madagascariensis	49	15 (30.6)
Vespertilionidae (I) (n = 80)	*Hypsugo bemainty*	2	0
	Myotis goudoti	48	21 (43.8)
	Neoromicia malagasyensis	2	0
	Neoromicia matroka	4	0
	Neoromicia robertsi	1	1 (100.0)
	Pipistrellus / Neoromicia sp.	8	0
	Pipistrellus hesperidus	11	0
	Pipistrellus raceyi	3	0
	Scotophilus marovaza	1	1 (100.0)

individuals and the remaining sequences (20.5%) could not be assigned to any known *Leptospira* taxon.

We identified leptospiral infection in seven bat species that have not been previously investigated: *Coleura kibomalandy* (Emballonuridae), *Hipposideros commersoni* (Hipposideridae), *Miniopterus manavi* (Miniopteridae), *Chaerephon leucogaster* (Molossidae), *Rousettus madagascariensis* (Pteropodidae), and *Neoromicia robertsi* and *Scotophilus marovaza* (Vespertilionidae). All tested species within the Hipposideridae and Rhinonycteridae were found positive for *Leptospira*. Similarly, all but two *Miniopterus* spp. (*M. aelleni* and *M. gleni*) were found infected with *Leptospira*. Within the Molossidae, *Chaerephon* spp. and *Mops* spp. showed lower prevalence of infection than *Mormopterus jugularis* and *Otomops madagascariensis* (P-value < 0.01, Fisher's exact test). Noteworthy, *M. jugularis* was the only species sampled both in caves (n = 31) and synanthropic sites (n = 108). A Pearson's Chi-square test indicated that there was no significant difference in prevalence of infection of this species sampled in both types of habitat (P-value = 0.54).

Phylogeny of *Leptospira*

Amplification of *secY*, *adk* and *rrs2* loci was performed on all *Leptospira* positive samples and produced 73, 36 and 116 sequences of 482, 500 and 503-504 base pairs, respectively. All sequences were deposited in GenBank (see Supplementary Table 4). The number of polymorphic sites was higher in *secY* (38%) and *adk* (37%) than in *rrs2* (8%). Phylogenies of *Leptospira* based on *secY*, *adk* and *rrs2* markers are depicted in Figures 1, S1 and S2, respectively. No concatenated phylogeny was produced since the ILD test did not validate the congruence of topologies obtained with the different markers (P-value < 0.01). The *secY*-based phylogeny displays seven well-supported pathogenic *Leptospira* clades (A to G)

Fig. 1 Phylogenetic tree based on pathogenic *Leptospira secY (*482 bp) gene. The analysis was carried out using Bayesian Inference under the HKY+I substitution model. Nodal values correspond to posterior probabilities. The letters (A to G) designate the main well-supported *Leptospira* clades. *Leptospira* haplotypes are coloured according to host family (red: Hipposideridae, blue: Miniopteridae, purple: Molossidae, orange: Pteropodidae, light blue: Rhinonycteridae and green: Vespertilionidae). Names typed in black refer to *Leptospira* (L.) sequences accessible from GenBank (see Supplementary Table 1). *Hi: Hipposideros, Mi: Miniopterus, Mo: Mormopterus, My: Myotis, Ne: Neoromicia, Ot: Otomops, Ro: Rousettus* and *Tr: Triaenops.*

infecting Malagasy bats (Fig. 1). Clade A corresponds to *L. borgpetersenii* and is composed of sequences obtained only from *Miniopterus* spp. and *Myotis goudoti*. Bacterial sequences obtained from Molossidae (*Mormopterus jugularis* and *Otomops madagascariensis*) and Pteropodidae (*Rousettus madagascariensis*) taxa cluster in two distinct monophyletic clades C and D, respectively, both being closely related to *L. borgpetersenii* clade A. Clade B includes *L. kirschneri* haplotypes and, as for clade A, is composed only of *Leptospira* infecting *Miniopterus* spp. and *Myotis goudoti*. Clade E is composed of haplotypes obtained from *Hipposideros commersoni* and clades F and G are composed of haplotypes obtained from *Triaenops menamena*. Interestingly, these three later clades do not cluster with any known pathogenic *Leptospira* taxon, although clade G is embedded into a clade containing *L. noguchii, L. kirschneri* and *L. interrogans*. Finally, a sequence from *Neoromicia robertsi* clustered with a sequence obtained from *H. commersoni*.

Phylogenetic reconstruction with *adk* displayed comparable information to that obtained with *secY* despite a more limited number of sequences (see Supplementary Figure 1). A greater number of sequences were obtained from *rrs2*, but the resulting phylogeny was poorly resolved (see Supplementary 2).

Genetic population structure of *Leptospira*

The AMOVA indicated that the genetic diversity of bat-borne *Leptopsira* was not dependent on geoclimatic parameters, specifically the distinct bioclimatic regions of Madagascar (F_{CT} = -0.02769, P-value = 0.63734) or the geographic location of sampling sites (F_{CT} = -0.08457, P-value = 0.67449) (Table 2). Moreover, bats of the same species and sampled at distant sites shared closely related bacterial haplotypes (Fig. 2). This aspect was observed with *Leptospira* spp. detected in *Myotis goudoti*, *Miniopterus* spp., *Hipposideros commersoni*, *Triaenops*

Table 2. Analysis of molecular variance (AMOVA) based on *Leptospira secY* gene. In each analysis, a population is referred to all *Leptospira* sequences detected in a single bat species at a given sampling site.

Comparison	Source of variation	d.f.	Fixation indices (F)	P-value	% of variation
Bioclimatic regions	Among regions (n = 4)	3	Φ_{CT} = -0.02769	0.63734	-2.77
	Among host species within regions	36	Φ_{SC} = 0.43875	< 0.001	45.09
	Within host species	33	Φ_{ST} = 0.42321	< 0.001	57.68
Sampling sites	Among sites (n = 23)	22	Φ_{CT} = -0.08457	0.67449	-8.46
	Among host species within sites	17	Φ_{SC} = 0.47232	< 0.01	51.23
	Within host species	33	Φ_{ST} = 0.42769	< 0.001	57.23
Host families	Among families (n = 6)	5	Φ_{CT} = 0.51451	< 0.001	51.45
	Among host species within families	34	Φ_{SC} = -0.02617	0.48680	-1.71
	Within host species	33	Φ_{ST} = 0.50180	< 0.001	49.82

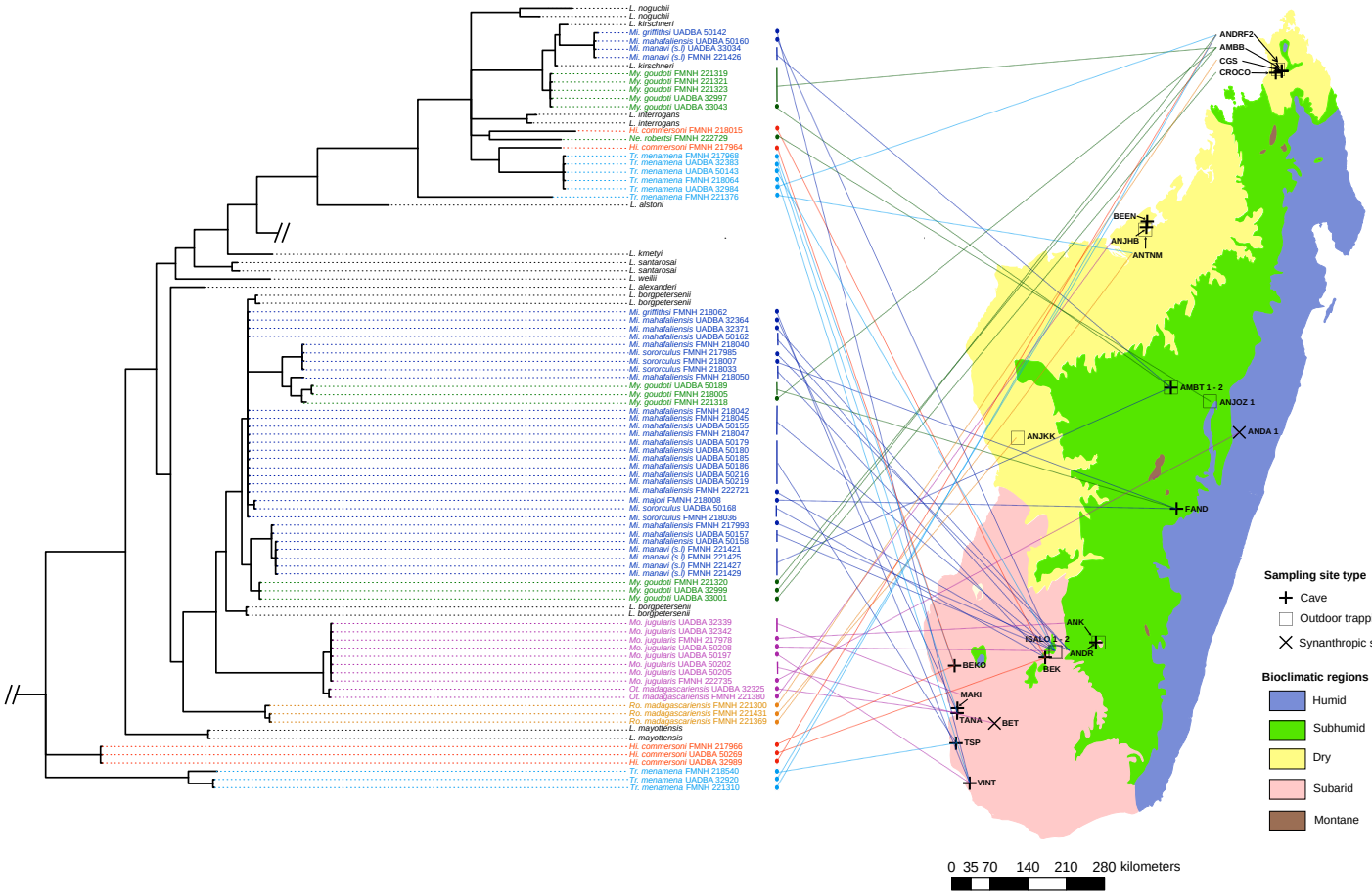

Fig. 2 Distribution of pathogenic *Leptospira* spp. in bats sampled in different sites and bioclimatic regions (Cornet 1974) of Madagascar. *Leptospira* phylogeny is based on *secY* sequences. For details on code colours and acronyms see Figure 1 and for sampling sites (example: BET, TSP, VINT…) see Supplementary Table 3.

menamena, *Otomops madagascariensis* and *Rousettus madagascariensis*. In keeping with this pattern, *Mormopterus jugularis* was infected with a unique *Leptospira* haplotype regardless of the roost site type (synanthropic or natural) and bioclimatic zone (dry, sub-arid, sub-humid or humid). In contrast, we detected that global leptospiral genetic diversity was strongly associated with the host, as differences between host families accounted for 51.45% of the variation (F_{CT} = 0.51451, P-value < 0.001). The AMOVA test also indicated a high genetic variation (49.82%) within host species (Table 2), associated with the observation that certain bat species can harbour several distinct *Leptospira* lineages, as exemplified for *Myotis goudoti*, *Miniopterus* spp., *H. commersoni* and *T. menamena*. Finally, we found a significant positive correlation between host species richness at a given site and the associated genetic diversity of *Leptospira* (r^2 = 0.87, df = 12, P-value < 0.001) (Fig. 3).

Co-phylogeny

ParaFit and PACo did no support co-evolution between bats and their respective *Leptospira* based on *secY* (ParaFitGlobal = 4.5128, P-value = 0.09; m2 global value = 25.9503, P-value = 0.06) and *adk* data sets (ParaFitGlobal = 0.4922, P-value = 0.47; m2 global value = 16.5497, P-value = 0.26). When *rrs2* data were used, ParaFit analysis was consistent with the absence of global co-evolution between bats and *Leptospira* (ParaFitGlobal = 0.0249, P-value = 0.07), while PACo analysis indicated a co-evolution pattern (m2 global value = 7.4856, P-value < 0.01).

Analyses of co-evolutionary scenarios

The CoRe-PA analysis was not performed on *adk* as branch lengths were notably different and did not allow length adjustment, or on *rrs2* because of the poor resolution of this marker

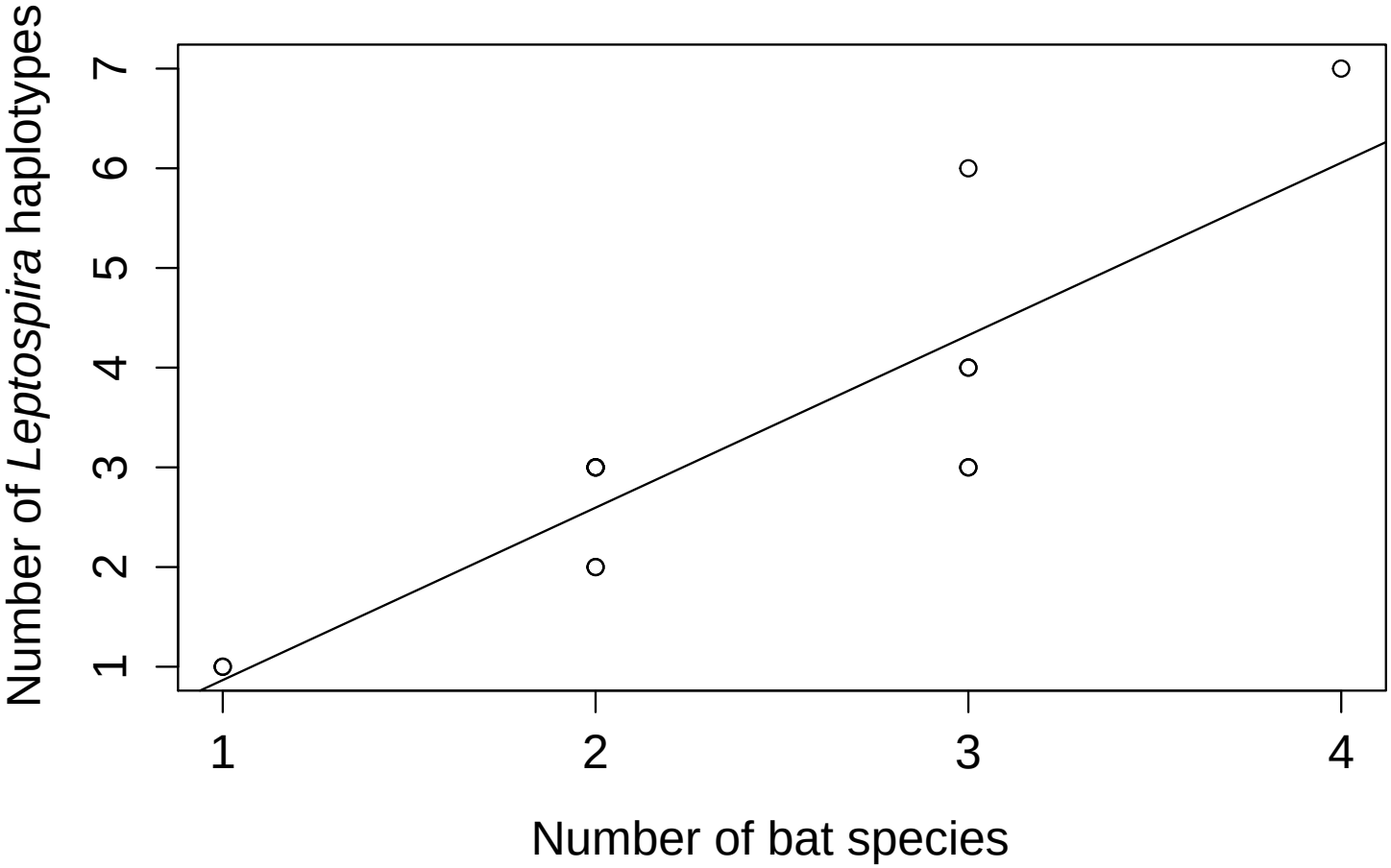

Fig. 3 Correlation between *Leptospira* haplotype diversity (based on the *secY* gene) and host species richness within sampling sites (r2 = 0.87, df = 12, P-value < 0.001, Pearson's R correlation test). At a given sampling site, the number of host corresponds to the number of bat species from which at least one *secY* sequence was obtained.

(see Supplementary Figure 2). Based on the *secY* data set, the *Leptospira* and bat co-phylogeny analysis using CoRe-PA produced 384 distinct co-evolutionary reconstructions. Fifteen solutions displayed the same quality value (0.108) and corresponded to the preferred reconstruction. All of the 15 reconstructions had the same event frequencies: seven co-speciation, 25 sorting, 11 duplication and seven host-switching events. For the remaining reconstructions, the quality value varied from 0.122 to 1.029. The randomized data sets showed more co-speciation events (8.21 on average) and a better quality value (0.040) than the original data set (0.108). Moreover, 69% of the randomized reconstructions showed more co-speciation events than did the original data set. These results do not support co-phylogeny between bats and their associated *Leptospira*. Lastly, five of the seven detected host-switching events involved *Myotis goudoti* and three occurred between *Miniopterus* spp. and *Myotis goudoti* (Fig. 4).

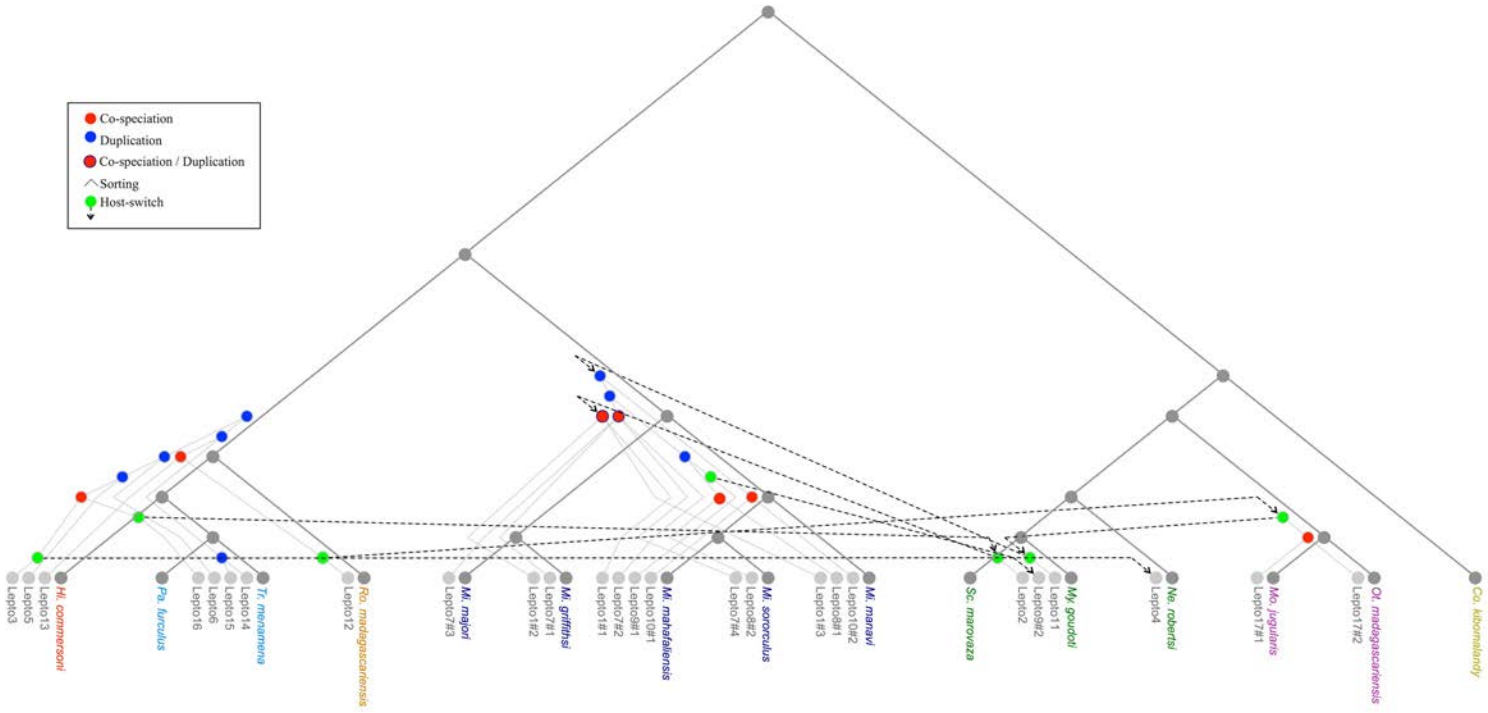

Fig. 4 Representation of the most parsimonious co-evolutionary scenario for *Leptospira* (light grey) and bat lineages (dark grey) proposed by CoRe-Pa. *Leptospira* and bat phylogenies were based on *secY* and *cyt b*, respectively. As for *Leptospira* lineages, 17 distinct groups were defined based on the *secY* phylogeny and numbered accordingly. A given *Leptospira* can be found in different bat species and is annotated as for example: Lepto8#1 in *Miniopterus manavi* and Lepto8#2 in *Miniopterus sororculus*. Each colour corresponds to a bat family (yellow: Emballonuridae, red: Hipposideridae, blue: Miniopteridae, purple: Molossidae, orange: Pteropodidae, light blue: Rhinonycteridae and green: Vespertilionidae). *Co: Coleura, Hi: Hipposideros, Mi: Miniopterus, Mo: Mormopterus, My: Myotis, Ne: Neoromicia, Pa: Paratriaenops, Ot: Otomops, Ro: Rousettus, Sc: Scotophilus* and *Tr: Triaenops.*

DISCUSSION

A high genetic diversity of pathogenic *Leptospira* is detected in several Malagasy bat species

Our study highlights that *Leptospira* infection is widespread in Malagasy bats with new documented cases of infection in species belonging to seven families. Interestingly, as previously reported from the New World tropics (Matthias et al. 2005), we detected considerable variation in prevalence of leptospiral infection among different Malagasy bat species. Our data also showed either absence or low prevalence of infection in the genera *Chaerephon* and *Mops* (Molossidae), whereas other members of this family, *Mormopterus jugularis* and *Otomops madagascariensis*, showed high prevalence of infection. This may be associated with differences in roosting ecology: our sampled individuals of *Chaerephon* spp. and *Mops* spp. were predominantly from synanthropic roost sites, whereas *M. jugularis* and *O. madagascariensis* where at least in part from natural cave roost sites. We did not find significant differences in prevalence of infection among *M. jugularis* sampled in natural as compared to synanthropic roost sites.

The present study reveals a higher genetic diversity of pathogenic *Leptospira* spp. in Malagasy bats than previously documented (Lagadec et al. 2012; Dietrich et al. 2014). *Leptospira borgpetersenii* has been already reported in Madagascar bats (Lagadec et al. 2012; Dietrich et al. 2014) and appear herein as a dominant *Leptospira* taxon. It is interesting to note that *L. borgpetersenii* and *L. kirschneri* (the latter is reported here for the first time in Malagasy bats) have been also reported in South American and African bats (Matthias et al. 2005; Ogawa et al. 2015). We also detected undescribed *Leptospira* lineages hosted notably by *Hipposideros commersoni*, *Neoromicia robertsi*, *Rousettus madagascariensis* and *Triaenops menamena*. Lastly, even though we failed to amplify and sequence the *secY* and

adk genes of *Leptospira* infecting some bat species within our sample (*Paratriaenops furculus*, *Coleura kibomalandy* and *Scotophilus marovaza*), the obtained *rrs2* sequences were indicative of other potentially undescribed bacterial lineages. The presence of divergent *Leptospira* lineages and/or the low bacterial loads could explain the PCR failures from certain *Leptospira*–positive bats. For example, we could not genotype *Leptospira* from *Miniopterus griveaudi* samples for which RT-PCR Cycle Threshold (CT) values were high (40.0 ± 1.19, n = 10). In contrast, we obtained *Leptospira* sequences from the other Miniopteridae species, which displayed lower CT values ranging from 29.7 ± 6.24 (n = 5) to 33.3 ± 5.19 (n = 2). Altogether, our work suggests that the actual bacterial genetic diversity in Malagasy bats is likely even higher than documented herein.

Leptospira spp. display a notable host-specificity pattern

Although serological tools have indicated some specificity of *Leptospira* towards host reservoirs with certain serovars commonly associated to specific animals (Bharti et al. 2003), only a few studies have addressed the question of *Leptospira* host-specificity at the molecular level (Cameron et al. 2008; Cosson et al. 2014; Dietrich et al. 2014; Koizumi et al. 2015). Based on species-specific primers, Cameron et al. (2008) highlighted a *Leptospira* host-specificity pattern in pinniped populations with sea lions (*Eumetopias jubatus* and *Zalophus californianus*) infected only by *L. interrogans* and northern elephant seals (*Mirounga angustirostris*) infected only by *L. kirschneri*. Similarly, endemic Malagasy small terrestrial mammals and bats have been shown to be infected by distinct *Leptospira* lineages (Dietrich et al. 2014).

Using molecular approaches and Malagasy bats, which provide a powerful biological model of high levels of endemicity and diverse geographical distribution for host species, the

present study looks deeper into patterns of *Leptospira* host-specificity. Our data indicate that *Leptospira* spp. display a high degree of host-specificity for Malagasy bats at different taxonomic levels. Generally, each bat species harbours its own *Leptospira* lineage or lineages. For the Miniopteridae, the bacteria host-specificity pattern has to be considered at the level of host genus, as there is no evidence of specific *Leptospira* haplotypes (or clades) restricted to a given *Miniopterus* species. Overall, the host-specificity pattern together with the lack of geographical structure of leptospiral genetic diversity indicate that (i) co-roosting bat species do not share the same *Leptospira* community (except in the case of *Miniopterus* in which different species within the genus can harbour the same *Leptospira* community) and (ii) the genetic diversity of *Leptospira* depends on the bat host species richness at a given site (Fig. 3). Such bacteria host associations imply that the geographical distribution of a given *Leptospira* haplotype depends on the dispersal patterns of the host species. For instance, *Mormopterus jugularis* harbours a unique *Leptospira* haplotype across a broad area of the island, irrespective of the roost site type or bioclimatic zone. The maintenance of this unique haplotype could be also reinforced by the fact that *M. jugularis* is genetically panmictic across its distribution and indicating broad patterns of dispersal (Ratrimomanarivo et al. 2009).

Globally, analyses with *secY* and *adk* genes did not show evidence of a co-evolution signal between Malagasy bats and their respective *Leptospira*. Using the same global-fit methods employed herein (ParaFit and PACo), Lei and Olival (2014) conducted a similar study using previously published *rrs2* sequences (26 sequences belonging to 20 bat species) and did not find evidence of co-evolution between bats and *Leptospira*. Here, with 116 *rrs2* *Leptospira* sequences from 31 bat species, we come to a similar conclusion based on the ParaFit analyses while PACo analyses provide evidence of co-evolution. This result may be biased because of the poor resolution of the *rrs2* tree and the presence of many related

Leptospira in the same host. These findings underline that results provided by co-evolutionary analyses should be interpreted cautiously as they strongly depend on the chosen method and the resolution of the used genetic marker. Lastly, an increase in sample size (*i.e.* number of sequences in the data set) may help clarify the significance of co-evolution signals obtained from those markers showing P-values close to 0.05.

The leptospiral genetic diversity related to the ecology and the evolution history of bats

Based on the *secY* gene, the CoRe-PA analysis indicated that the Malagasy bat–*Leptospira* evolutionary history results from a combination of duplication, co-speciation, host-switching as well as numerous sorting events (Fig. 4). These events could be explained by the ecology of the hosts, as for example their roosting behaviour, particularly when species occur in sympatry or syntony (*i.e.* with physical contact within the colony) in day roost sites. Such roosting behaviour could favour parasite transmission between co-roosting bat species (Hayman et al. 2013) and in turn favour host-switching events. In the present study, this assumption was substantiated by three host-switching events detected between *Miniopterus* spp. (Miniopteridae) and *Myotis goudoti* (Vespertilionidae), both bat taxa belonging to sister families and known to occur syntopically in mixed day roost sites (Goodman 2011; Miller-Butterworth et al. 2007). Within the sampling sites, six caves harboured at least two different *Miniopterus* spp. and in five caves, *Myotis goudoti* occurred in sympatry with at least one *Miniopterus* sp. (see Supplementary Table 3). The level of direct contact of *M. goudoti* with different species of *Miniopterus* might be a key element in the transmission of bacteria between non-sympatric *Miniopterus*. For instance, *M. mahafaliensis*, *M. griffithsi* and *M. manavi* share the same *L. kirschneri* haplotype (Fig. 1), although *M. manavi* is not living in sympatry with the other two *Miniopterus* spp., but in each case with *Myotis goudoti*. Thus, as

this latter is a widespread species (Goodman and Ramasindrazana 2013), occurs in syntopy with different *Miniopterus* spp. and does not show very pronounced patterns of genetic variation (Weyeneth et al. 2010), we can hypothesize that *Myotis* plays a bridging role for *Leptospira* host-switch between non-sympatric *Miniopterus* spp.

The host-switches are not restricted to syntopic bat species (*Miniopterus* spp. and *Myotis goudoti*) since such events were also detected between other sympatric (Molossidae – *M. goudoti*, *Rousettus madagascariensis* – Molossidae, *Triaenops menamena – M. goudoti*) and non-sympatric species (*Hipposideros commersoni* and *Neoromicia robertsi*) (Fig. 4 and see Supplementary Table 3). These findings underline that physical close contact is not a prerequisite for host-switching events and there are probably other means for horizontal transmission of *Leptospira*.

Beside ecological behaviour, parasite-host specificity may depend on the evolutionary and natural history traits of both parasites and hosts (Poulin and Keeney 2008). On Madagascar, the contemporary bat fauna is the result of at least 20 different colonization events from the mid-Miocene to the modern era and mainly from Africa (Samonds et al. 2013). In several cases, an adaptive radiation followed successful colonization by an ancestral population, as best illustrated by the genus *Miniopterus* whose initial divergence took place an estimated 4.5 Mya (Christidis et al. 2014). This relatively recent radiation and the syntopic behaviour displayed by several *Miniopterus* spp. can explain the absence of bacteria-host specificity at the species level within the genus. Whether the ancestral *Miniopterus* brought over different bacterial lineages from continental Africa or got infected following successful colonization of Madagascar by phylogenetically different bat groups may be addressed through a thorough investigation of *Miniopterus*-*Leptospira* associations in continental Africa.

Epidemiological implications

The tight host-specificity reported here between *Leptospira* and bat species may have broad implications in understanding patterns of maintenance in reservoir hosts. Our study is based on the PCR detection of bacterial DNA in a pool of tissue biopsies including kidney. Although it cannot be ruled out that PCR positive animals were actually experiencing acute systemic infection, we propose that the high prevalence of infection reported herein is a signature of the carrier state of the positive animals, *i.e.* their capacity to maintain a bacterial biofilm in the proximal convoluted tubule of the kidney with bacteria being excreted upon urination. The bacteria host-specificity pattern reported in our study might reflect the selection for an appropriate bacterial genotype able to develop and be maintained in the host. Previous studies hypothesized that biofilms formed by *Leptospira* play an important role in the maintenance of bacteria in the kidneys of reservoir hosts (Ristow et al. 2008; Matsui et al. 2015). Further, it can be suggested that some specific interactions between the tubular epithelial cell and the *Leptospira* bacterial cell wall might trigger the first steps of the biofilm formation and act as the selection force. As mammals can be infected by different pathogenic *Leptospira*, the genotype of the selected bacteria, which defines the carrier state of the specific host, does not preclude multiple infections of the same individual by different *Leptospira* species, and that at a fine level are controlled by the immune responses of the host. Serological analyses are warranted to provide information on the actual exposure of bats to leptospiral infection and assess the decoupling between the carrier state, defining strict host specificity and the infection state, which is likely less restricted with regard to infecting genotypes.

CONCLUSIONS

The data presented herein help to complete available information on *Leptospira* infection and genetic diversity in bats from different regions of the world, in this case from Madagascar. Our results highlight a notable level of specificity of *Leptospira* with regards to their bat hosts; this relation shows sufficient correlation to host identity of the different *Leptospira* taxa described herein. In other words, "*tell me who you walk with and I will tell you who you are*" (Spanish proverb). The present study should help stimulate molecular characterization of pathogenic *Leptospira* and structuration within different animal reservoirs. Such approaches will provide insightful data regarding the ecology of *Leptospira* and the medical and/or veterinary importance of local animal reservoirs.

DATA ACCESSIBILITY

Accession numbers: All *cyt b* sequences obtained from bats in the frame of the present work were deposited on GenBank under accession numbers KR606331 – KR606336 (see Supplementary Table 2). *Leptospira* sequences were deposited on GenBank under accession numbers KP822572 - KP822607, KP822608 - KP822680, KP822681 - KP822796, for *adk*, *secY*, *rrs2* genes, respectively (see Supplementary Table 4).

Sequences alignment: Sequences alignment of each gene is available on Dryad (doi: XXXX)

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Supplementary Table 1. Details on *Leptospira* sequences from GenBank used in the present study. NA: not available or not used in the present study.

Leptospira species	***secY***	***adk***	***rrs2***	**Isolation source**	**Site / Country**
Leptospira alexanderi	AHMT02000039	NA	AY631880	*Homo sapiens*	China
Leptospira alstoni	NZ_AOHD02000041	NA	DQ991480.1	Frog	Pingchang (China)
Leptospira biflexa	CP000786	CP000786	NR_102883	NA	NA
Leptospira borgpetersenii	JN683933	JN683882	JN683865	*Homo sapiens*	Mayotte (France)
Leptospira borgpetersenii	JN683939	JN683888	JN683871	*Homo sapiens*	Mayotte (France)
Leptospira borgpetersenii	JN683944	JN683893	JN683876	*Homo sapiens*	Mayotte (France)
Leptospira borgpetersenii	JN683943	JN683892	JN683875	*Homo sapiens*	Mayotte (France)
Leptospira borgpetersenii	NA	NA	AY995713	*Sturnira lilium*	Peru
Leptospira borgpetersenii	NA	NA	AY995714	*Sturnira tildae*	Peru
Leptospira borgpetersenii	NA	NA	AY995715	*Desmodus rotundus*	Peru
Leptospira borgpetersenii	NA	NA	AY995716	*Carollia perspicillata*	Peru
Leptospira broomii	AHMO02000008	AHMO02000008	AHMO02000008	*Homo sapiens*	Denmark
Leptospira fainei	AKWZ02000010	AKWZ02000010	AY631885	*Sus scrofa*	Australia
Leptospira fainei	NA	NA	AY995712	*Uroderma bilobatum*	Peru
Leptospira inadai	AHMM02000015	AHMM02000015	AHMM02000015	*Homo sapiens*	USA
Leptospira interrogans	JN683938	JN683887	JN683870	*Homo sapiens*	Mayotte (France)
Leptospira interrogans	JF509188	JF509328	JF509216	NA	Thailand

Leptospira interrogans	NA	NA	AY995729	*Promops nasutus*	Peru
Leptospira kirschneri	JN683930	JN683879	JN683862	*Homo sapiens*	Mayotte (France)
Leptospira kirschneri	JN683932	JN683881	JN683864	*Homo sapiens*	Imported from Nosy Be (Madagascar)
Leptospira kirschneri	NA	NA	AY995730	*Phyllostomus hastatus*	Peru
Leptospira kmetyi	AHMP02000003	NA	NR_041544	soil	Malaysia
Leptospira licerasiae	NZ_AFLO01000002	NA	EF612287	*Philander opossum*	Peru
Leptospira mayottensis	JN683931	JN683880	JN683863	*Homo sapiens*	Mayotte (France)
Leptospira mayottensis	JN683934	JN683883	JN683866	*Homo sapiens*	Mayotte (France)
Leptospira meyeri	NZ_AKXE01000001	NZ_AKXE01000001	AY631889	*Homo sapiens*	Canada
Leptospira meyeri	ANIL01000018	ANIL01000018	FJ154599	*Rattus* sp.	Indonesia
Leptospira noguchii	NZ_AKWY02000014	NZ_AKWY02000014	AY631886	*Didelphis marsupialis*	Panama
Leptospira noguchii	AHMF02000105.1	NA	NA	*Homo sapiens*	Hawaii (USA)
Leptospira santarosai	NZ_ANNW01000041	NZ_ANNW01000041	NA	*Homo sapiens*	Iquitos, (Peru)
Leptospira santarosai	NZ_AOHB02000045	NZ_AOHB02000045	FJ154576	*Homo sapiens*	Iquitos, (Peru)
Leptospira sp.	NA	NA	AY995717	*Lonchophylla thomasi*	Peru
Leptospira sp.	NA	NA	AY995718	*Artibeus planirostris*	Peru
Leptospira sp.	NA	NA	AY995719	*Artibeus planirostris*	Peru
Leptospira sp.	NA	NA	AY995720	*Rhinophylla pumilio*	Peru
Leptospira sp.	NA	NA	AY995721	*Glossophaga soricina*	Peru
Leptospira sp.	NA	NA	AY995722	*Mimon crenulatum*	Peru
Leptospira sp.	NA	NA	AY995723	*Myotis riparius*	Peru

Leptospira sp.	NA	NA	AY995724	*Lonchophylla thomasi*	Peru
Leptospira sp.	NA	NA	AY995725	*Uroderma bilobatum*	Peru
Leptospira sp.	NA	NA	AY995726	*Glossophaga soricina*	Peru
Leptospira sp.	NA	NA	AY995727	*Artibeus obscurus*	Peru
Leptospira sp.	NA	NA	AY995728	*Rhinophylla pumilio*	Peru
Leptospira sp.	NA	NA	JQ288729	*Otomops madagascariensis*	Madagascar
Leptospira sp.	NA	NA	JQ288730	*Miniopterus mahafaliensis*	Madagascar
Leptospira sp.	NA	NA	JQ288731	*Triaenops menamena*	Madagascar
Leptospira sp.	NA	NA	JQ288732	*Rousettus obliviosus*	Comoros
Leptospira sp.	NA	NA	JQ288733	*Rousettus obliviosus*	Comoros
Leptospira sp.	NA	NA	JQ288734	*Miniopterus griveaudi*	Comoros
Leptospira sp.	NA	NA	JQ302791	*Myotis goudoti*	Madagascar
Leptospira terpstrae	AOGW02000010	AOGW02000010	AY631888	NA	China
Leptospira vanthielii	NZ_AOGY02000051	NZ_AOGY02000051	AY631897	Isolated from water	Netherlands
Leptospira weilii	NZ_AHNC02000010	AFLV02000058	JQ906670	*Homo sapiens*	Laos
Leptospira wolbachii	AOGZ02000008	AOGZ02000008	Z21638	NA	USA
Leptospira wolffii	NZ_AKWX02000023	NZ_AKWX02000023	EF025496	*Homo sapiens*	Thailand
Leptospira yanagawae	NZ_AOGX02000024	NA	AY631882	Isolated from water	Brazil

Supplementary Table 2. GenBank accession number of Cytochrome b *(cyt b)* sequences from bat species used in this study, the asterisk indicates the sequences of *cyt b* generated in this study. Asterisks indicate the sequences produced in the present study.

Bat family	Bat species	Cytochrome b GenBank accesion number
Emballonuridae	*Coleura kibomalandy*	JQ710748
Hipposideridae	*Hipposideros commersoni*	KR606333 *
Miniopteridae	*Miniopterus griffithsi*	JF440240
	Miniopterus mahafaliensis	FJ383166
	Miniopterus majori	HQ619939
	Miniopterus manavi	HQ619934
	Miniopterus sororculus	JF440282
Molossidae	*Mormopterus jugularis*	KR606332 *
	Otomops madagascariensis	KR606335 *
Rhinonycteridae	*Paratriaenops furculus*	KR606331 *
	Triaenops menamena	KR606334
Pteropodidae	*Rousettus madagascariensis*	GU228727
Vespertilionidae	*Myotis goudoti*	GU116769
	Neoromicia robertsi	KR606336 *
	Scotophilus marovaza	EU750943

Supplementary Table 3. Details of sampling sites: locality 17 code, province, description, type of site and number of specimens investigated at each site. (See Supplementary Table 3 in Excel)

Supplementary Table 3

CODE	Province	Locality description	Type of site	*Coleura kibomalandy*	*Paremballoneura tiavato*	*Hipposideros commersoni*	*Miniopterus aelleni*	*Miniopterus manavi sensu lato*	*Miniopterus gleni*	*Miniopterus griffithsi*	*Miniopterus griveaudi*	*Miniopterus mahafaliensis*	*Miniopterus majori*	*Miniopterus sororculus*	*Chaerephon ashinanana*	*Chaerephon leucogaster*	*Mops leucostigma*	*Mops midas*	*Mormopterus jugularis*	*Otomops madagascariensis*	*Paratriaenops furculus*	*Triaenops menamena*	*Eidolon dupreanum*	*Pteropus rufus*	*Rousettus madagascariensis*	*Hypsugo nov. sp.*	*Myotis goudoti*	*Neoromicia malagasyensis*	*Neoromicia matroka*	*Neoromicia robertsi*	*Pipistrellus hesperidus*	*Pipistrellus raceyi*	*Pipistrellus /Neoromicia sp.*	*Scotophilus marovaza*
AMBT	Antananarivo	Réserve Spéciale d'Ambohitantely - Grotte des chauve-souris	Cave					**19**																										
AMBT 2	Antananarivo	Réserve Spéciale d'Ambohitantely - Début sentier touristique	Outdoor trapping																								**2**							
ANJOZ 1	Antananarivo	Sous prefecture d'Anjozorobe, Forêt d'Antsahabe - Andohasahabe	Outdoor trapping																										**2**	**1**				
ANJOZ 2	Antananarivo	Ambohibeloma, 3,2km W. Anjozorobe	Synanthropic															**20**																
AMBB	Antsiranana	Parc National d'Ankarana, Ambahibe Cave, 2 km W. Mahamasina	Cave		**4**					**24**																	**11**							
ANDOK	Antsiranana	Parc National d'Ankarana, Grotte du troisième Canyon, along Andokotokana River	Cave																				**10**											
ANDRF 1	Antsiranana	Parc National d'Ankarana, Grotte d'Andrafiabe, 3.3 ESE Andrafiabe	Cave							**14**																	**1**							
ANDRF 2	Antsiranana	Parc National d'Ankarana, 2,6 km E Andrafiabe, in forest near Andrafiabe Cave	Outdoor trapping		**2**	**5**																**5**					**7**							
CGS	Antsiranana	Parc National d'Ankarana, Grotte des chauves-souris, 3 km NW Mahamasina	Cave																						**15**									
CROCO	Antsiranana	Parc National d'Ankarana, 2.2 km ESE Amboandriky, Grotte d'Ambatoharanana	Cave	**3**			**2**		**10**		**6**									**1**								**5**							
ANDR	Fianarantsoa	Grotte d'Andranomilitra, west of Ihosy off RN7	Cave									**7**		**3**																				
ANK	Fianarantsoa	Commune rurale d'Ankily, west of Ihosy off RN7	Synanthropic															**10**																
BEK	Fianarantsoa	Parc National d'Isalo, Grotte de Bekapity	Cave			**12**			**7**			**1**		**14**																				
FAND	Fianarantsoa	Grotte de Fandanana, 4.1 km NE de Fandriana	Cave										**7**	**3**													**6**							
IHO	Fianarantsoa	Ihosy, Bureau du chef de la Région,	Synanthropic															**25**																
ISALO 1	Fianarantsoa	Parc National d'Isalo, 3.8 km NW de Ranohira, along Namaza river	Outdoor trapping			**2**						**22**		**2**				**8**				**1**	**1**				**7**							
ISALO 2	Fianarantsoa	At the edge of Parc National of Isalo, 7.8Km N. Ranohira, along menamaty river	Outdoor trapping									**7**						**1**									**1**	**1**						
ISALO 3	Fianarantsoa	Parc National de l'Isalo, Ambinanindranohira-Bas, Andranomboalavo	Outdoor trapping															**2**										**1**						
VOHI	Fianarantsoa	Vohiposa, CSB II	Synanthropic															**25**																
ZAZA	Fianarantsoa	Zazafotsy	Synanthropic																										**2**					
AMB0	Mahajanga	Ambovondramanesy village near Berivotra, along RN4	Outdoor trapping																				**20**											
ANJHB	Mahajanga	Grotte d'Anjohibe - 3.7 km NE Antanamarina	Cave			**1**				**16**									**12**	**1**				**20**										
ANJHK 1	Mahajanga	Grotte d'Anjohikely (south entrance), 1.5 km NE Antanamarina	Cave							**26**																								
ANJHK 2	Mahajanga	Grotte d'Anjohikely 2, 1.6 km NE Antanamarina	Cave							**10**																								
ANJKK	Mahajanga	Parc National de Bemaraha, Anjohikinakina 15.5 km N Bekopaka	Cave				**5**			**20**									**10**					**1**		**5**								
ANKPK	Mahajanga	Just outside the limit of Parc National de Bemaraha, Ankapoka	Outdoor trapping																					**2**										
ANTNM	Mahajanga	Cascade d'Antanamarina	Outdoor trapping													**9**					**2**	**2**			**11**								**8**	**1**
BEEN	Mahajanga	Grotte de Beenta, 2 km W Mitsinjo	Cave																		**1**	**3**												
MAHA	Mahajanga	North of Mahajanga, petite plage	Synanthropic													**21**																		
ANDA 1	Toamasina	CEG Andasibe	Synanthropic												**34**			**2**																
ANDA 2	Toamasina	Outskirt of Andasibe, Mangarivotra, Ambany Atsinanana	Outdoor trapping																														**1**	
AMB	Toliara	Grotte d'Ambanilia 3.7 km SSE Sarodrano	Cave								**10**																							
ANDRO	Toliara	Grotte d'Androimpano, 4.2 km NE Itampolo (village), on old road to Ejeda	Cave						**1**												**1**	**6**												
ANTAN	Toliara	Antanandava, Eglise FLM 5.8 km NE Beroboka Sud	Synanthropic														**11**																	
BEKO	Toliara	Grotte de Bekoaky, 9.4 km SSE Ankililaoka	Cave			**4**			**4**			**2**							**3**	**3**	**1**						**3**							
BELO	Toliara	Belo Tsiribihina, central hospital	Synanthropic													**17**	**5**																	
BET	Toliara	Betioky Sud, New Lutherian church	Synanthropic															**20**																
ITA	Toliara	Itampolo village	Synanthropic															**5**																
KIR 1	Toliara	Kirindy village	Outdoor trapping													**1**														**7**				
KIR 2	Toliara	0.8 km N Kirindy (village)	Outdoor trapping																					**2**						**2**	**2**			
MAH	Toliara	Mahabo, EPP de Mahabo	Synanthropic													**30**	**4**																	
MAKI	Toliara	Grotte de Makis (Mikea), near Hotel la Mangrove on Toliara-St Augustin Road	Cave			**1**												**27**																
MARFA	Toliara	Marofandilia (village), Ecole primaire	Synanthropic														**14**																	
MRFTT	Toliara	Marofototra, Tsarafototra FLM	Synanthropic													**2**	**7**	**4**																
SAK 1	Toliara	Sakaraha, Direction des Eaux et forêt, Bureau chef de cantonnement	Synanthropic															**15**																
SAK 2	Toliara	Sakaraha, Near Direction des Eaux et forêts Complex at edge of town	Synanthropic														**2**		**2**																
SAR	Toliara	Grotte de Sarodrano (sea cave)	Cave					**1**										**1**												**2**				
STAUG	Toliara	St Augustin, in Lycée Building	Synanthropic													**9**		**1**																
TANA	Toliara	Grotte de Tanambao (Bishiko) 0.75 km E St Augustin	Cave			**2**						**10**							**13**		**1**													
TSMIF	Toliara	Tsimafana, CEG de Tsimafana	Synanthropic													**5**	**25**																	
TSP	Toliara	Parc National de Tsimanampetsotsa, Grotte d'Andranoilovy	Cave									**29**								**6**	**12**													
VINT	Toliara	Grotte de Vintane, (Vintany) 4.1 km SE Itampolo	Cave						**6**			**1**						**3**			**11**													

Supplementary Table 4. Details on *Leptospira* sequences produced used in this study; GenBank accession number for three genes: *secY*, *adk*, and *rrs2;* information on the host: museum number, bat species and family and locality of sampling. For locality details see Supplementary Table 3. NA: not available.

Bat family	**Bat species**	**Collection date**	**Museum Number**	**Locality**	***Leptospira* isolate**	**GenBank accesion number of *Leptospira* marker**		
						secY	***adk***	***rrs2***
Emballonuridae	*Coleura kibomalandy*	17/09/12	FMNH 221311	CROCO	580MG	NA	NA	KP822681
Hipposideridae	*Hipposideros commersoni*	10/02/12	FMNH 217964	BEKO	43MG	KP822608	KP822572	KP822682
	Hipposideros commersoni	10/02/12	FMNH 217965	BEKO	44MG	NA	NA	KP822683
	Hipposideros commersoni	10/02/12	FMNH 217966	BEKO	45MG	KP822609	KP822573	KP822684
	Hipposideros commersoni	10/02/12	UADBA 32377	BEKO	46MG	NA	NA	KP822685
	Hipposideros commersoni	16/03/12	UADBA 50269	BEK	792MG	KP822610	KP822574	KP822686
	Hipposideros commersoni	16/03/12	FMNH 218015	BEK	794MG	KP822611	NA	KP822687
	Hipposideros commersoni	17/03/12	FMNH 218016	BEK	800MG	NA	NA	KP822688
	Hipposideros commersoni	17/03/12	FMNH 218018	BEK	801MG	NA	NA	KP822689
	Hipposideros commersoni	15/09/12	UADBA 32989	ANDRF2	562MG	KP822612	KP822575	KP822690
	Hipposideros commersoni	12/02/12	UADBA 32919	TANA	92MG	NA	NA	KP822757
Miniopteridae	*Miniopterus manavi* sensu lato	20/09/12	UADBA 33034	AMBT	607MG	KP822622	KP822578	KP822711
	Miniopterus manavi sensu lato	20/09/12	UADBA 33035	AMBT	608MG	NA	NA	KP822712
	Miniopterus manavi sensu lato	20/09/12	UADBA 33037	AMBT	610MG	NA	NA	KP822713
	Miniopterus manavi sensu lato	20/09/12	FMNH 221421	AMBT	615MG	KP822623	NA	KP822714

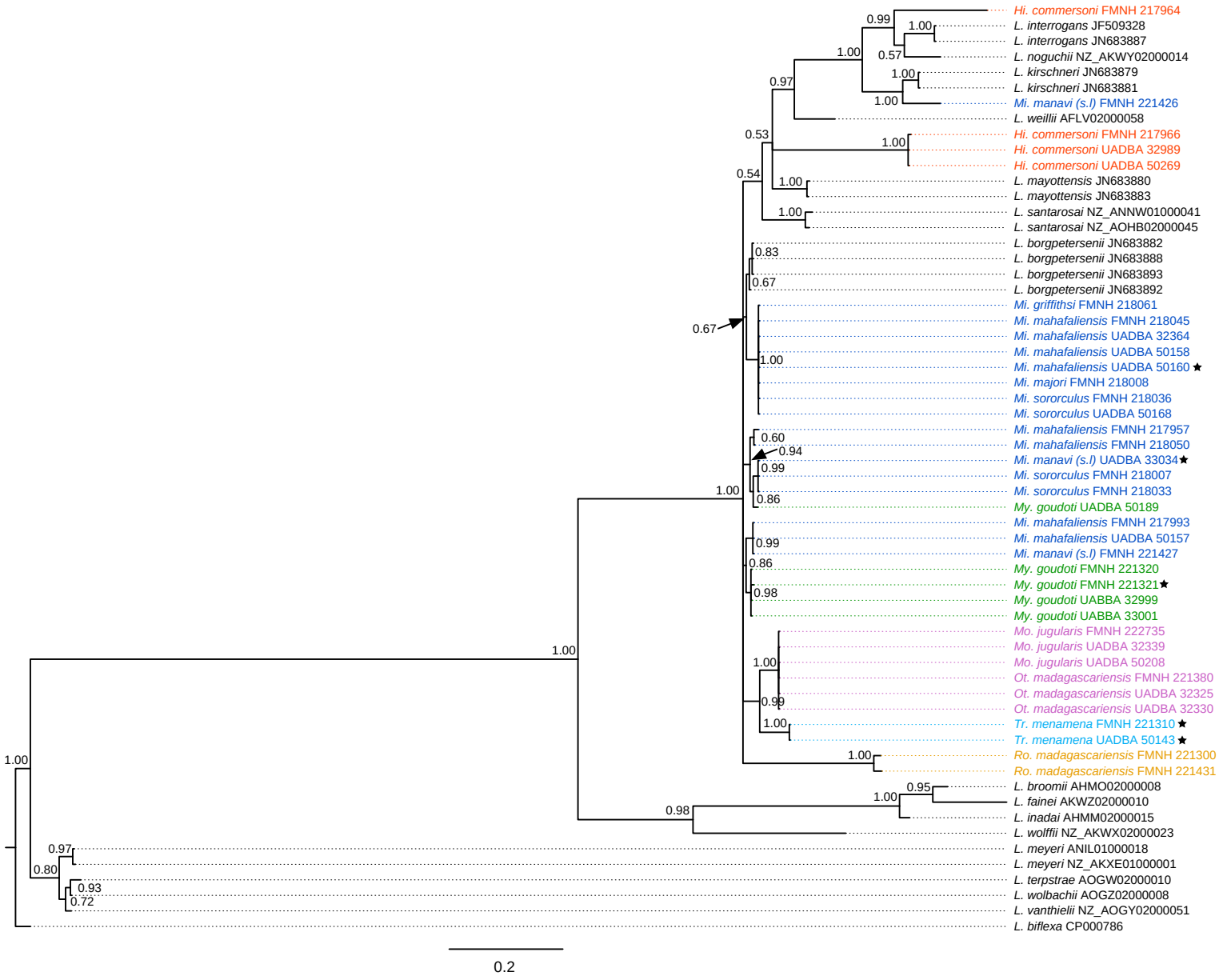
	Miniopterus manavi sensu lato	20/09/12	FMNH 221425	AMBT	52MG	KP822624	NA	KP822715
	Miniopterus manavi sensu lato	20/09/12	FMNH 221426	AMBT	616MG	KP822625	KP822579	KP822716
	Miniopterus manavi sensu lato	20/09/12	FMNH 221427	AMBT	968MG	KP822626	KP822580	KP822717
	Miniopterus manavi sensu lato	20/09/12	FMNH 221429	AMBT	53MG	KP822627	NA	KP822718
	Miniopterus griffithsi	24/04/12	FMNH 218061	VINT	170MG	NA	KP822581	KP822719
	Miniopterus griffithsi	24/04/12	FMNH 218062	VINT	174MG	KP822628	NA	KP822720
	Miniopterus griffithsi	24/04/12	UADBA 50142	VINT	183MG	KP822629	NA	KP822721
	Miniopterus mahafaliensis	10/02/12	FMNH 217957	BEKO	36MG	NA	KP822582	KP822722
	Miniopterus mahafaliensis	12/02/12	UADBA 32355	AMB	76MG	NA	NA	KP822723
	Miniopterus mahafaliensis	12/02/12	UADBA 32364	TANA	99MG	KP822630	KP822583	KP822724
	Miniopterus mahafaliensis	15/02/12	FMNH 217988	ANDR	118MG	NA	NA	KP822725
	Miniopterus mahafaliensis	15/02/12	UADBA 32371	ANDR	50MG	KP822631	NA	KP822726
	Miniopterus mahafaliensis	15/02/12	FMNH 217990	ANDR	120MG	NA	NA	KP822727
	Miniopterus mahafaliensis	15/02/12	FMNH 217992	ANDR	122MG	NA	NA	KP822728
	Miniopterus mahafaliensis	15/02/12	FMNH 217993	ANDR	123MG	KP822632	KP822584	KP822729
	Miniopterus mahafaliensis	14/03/12	UADBA 50162	ISALO1	741MG	KP822633	NA	KP822730
	Miniopterus mahafaliensis	14/03/12	FMNH 218040	ISALO1	742MG	KP822634	NA	NA
	Miniopterus mahafaliensis	14/03/12	FMNH 218042	ISALO1	743MG	KP822635	NA	KP822731
	Miniopterus mahafaliensis	14/03/12	FMNH 218045	ISALO1	749MG	KP822636	KP822585	KP822732
	Miniopterus mahafaliensis	14/03/12	UADBA 50155	ISALO1	750MG	KP822637	NA	KP822733
	Miniopterus mahafaliensis	14/03/12	UADBA 50157	ISALO1	753MG	KP822638	KP822586	KP822734
	Miniopterus mahafaliensis	14/03/12	UADBA 50158	ISALO1	950MG	KP822639	KP822587	KP822735

	Miniopterus mahafaliensis	14/03/12	UADBA 50160	ISALO1	755MG	KP822640	KP822588	KP822736
	Miniopterus mahafaliensis	14/03/12	FMNH 218047	ISALO1	757MG	KP822641	NA	KP822737
	Miniopterus mahafaliensis	17/03/12	FMNH 218050	BEK	796MG	KP822642	KP822589	KP822738
	Miniopterus mahafaliensis	21/04/12	UADBA 50176	TSP	142MG	NA	NA	KP822739
	Miniopterus mahafaliensis	21/04/12	UADBA 50179	TSP	145MG	KP822643	NA	KP822740
	Miniopterus mahafaliensis	21/04/12	UADBA 50180	TSP	146MG	KP822644	NA	KP822741
	Miniopterus mahafaliensis	21/04/12	UADBA 50184	TSP	150MG	NA	NA	KP822742
	Miniopterus mahafaliensis	21/04/12	UADBA 50185	TSP	151MG	KP822645	NA	KP822743
	Miniopterus mahafaliensis	22/04/12	UADBA 50186	TSP	152MG	KP822646	NA	KP822744
	Miniopterus mahafaliensis	26/04/12	UADBA 50216	TSP	188MG	KP822647	NA	KP822745
	Miniopterus mahafaliensis	26/04/12	UADBA 50219	TSP	191MG	KP822648	NA	KP822746
	Miniopterus mahafaliensis	15/03/13	FMNH 222721	ISALO2	897MG	KP822649	NA	KP822747
	Miniopterus majori	12/03/12	FMNH 218008	FAND	728MG	KP822650	KP822590	KP822748
	Miniopterus sororculus	15/02/12	FMNH 217985	ANDR	115MG	KP822651	NA	KP822749
	Miniopterus sororculus	12/03/12	UADBA 50173	FAND	724MG	NA	NA	KP822750
	Miniopterus sororculus	12/03/12	FMNH 218007	FAND	736MG	KP822652	KP822591	KP822751
	Miniopterus sororculus	16/03/12	UADBA 50344	BEK	772MG	NA	NA	KP822752
	Miniopterus sororculus	16/03/12	FMNH 218033	BEK	773MG	KP822653	KP822592	KP822753
	Miniopterus sororculus	16/03/12	FMNH 218036	BEK	783MG	KP822654	KP822593	KP822754
	Miniopterus sororculus	16/03/12	FMNH 218038	BEK	785MG	NA	NA	KP822755
	Miniopterus sororculus	16/03/12	UADBA 50168	BEK	790MG	KP822655	KP822594	KP822756
Molossidae	*Mormopterus jugularis*	08/02/12	UADBA 32335	MAKI	8MG	NA	NA	KP822758

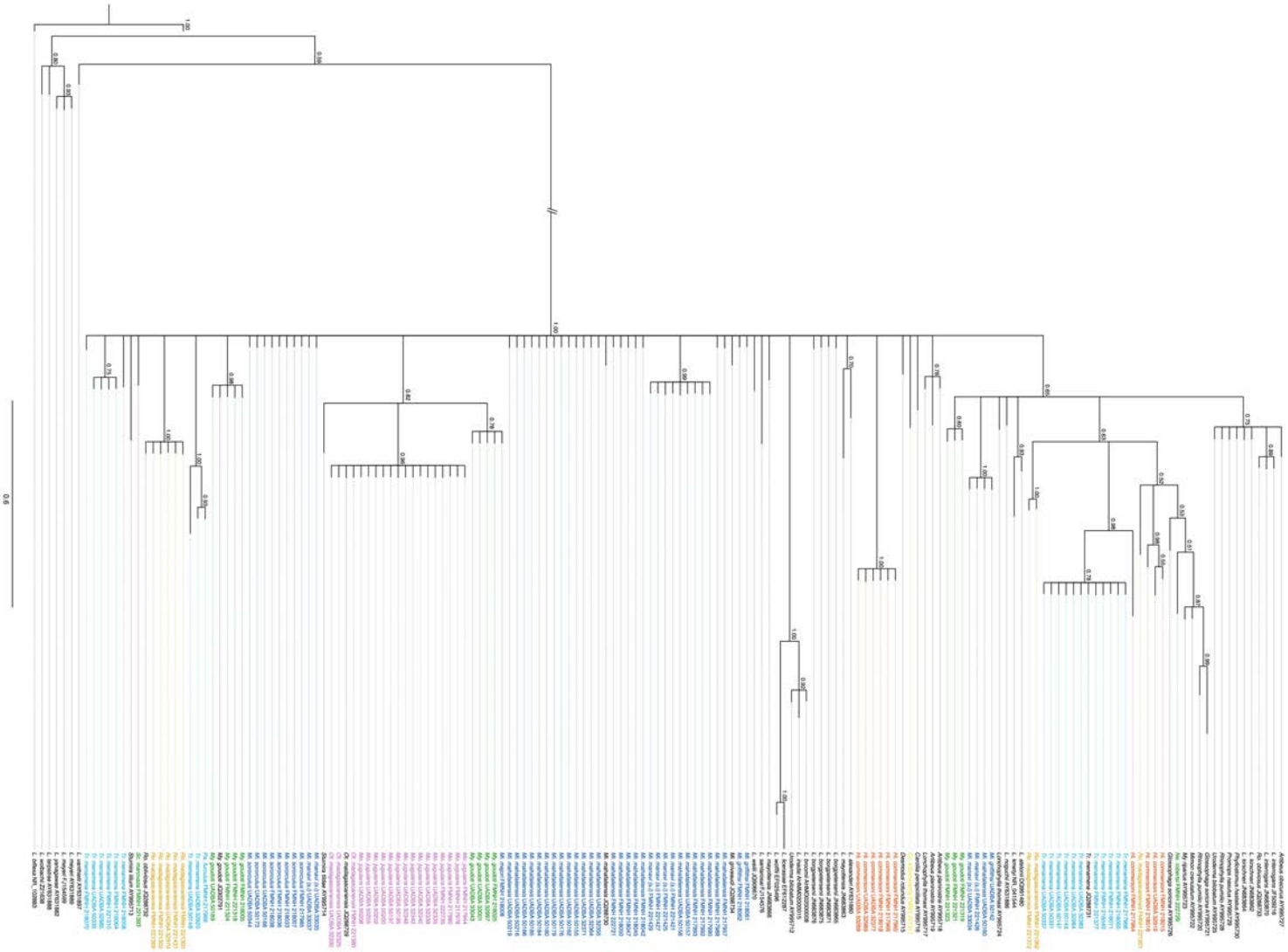
	Mormopterus jugularis	08/02/12	UADBA 32339	MAKI	12MG	KP822656	KP822595	KP822759
	Mormopterus jugularis	08/02/12	UADBA 32340	MAKI	13MG	NA	NA	KP822760
	Mormopterus jugularis	08/02/12	UADBA 32342	MAKI	15MG	KP822657	NA	KP822761
	Mormopterus jugularis	08/02/12	UADBA 32346	MAKI	31MG	NA	NA	KP822762
	Mormopterus jugularis	08/02/12	FMNH 217944	MAKI	20MG	NA	NA	KP822763
	Mormopterus jugularis	15/02/12	FMNH 217978	ANK	108MG	KP822658	NA	KP822764
	Mormopterus jugularis	15/02/12	FMNH 217980	ANK	110MG	NA	NA	KP822765
	Mormopterus jugularis	15/03/12	UADBA 50208	ISALO1	762MG	KP822659	KP822596	KP822766
	Mormopterus jugularis	24/04/12	UADBA 50195	ITA	165MG	NA	NA	KP822767
	Mormopterus jugularis	24/04/12	UADBA 50197	VINT	172MG	KP822660	NA	KP822768
	Mormopterus jugularis	26/04/12	UADBA 50200	BET	204MG	NA	NA	KP822769
	Mormopterus jugularis	26/04/12	UADBA 50202	BET	206MG	KP822661	NA	KP822770
	Mormopterus jugularis	26/04/12	UADBA 50205	BET	209MG	KP822662	NA	KP822771
	Mormopterus jugularis	10/03/13	FMNH 222735	ANDA 1	911MG	KP822663	KP822597	KP822772
	Otomops madagascariensis	10/02/12	UADBA 32325	TANA	68MG	KP822664	KP822598	KP822773
	Otomops madagascariensis	11/02/12	UADBA 32330	TANA	71MG	NA	KP822599	KP822774
	Otomops madagascariensis	08/09/12	FMNH 221380	ANJHB	418MG	KP822665	KP822600	KP822775
Pteropodidae	*Rousettus madagascariensis*	07/09/12	FMNH 221362	ANJHB	407MG	NA	NA	KP822776
	Rousettus madagascariensis	09/09/12	FMNH 221369	ANTNM	453MG	KP822666	NA	KP822777
	Rousettus madagascariensis	09/09/12	FMNH 221372	ANTNM	459MG	NA	NA	KP822778
	Rousettus madagascariensis	14/09/12	UADBA 32970	CGS	525MG	NA	NA	KP822779
	Rousettus madagascariensis	14/09/12	FMNH 221300	CGS	530MG	KP822667	KP822601	KP822780

	Rousettus madagascariensis	14/09/12	FMNH 221301	CGS	531MG	NA	NA	KP822781
	Rousettus madagascariensis	14/09/12	FMNH 221302	CGS	532MG	NA	NA	KP822782
	Rousettus madagascariensis	06/11/12	FMNH 221431	ANJKK	632MG	KP822668	KP822602	KP822783
Rhinonycteridae	*Paratriaenops furculus*	10/02/12	FMNH 217969	BEKO	49MG	NA	NA	KP822710
	Triaenops menamena	10/02/12	FMNH 217968	BEKO	48MG	KP822613	NA	KP822691
	Triaenops menamena	12/02/12	UADBA 32383	TANA	95MG	KP822614	NA	KP822692
	Triaenops menamena	14/03/12	UADBA 50143	ISALO1	733MG	KP822615	KP822576	KP822693
	Triaenops menamena	21/04/12	UADBA 50147	TSP	133MG	NA	NA	KP822694
	Triaenops menamena	21/04/12	UADBA 50148	TSP	134MG	NA	NA	KP822695
	Triaenops menamena	21/04/12	FMNH 218540	TSP	138MG	KP822616	NA	KP822696
	Triaenops menamena	21/04/12	FMNH 218058	TSP	141MG	NA	NA	KP822697
	Triaenops menamena	23/04/12	UADBA 50331	ANDRO	157MG	NA	NA	KP822698
	Triaenops menamena	23/04/12	UADBA 50335	ANDRO	161MG	NA	NA	KP822699
	Triaenops menamena	24/04/12	UADBA 50337	VINT	176MG	NA	NA	KP822700
	Triaenops menamena	24/04/12	FMNH 218064	VINT	177MG	KP822617	NA	KP822701
	Triaenops menamena	24/04/12	FMNH 218065	VINT	178MG	NA	NA	KP822702
	Triaenops menamena	24/04/12	FMNH 218070	VINT	184MG	NA	NA	KP822703
	Triaenops menamena	24/04/12	FMNH 218071	VINT	185MG	NA	NA	KP822704
	Triaenops menamena	09/09/12	FMNH 221376	ANTNM	460MG	KP822618	NA	NA
	Triaenops menamena	09/09/12	FMNH 221377	ANTNM	461MG	NA	NA	KP822705
	Triaenops menamena	10/09/12	UADBA 32920	BEEN	483MG	KP822619	NA	KP822706
	Triaenops menamena	15/09/12	UADBA 32984	ANDRF2	566MG	KP822620	NA	KP822707

	Triaenops menamena	15/09/12	UADBA 32985	ANDRF2	567MG	NA	NA	KP822708
	Triaenops menamena	15/09/12	FMNH 221310	ANDRF2	568MG	KP822621	KP822577	KP822709
Vespertilionidae	*Myotis goudoti*	12/03/12	UADBA 50189	FAND	718MG	KP822669	KP822603	KP822784
	Myotis goudoti	12/03/12	FMNH 218005	FAND	721MG	KP822670	NA	KP822785
	Myotis goudoti	14/03/12	FMNH 218025	ISALO1	739MG	NA	NA	KP822786
	Myotis goudoti	13/09/12	FMNH 221318	AMBB	490MG	KP822671	NA	KP822787
	Myotis goudoti	13/09/12	FMNH 221319	AMBB	491MG	KP822672	NA	KP822788
	Myotis goudoti	13/09/12	FMNH 221320	AMBB	492MG	KP822673	KP822604	NA
	Myotis goudoti	13/09/12	FMNH 221321	AMBB	493MG	KP822674	KP822605	KP822789
	Myotis goudoti	13/09/12	FMNH 221323	AMBB	494MG	KP822675	NA	KP822790
	Myotis goudoti	13/09/12	FMNH 221324	AMBB	495MG	NA	NA	KP822791
	Myotis goudoti	15/09/12	UABBA 32997	AMBB	556MG	KP822676	NA	NA
	Myotis goudoti	15/09/12	UABBA 32999	ANDRF2	558MG	KP822677	KP822606	KP822792
	Myotis goudoti	17/03/12	UABBA 33001	CROCO	594MG	KP822678	KP822607	KP822793
	Myotis goudoti	20/09/12	UADBA 33043	AMBT2	55MG	KP822679	NA	KP822794
	Neoromicia robertsi	19/03/13	FMNH 222729	ANJOZ1	857MG	KP822680	NA	KP822795
	Scotophilus marovaza	09/09/12	FMNH 221393	ANTNM	464MG	NA	NA	KP822796



Supplementary Figure 1. Phylogenetic tree based on the *adk* (435 bp) of pathogenic *Leptospira* spp. from Malagasy bat species. The analysis was carried out using Bayesian Inference under the HKY+I+G substitution model. Nodal supports values correspond to posterior probabilities. The detected Leptospira spp. are coloured according to the family of bats host species (red: Hipposideridae, blue: Miniopteridae, purple: Molossidae, orange: Pteropodidae, light–blue: Rhinonycteridae and green: Vespertilionidae). Names typed in black refer to *Leptospira* (*L.)* sequences accessible from GenBank (see Supplementary Table 1). Stars indicate *Leptospira* sequences, which do not group within the same position as previously described in secY phylogeny. *Hi: Hipposideros, Mi: Miniopterus, Mo: Mormopterus, My: Myotis, Ot: Otomops, Ro: Rousettus* and *Tr: Triaenops.*



Supplementary Figure 2. Phylogeny based on *Leptospira* spp. *rrs2* sequences (456 bp) from Malagasy bat species. The analysis was carried out using Bayesian Inference under the K2P+I+G substitution model. Nodal supports values correspond to posterior probabilities. The detected Leptospira spp. are coloured according to the family of bats host species (yellow: Emballonuridae, red: Hipposideridae, blue: Miniopteridae, purple: Molossidae, orange: Pteropodidae, light–blue: Rhinonycteridae and green: Vespertilionidae). Names typed in black refer to *Leptospira (L.)* sequences accessible from GenBank (see Supplementary Table 1). *Co: Coleura, Hi: Hipposideros, Mi: Miniopterus, Mo: Mormopterus, My: Myotis, Ne: Neoromicia, Pa: Paratriaenops, Ot: Otomops, Ro: Rousettus, Sc: Scotophilus* and *Tr: Triaenops.*