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# Virulence et spéciicité d'hôte de leptospires pathogènes endémiques de Madagascar et ses îles voisines

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## **Université de La Réunion**

Faculté des Sciences et Technologies

Ecole Doctorale (542) : Sciences Technologies et Santé

UMR PIMIT (Processus Infectieux en Milieu Insulaire Tropical)

Thèse de Sciences  
en vue de l'obtention du grade de  
**Docteur en Biologie Moléculaire**

Présentée par  
**Colette CORDONIN**

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**Virulence et spécificité d'hôte de leptospires pathogènes endémiques  
de Madagascar et ses îles voisines**

**Virulence and host-specificity of pathogenic *Leptospira* endemic to  
Madagascar and surrounding islands**

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## Abstract

Leptospirosis is a zoonosis of main medical concern on several islands of southwestern Indian Ocean (SWIO), some of which recording among the highest human incidence worldwide. Over the last decade, molecular epidemiology investigations carried out under a One Health framework have revealed a wide variety of *Leptospira* lineages and distinct transmission chains throughout the islands of the region. These islands are home to pathogenic *Leptospira* lineages and animal reservoirs that are either introduced or endemic to the SWIO region. Interestingly, the regional distribution of *Leptospira* diversity is associated with (i) a contrasted severity of human cases and (ii) distinct levels of specificity of *Leptospira* towards their mammalian hosts. Specifically, endemic *Leptospira* appear less pathogenic in humans and display higher specificity towards their animal reservoirs than their cosmopolitan counterparts.

To complete the dataset of *Leptospira* diversity in the SWIO region, we produced data from bats of eastern Africa. Results support the previously observed pattern of host specificity of *Leptospira* towards their bats hosts and, overlaid upon the biogeographic history of Malagasy bats, suggest that these volant mammals have colonized Madagascar from continental Africa while hosting pathogenic *Leptospira*.

To better understand the role of distinct *Leptospira* lineages in the contrasted epidemiology observed in the SWIO, we investigated the **pathogenicity** of three *Leptospira* isolates from this region using a hamster model. *Leptospira mayottensis* and *Leptospira borgpetersenii* isolates were obtained from *Tenrec ecaudatus* (tenrec) on Mayotte and *Triaenops menamena* (bat) in Madagascar, respectively, both mammals being endemic to the SWIO region. A *Leptospira interrogans* strain, which genotype has been reported in the majority of human acute cases on La Réunion, was isolated from the introduced *Rattus rattus* (rat). In keeping with a distinct severity of leptospirosis on Mayotte and La Réunion, endemic bat-borne and tenrec-borne *Leptospira* were significantly less pathogenic than the control cosmopolitan rat-borne isolate.

The **host specificity** of the isolates obtained from endemic hosts was addressed using experimental infection of *Rattus norvegicus*, a known reservoir of pathogenic *Leptospira*. This animal model was challenged with all three isolates and mostly failed in supporting chronic infection with bat-borne and tenrec-borne *Leptospira*. Hence, the strong host-specificity of endemic *Leptospira* towards their hosts observed in the wild likely results from genetic determinants shaped by long-term co-evolutionary processes rather than from ecological constraints such as a lack of physical contact between introduced and endemic animal reservoirs.

Finally, we undertook full genome sequencing of regional strains in order to highlight genomic features that may be associated with virulence and host specificity. Whole genome sequencing allowed the accurate classification of *Leptospira* isolates obtained on SWIO islands. Comparative genomics allowed to identify genes specific to a group or species of *Leptospira* but complex changes in *Leptospira* genome make difficult the identification of genomic elements responsible for specific traits such as virulence and host specificity.

Keywords: *Leptospira*, leptospirosis, southwestern Indian Ocean, rats, tenrecs, bats, hamsters, pathogenicity, host specificity, whole genome sequencing

## Résumé

La leptospirose est une zoonose d'importance médicale majeure dans les îles du Sud-Ouest de l'Océan Indien (SOOI) dont certaines enregistrent des incidences parmi les plus élevées au monde. Durant la dernière décennie, les données épidémiologiques moléculaires obtenues avec une approche « One Health » ont mis en évidence une grande diversité de lignées de leptospires ainsi que différentes chaînes de transmission sur les différentes îles de la région. Les données moléculaires montrent la présence de différents leptospires pathogènes et de réservoirs animaux introduits ou endémiques de cette région. La distribution de ces différentes lignées de leptospires est associée à (i) un contraste épidémiologique incluant des différences dans la sévérité des cas humains et (ii) des niveaux de spécificité d'hôtes différents selon les leptospires considérés. Plus particulièrement, les leptospires endémiques du SOOI semblent être moins pathogènes chez les humains et montrent une plus forte affinité pour leur réservoir que les leptospires cosmopolites.

Pour compléter nos connaissances sur l'histoire évolutive des leptospires du SOOI, nous avons produit des données provenant de chauves-souris de l'Afrique de l'Est. Ces données confirment la spécificité de certaines lignées de leptospires envers leurs hôtes chiroptères et suggèrent que les chauves-souris d'Afrique ont colonisé Madagascar tout en étant infectées par leurs leptospires.

Afin de mieux comprendre le rôle des différents leptospires dans l'épidémiologie régionale de la leptospirose, nous avons mesuré la **pathogénicité** de trois souches de leptospires retrouvées dans cette région à l'aide d'un modèle hamster. Des souches de *Leptospira mayottensis* et *Leptospira borgpetersenii* ont été isolés respectivement de *Tenrec ecaudatus* (tenrec) et *Triaenops menamena* (chauve-souris), deux mammifères endémiques du SOOI. Une souche de *Leptospira interrogans*, dont le génotype est retrouvé dans la majorité des cas humains graves à la Réunion, a été isolée de *Rattus rattus* (rat). En cohérence avec les données épidémiologiques humaines de Mayotte et de La Réunion, les leptospires endémiques se sont révélées être significativement moins pathogènes que la souche cosmopolite *L. interrogans*.

La **spécificité d'hôte** des deux souches isolées de mammifères endémiques a été mise à l'épreuve par des infections expérimentales de *Rattus norvegicus*, connu comme un réservoir important de leptospires. Les rats ont été infectés avec les trois isolats précédemment utilisés. Les rats infectés par les souches endémiques n'ont pas développé d'infection rénale chronique contrairement à la souche cosmopolite. Ces résultats montrent que la spécificité d'hôte des leptospires endémiques observée *in natura* est probablement due à des facteurs génétiques plutôt qu'à des facteurs écologiques, comme un manque de contacts physiques entre les réservoirs animaux endémiques et introduits.

Enfin, le séquençage complet de souches de leptospires du SOOI a été réalisé afin d'identifier des caractéristiques génétiques pouvant être associées à la pathogénicité et la spécificité d'hôte des leptospires pathogènes. Une classification précise de souches de leptospires du SOOI a pu être réalisée sur la base des génomes complets. La comparaison de ces génomes a permis d'identifier des gènes spécifiques à un groupe ou une espèce de leptospires. Cependant des modifications génomiques complexes rendent difficiles l'identification de caractéristiques génomiques responsables d'un phénotype particulier tel que la virulence ou la spécificité d'hôte.

Mots-clés : *Leptospira*, leptospirose, Sud-Ouest de l'Océan Indien, rats, tenrecs, chauves-souris, hamsters, séquençages de génomes complets

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## List of abbreviations

5-Fu	5-Fluorouracil
AFAS	Albumin fatty acid supplement
AMOVA	Analysis of molecular variance
COG	Cluster of orthologous genes
DAPC	Discriminant analysis in principal components
EMJH	Ellinghausen-McCullough-Johnson-Harris
GWAS	Genome wide association study
ICU	Intensive care unit
Ma	Million years ago
MAT	Microscopic agglutination test
Mb	Megabase pair
MLST	Multilocus sequence typing
qPCR	Quantitative polymerase chain reaction
SNP	Single nucleotide polymorphism
ST	Sequence type
SWIO	Southwestern Indian Ocean
WGS	Whole genome sequencing

# INTRODUCTION

## I. General introduction on leptospirosis

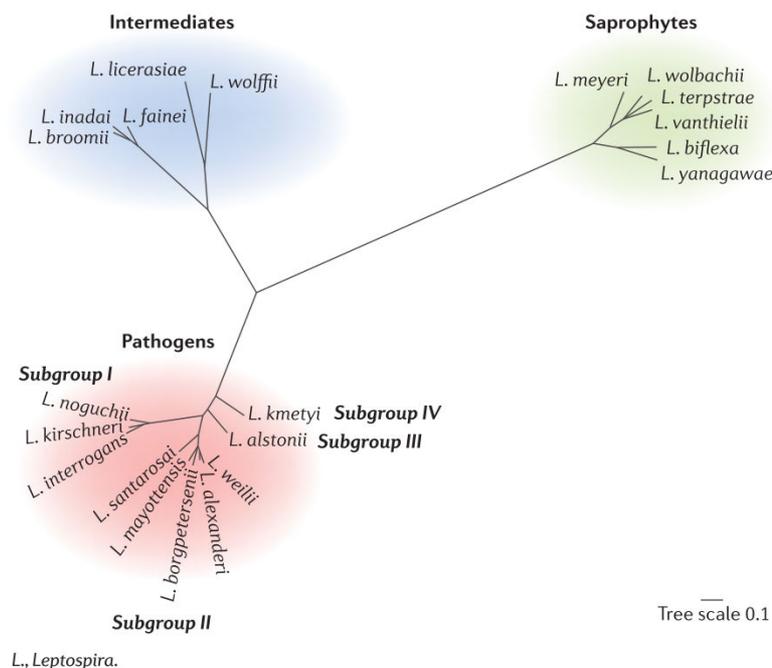
Leptospirosis is one of the most widespread zoonosis worldwide. It is caused by a spirochete from the genus *Leptospira* and can be asymptomatic or cause a wide range of symptoms ranging from a febrile illness to the most severe form known as Weil's disease, and characterized by high fever, jaundice and multi-organ failure. The disease can be transmitted from animals to humans through direct or indirect contact with the tissues or urine of infected animals. Wounded skin and mucosal membranes may facilitate the entry of the bacteria into a human host (Evangelista and Coburn, 2010; WHO, 2003), such infections being recorded in a number of cases reporting occupational (e.g. veterinarian, slaughterhouse workers) or recreational activities (e.g. water sports, caving).

Leptospirosis can be diagnosed employing different tests including bacteriological (i.e. isolation of leptospire from patients' blood and their observation under a dark-field microscope), serological (e.g. microscopic agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA)) or molecular methods (Allan et al., 2015; Vijayachari et al., 2008). Antibiotics are used to treat the disease in the early phase: doxycycline or amoxicillin are used to treat the mild forms of leptospirosis and penicillin or ceftriaxone may be used in the most severe forms (Lau et al., 2018). However the treatment of late and severe forms with antibiotherapy may be not efficient and generate complications such as Jarisch-Herxheimer reaction in which high amounts of cytokines are released and induce an acute inflammatory response (Guerrier et al., 2017; Pappas and Cascio, 2006). Detection, prevention, clinical manifestation and treatment of the disease in humans and domestic animals have been the subject of a number of comprehensive reviews and books (Adler, 2015; Bharti et al., 2003; Lau et al., 2018; Levett and Haake, 2015).

Although the global morbidity of the disease is unknown, a recent modeling study incorporating information from several databases and publications suggests that the disease indeed affects more than one million people worldwide and causes 58,900 deaths annually (Costa et al., 2015). These figures place leptospirosis as a leading zoonotic cause of morbidity and mortality worldwide. However, because of the asymptomatic forms of the disease, self-resolving illness, non-specific symptoms or the lack of accessibility to robust diagnostic facilities, incidence of leptospirosis is currently largely underestimated (Lau et al., 2018; Picardeau, 2017). Higher morbidity is reported in studies exploring the disease in rural or tropical regions, in which climates and environments are conducive to survival of the bacterium (Cassadou et al., 2016; Costa et al., 2015; de Vries et al., 2014; WHO, 2003). The risk of contamination is thus expected to increase in the near future due to the growing popularity of water sports and the higher frequency of leisure travels to tropical countries

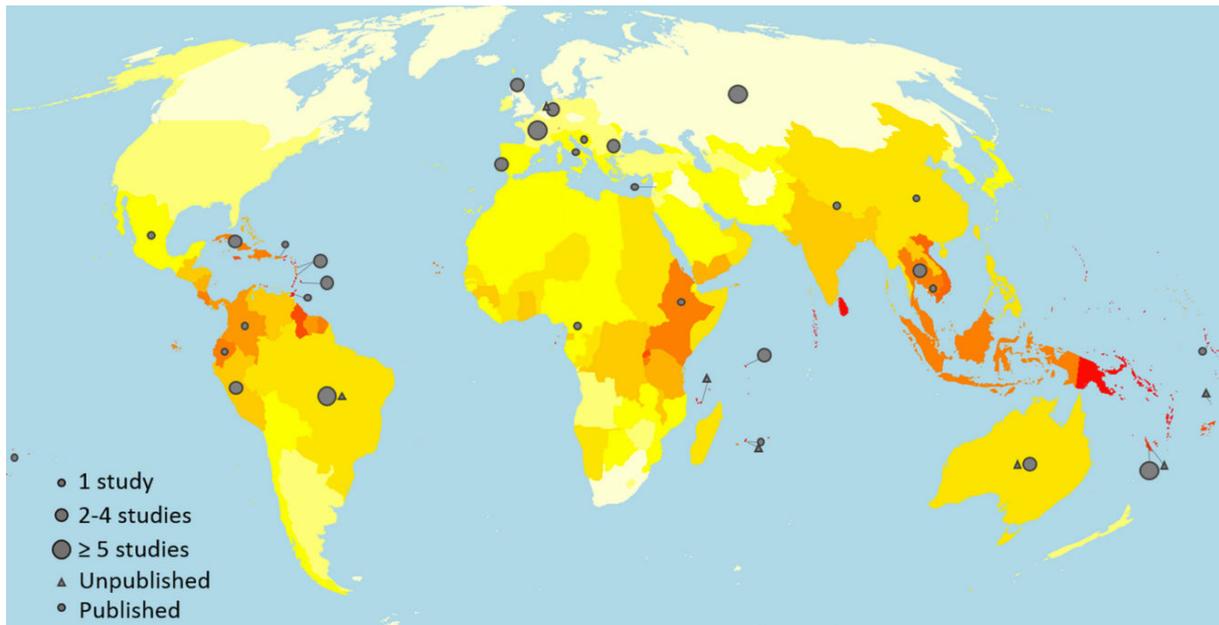
(Haake and Levett, 2015). For example, in Germany, the proportion of leptospirosis human cases associated to travel increased from 15.7% in 2003 to 40.7% in 2008 (Bandara et al., 2014). Climate is also an important factor for leptospirosis transmission. Several outbreaks have been reported after heavy rainfalls or flooding events (Amilasan et al., 2012; Balbuena et al., 2010; Dechet et al., 2012; Pagès et al., 2015b; Sanders et al., 1999; Sehgal et al., 2002; Smith et al., 2013). Hence, global warming is also expected to induce an increase in the number of leptospirosis cases in Europe by creating environmental conditions favorable for the development and/or the maintenance of the bacteria (Picardeau, 2017).

*Leptospira* are highly diversified bacteria. A classification based on the antigenic properties of the bacteria describes more than 250 pathogenic serovars grouped into 24 antigenically-related serogroups (Evangelista and Coburn, 2010). However, the rapid development of genotyping and genomics is accelerating a switch to molecular classifications. The genus *Leptospira* currently comprises at least 21 species and is divided into three clusters: the pathogenic cluster includes at least ten *Leptospira* species, a second intermediate cluster is composed of species of unknown pathogenicity or causing mild illness while a third cluster includes free-living saprophytic species (**Figure 1**). The bacterium harbors a large genome size, ranging from 3.8 to 4.7 Mb, which is associated to its ability to survive in diverse environments (Bharti et al., 2003; Picardeau, 2017) and consisting of two chromosomes in addition to a variable number of smaller circular replicons.



**Figure 1.** *Leptospira* phylogeny based on the concatenation of 491 selected core genes. (Picardeau, 2017)

Although leptospirosis has a worldwide distribution in both industrialized and developing countries (Bharti et al., 2003), the incidence of the disease is higher in tropical and subtropical areas with 73% of global reported cases occurring in tropical regions (Costa et al., 2015). As shown in **Figure 2**, the burden of the disease is particularly high in the Caribbean islands, East sub-Saharan Africa, South-East Asia and Oceania (Costa et al., 2015; Evangelista and Coburn, 2010; Pappas et al., 2008; Yang, 2018).



**Figure 2.** Worldwide incidence distribution of leptospirosis cases. 0-3 (white), 7-10 (yellow), 20-25 (orange), > 100 (red) cases per 100,000 people (Costa et al., 2015)

Noteworthy, disease incidence is maximal in tropical islands (Pappas et al., 2008) where the limited diversity of animal reservoirs typical of insular ecosystems may enhance *Leptospira* transmission (Derne et al., 2011).

This thesis will focus on southwestern Indian Ocean (SWIO) region, which records some of the highest incidence worldwide. In the last 20 years, a large body of serological and molecular data gathered on the different islands of the region has shown that these territories actually display distinct epidemiological patterns in terms of incidence and severity of the disease (Biscornet et al., 2017; Bourhy et al., 2010, 2012, 2014; Bovet et al., 1999; Desvars et al., 2011, 2012, 2013a; Gomard et al., 2014; Guernier et al., 2016; Lagadec et al., 2016; Yersin et al., 1998). In addition, a wide diversity of pathogenic *Leptospira* lineages has been reported in both human acute cases and reservoir animals. These epidemiological patterns may result from the co-evolution of *Leptospira* spp. and their animal hosts in a multi insular ecosystem considered as a leading biodiversity hotspot (Dietrich et al., 2018; Myers et al., 2000). Indeed, the unique biodiversity hosted by SWIO islands may

have led to the emergence of zoonotic pathogens that are unique to this region, including endemic *Leptospira* lineages and species, with consequences on human health that are key to this thesis. A large part of the following introduction synthesizes bibliographic together with original research data on *Leptospira* and leptospirosis in the SWIO. *Leptospira* diversity and evolution are reviewed and their consequences on the contrasted epidemiology of human disease in the region are discussed.

## **II. The biogeography of *Leptospira* in SWIO is intimately linked to the evolutionary history of animal reservoirs.**

### **1. SWIO islands are home to a unique biodiversity shaped by colonization and radiation events.**

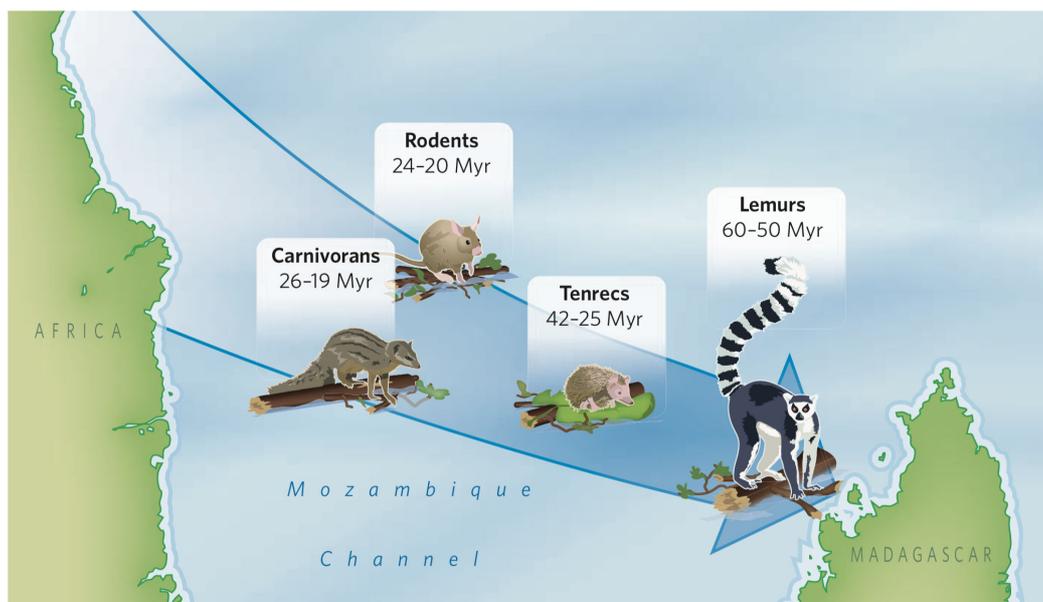
The SWIO is considered one of the five leading biodiversity hotspots in the world with Malagasy region hosting 3.2% of global endemic plants and 2.8% of global endemic vertebrates, all encompassed in 0.4% of Earth's land surface (Myers et al., 2000). The region is composed of islands, including Madagascar, Mauritius, Mayotte, La Réunion, the Seychelles, and the Union of the Comoros, with different surface areas, geographical isolation and geological histories; these aspects have in turn contributed to the evolution of the biodiversity sheltered by each island. True oceanic islands including the Comoros and Mascarene archipelagos, as well as coralline Seychelles, have emerged *de novo* from volcanic hot spots. They are distinctly younger than landmasses with a Gondwana origin, such as Madagascar and the granitic Seychelles. The composite structure of this multi-insular ecosystem is associated with distinct levels of species richness and endemism, as best exemplified by plant and animal species that have been investigated in the region (Myers et al., 2000).

The diversity of microorganisms, including zoonotic pathogens, has been more recently explored. In animals, these investigations have shown that microbial diversity and distribution result in part from the occurring reservoir species that include endemic and introduced taxa (Dietrich et al., 2014a, 2018, Wilkinson et al., 2014a, 2014b). Herein, we review the general drivers of species diversification and structuration with a special focus on Madagascar mammalian diversity. Indeed, the natural history of Malagasy mammals is complex but well established, and has directly influenced the diversification and distribution of associated zoonotic microorganisms. Using leptospirosis as a model of zoonotic disease, we present epidemiological evidence highlighting the links existing between the extant mammalian diversity and associated pathogenic *Leptospira*, together with the consequences of these biogeographical patterns on human health at a regional level.

### **Origin of Madagascar's unique biodiversity**

The high biotic endemism on Madagascar results from geographical isolation in deep geological time associated with plate tectonics, and multiple colonization events that led to some of the more pronounced adaptive radiations in the world. Madagascar was part of the Gondwana supercontinent until it started to separate during the Mesozoic era. Indo-Madagascar, which included what is today the Indian subcontinent, was isolated from Africa between 130 and 160 Ma. India separated 88 Ma from Indo-Madagascar and subsequently drifted towards its current position (Bukontaite et al., 2015; Samonds et al., 2013; Wirta et al., 2008). While drifting northwards, large portions splintered off the Indian landmass and today form the granitic islands of the Seychelles.

The vast majority of the extant fauna of Madagascar is not related to what is known in the Mesozoic fossil record, but to the Cenozoic period when the ancestors of extant animals colonized the island following its isolation. Reptiles and fishes were the predominant fauna on Madagascar before the mass extinction at the K-T boundary, after which mammals and birds became more prevalent (Ganzhorn et al., 2013; Samonds et al., 2013). According to the most parsimonious hypothesis, the ancestors of all four extant groups of terrestrial mammals successfully colonized Madagascar from continental Africa through four asynchronous arrival events taking place during the Cenozoic. Lemurs (Lemuridae) established first (70-41 Ma), followed by tenrecs (Tenrecidae) (50-20 Ma), carnivores (Carnivorans) (33-14 Ma) and nesomyine rodents (Nesomyinae) (30-15 Ma) (Ali and Huber, 2010; Cox, 2000; Poux et al., 2005) (**Figure 3**).



**Figure 3.** Timing of the colonization events involving terrestrial mammals from Africa to Madagascar (Krause, 2010)

Two alternative hypotheses have been proposed to explain these four successful ancestral colonization events. According to the land bridge hypothesis, Madagascar estrangement from Africa was followed by the emergence of small land areas located in the Mozambique Channel that may have represented rest areas and thus helped mammals to achieve and colonize Madagascar (McCall, 1997). However, such rest areas would have been located along the Davie Ridge, a 1200-km long North-South high rise located in the Mozambique Channel, and the different colonization events would have occurred in a limited area of Madagascar near to landmasses on the Davie Ridge, while mammalian colonization of Madagascar seems to have occurred at several randomly distributed sites (Ali and Huber, 2010). Moreover, it is recognized that these land areas would have been separated from each other from at least 230 km. Lastly, this stepping stone scenario should have led to a higher taxonomic diversity of Quaternary or extant mammals on Madagascar than currently recognized.

According to the second and alternative sweepstakes hypothesis, mammals could have crossed the Mozambique Channel by rafting on floating vegetation mats or large trees. The premise of this hypothesis was considered improbable because present oceanic current would not have allowed rafting from Africa to Madagascar. In 2010, Ali and Huber demonstrated that oceanic currents in the Mozambique Channel were different from the present situation. Indeed, during the Eocene, Africa, Madagascar and Australia were much further South, meaning that Madagascar was located within the convergence zone under the influence of subtropical anticlockwise gyre, which resulted in oceanic currents passing from eastern Africa towards Madagascar. Hence, this would have allowed rafts of vegetation to drift across the Mozambique Channel from Africa to Madagascar in less than a month (Ali and Huber, 2010; Samonds et al., 2013). However, such colonization might have also been facilitated by special physiological traits of land vertebrates, such as torpor and/or the ability to lower metabolism that would have facilitated successful arrival on Madagascar. Madagascar and Africa slowly drifted northwards until the Early Miocene. At that time, colonization of Madagascar via vegetation mats became virtually impossible given the change in ocean currents from Madagascar to continental Africa (Ali and Huber, 2010).

Unlike terrestrial mammals, bats, which fly, were only marginally influenced by oceanic currents and could have colonized Madagascar from both African and Asian sources (Samonds et al., 2012, 2013). Recent investigations support that bats have arrived on Madagascar within the last 15 Ma (Samonds et al., 2013), and within the modern fauna there is evidence for at least 24 colonization events (Samonds et al., 2012), mostly of African origin.

### ***Madagascar has sheltered intense biota radiation.***

Following these colonization events, Madagascar held considerable ecological diversity with numerous different biomes that facilitated an extraordinary series of adaptive radiations and gave rise to the modern taxa present on the island (Bukontaite et al., 2015; Eger and Mitchell, 1996). The mountainous landscapes of Madagascar provide different climates and three broad types of forest formations: eastern humid, western dry-deciduous forests and spiny forests (Ganzhorn et al., 2013). The diversity of climates and vegetation types, superimposed on broad elevational and latitudinal clines, provides key aspects for animal adaptation to each specific environment and resulting in large-scale speciation. For instance, tenrecs underwent a high level of adaptive radiation 29 Ma associated with changes in their morphology, physiology, behavior and ecology (Poux et al., 2008). The subsequent colonization of Madagascar by carnivores and rodents added more ecological pressure, requiring additional adaptive specialization within Tenrecidae (Poux et al., 2008).

Madagascar possesses a notable diversity of bats, with around 46 species reported from the island, included in nine different families (Goodman and Ramasindrazana, 2018). A majority of bats species are endemic to Madagascar and/or neighboring islands, most of them originating from Africa (Cardiff and Jenkins, 2016). For instance, nine of the 11 *Miniopterus* spp. currently recognized on Madagascar are endemic to the island, while the two remaining species are also found in the Comoros archipelago. This genus underwent two main radiation events circa 4-2 Ma and 1.25-0.75 Ma, probably related in part to climatic changes in mountainous areas of Madagascar (Christidis et al., 2014).

### **2. Mammals from SWIO islands host several zoonotic pathogens including pathogenic *Leptospira* that are endemic to this insular ecosystem.**

Volant and terrestrial mammals are known reservoirs for a number of viral and bacterial zoonotic pathogens (Brook and Dobson, 2015; Calisher et al., 2006; Helmy et al., 2017; Heredia and García, 2018; Mostafavi et al., 2018; Pacheco et al., 2017). Recent studies carried out in Madagascar and surrounding islands have shown that the regional mammalian diversity is no exception. Hantaviruses, responsible for hemorrhagic fever with renal syndrome and hantavirus cardiopulmonary syndrome, have been described in rodents on Mayotte (Filippone et al., 2016). Astroviruses, responsible for gastroenteritis among children less than two years old, are found in several bat species from Mozambique and Madagascar (Hoarau et al., 2018; Lebarbenchon et al., 2017). A high diversity of paramyxoviruses responsible for respiratory tracts infection are widespread on Indian Ocean islands and are hosted by bats, rodents, tenrecs as well as introduced terrestrial small mammals (Ghawar et al., 2017; Mélade et al., 2016; Wilkinson et al., 2012, 2014b). Among the documented bacterial zoonoses occurring on Indian Ocean islands, plague is both emblematic and of

regional concern as Madagascar is considered as one of the leading foci of plague re-emergence. Further, rickettsioses and bartonellosis transmitted through flea bites have been documented in Madagascar and La Réunion (Dieme et al., 2015; Kreppel et al., 2014), the former recently being associated with several human cases of murine typhus (Balleydier et al., 2015). Finally, leptospirosis, considered as the most prevalent bacterial zoonosis worldwide, is a major public health issue in the southwestern Indian Ocean (Pappas et al., 2008). Molecular data produced from mammals' samples have recently highlighted the biogeography of *Leptospira* on some southwestern Indian Ocean islands, and certain islands have phylogenetically distinct *Leptospira* species/lineages, presumably associated with different land mammalian adaptive radiations in relatively deep geological time (Dietrich et al., 2018). Altogether, data accumulated through multidisciplinary research programs have shown that the biogeography of mammalian species actually shapes the diversity of occurring *Leptospira* (Dietrich et al., 2018; Gomard et al., 2016).

***The natural history of pathogenic Leptospira on SWIO islands is tightly associated to the evolutionary history of mammalian reservoirs.***

During the last decade, several studies have explored the diversity of pathogenic *Leptospira* in the wild endemic and introduced fauna of SWIO islands. These studies have revealed that small mammals endemic to Madagascar harbor a broad diversity of *Leptospira* lineages mostly unique to the island and grouping within 3 distinct major species, namely *L. borgpetersenii*, *L. mayottensis* and *L. kirschneri*. In addition, these bacterial lineages express strong levels of specificity towards their mammalian reservoir hosts, particularly for bat-borne *Leptospira*. Indeed, Malagasy bats and their associated *Leptospira* are an interesting biological model to study the evolution of host-pathogen interactions as the island's bat fauna is composed of more than 40 distinct species, of which 80% are endemic. The investigation of Malagasy bats showed that each genus or family is reservoir of at least one and in certain cases a handful of *Leptospira* lineages (Dietrich et al., 2014b; Gomard et al., 2016; Lagadec et al., 2012).

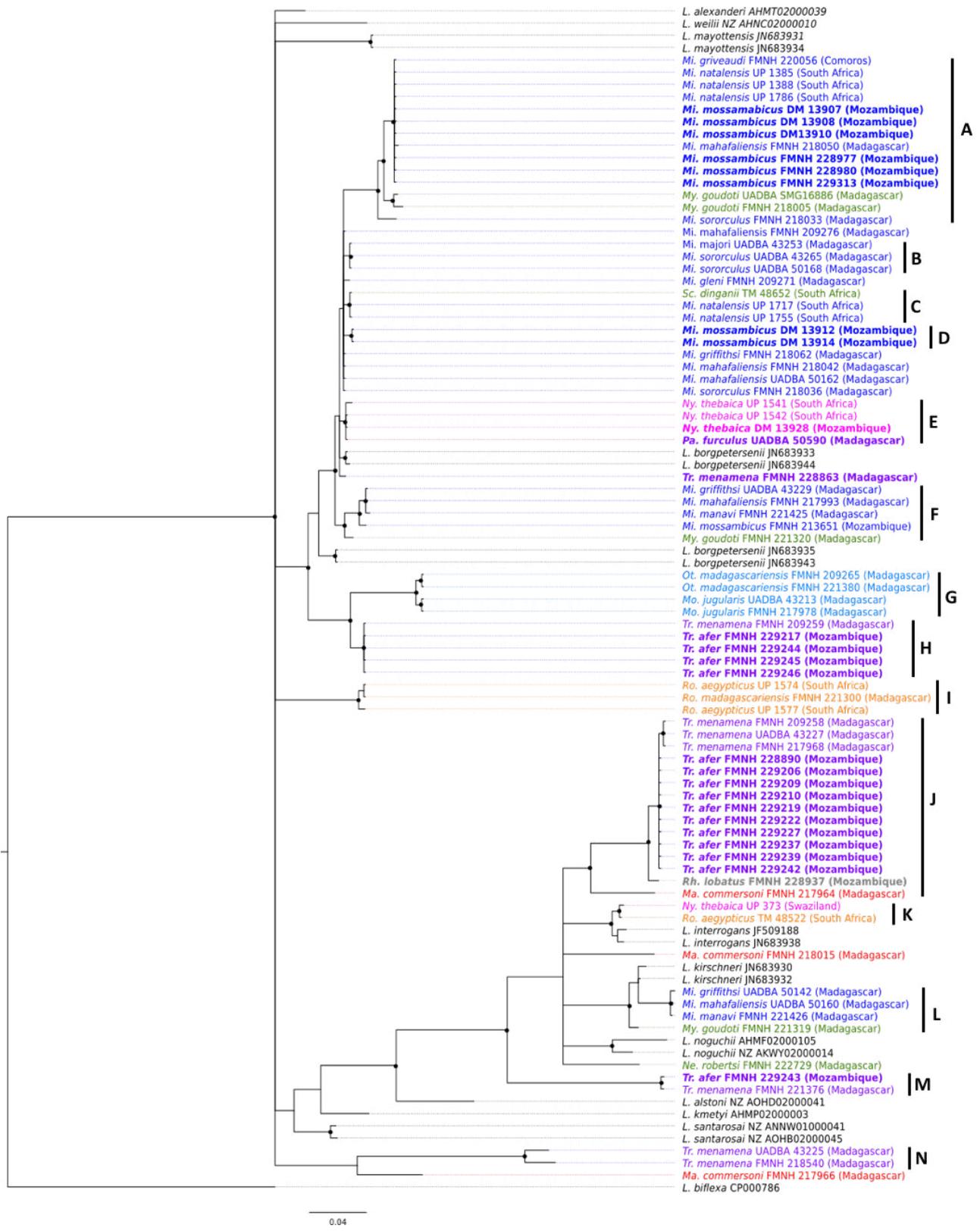
At an evolutionary scale, the detected diversity of bat-borne *Leptospira* is best explained by (i) co-diversification of *Leptospira* and bats and (ii) a few host-switches that may have been facilitated by the ecology and behavior of these animals, particularly physical contact in day-roost sites between genetically distinct species such as *Myotis goudoti* and *Miniopterus* spp. (Gomard et al., 2016). Such a scenario required further exploration of bats from continental Africa, considered as the source of colonizing lineages and including common ancestors with the majority of the extant Malagasy bat fauna (Cardiff and Jenkins, 2016). For this, we recently investigated pathogenic *Leptospira* sheltered by bats from Mozambique, revealing a wide diversity related to *L. borgpetersenii*, *L. interrogans*, *L. kirschneri* and *L. noguchii*, a bacterial species community also reported in Malagasy bats. Using the

polymorphic *secY* gene marker, we constructed a phylogeny comprising samples from SWIO islands and continental Africa. The phylogenetic tree depicted in **Figure 4** shows that the topology is driven by mammalian host community rather than geography. Indeed, well-supported clades are composed of lineages shared by closely related bats species collected on Mozambique, Madagascar or Comoros. This pattern is best exemplified by several monophyletic clades composed of *Leptospira* lineages shed by bat genera common to Mozambique and the Malagasy Region (**Figure 4**). For instance, clades A and F contain *Leptospira* genotypes found in the family Miniopteridae from Mozambique, Madagascar and/or Comoros, while clades H, I and J are composed of *Leptospira* lineages sheltered by *Triaenops afer* and *Triaenops menamena*, from Mozambique and Madagascar, respectively. There are some exceptions to this pattern, such as *Scotophilus dinganii* and *Miniopterus natalensis* carrying closely related *Leptospira* as well as *Nycteris thebaica* and *Paratriaenops furculus* shedding *Leptospira* with genotypes embedded in the same clade. However, the analysis of molecular variance (AMOVA) performed on molecular data of *Leptospira* in SWIO does not show any geographical structuration of *Leptospira* lineages ( $p = 0.52$ ) but rather suggests a structuration according to their bat host families ( $p < 0.001$ ) (**Table 1**). These results raise the hypothesis that certain lineages of ancestral bats colonizing Madagascar from eastern Africa were actually carrying *Leptospira*. Altogether, the natural history of Malagasy mammals and their associated microbial biomes suggest that the current diversity of pathogenic *Leptospira* on the island results from colonization of mammalian reservoirs from continental Africa and subsequent co-diversification processes. To our knowledge, this is the first documented dataset supporting the existence of endemic zoonotic pathogens resulting from colonization and subsequent radiation of their mammalian hosts.

**Table 1.** Analysis of molecular variance (AMOVA) based on *Leptospira secY* gene.

In each analysis, a population is defined as all *Leptospira* sequences detected in a single bat species at a single sampling site.

Comparison	Source of variation	df	Fixation indices (F)	p-value	% of variation
Sampling sites	Among sites (n = 5)	4	FCT = -0.00275	0.51992	-0.28
	Among host species within sites	19	FSC = 0.20315	0.00285	20.37
	Within host species	32	FST = 0.02096	0.00285	79.9
Host families	Among families (n = 8)	7	FCT = 0.28539	< 0.001	28.64
	Among host species within families	16	FSC = 0.05958	0.041082	-4.26
	Within host species	32	FST = 0.24282	0.00569	75.72



**Figure 4.** Phylogenetic tree based on a 473-bp-fragment of pathogenic *Leptospira secY* gene

Bayesian inference with HKY+I+G substitution model was used for analysis. Nodes with a black dot correspond to posterior probabilities  $\geq 0.80$ . *Leptospira* haplotypes are colored according to host family (blue: Miniopteridae, green: Vespertilionidae, pink: Nycteridae, red: Hipposideridae, purple: Rhinonycteridae, light blue: Molossidae, orange: Pteropodidae). *Mi*: *Miniopterus*, *My*: *Myotis*, *Sc*: *Scotophilus*, *Ny*: *Nycteris*, *Pa*: *Paratriaenops*, *Tr*: *Triaenops*, *Ot*: *Otomops*, *Mo*: *Mormopterus*, *Ro*: *Rousettus*, *Ma*: *Macronycteris*.

### ***Endemic and introduced Leptospira display distinct levels of host-specificity.***

Although bat-borne *Leptospira* show tight host-specificity, a detailed exploration of terrestrial small mammals also brings forth interesting insights (**Table 2**). Indeed, the investigation of terrestrial mammals in different forests of Madagascar showed that tenrecs are the reservoirs of several distinct *L. mayottensis* lineages (Dietrich et al., 2015, 2018). Importantly, among the wild mammal fauna on Mayotte (Lagadec et al., 2016), *L. mayottensis* was isolated only from *Tenrec ecaudatus*, a spiny tenrec introduced to the island and other neighboring islands from Madagascar and probably in the context of bush. Hence, co-diversification appears as a major driver of *Leptospira* host-specificity in volant and terrestrial mammals occurring on SWIO islands.

Synanthropic and wild introduced mammals have been investigated on La Réunion and the Seychelles archipelago as well, showing that *Rattus rattus* and *Rattus norvegicus* harbor a limited diversity of *Leptospira*, mostly restricted to *L. interrogans* (Rahelinirina et al., 2010). Interestingly, the most prevalent Sequence Type (ST) found in rats from both islands has been reported in several other parts of the world (according to scheme 3 of MultiLocus Sequence Typing (MLST), see <http://www.pubmlst.org/leptospira/>). The limited genetic diversity of *Rattus*-borne *Leptospira* and the cosmopolitan distribution of the most prevalent ST are coherent with its recent introduction (Tortosa et al., 2017). Recently, a study investigated the presence of *Leptospira* by sampling both introduced and endemic small mammals at the same forest sites on Madagascar. *Leptospira* co-infections in introduced and endemic small rodents were highlighted following the implementation of sophisticated detection schemes focusing on possible co-infections (Moseley et al., 2018). Although only *L. interrogans* had thus far been detected in introduced *Rattus* on Madagascar (Rahelinirina et al., 2010), these newly described co-infections in introduced and endemic rodents also involved *L. borgpetersenii* and *L. mayottensis* (Moseley et al., 2018). It was suggested that introduced rodents may indeed support multiple infections composed of introduced *L. interrogans* lineages and endemic *Leptospira* such as *L. mayottensis* shed by tenrecs. Rats may have acquired *L. mayottensis* infection through direct or indirect contact with tenrecs (Moseley et al., 2018). Symmetrically, the presence of *L. interrogans* in co-infections in endemic small nesomyine rodents from these same areas were reported although much more rarely (Moseley et al., 2018). A relaxed specificity was also reported in *R. Rattus* from Mayotte with *L. interrogans* and *L. borgpetersenii* being the most prevalent *Leptospira* spp. and *L. mayottensis* and *L. kirschneri* being present at a less extent (Desvars et al., 2012). These studies place *Rattus* spp. as potential reservoirs of several *Leptospira* spp. but more detailed description of *Leptospira* strains and their hosts is necessary to assess host specificity of these bacterial strains. Altogether, gathered data support that the genetic diversity of pathogenic *Leptospira* on SWIO islands is composed of endemic and introduced

*Leptospira* displaying distinct levels of host-specificity ranging from strict specificity for the former to more relaxed associations for the latter.

**Table 2.** Potential hosts of *Leptospira* spp. in SWIO islands recorded during the last decade.

Host families	Host species	<i>Leptospira</i> sp	Island	Reference
<b>Domestic animals</b>				
	cat	<i>Li</i>	Seychelles	(Biscornet et al., 2017)
	cat	NA	Reunion	(Guernier et al., 2016)
	cattle	<i>Lb</i>	Reunion	(Guernier et al., 2016)
	dog	<i>Li</i>	Seychelles	(Biscornet et al., 2017)
	dog	<i>Li</i>	Reunion	(Guernier et al., 2016)
	dog	<i>Lb, Lk</i>	Mayotte	(Lagadec et al., 2016)
<b>Chiroptera</b>				
Emballonuridae	<i>Coleura kibomalandy</i>	NA	Madagascar	(Gomard et al., 2016)
Hipposideridae	<i>Hipposideros commersoni</i>	NA	Madagascar	(Gomard et al., 2016)
Miniopteridae	<i>Miniopterus gleni</i>	<i>Lb</i>	Madagascar	(Dietrich et al., 2014b, 2018)
Miniopteridae	<i>Miniopterus gleni</i>	NA	Madagascar	(Lagadec et al., 2012)
Miniopteridae	<i>Miniopterus griffithsi</i>	<i>Lb, NA</i>	Madagascar Union of the	(Dietrich et al., 2014b, 2018; Gomard et al., 2016; Lagadec et al., 2012)
Miniopteridae	<i>Miniopterus griveaudi</i>	<i>Lb, NA</i>	Comoros	(Dietrich et al., 2018; Lagadec et al., 2012)
Miniopteridae	<i>Miniopterus griveaudi</i>	NA	Madagascar	(Gomard et al., 2016)
Miniopteridae	<i>Miniopterus mahafaliensis</i>	<i>Lb, Lk</i>	Madagascar	(Dietrich et al., 2014b, 2018; Gomard et al., 2016; Lagadec et al., 2012)
Miniopteridae	<i>Miniopterus majori</i>	<i>Lb</i>	Madagascar	(Dietrich et al., 2014b, 2018; Gomard et al., 2016)
Miniopteridae	<i>Miniopterus manavi sensu lato</i>	<i>Lb, Lk</i>	Madagascar	(Gomard et al., 2016)
Miniopteridae	<i>Miniopterus sororculus</i>	<i>Lb</i>	Madagascar	(Dietrich et al., 2014b, 2018; Gomard et al., 2016)
Molossidae	<i>Chaerophon leucogaster</i>	NA	Madagascar Union of the	(Gomard et al., 2016)
Molossidae	<i>Chaerophon pusillus</i>	NA	Comoros	(Lagadec et al., 2012)
Molossidae	<i>Mopmopterus jugularis</i>	NA	Madagascar	(Lagadec et al., 2012)
Molossidae	<i>Mormopterus acetabulosus</i>	<i>Lb</i> -related	Mauritius	(Dietrich et al., 2018)
Molossidae	<i>Mormopterus francoismoutoui</i>	<i>Lb</i> -related	Reunion	(Dietrich et al., 2018)
Molossidae	<i>Otomops madagascariensis</i>	<i>Lb, Lb</i> -related	Madagascar	(Dietrich et al., 2018; Gomard et al., 2016; Lagadec et al., 2012)
Molossidae	<i>Mormopterus jugularis</i>	<i>Lb, Lb</i> -related	Madagascar	(Dietrich et al., 2018; Gomard et al., 2016)
Pteropodidae	<i>Pterofus rufus</i>	NA	Madagascar	(Dietrich et al., 2018; Gomard et al., 2016)
Pteropodidae	<i>Pteropus seychellensis</i>	<i>Lk, NA</i>	Seychelles	(Dietrich et al., 2018; Lagadec et al., 2016)
Pteropodidae	<i>Rousettus madagascariensis</i>	<i>Lb</i>	Madagascar Union of the	(Gomard et al., 2016)
Pteropodidae	<i>Rousettus obliviosus</i>	<i>Lb, Li</i>	Comoros	(Dietrich et al., 2018; Lagadec et al., 2012)
Rhinonycteridae	<i>Paratriaenops furculus</i>	NA	Madagascar	(Gomard et al., 2016)
Rhinonycteridae	<i>Triaenops menamena</i>	<i>Li, Lb</i> -related, NA	Madagascar	(Dietrich et al., 2018; Gomard et al., 2016; Lagadec et al., 2012)

Vespertilionidae	<i>Myotis goudoti</i>	Lb, Lk, NA	Madagascar	(Dietrich et al., 2014b, 2018; Gomard et al., 2016; Lagadec et al., 2012)
Vespertilionidae	<i>Neoromicia robertsi</i>	NA	Madagascar	(Gomard et al., 2016)
Vespertilionidae	<i>Scotophilus marovaza</i>	NA	Madagascar	(Gomard et al., 2016)
<b>Rodentia</b>				
Nesomyidae	<i>Eliurus minor</i>	Lb, Li + Lm or Lb *	Madagascar	(Dietrich et al., 2014b, 2018; Moseley et al., 2018)
Nesomyidae	<i>Nesomys rufus</i>	Li + Lm or Lb *	Madagascar	(Moseley et al., 2018)
Muridae	<i>Mus musculus</i>	NA	Madagascar	(Moseley et al., 2018; Rahelinirina et al., 2010)
Muridae	<i>Mus musculus</i>	Lb, Lk	Reunion	(Guernier et al., 2016)
Muridae	<i>Rattus spp.</i>	Lb, Li, Lk, Lm	Mayotte	(Desvars et al., 2012; Dietrich et al., 2018; Lagadec et al., 2016)
Muridae	<i>Rattus spp.</i>	Li	Seychelles	(Biscornet et al., 2017; Dietrich et al., 2018)
Muridae	<i>Rattus spp.</i>	Li	Reunion	(Dietrich et al., 2018; Guernier et al., 2016)
Muridae	<i>Rattus spp.</i>	Lb, Li, Lm	Madagascar	(Dietrich et al., 2018; Moseley et al., 2018*; Rahelinirina et al., 2010)
<b>Soricomorpha</b>				
Soricidae	<i>Suncus murinus</i>	Li	Reunion	(Guernier et al., 2016)
Soricidae	<i>Suncus murinus</i>	NA	Madagascar	(Moseley et al., 2018; Rahelinirina et al., 2010)
<b>Afrosoricidae</b>				
Tenrecidae	<i>Hemicentetes nigriceps</i>	Lk	Madagascar	(Dietrich et al., 2014b, 2018)
Tenrecidae	<i>Hemicentetes semispinosus</i>	Lk, NA	Madagascar	(Dietrich et al., 2014b, 2018; Moseley et al., 2018)
Tenrecidae	<i>Microgale cowani</i>	Lb, Lm	Madagascar	(Dietrich et al., 2014b, 2018)
Tenrecidae	<i>Microgale dobsoni</i>	Lm	Madagascar	(Dietrich et al., 2014b, 2018)
Tenrecidae	<i>Microgale longicaudata</i>	Lb	Madagascar	(Dietrich et al., 2014b, 2018)
Tenrecidae	<i>Microgale majori</i>	Lb	Madagascar	(Dietrich et al., 2014b, 2018)
Tenrecidae	<i>Microgale principula</i>	Lb	Madagascar	(Dietrich et al., 2014b, 2018)
Tenrecidae	<i>Microgale spp</i>	NA	Madagascar	(Moseley et al., 2018)
Tenrecidae	<i>Tenrec ecaudatus</i>	Lm	Mayotte	(Dietrich et al., 2018; Lagadec et al., 2016)

NA = not available; Lb = *Leptospira borgpetersenii*; Li = *Leptospira interrogans*; Lk = *Leptospira kirschneri*; Lm = *Leptospira mayottensis*

\* = co-infection

### 3. The contrasted epidemiology of human leptospirosis in SWIO islands is associated with distinct transmission chains.

Leptospirosis is of considerable medical concern in most if not all of the western Indian Ocean islands. For instance, the Seychelles Archipelago has been considered the country with the highest human incidence worldwide (Pappas et al., 2008). Similarly, human incidence is 10 to 50 times higher on La Réunion and Mayotte than in continental France (Bourhy et al.; Centre national de référence de la leptospirose, 2015). This is consistent with an incidence of leptospirosis overall higher in tropical areas, which humid climates are known drivers of bacterial persistence in the environment

(Costa et al., 2015). Interestingly, human incidence is even higher on small tropical islands possibly because limited species richness typical of insular ecosystems may facilitate transmission between wild mammalian reservoirs (Derne et al., 2011). As far as SWIO insular ecosystem is concerned, serological data obtained on different islands indicate different epidemiological patterns (Bourhy et al., 2012; Bovet et al., 1999; Desvars et al., 2011, 2013b; Yersin et al., 1998). More recent investigations using molecular data and carried out through a One Health framework have illuminated the epidemiology of leptospirosis on several of these islands and highlighted the occurrence of sharply different transmission chains.

**Madagascar** has been previously considered as having very low levels of leptospirosis. In 2015, a human case of leptospirosis was reported from a traveler on La Réunion returning from Madagascar, probably infected with *L. interrogans* (Pagès et al., 2015a). The same year, a human seroprevalence of 2.9% was reported in the Moramanga District with Icterohaemorrhagiae identified as the most prevalent serogroup (Ratsitorahina et al., 2015). A 2018 study reported no case of leptospirosis from febrile patients based on PCR analysis of blood samples (Hagen et al., 2018). However, urine samples were not collected and these cases may have been misdiagnosed. This highlights the necessity of using correct protocols elaboration to better assess and understand the actual burden of human leptospirosis on Madagascar, which is still largely unknown.

Human leptospirosis is also poorly described in the **Union of the Comoros** besides a few cases reported among travelers returning home (Subiros et al., 2017). In 2011, a serological investigation carried out using sera from human febrile cases highlighted the presence of leptospirosis on all three islands of the Union of the Comoros with seroprevalence extending from 3.4% on Grande Comore to 10.3% on Mohéli (Gomard et al., 2014). Again, the actual burden of the disease in this country remains an open question.

The epidemiology of leptospirosis on **Mayotte** and **La Réunion** has been the subject of considerable studies. Further, the surveillance of leptospirosis is under the control of the same health system on both islands, Health Regional Agency (Agence Régionale de Santé, ARS). Hence, surveillance biases are expected to be limited, conferring to the epidemiological data produced on these French islands a good analysis power. Between 2010 and 2015, the annual mean incidence of human leptospirosis on Mayotte was 100 cases per 100,000 habitants. Three per cent of the acute cases were admitted to intensive care units (ICU) and 0.9% of total cases were lethal (Pagès et al., 2017a). Between 2015 and 2017, the incidence of leptospirosis on Mayotte increased from 38.8 to 66.3 cases per 100,000 habitants. In 2016, 22% of human cases were hospitalized, 4.6% of the cases were admitted to ICU and a mortality rate of 0.6% was reported (Subiros et al., 2017). In 2017, 19%

of leptospirosis human cases were hospitalized; among them, one third was admitted to ICU and one patient died from the disease (mortality rate of 0.6%) (Cire océan Indien, 2018b). The serogroup Mini was the most prevalent one in human cases of leptospirosis, while Icterohaemorrhagiae has not been detected since 2008. This latter serogroup was identified from human cases when more severe forms of leptospirosis were observed in patients and with a mortality rate reaching 23% (Subiros et al., 2017). Since 2008, Icterohaemorrhagiae serogroup has not been identified and the lethality rates dropped drastically to 0.6% in 2017 (Cire océan Indien, 2018b; Subiros et al., 2017). Molecular findings indicate that four species are involved in human leptospirosis on Mayotte: *L. borgpetersenii*, *L. interrogans*, *L. kirschneri* and *L. mayottensis*. The three former species can be found in introduced mammals, such as rats and dogs. The fourth form of *Leptospira* on the island is restricted to *T. ecaudatus*, which is endemic to Madagascar and introduced to Mayotte, and has been identified as the exclusive reservoir of *L. mayottensis* (Lagadec et al., 2016).

Between 2004 and 2015, a mean incidence of six cases of leptospirosis per 100,000 habitants was recorded on La Réunion, with hospitalization being significantly more frequent than on Mayotte. Indeed, within the same period, 93% of total cases were hospitalized and among them, 33% were admitted to ICU, while 3% of acute cases were lethal (Pagès et al., 2017b). Between 2014 and 2017, the annual incidence of leptospirosis varied between 4.7 and 7.2 cases per 100,000 habitants. The hospitalization rate was high (over 75%) and mortality rates averaged 5% except for the year 2017 when no person was reported to succumb to the disease. On La Réunion, Icterohaemorrhagiae is the most prevalent serovar and *L. interrogans* is largely dominant among human cases (Guernier et al., 2016; Pagès et al., 2017b). Both STs of *L. interrogans* detected in human cases on La Réunion (ST02 and ST34, MLST scheme 3) have a broad worldwide distribution. Introduced *Rattus* appear as a major reservoir of *Leptospira* on the island, although only one ST of *L. interrogans* (ST02), identical to one ST found in humans, is described in these animals. The absence of the second *L. interrogans* ST (ST34) in rats indicates that they are not the only source of *L. interrogans* infecting humans. Indeed, stray dogs may play a role in the transmission of the second haplotype of *L. interrogans*. *Mus musculus* and cattle may also act as disease reservoirs as they shelter *L. borgpetersenii* with STs identical to those characterized in a few human cases (Guernier et al., 2016).

Lastly, population-based studies carried out in the **Seychelles** since 1989 report high annual incidence rate of human leptospirosis, reaching 101 cases per 100,000 habitants between 1995 and 1996 (Yersin et al., 1998). In 2008, Seychelles was considered as having the highest incidence rate of human leptospirosis worldwide (Pappas et al., 2008). A longitudinal 12 months survey in 2014-2015 showed that this incidence decreased but remained high with a lethality rate of 11.8% (Biscornet et al., 2017). A large proportion of acute cases include severe manifestations of the disease with

patients harboring one or several symptoms including jaundice, renal failure or pulmonary hemorrhage, the last one being the most important cause of death (Yersin et al., 1998). As on La Réunion, human cases of leptospirosis in the Seychelles are caused by a narrow diversity of *Leptospira* lineages. Most human cases involve the serogroup Icterohaemorrhagiae and only *L. interrogans* is detected among human acute cases and *Rattus*. Genotyping identified three STs in humans and animals, one corresponding to the most common ST found in rats and human cases from La Réunion (ST02) (Biscornet et al., 2017; Guernier et al., 2016). Importantly, in the Seychelles, *Rattus* does not appear as the main reservoir of human leptospirosis: ST02 is the only haplotype reported among rats and is involved in only one third of acute cases. Stray dogs may be involved in disease transmission to humans as one tested dog was found shedding the ST involved in the majority of human cases. However, complementary investigations are required to address the importance of dogs in the epidemiology of human leptospirosis (Biscornet et al., 2017).

The contrasted epidemiology of leptospirosis on SWIO islands indicates different transmission chains. The presence of different strains responsible for the disease depends on the local fauna (**Table 2**) and its biogeographic history (Dietrich et al., 2018). For instance, cosmopolitan and presumably introduced *Leptospira* (mainly *L. interrogans*) are responsible for most, if not all, human cases in the Seychelles and La Réunion, while original *Leptospira* lineages including endemic *L. mayottensis* are involved in most human cases on Mayotte (Lagadec et al., 2016).

### **III. Experimental animal models used in *Leptospira* research**

The investigation of leptospirosis through a One Health framework allowed highlighting the epidemiology of leptospirosis and the different transmission chains involved in human infection. The use of experimental models helps understanding the mechanisms of infection underlying *Leptospira* epidemiology as currently recognized in SWIO islands. Indeed, experimental approach can focus the investigations either on the acute phase or the chronic phase of the infection, hence allowing disentangling the mechanisms at play in humans during acute infection or in animal reservoirs during chronic infection.

#### **1. Acute models of infection**

Guinea pigs and hamsters mimic the symptoms of human leptospirosis and are used to study the acute phase of *Leptospira* infection. Several *Leptospira* strains isolated around the world have been characterized in hamsters to describe the consequences of these infections. These studies revealed several organs of interest as targets of *Leptospira* infection. The mostly described damages include hemorrhage, congestion and infiltration of inflammatory cells in the kidneys, lungs and liver

of infected animals (da Silva et al., 1995; Diniz et al., 2011; Matsui et al., 2015). Histopathological studies report the presence of urinary casts, nephritis, and glomerular and tubular damage in the kidneys of infected hamsters (Da Silva et al., 2010; Diniz et al., 2011; Forster et al., 2013; Matsui et al., 2015; Silva et al., 2008; Tomizawa et al., 2017; Villanueva et al., 2014). Petechial hemorrhage and alveolar collapse are often observed in the lungs of hamsters infected with virulent strains of *Leptospira* (Da Silva et al., 2010; Silva et al., 2008; Tomizawa et al., 2017; Villanueva et al., 2014). Lastly, in the liver of infected animals, hepatocytes can be swollen and undergo degeneration and necrosis while the loss of cohesion between hepatocytes can lead to jaundice (Alves et al., 1992; Diniz et al., 2011; Forster et al., 2013; Miyahara et al., 2014; Villanueva et al., 2014).

Rodent models of acute leptospirosis have also been used to describe the immune and inflammatory mechanisms underlying *Leptospira* infection. Several studies highlighted that an overwhelming inflammatory response in hamsters following infection with virulent strains of *Leptospira* could contribute to the severity of the disease, leading to renal lesions and a fatal outcome in most cases (Nakornpakdee et al., 2018; Nally et al., 2004; Vernel-Pauillac and Goarant, 2010; Vernel-Pauillac and Merien, 2006).

Experimental models of acute leptospirosis also allowed the identification of markers for early diagnosis of leptospirosis (Segawa et al., 2014; Vanaja et al., 2001) and have a crucial role in the development of effective vaccines against the disease (Coutinho et al., 2011; Murray et al., 2018; Oliveira et al., 2019). Models of acute infection have also proven the efficiency of bioinformatics tools to better understand leptospiral antigens structure and further improve the efficiency of vaccines (Garba et al., 2018; Hsieh et al., 2017; Murray et al., 2013).

## **2. Chronic models of infection**

Rats are renowned reservoirs for leptospirosis worldwide and thus provide a relevant model to study the mechanisms of renal colonization. Rats are not resistant to leptospirosis at their youngest age. As hamsters and guinea pigs, rat pups are susceptible to *Leptospira* infection in the first two weeks of their lives and undergo weight loss, jaundice and several tissue damage, including pulmonary and cerebellum hemorrhage and hepatocytes disarrangement (Muslich et al., 2015). In these cases, leptospire can be detected in several organs, including the kidneys, liver, lung and cerebellum. Resistance to leptospirosis is expressed by rats aged of 23 days or more in which case leptospire are most often detected in the kidneys only. Experimental *Leptospira* infection in *R. norvegicus* mostly results in asymptomatic chronic renal infection (Athanzio et al., 2008; Chagas-Junior et al., 2012; Nally et al., 2005, 2015; Tucunduva de Faria et al., 2007; Zilber et al., 2016). Histological studies on rats reveal no tissue damage (Athanzio et al., 2008; Nally et al., 2005) except in cases where nephritis and infiltration of lymphocytes and macrophages can be observed in the

kidneys (Nally et al., 2015; Tucunduva de Faria et al., 2007). *Rattus norvegicus* does not exhibit identical affinities to all *Leptospira* serovars. In 1981, Thierman *et al* have shown that the renal carriage of the serovar Icterohaemorrhagiae lasted longer than that of serovar Grippotyphosa. Thus, *R. norvegicus* appears as a relevant model to study and understand the host specificity highlighted of leptospires by investigation of wild reservoirs in the field.

#### IV. Main conclusions and research hypotheses.

Molecular and serological data gathered from SWIO islands in the last 20 years indicate that the local diversity of *Leptospira* lineages is related to the diversity of local mammalian reservoirs. Besides the heterogeneity of *Leptospira* lineages/species occurring on each island, it appears that *Leptospira* lineages unique to the region show a strong affinity for their endemic hosts in contrast with cosmopolitan lineages, which can be sheltered by a variety of mostly introduced mammalian hosts, including rodents, dogs and cattle. The uniqueness of some *Leptospira* lineages/species shed by endemic mammals, such as tenrecs, likely results from long-term co-evolution. These unique *Leptospira* can be considered as endemic to Madagascar and neighboring islands ; this is in contrast with the *Leptospira* lineages prevailing on La Réunion or the Seychelles, which have limited diversity and cosmopolitan distribution likely associated with a recent introduction.

The patterns of *Leptospira* diversity on each island are in turn associated with distinct levels of severity in human cases. Indeed, cosmopolitan lineages such as *L. interrogans* ST02 commonly found in human cases on La Réunion and the Seychelles are associated with severe forms of leptospirosis. As far as endemic *Leptospira* are concerned, *L. mayottensis* is responsible for roughly 16% of human cases on Mayotte (suppl. mat. Bourhy et al., 2012), where severe forms of human leptospirosis are less frequent than on La Réunion and the Seychelles (Biscornet et al., 2017; Cire océan Indien, 2018a, 2018b). To date, no bat-borne *Leptospira* has been detected in acute human cases from Madagascar, La Réunion, Mayotte or the Seychelles, although high infection prevalence have been reported in these volant mammals (Biscornet et al., 2017; Dietrich et al., 2015; Gomard et al., 2016; Guernier et al., 2016; Lagadec et al., 2016).

Altogether, the data summarized in this introductory chapter raise questions about (i) a **higher virulence of cosmopolitan *Leptospira* lineages** as compared to lineages that are unique to the western Indian Ocean islands and (ii) a **strong host specificity of endemic *Leptospira*** possibly resulting from co-diversification of *Leptospira* and their hosts at evolutionary time scales.

The **virulence** of cosmopolitan and endemic strains of *Leptospira* can be assessed through experimental infection using different models to mimic and ascertain the impact of each lineage on human health. In the context of this thesis, we assessed the virulence of three strains of *Leptospira*

using a hamster model and strains isolated on regional islands: one cosmopolitan strain of *L. interrogans* isolated from *R. Rattus* on La Réunion, which genotype (ST02) is involved in the majority of human cases of leptospirosis on the island; one *L. mayottensis* isolate obtained from a tenrec and which genotype was previously found in human cases on Mayotte (Bourhy et al., 2014; Lagadec et al., 2016); and one *L. borgpetersenii* endemic isolate obtained from a Malagasy bat and which genotype has not been reported in any human case thus far.

The tight **host specificity** observed for endemic *Leptospira* lineages/species towards their mammalian reservoir can be challenged experimentally in order to determine whether this pattern results from ecological or genetic determinants. Since rats are considered as a major reservoir of leptospirosis, they were used as animal models to investigate the host specificity of *Leptospira* and were challenged with all three *Leptospira* isolates. If experimentally infected rats fail to chronically maintain and shed *L. mayottensis* and/or *L. borgpetersenii*, the host specificity observed in the field likely involves genetic factors responsible for host-pathogen interaction. Symmetrically, if experimentally infected rats are found able to chronically excrete *L. mayottensis* and *L. borgpetersenii*, hence the host specificity may actually result from ecological factors including the lack of physical contact between endemic and introduced reservoirs.

Finally, the exploration and comparison of genomic features of *Leptospira* strains from SWIO islands and strains found elsewhere worldwide may shed light on the phenotypic contrasts observed among human acute cases. Altogether, this thesis aims at enlightening the contrasted epidemiology of leptospirosis observed in this region through experimental approaches that are complementary to the One Health approach implemented on several islands in the last decade.

The results produced in the frame of this thesis are presented according to three chapters that include the investigation of (i) the pathogenicity of three *Leptospira* strains isolated in SWIO islands, (ii) the specificity of these strains towards their mammalian reservoirs and (iii) genomic components possibly associated to these features.

## CHAPTER 1

# Investigation of the pathogenicity of three *Leptospira* isolates from southwestern Indian Ocean islands

## I. Medical importance of *Leptospira* lineages in the SWIO region

Leptospirosis is of major medical concern on several islands of western Indian Ocean and shows different epidemiological features on each island. For instance, the human disease is distinctly more severe on La Réunion than on Mayotte. Indeed, the proportion of human cases admitted in ICU is higher on La Réunion than on Mayotte (33% and 3% of acute cases, respectively). Furthermore, 5% of acute cases are lethal on La Réunion vs. less than 1% on Mayotte. This contrasted epidemiology may result from the presence of different *Leptospira* communities hosted by each island. Indeed, human cases on La Réunion are mainly caused by a single *L. interrogans* strain (Guernier et al., 2016) while four distinct *Leptospira* species (*L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, and *L. mayottensis*) with original genetic profiles have been reported in human acute cases on Mayotte (Bourhy et al., 2012). This observation led us to hypothesize that some of the original leptospires, probably endemic to the Malagasy region, may be less pathogenic than the single *L. interrogans* strain responsible for most human cases on La Réunion.

This first chapter aims at exploring virulence features of three *Leptospira* strains found in western Indian Ocean region:

- a. one strain of *L. interrogans* isolated from *Rattus rattus* on La Réunion,
- b. one strain of *L. mayottensis* isolated on Mayotte from *Tenrec ecaudatus*, a small mammal endemic to the Malagasy region,
- c. one strain of *L. borgpetersenii* strain isolated from *Triaenops menamena*, a bat endemic to Madagascar.

Classifying these isolates by their MLST profiles, the *L. interrogans* isolate belongs to ST that is known to have a worldwide distribution while both the *L. mayottensis* and *L. borgpetersenii* isolates have STs that have not been observed elsewhere. Note worthily, the *L. interrogans* and *L. mayottensis* isolates are of medical importance on La Réunion and Mayotte, respectively, while the *L. borgpetersenii* isolate has never been associated with human disease.

The virulence of all three strains was first restored (**Figure 5**) and then evaluated (**Figure 6**) through experimental infection of golden Syrian hamsters. This animal model can mimic the symptoms of acute human leptospirosis and is commonly used to characterize pathogenic *Leptospira* (Coutinho et al., 2014; Diniz et al., 2011; Forster et al., 2013; Haake, 2006; Silva et al., 2008; Villanueva et al., 2014). We inoculated each hamster intraperitoneally (i.p.) with  $2 \times 10^8$  bacteria monitored them for one month. *Leptospira* DNA was then quantified in the urine collected at different time points post-infection and in different organs (brain, kidney, liver, lung, spleen) one month after the infection or earlier when hamsters were moribund, requiring euthanasia (**Figure 6**).

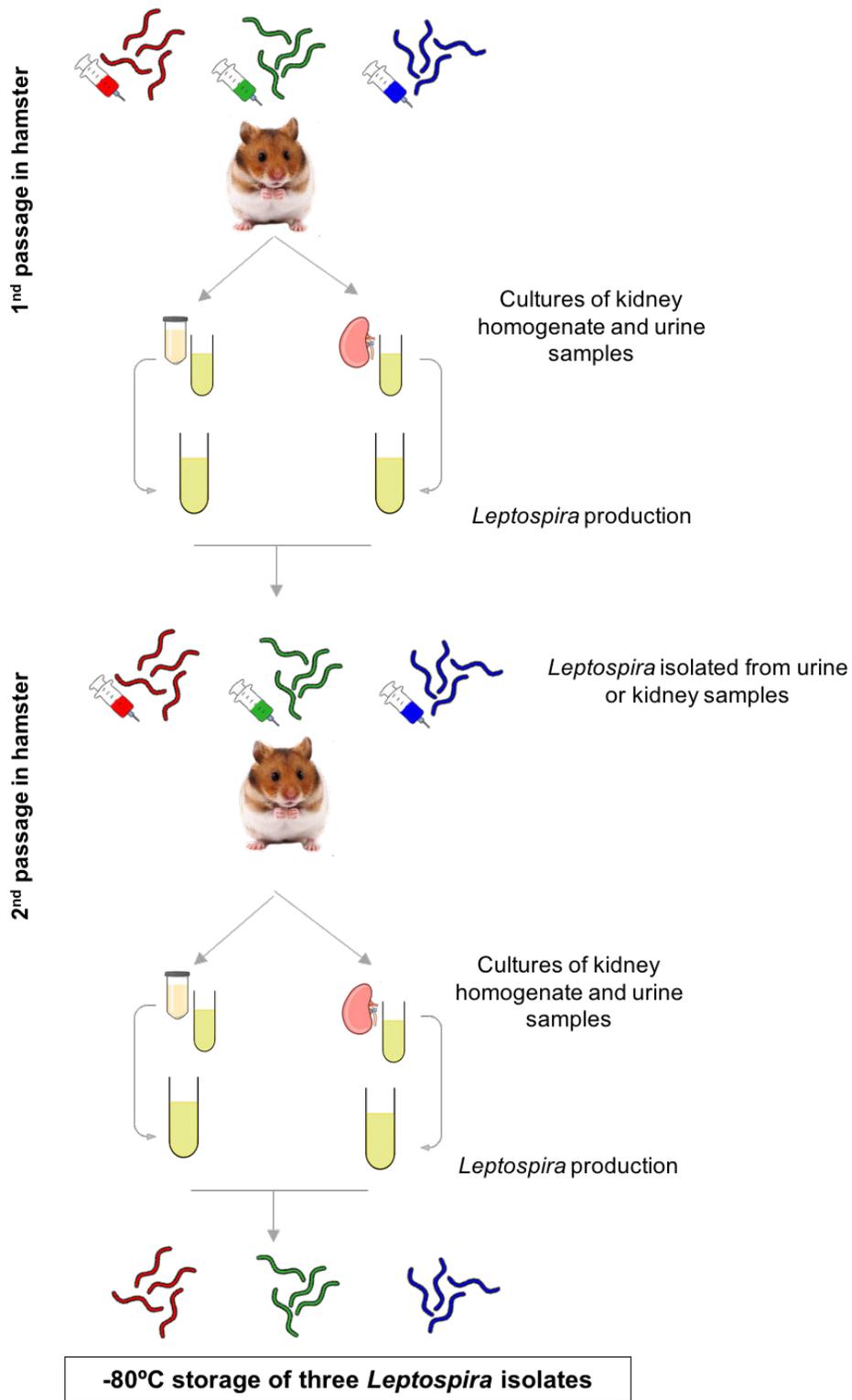


Figure 5. Experimental procedure to restore the virulence of the *Leptospira* isolates

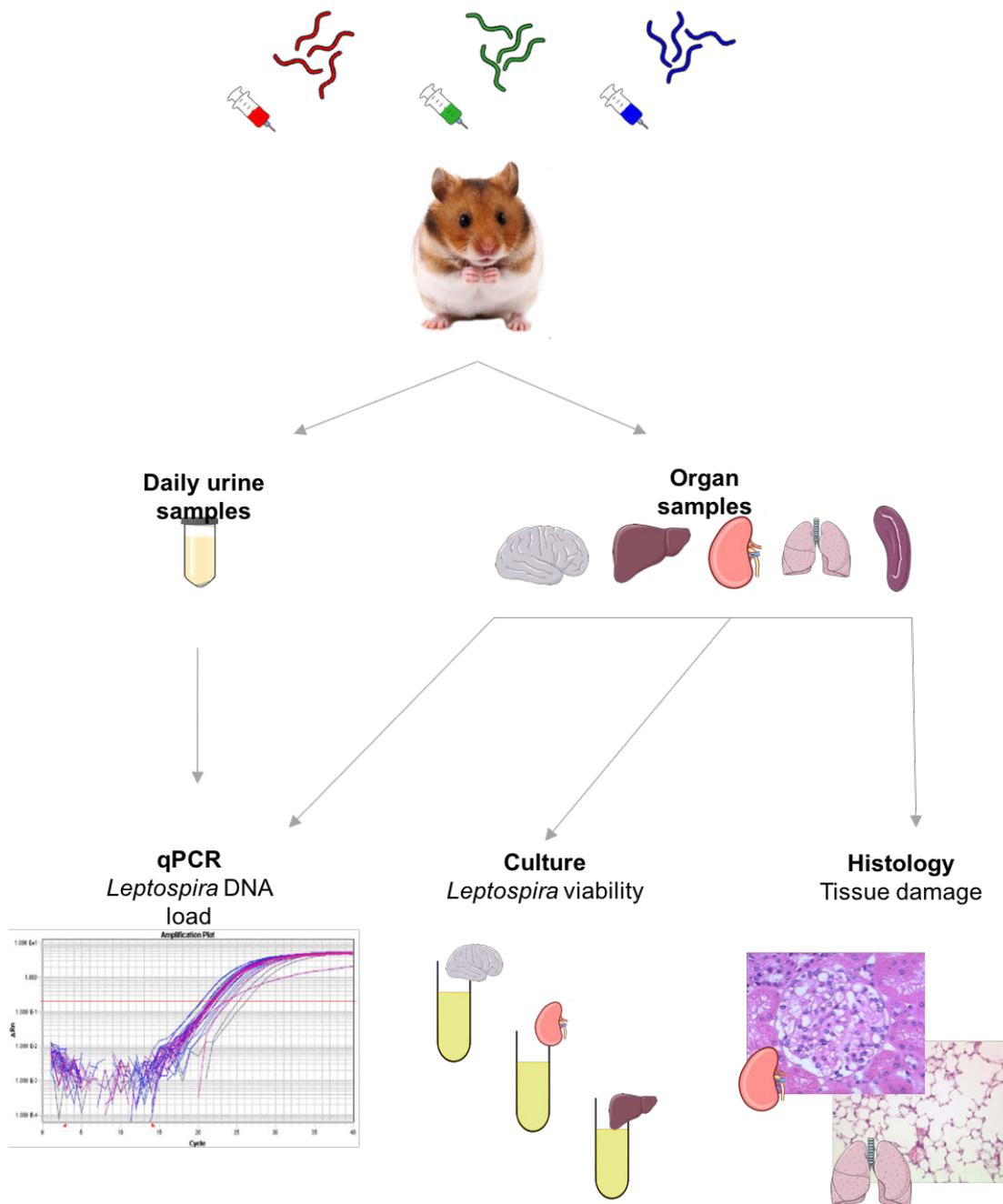
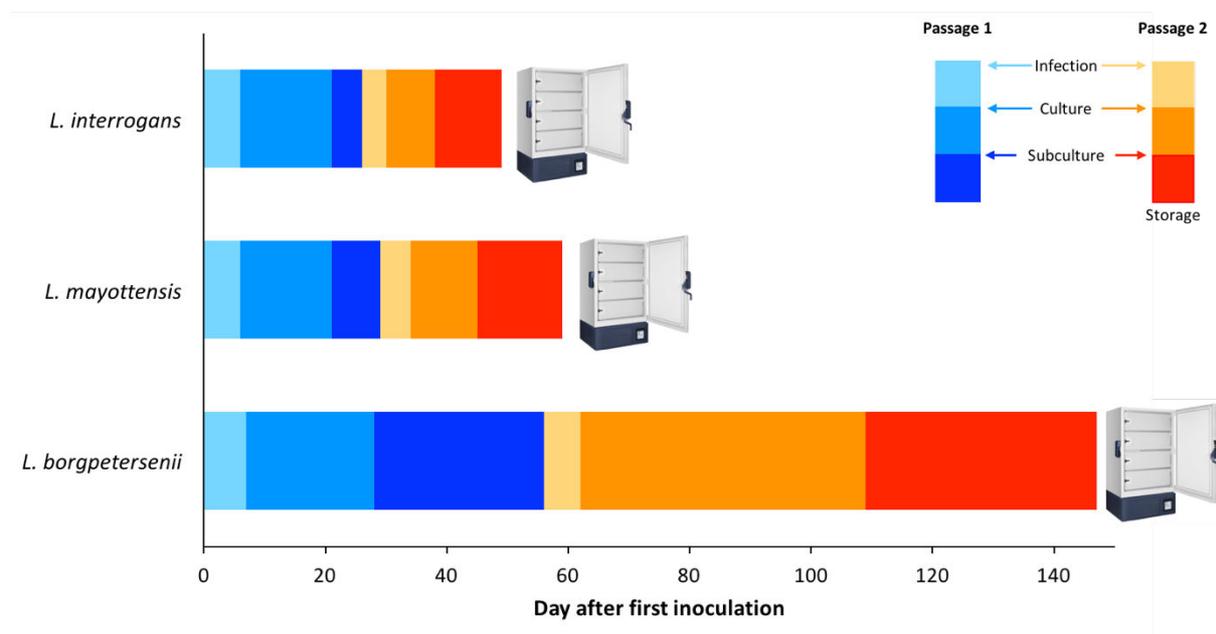


Figure 6. Experimental procedure to investigate the virulence of *Leptospira* isolates.

## II. Restoration of virulence of three *Leptospira* isolates

The strains used in this study were isolated on the field less than five years ago and parallel cultures were stored at  $-80^{\circ}\text{C}$  and in liquid nitrogen. As the virulence of *Leptospira* decreases with long-term storage (Reed et al., 2000), the first step of the infection protocol was to restore the virulence of the different strains by two serial passages in hamsters. This is standard practice with *Leptospira* infection (Haake, 2006). Briefly hamsters were inoculated i.p. a first time with  $1.5\text{-}2 \times 10^8$  leptospires ( $n = 1$  hamster for each *Leptospira* isolate). Hamsters were sacrificed during the

bacteremic phase of infection usually occurring up to 7 days after the infection. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with albumin fatty acid serum (AFAS) and 5-fluorouracil (5-Fu) was then inoculated with either a kidney homogenate or a urine sample of infected hamsters to allow the isolation and growth of leptospires. After a subculture in EMJH + AFAS medium without 5-Fu, leptospires were inoculated to a second set of hamsters (n=1 for each *Leptospira* isolate). The same methodology was then used to obtain a production of each *Leptospira* strain, which was eventually stored at -80°C. After the second passage, DNA was extracted from *Leptospira* cultures and *secY* gene was sequenced to verify the species of the isolated *Leptospira*. As shown in **Figure 7**, the restoration of virulence took approximately the same time for the isolates of *L. interrogans* and *L. mayottensis* while it took more than twice longer for the *L. borgpetersenii* isolate. This first observation indicates that the growth of the *L. borgpetersenii* isolate was much slower than that of *L. interrogans* and *L. mayottensis* isolates.



**Figure 7.** Restoration of virulence of three *Leptospira* isolates. Passage 1 (light to dark blue ) and passage 2 (yellow to dark red ) of *Leptospira* isolates in hamsters were performed in the same way. Hamsters were euthanized a few days after the infection and urine and kidney samples were inoculated to EMJH medium + AFAS + 5-Fu. *Leptospira* cultures were then subcultured in EMJH medium + AFAS. When *Leptospira* subcultures were dense enough, they were either used for the second infection or stored at -80°C.

**III. Article 1:** Three *Leptospira* strains from western Indian Ocean wildlife show highly distinct virulence phenotypes through hamster experimental infection (*Frontiers in Microbiology*, in press)

# Three *Leptospira* Strains from Western Indian Ocean Wildlife Show Highly Distinct Virulence Phenotypes Through Hamster Experimental Infection

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26 **Abstract (227 words)**

27 Leptospirosis is one of the most widespread zoonoses worldwide, with highest incidence reported  
28 on tropical islands. Recent investigations carried out in a One-Health framework have revealed a  
29 wide diversity of pathogenic *Leptospira* lineages on the different islands of Western Indian  
30 Ocean carried out by a large diversity of mammal reservoirs, including domestic and wild fauna.  
31 Using golden Syrian hamsters as a model of acute infection, we studied the virulence of  
32 *Leptospira interrogans*, *Leptospira mayottensis* and *Leptospira borgpetersenii* isolates obtained  
33 from rats, tenrecs and bats, respectively. Hamsters were inoculated with  $2.10^8$  bacterial cells and  
34 monitored for one month. The *L. interrogans* isolate proved to be the most pathogenic while *L.*  
35 *mayottensis* and *L. borgpetersenii* isolates induced no clinical symptoms in the infected hamsters.  
36 High leptospire DNA amounts were also detected in the urine and organs of hamsters infected  
37 with the *L. interrogans* isolate while *L. mayottensis* and *L. borgpetersenii* isolates mostly failed  
38 to disseminate into the organism. In addition, histological damage was more pronounced in the  
39 kidneys and lungs of hamsters infected with the *L. interrogans* isolate. Altogether, these data  
40 support that *Leptospira* strains shed by mammals endemic to this insular ecosystem (*L.*  
41 *mayottensis* and *L. borgpetersenii* isolates) are less pathogenic than the *L. interrogans* rat-borne  
42 isolate. These results may provide a relevant framework for understanding the contrasting  
43 epidemiology of human leptospirosis observed among Western Indian Ocean islands.  
44

45 **Keywords**

46 *Leptospira*, leptospirosis, virulence, hamster model, kidney, bats, tenrecs, Western Indian Ocean.  
47

## 47 1 Introduction

48 Leptospirosis is a neglected re-emerging bacterial zoonosis affecting over one million persons  
49 annually and causing nearly 60,000 deaths worldwide (Costa et al., 2015). This disease is caused  
50 by a spirochete from the genus *Leptospira* (Adler, 2015) and human infection most commonly  
51 results from indirect environmental contamination with leptospire shed by the urine of infected  
52 reservoir animals. In humans, asymptomatic infection is more common than the symptomatic  
53 disease which may be associated with mild symptoms such as fever, headache, vomiting or more  
54 severe symptoms including pulmonary hemorrhage, liver and renal failure, also known as Weil's  
55 syndrome (Evangelista and Coburn, 2010).

56  
57 Leptospirosis can reach high incidence in human populations especially in the tropics where  
58 humid and warm climate can promote survival of bacteria in the environment (Simbizi et al.,  
59 2016). The tropical islands of Western Indian Ocean report some of the highest incidence  
60 worldwide (Derne et al., 2011; Pappas et al., 2008). Despite their importance, unfortunately little  
61 is known about the precise nature of pathogenic strains in this region, partly due to the fact that  
62 very few have been successfully isolated from either human clinical cases or animal hosts. Rats  
63 are well-known reservoirs of leptospirosis worldwide, including Western Indian Ocean islands,  
64 but they are not the only mammals at play. For example, dogs and cattle were also identified as  
65 potential reservoirs for the disease on Reunion while *Tenrec ecaudatus*, introduced from  
66 Madagascar to Mayotte as game meat, may play a role in human leptospirosis (Guernier et al.,  
67 2016; Lagadec et al., 2016). In Madagascar, a wide range of endemic mammals, including several  
68 tenrec and bat species, has been reported to shelter a wide diversity of *Leptospira* lineages  
69 although there is no evidence of their role as disseminators of the disease (Dietrich et al., 2014;  
70 Gomard et al., 2016; Guernier et al., 2016; Lagadec et al., 2012).

71  
72 Recently, molecular studies have described contrasting epidemiology of leptospirosis between the  
73 islands of Western Indian Ocean. On Reunion Island, most cases of leptospirosis are caused by  
74 *Leptospira interrogans* with Sequence Types (STs) also distributed in other countries worldwide  
75 (Guernier et al., 2016). By contrast, studies conducted on human cases from Mayotte reported a  
76 variety of original STs belonging to *L. interrogans*, *L. kirschneri*, *L. borgpetersenii* and *L.*  
77 *mayottensis* species (Bourhy et al., 2012, 2014). In addition, severity of human leptospirosis is  
78 significantly different on the two islands with 4.6% admission in intensive care unit and 0.7%  
79 mortality rate in Mayotte while Reunion records 30% admission in intensive care unit and 5 %  
80 mortality (Cire océan Indien, 2017; Pagès et al., 2017; Subiros et al., 2017).

81  
82 In this study we aim to provide a first insight into differences in virulence between *Leptospira*  
83 strains from Western Indian Ocean islands shed by different mammal hosts through experimental  
84 infection of golden Syrian hamsters. This rodent model mimics the clinical symptoms of human  
85 leptospirosis and has already allowed the description of several virulence phenotypes of different  
86 *Leptospira* strains (Matsui et al., 2015; Silva et al., 2008; Tucunduva de Faria et al., 2007;  
87 Villanueva et al., 2014). To pursue our goal, we tested three available *Leptospira* isolates  
88 obtained from wild fauna during a previous research program. The *L. interrogans* isolate was  
89 obtained from a rat on Reunion and displays a ST associated with the majority of acute human  
90 cases on Reunion Island (Guernier et al., 2016) and the *L. mayottensis* isolate obtained from a  
91 tenrec on Mayotte has a ST previously reported in several human acute cases on the island

92 (Lagadec et al., 2016). By contrast, the *L. borgpetersenii* isolate was obtained from a bat endemic  
93 to Madagascar and has a genotype that has not been reported from any human case thus far.

94

## 95 **2 Materials and methods**

### 96 **2.1 Animal experiments and ethical statement**

97 Experiments were conducted following the guidelines of the Office Laboratory of Animal Care at  
98 the CYROI platform. Female golden Syrian hamsters (Janvier Labs, Le Genest, France), aged  
99 from 6 to 8 weeks, were housed by 3 in enriched cages. The appropriate number of animals was  
100 calculated using the Resource equation method (Festing, 2006). All animal procedures carried out  
101 in this study were performed in accordance with the European Union legislation for the  
102 protection of animals used for scientific purposes (Directive 2010/63/EU). Experimental  
103 procedures were approved by the French Ministry of Sciences and Higher Education under the  
104 number APAFIS#8773-2016111615105111 v2.

105

### 106 **2.2 Bacterial isolates**

107 *Leptospira* strains used in this study were isolated from the kidneys of animals trapped in the  
108 field. *Leptospira interrogans* was isolated on Reunion Island from *Rattus rattus* (2013RR  
109 GLM983) (Guernier et al., 2016), *L. mayottensis* on Mayotte from *Tenrec ecaudatus* (2014TE  
110 MDI222) (Lagadec et al., 2016) and *L. borgpetersenii* on Madagascar from *Triaenops menamena*  
111 (2014TM FMNH228863), an insectivorous bat endemic to Madagascar (Lebarbenchon et al.,  
112 2017). Leptospire were grown at 28°C for 14 to 70 days in Ellinghausen-McCullough-Johnson-  
113 Harris (EMJH) liquid medium (Difco, Detroit, MI, USA) supplemented with albumin fatty acid  
114 supplement (AFAS) and 5-fluorouracil (5-Fu) as previously described (Biscornet et al., 2017;  
115 Lagadec et al., 2016). Of note, all used isolates were passaged less than ten times *in vitro* before  
116 restauration of virulence.

117

### 118 **2.3 Restauration of virulence**

119 As the virulence of pathogenic *Leptospira* tends to lower during *in vitro* passages and freeze  
120 storage (Reed et al., 2000; Samir and Wasfy, 2013), the assessment of virulence requires a  
121 restauration step in hamsters by serial infections (Haake, 2006). For this, two serial passages in  
122 hamsters were performed and the resulting isolates were subsequently used for experimental  
123 infection. Cultures were centrifuged at 9,000 rpm for 30 minutes, pellets resuspended in 500 µl  
124 sterile phosphate buffer saline (PBS) 1X and bacterial cells counted using an improved Neubauer  
125 cell counting chamber (Marienfeld Superior, Germany). Animals were inoculated  
126 intraperitoneally (i.p.) with  $1.5 - 3 \times 10^8$  leptospire in 500 µl sterile PBS 1X and sacrificed 3 to 7  
127 days post-infection (dpi). Urine and kidney samples were used to inoculate EMJH liquid medium  
128 supplemented with AFAS and 200 µg/ml 5-Fu as previously detailed  
129 (<http://dx.doi.org/10.17504/protocols.io.ifccbiw>), followed by a subculture in the same fresh  
130 medium without 5-Fu. Each strain was subcultured less than 5 times *in vitro* in order to maintain  
131 the virulence of leptospire used for experimental infection (Reed et al., 2000).

132

### 133 **2.4 Infection and sample collection**

134 Hamsters were inoculated i.p. with  $2 \times 10^8$  low-passaged *L. interrogans* (n=9 animals), *L.*  
135 *mayottensis* (n=7) or *L. borgpetersenii* (n=7) resuspended in 500 µl sterile PBS 1X. Control

136 animals (n=7) were injected i.p. with 500 µl sterile PBS 1X. Animals were monitored for four  
137 weeks. Urine samples were collected from each hamster once a day and subsequently frozen at -  
138 80°C. After four weeks or earlier when moribund, hamsters were anesthetized with 5 mg/kg  
139 xylazine and 25 mg/kg ketamine injected i.p. and subsequently euthanized by cardiac puncture.  
140 The lungs, liver, spleen, kidneys and brain were collected and processed for further experiments  
141 or immediately frozen at -80°C.

142

## 143 **2.5 Bacterial load measurement**

144 Up to 25 mg of frozen kidney, lung, liver, brain, and up to 10 mg of frozen spleen tissue were  
145 collected and lysed in ATL buffer (Qiagen, Germany) for at least 3 hours. DNA was extracted  
146 from frozen organs and from up to 100 µl frozen urine samples using the DNEasy Blood &  
147 Tissue kit (Qiagen, Germany). DNA amounts were quantified using a probe-specific real-time  
148 PCR (Smythe et al., 2002) and a Quantinova probe PCR mix (Qiagen, Germany). Each sample  
149 was triplicated and considered positive if at least two out of the three triplicates led to positive  
150 PCR with Ct < 45. For organ samples, results are expressed as mean genome copies per  
151 milligram of tissue ± standard error of mean (SEM), determined using data of all hamsters within  
152 a group. DNA amounts in urine were determined for each animal and are reported as genome  
153 copies per microliter of urine. The number of genome copies was calculated considering the  
154 genome size of *L. interrogans* strain Fiocruz L1-130 (Nascimento et al., 2004).

155

## 156 **2.6 Viability of leptospire assay**

157 A portion of fresh liver, kidney or brain was used to inoculate fresh EMJH medium as previously  
158 detailed (<http://dx.doi.org/10.17504/protocols.io.ifccbiw>). Cultures were observed once a week  
159 under a dark-field microscope (Axio Lab.A1, Zeiss France) and considered negative if no  
160 leptospire were visible after four months.

161

## 162 **2.7 Histological studies**

163 A portion of fresh lung and one whole kidney were fixed in 4% paraformaldehyde for 24-48h,  
164 dehydrated in five successive ethanol baths (70%, 90%, 95%, 2 × 100%, 1 hour each) and two  
165 successive xylene baths, 1 hour each. Dehydrated organs were incubated in 2 successive melted  
166 paraffin baths for 1 hour and overnight, respectively, and eventually embedded in paraffin. Four-  
167 to six-µm-thick paraffin sections of kidney were deparaffinized in three successive xylene baths,  
168 10 minutes each, and rehydrated in three successive ethanol baths (100%, 70% and 50%, 2  
169 minutes each) and one bath of distilled water, 2 minutes. Rehydrated sections were stained with  
170 either periodic acid Schiff or Sirius Red and sections of lung were stained with haematoxylin and  
171 eosin. Stained sections were observed with a digital slide scanner (NanoZoomer S60, Hamamatsu  
172 France).

173

## 174 **2.8 Statistical analysis**

175 Log-rank test was used to compare survival curves of the different groups of hamsters. Non-  
176 parametric Kruskal-Wallis followed by non-parametric Dunn's multiple comparison tests were  
177 used to compare mean body weight percentage and *Leptospira* DNA amounts in organs of  
178 hamsters. Statistical analyses were conducted using RStudio 1.0.153 (RStudio Team, 2015).

179

## 180 3 Results

### 181 3.1 Body weight monitoring, clinical signs and survival analysis

182 Groups of hamsters were inoculated i.p. with  $2 \times 10^8$  bacteria and subsequently monitored for  
183 four weeks. None of the hamsters infected with *L. borgpetersenii* and *L. mayottensis* exhibited  
184 any clinical sign and all challenged animals survived throughout the whole experiment with a  
185 mean weight increase of 24% and 22%, respectively, comparable with the weight increase  
186 measured on control hamsters (27%) (Fig 1A). By contrast, the survival rate of hamsters infected  
187 with *L. interrogans* dropped drastically from seven days after the infection compared to hamsters  
188 infected with *L. mayottensis* and *L. borgpetersenii* ( $p=0.0019$ ) (Fig 1B): only one out of the nine  
189 *L. interrogans*-infected hamsters actually survived during the four weeks of experiment without  
190 any clinical sign and a weight increase of 23%. Moribund *L. interrogans*-infected hamsters  
191 showed weight loss averaging 7.08% in the first week, and reaching 16.65% between 7 and 12  
192 dpi (Fig 1A). Other clinical signs included ruffled fur, lethargy and isolation.

### 194 3.2 Dynamics of *Leptospira* urinary shedding

195 The urine of hamsters was collected once a day whenever possible in order to follow the  
196 dynamics of *Leptospira* shedding following infection (Fig 2A). *Leptospira* DNA was detected  
197 discontinuously in the urine of all *L. interrogans*-infected hamsters: high peaks of *Leptospira*  
198 DNA, ranging from 820.62 ( $\pm 159.79$ ) to 1839.19 ( $\pm 414.13$ ) genome copies per microliter of  
199 urine, were detected in the urine of five out of nine hamsters as early as 5 dpi. Among these  
200 animals, the single hamster surviving *L. interrogans* infection excreted high amounts of  
201 *Leptospira* DNA throughout the four weeks of infection. The other four *L. interrogans*-infected  
202 animals showed limited leptospiral excretion during the whole experiment ( $<80$  *Leptospira*  
203 genome copies/ $\mu$ l). By contrast, hamsters infected with *L. borgpetersenii* and *L. mayottensis*  
204 isolates shed little or no *Leptospira* DNA in their urine (up to 2.54 and 35.72 genome copies/ $\mu$ l,  
205 respectively).

### 207 3.3 Quantification of *Leptospira* DNA in organs

208 *Leptospira* DNA was quantified in brain, kidney, liver, lung and spleen tissues of euthanized  
209 animals. As shown on Table 1, highest DNA amounts were obtained in kidney tissues, the  
210 highest amount being detected in the single hamster surviving *L. interrogans* infection ( $1.7 \times 10^6$   
211  $\pm 2.9 \times 10^5$  genome copies/mg). *Leptospira* DNA amounts were higher in *L. interrogans*-  
212 infected animals for all tested tissues: DNA amount was increased by over 10 fold in the kidneys  
213 and over 100 fold in the brain, lungs and spleen tissues of animals infected with *L. interrogans* as  
214 compared to those infected with *L. mayottensis* or *L. borgpetersenii* (Fig 2B). *Leptospira* DNA  
215 was detected in the liver of *L. interrogans*-infected animals only. All kidney tissues from *L.*  
216 *interrogans*-infected hamsters tested positive through PCR, while only three out of seven *L.*  
217 *mayottensis*- and none of the *L. borgpetersenii*-infected animals tested positive using DNA from  
218 their kidney tissues (Table 1). DNA was detected at low levels ( $< 40$  genome copies/mg) in the  
219 spleen and brain of hamster infected with either *L. mayottensis* or *L. borgpetersenii* (Fig 2B).

### 221 3.4 Viability of leptospire

222 Considering previous detection of high levels of *Leptospira* DNA in the kidneys and liver of  
223 infected hamsters (Coutinho et al., 2014; Wunder et al., 2016), possible brain damage induced by

224 the disease (Chang et al., 2003; Panicker et al., 2001), and cultures constraints, we selected these  
225 three organs for detection of live leptospires through culture (Table 1). Live leptospires were  
226 obtained from almost all kidney and brain homogenate cultures and in one third of liver  
227 homogenate cultures from *L. interrogans*-infected hamsters. Intriguingly, no leptospires were  
228 seen in any tissue culture prepared from the single surviving *L. interrogans*-infected hamster.  
229 Organs from *L. mayottensis*-infected hamsters led to lower isolation success with no positive  
230 brain or liver homogenate cultures and only 2 out of 7 positive kidney homogenate cultures.  
231 Although lost after one passage *in vitro*, live leptospires were obtained from animals infected  
232 with *L. borgpetersenii* mainly using brain tissue and to a lesser extent using kidney tissue (Table  
233 1).

234

### 235 3.5 Differential tissue damages revealed by histological monitoring

236 The renal tropism of pathogenic *Leptospira* and the pulmonary involvement in most severe  
237 human forms of leptospirosis (Vijayachari et al., 2008) led us to consider kidneys and lungs for  
238 histological studies. Gross external examination of organs showed important lesions in the  
239 kidneys of hamsters infected with *L. interrogans* (Fig 3, see lower right inserts). Lungs of *L.*  
240 *interrogans*- and *L. mayottensis*-infected hamsters harbored some lesions while the lungs of *L.*  
241 *borgpetersenii*-infected hamsters showed a normal aspect, indistinguishable from control lungs  
242 (data not shown).

243

244 Microscopic sections of kidneys from control hamsters and from hamsters infected with *L.*  
245 *borgpetersenii* stained with periodic acid Schiff showed glomeruli and tubules of regular shape  
246 four weeks after infection. Slightly dilated tubules and some hyaline casts were visible in kidney  
247 sections from *L. mayottensis*-infected hamsters. Kidneys from hamsters infected with *L.*  
248 *interrogans* showed retracted glomeruli with large urinary space, focal infiltrates of inflammatory  
249 cells mainly around vessels and very important tubular damages characterized by tubular dilation,  
250 atrophy and hyaline casts (Fig 3). Sirius red staining further highlighted interstitial fibrosis in  
251 kidneys from these animals (Fig 4).

252

253 Lung sections stained with hematoxylin and eosin revealed a mild to moderate thickening of  
254 alveolar septa in the lungs of hamsters infected with *L. interrogans* and *L. borgpetersenii*, at the  
255 time of death and after four weeks of infection, respectively. Dilated alveoli were also observed  
256 in the lungs of *L. interrogans*-infected hamsters. By contrast, lungs of control and *L. mayottensis*-  
257 infected hamsters euthanized after four weeks showed a regular structure (Fig 5).

258

259 Given that no clinical sign was observed in the first week of infection in hamsters infected with  
260 either *L. mayottensis* or *L. borgpetersenii*, we chose to sacrifice only one hamster from each  
261 group at two different intermediate timepoints, 10 and 17 (*L. mayottensis* group) or 18 (*L.*  
262 *borgpetersenii* group) dpi, in order to explore possible internal damages. At 10 dpi, the hamster  
263 infected with *L. mayottensis* harbored several petechial hemorrhages in the lungs and histological  
264 analysis revealed focal areas of hemorrhage and thickening of alveolar septa. At 17 dpi, no  
265 macroscopic lesion but thickening of alveolar septa were observed in the lungs; no area of  
266 hemorrhage was observed (Fig 6). Lungs of *L. borgpetersenii*-infected hamsters sacrificed at 18  
267 dpi harbored the same features as at the end of the experiment (data not shown).

268

#### 269 4 Discussion

270 Western Indian Ocean islands harbor a large diversity of pathogenic *Leptospira* lineages carried  
271 out by a wide range of animal reservoirs (Dietrich et al., 2018). In this study, we selected three  
272 regional *Leptospira* strains and evaluated their virulence phenotype using golden Syrian hamsters  
273 as a model of acute disease. The obtained data show that *L. mayottensis* 2014TE MDI222 and *L.*  
274 *borgpetersenii* 2014TM FMNH228863 isolates endemic to Madagascar and surrounding islands  
275 are both less virulent than *L. interrogans* 2013RR GLM983 isolate from Reunion. All but one  
276 hamster infected with *L. interrogans* isolate showed significant weight loss and had to be  
277 euthanized a few days after the challenge, in keeping with previous experimental infections  
278 (Forster et al., 2013; Villanueva et al., 2014). It was either difficult or impossible to collect urine  
279 from these hamsters, likely as a result of kidney failure, which was further confirmed by  
280 histological examinations that showed tissue damages. Unexpectedly, no areas of intensive  
281 hemorrhage were observed in the lungs of challenged animals, which differs from previous  
282 studies reporting foci of intensive hemorrhage in *L. interrogans*-infected hamsters (Nally et al.,  
283 2004; Villanueva et al., 2014). The absence of profuse pulmonary lesions could result from the  
284 high infectious dose used in our experiments: the hamsters could have died before the infection  
285 invaded the lungs (Diniz et al., 2011).

286  
287 By contrast, *L. mayottensis* and *L. borgpetersenii* isolates failed to induce clinical symptoms on  
288 infected hamsters. Intriguingly, necropsy at four weeks revealed macroscopic pulmonary lesions  
289 in some *L. mayottensis*-hamsters but not in *L. borgpetersenii*-hamsters although histological  
290 analysis revealed a thickening of alveolar septa in these latter. The absence of early symptoms in  
291 these two groups led us to euthanize animals at intermediate time points in order to address  
292 possible tissue damage in the course of the experiment. No damage was observed at 10 and 18  
293 dpi in *L. borgpetersenii*-infected hamsters. *Leptospira mayottensis*-infected hamsters showed  
294 pulmonary damage at 10 dpi but not at 17 dpi. Histological findings confirmed these observations  
295 with the presence of focal hemorrhage and alveolar septal thickening at 10 dpi and only alveolar  
296 septal thickening at 17 dpi. This pattern is suggestive of an attempt of lung repair, a phenomenon  
297 previously described in hamsters experimentally infected with *L. interrogans* (Marinho et al.,  
298 2009). The confirmation of such pattern will require the implementation of an experimental setup  
299 allowing to monitor the dynamics of infection and tissue lesions induced by *L. mayottensis*.

300  
301 Leptospiral tissue tropism following experimental infection differed among bacterial isolates. The  
302 high *L. interrogans* DNA amounts and the presence of live leptospires in the brain and kidneys of  
303 infected hamsters suggest that these organs are preferential targets of *L. interrogans*. Retracted  
304 glomeruli, dilated tubules and an abundance of hyaline casts in the kidneys have commonly been  
305 observed in *L. interrogans* infection (Arean, 1962; Forster et al., 2013; Zhang et al., 2012).  
306 Although the induction of nervous system damage by *L. interrogans* has been previously reported  
307 in human cases (Brown et al., 2003; Chang et al., 2003; El Bouazzaoui et al., 2011; Forwell et al.,  
308 1984), experimental infection studies mainly report damage and high leptospiral load in the  
309 kidneys, lungs and liver only (Fujita et al., 2015; Lourdault et al., 2009; Silva et al., 2008). Four  
310 weeks following infection, *L. mayottensis* was present only in the kidneys as indicated by high  
311 leptospiral DNA amounts and the presence of live leptospires. However, at 10 dpi the presence of  
312 live leptospires in cultures inoculated with kidney, brain and liver homogenates indicates that *L.*  
313 *mayottensis* could actually disseminate into several organs in the early phases of infection.

314

315 *Leptospira borgpetersenii* isolate was found to be the least virulent strain used in this study. This  
316 isolate induced no clinical sign and very low leptospiral DNA amounts were detected in the  
317 different organs. Leptospire were observed in some cultures from brain and kidney homogenates  
318 albeit with low density and with bacteria being lost after one passage *in vitro*. The low *Leptospira*  
319 DNA amounts and the absence of cultivable leptospire in the organs from hamsters sacrificed at  
320 intermediate timepoints are suggestive of poor dissemination although observation of alveolar  
321 thickening indicates that the lungs can be affected by this *Leptospira* lineage. This differs from  
322 infection patterns observed with other *L. borgpetersenii* strains inducing important systemic  
323 damage and death in hamsters (Matsui et al., 2015; Villanueva et al., 2014). A previous study  
324 comparing the virulence of two *L. borgpetersenii* strains, both serovar Hardjo, reported that the  
325 avirulent strain was able to efficiently colonize the kidneys of hamsters even at a lower dose  
326 (Zuerner et al., 2012). Hence, the bat-borne *Leptospira* used herein clearly behaves differently.  
327 Although genotyping defines this isolate as *L. borgpetersenii*, Malagasy bats shelter a huge  
328 diversity of unique *L. borgpetersenii* lineages (Gomard et al., 2016) and a complete genome  
329 sequencing of this particular isolate is required to determine its exact phylogenetic position.  
330

331 We can hypothesize that these distinct virulence phenotypes may partly explain the contrasted  
332 epidemiology of human leptospirosis in Western Indian Ocean region. Indeed, MLST profile of *L.*  
333 *interrogans* 2013RR GLM983 isolate, found in other countries worldwide, is the same as that of  
334 *L. interrogans* identified in most human leptospirosis acute cases on Reunion while *L.*  
335 *mayottensis* 2014TE MDI222 isolate, considered as endemic to Western Indian Ocean, has the  
336 same MLST profile as *L. mayottensis* identified in clinical cases on Mayotte. By contrast, *L.*  
337 *borgpetersenii* 2014TM FMNH228863 isolate, which induced no clinical sign in hamsters, has  
338 not yet been identified in human cases of leptospirosis. On Reunion, a narrow diversity of mainly  
339 *L. interrogans* is involved in clinical cases while a wider diversity of *Leptospira*, distributed in  
340 four distinct species, is identified in clinical cases on Mayotte. On Reunion, mostly introduced  
341 mammals like rats, mice or dogs, were identified as *Leptospira* carriers (Guernier et al., 2016)  
342 while on Mayotte endemic small mammals like tenrecs may also have epidemiological  
343 importance in human leptospirosis (Lagadec et al., 2016). The French islands of Mayotte and  
344 Reunion hence provide an interesting environmental setup, as transmission chains are distinct on  
345 both islands and associated with human infection of contrasting severity. The understanding of  
346 such epidemiological contrast could be addressed through a prospective study to be conducted on  
347 Mayotte in which the symptoms of each human case are described together with each associated  
348 *Leptospira* lineage/species. This will allow addressing whether one species or lineage is  
349 responsible for most of the fatal cases, and testing whether the remaining endemic lineages may  
350 be associated with milder symptoms.  
351

352 In conclusion, our data suggest that the *L. interrogans* 2013RR GLM983 isolate is significantly  
353 more virulent than both *L. mayottensis* 2014TE MDI222 and *L. borgpetersenii* 2014TM  
354 FMNH228863 isolates, endemic to Western Indian Ocean. Golden Syrian hamsters provide a  
355 good experimental model for exploring virulence features of *Leptospira* strains found in Western  
356 Indian Ocean and the contrasted epidemiology of leptospirosis observed in the islands of this  
357 region. The determinants of virulence are poorly known in *Leptospira* (Buyuktimkin and Jolla,  
358 2015; Fouts et al., 2016; Lehmann et al., 2014; Patra et al., 2015), notably because genetic tools  
359 are scarce hence impairing reverse genetics approaches and because the basal position of  
360 spirochetes is associated with a high number of proteins of unknown function. The unique  
361 diversity of pathogenic *Leptospira* sheltered by mammals mostly endemic to Western Indian

362 Ocean region provides a biological material that is suitable for the investigation of virulence  
363 determinants through genome-wide approaches such as comparative genomics.

364

365 **Conflict of interests.**

366 Authors certify no conflict of interests.

367

368 **Author contributions**

369 Conceived and designed the experiments: CC, KD, PT, MR, PM. Performed the experiments: CC,  
370 MR, PT, MT, JB. Contributed resources/reagents/materials/analysis tools: CC, PT, PM, MR, JB.

371 Wrote the paper: CC. Revised the paper: all co-authors

372

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381

381  
382

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540

540 **Tables**

541 **Table 1. Rate of *Leptospira* positive samples using qPCR or culture on organs of experimentally infected hamsters.**

	Positive qPCRs/Tested animals (%)					Positive cultures/Tested animals (%)		
	Brain	Kidney	Liver	Lung	Spleen	Brain	Kidney	Liver
<i>L. interrogans</i>	8/9 (88.9)	9/9 (100.0)	4/9 (44.4)	3/9 (33.3)	7/8 (87.5)*	8/9 (88.9)	8/9 (88.9)	3/9 (33.3)
<i>L. mayottensis</i>	2/7 (28.6)	4/7 (57.1)	0/7 (0.0)	1/7 (14.3)	1/7 (14.3)	0/7 (0.0)	2/7 (28.6)	0/7 (0.0)
<i>L. borgpetersenii</i>	3/7 (42.9)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	1/7 (14.3)	5/7 (71.4) <sup>#</sup>	2/7 (28.6) <sup>#</sup>	0/7 (0.0)

542 \*Data missing for one hamster

543 <sup>#</sup> The isolates were lost after one passage *in vitro*

544

545 Data indicate number of positive qPCRs and cultures at the time of death for *L. interrogans*-infected animals and at four weeks post-  
 546 infection for *L. mayottensis*- and *L. borgpetersenii*-infected hamsters.

547 **FIGURE LEGENDS**

548 **Figure 1. Hamsters monitoring.**

549 (A) Growth curves of control hamsters (black, n=7) and hamsters infected with *L. interrogans*  
550 (red, n=8), *L. mayottensis* (green, n=7) or *L. borgpetersenii* (blue, n=7). Results are expressed as  
551 means ± SEM. Asterisks (\*) indicate significant differences in body weight percentage between  
552 hamsters infected by *L. interrogans* and control hamsters. Plus signs (+) indicate significant  
553 differences in body weight percentage between hamsters infected with *L. interrogans* and two  
554 other groups of hamsters: control and *L. borgpetersenii*-infected hamsters. The hamster surviving  
555 the infection with *L. interrogans* was considered as an outlier and was not included in the growth  
556 curve data. (B) Survival rates of hamsters infected with *L. interrogans* (red, n=9), *L. mayottensis*  
557 (green, n=7) or *L. borgpetersenii* (blue, n=7).

558  
559 **Figure 2. Quantification of *Leptospira* DNA in urine and organs**

560 (A) Pathogenic *Leptospira* urinary shedding in hamsters infected with *L. interrogans* (red, n=9),  
561 *L. mayottensis* (green, n=7) or *L. borgpetersenii* (blue, n=7). Individual data are presented for  
562 each hamster. (B) Hamsters infected with *L. interrogans* are represented in red (n=8), *L.*  
563 *mayottensis* in green (n=7) and *L. borgpetersenii* in blue (n=7). DNA amounts in organs were  
564 calculated at the time of death for *L. interrogans*-infected hamsters and after four weeks of  
565 experiment for *L. mayottensis*- and *L. borgpetersenii*-infected hamsters. Results are expressed as  
566 means ± SEM. Differences in DNA amount were tested with non-parametric Kruskal-Wallis test  
567 followed by Dunn's multiple comparison test. After Dunn's test a p-value below 0.025 indicated  
568 a significant difference; \*\* p < 0.01; \*\*\* p < 0.001. The hamster surviving *L. interrogans*  
569 infection was considered as an outlier and was not included in the DNA amounts data.

570  
571 **Figure 3. Kidney structure from control and infected hamsters.**

572 Microscopic observations of kidney sections stained with periodic acid Schiff obtained from  
573 control animals (A) and animals infected with *L. interrogans* (B), *L. mayottensis* (C) or *L.*  
574 *borgpetersenii* (D) four weeks after infection. Atrophic tubules (arrow), hyaline casts (+) and  
575 cellular infiltrates (\*) are indicated. Scale bars: 150 µm. Macroscopic observations of kidneys are  
576 shown in inserts.

577  
578 **Figure 4. *Leptospira interrogans*-induced interstitial fibrosis.**

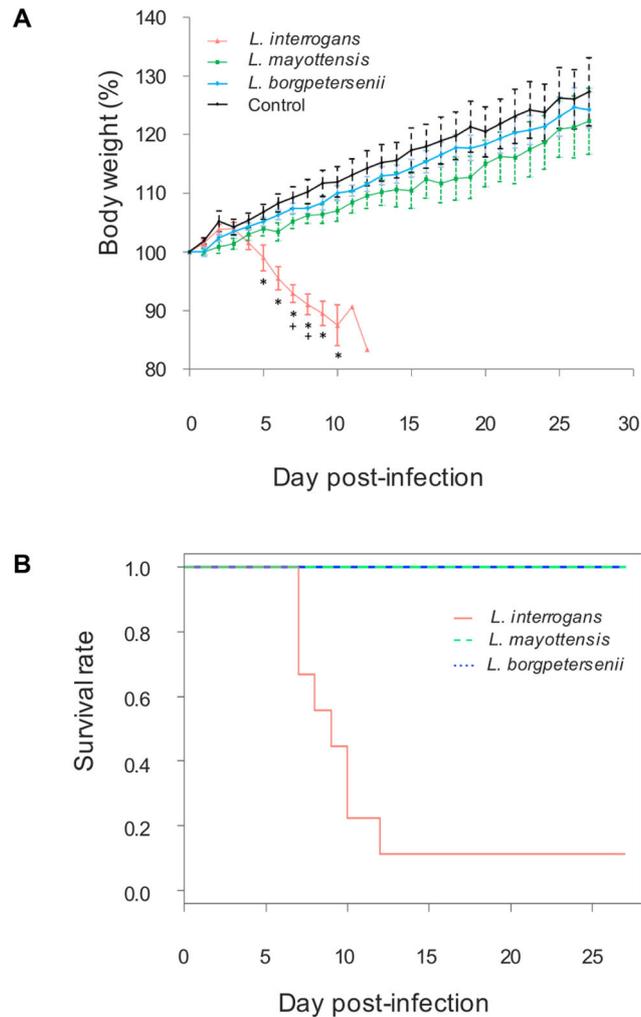
579 Microscopic observations of kidney sections stained with Sirius red obtained from control  
580 animals (A) and animals infected with *L. interrogans* (B), *L. mayottensis* (C) or *L. borgpetersenii*  
581 (D) four weeks after infection. Arrows indicate areas of interstitial fibrosis are indicated. Scale  
582 bars: 150 µm.

583  
584 **Figure 5. Lung structure from control and infected hamsters.**

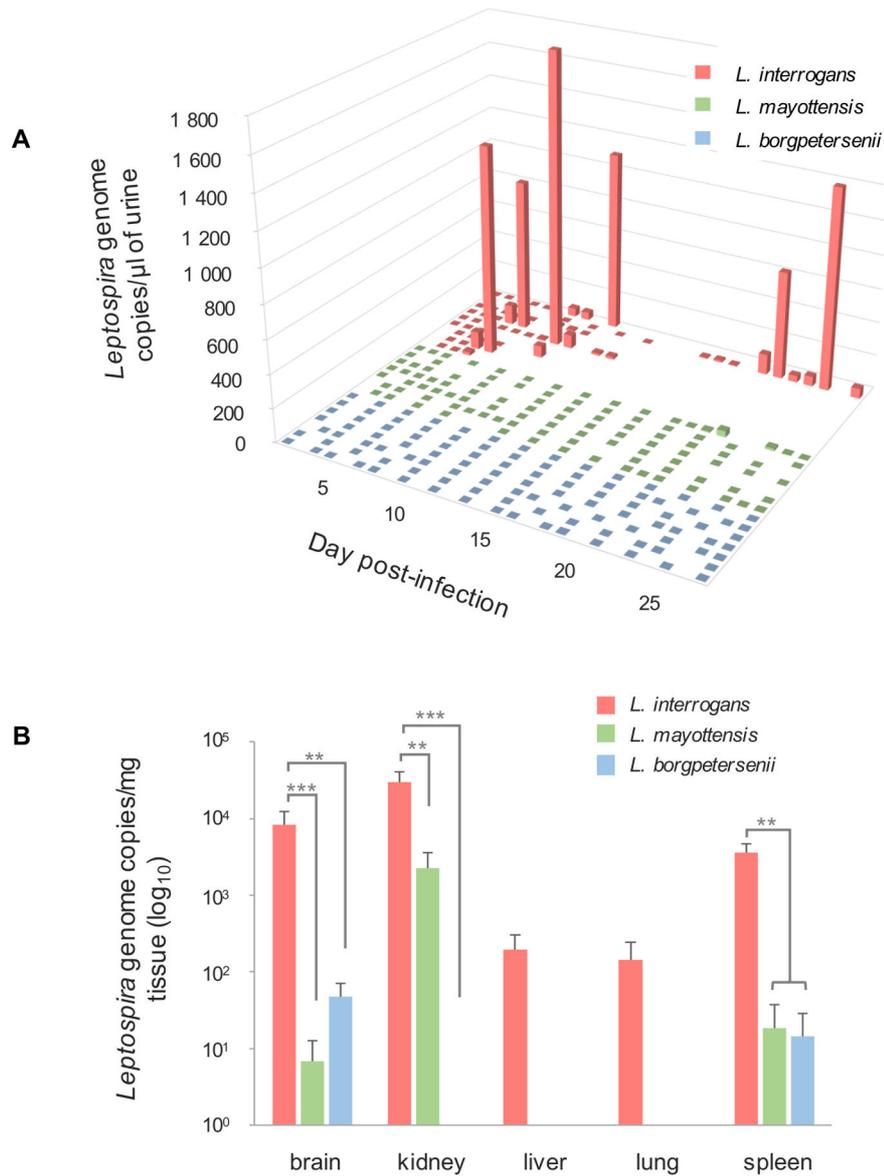
585 Microscopic observations of lung sections stained with hematoxylin and eosin obtained from  
586 control animals (A) and animals infected with *L. interrogans* (B), *L. mayottensis* (C) or *L.*  
587 *borgpetersenii* (D) four weeks after infection. Scale bars = 150 µm.

588  
589 **Figure 6. Lung structure from *L. mayottensis*-infected hamsters sacrificed at intermediate  
590 timepoints.**

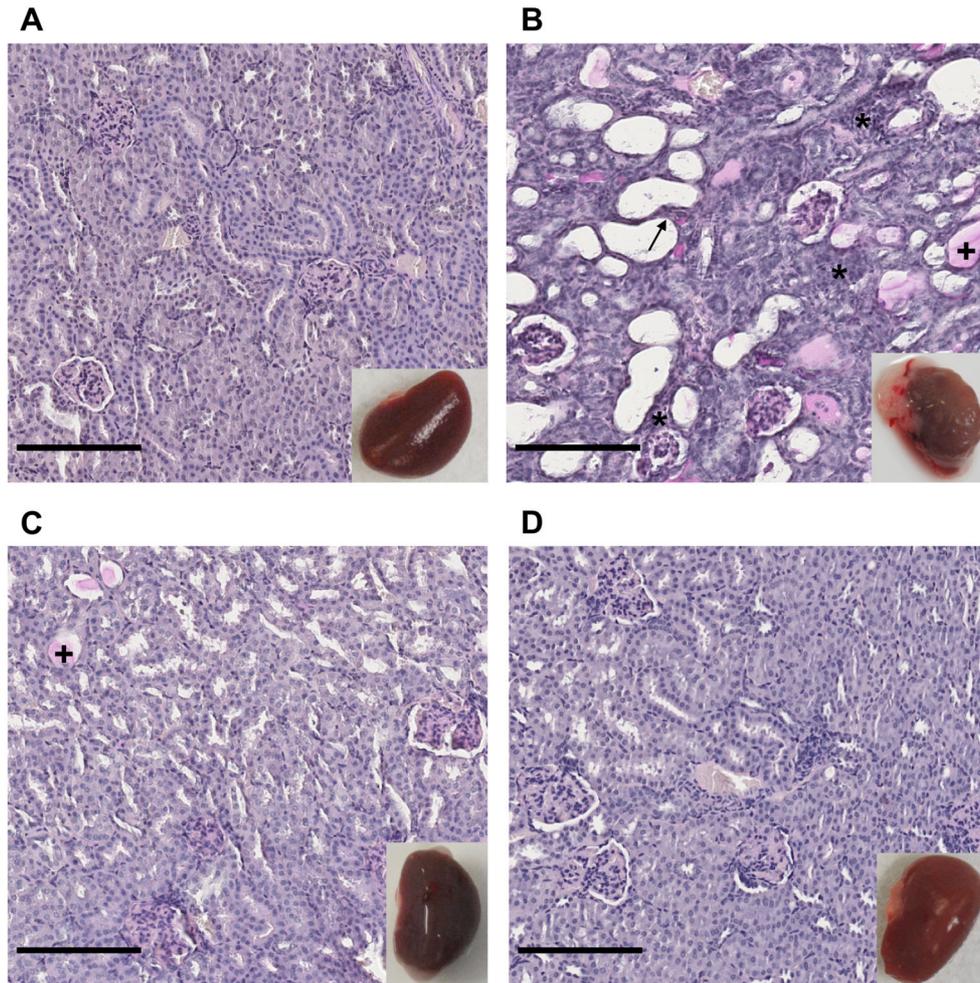
591 Macroscopic examination of lungs (A, B) and lung sections stained with hematoxylin and eosin  
592 (C, D) from *L. mayottensis*-infected hamsters sacrificed at 10 (A, C) or 17 dpi (B, D). Scale bars  
593 = 150 µm.



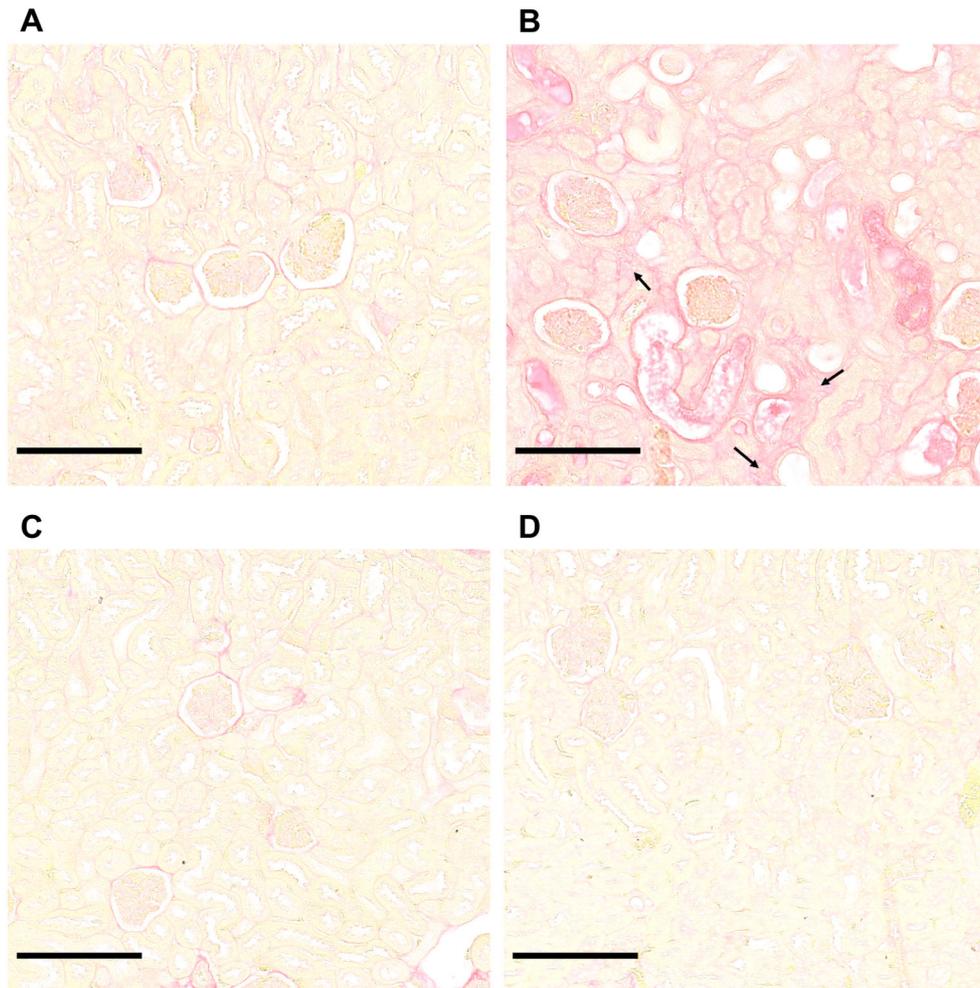
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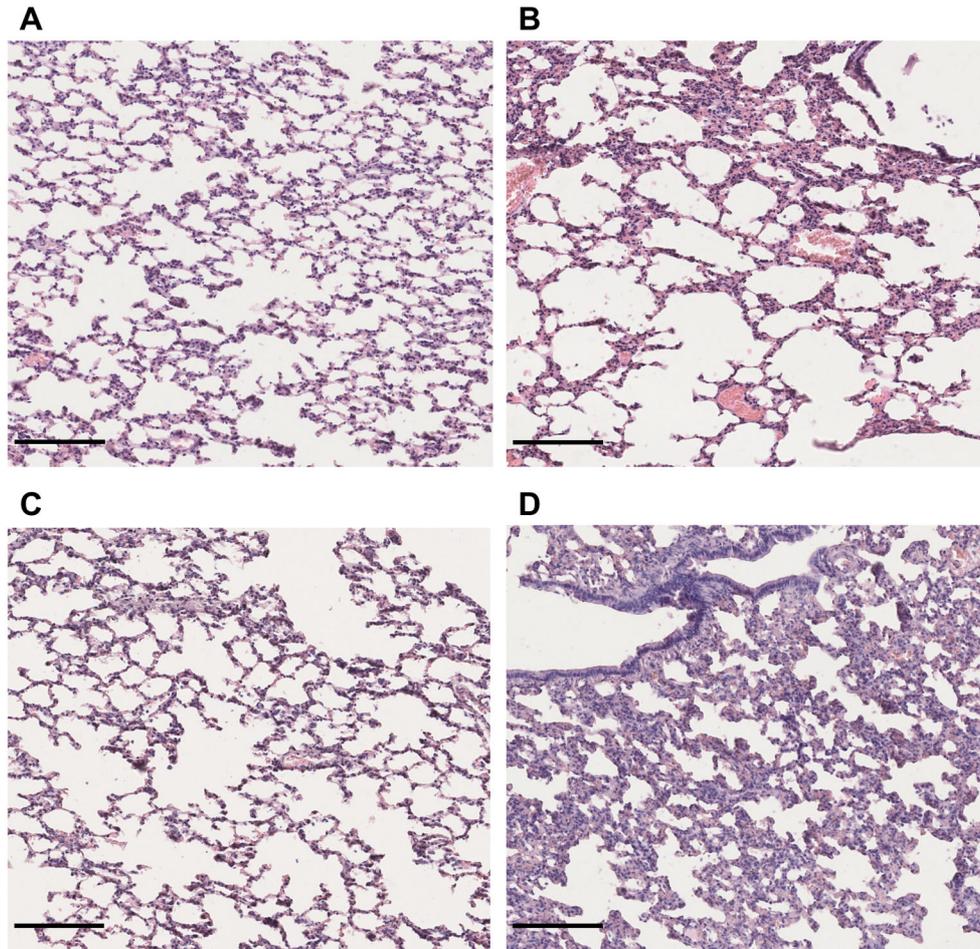
**Figure 2. Quantification of *Leptospira* DNA in urine and organs.** (A) Pathogenic *Leptospira* urinary shedding in hamsters infected with *L. interrogans* (red, n=9), *L. mayottensis* (green, n=7) or *L. borgpetersenii* (blue, n=7). Individual data are presented for each hamster. (B) Hamsters infected with *L. interrogans* are represented in red (n=8), *L. mayottensis* in green (n=7) and *L. borgpetersenii* in blue (n=7). DNA amounts in organs were calculated at the time of death for *L. interrogans*-infected hamsters and after four weeks of experiment for *L. mayottensis*- and *L. borgpetersenii*-infected hamsters. Results are expressed as means  $\pm$  SEM. Differences in DNA amount were tested with non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test. After Dunn's test a p-value below 0.025 indicated a significant difference; \*\* p < 0.01; \*\*\* p < 0.001. The hamster surviving *L. interrogans* infection was considered as an outlier and was not included in the DNA amounts data.



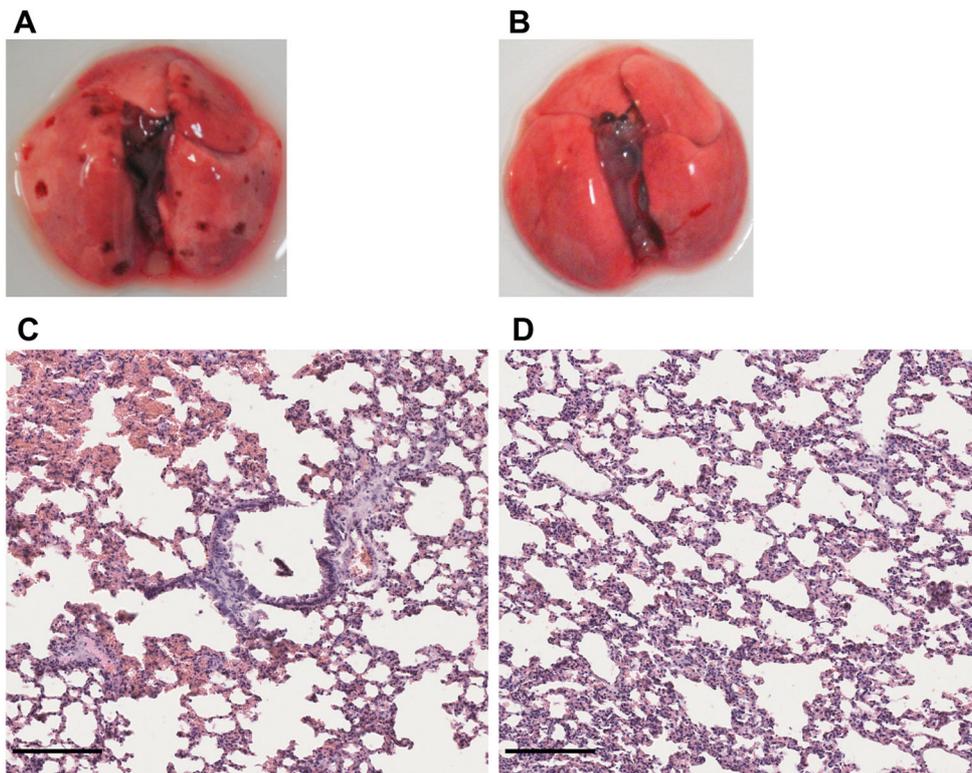
**Figure 3. Kidney structure from control and infected hamsters.** Microscopic observations of kidney sections stained with periodic acid Schiff obtained from control animals (A) and animals infected with *L. interrogans* (B), *L. mayottensis* (C) or *L. borgpetersenii* (D) four weeks after infection. Atrophic tubules (arrow), hyaline casts (+) and cellular infiltrates (\*) are indicated. Scale bars: 150 µm. Macroscopic observations of kidneys are shown in inserts.



**Figure 4. *Leptospira interrogans*-induced interstitial fibrosis.** Microscopic observations of kidney sections stained with Sirius red obtained from control animals (A) and animals infected with *L. interrogans* (B), *L. mayottensis* (C) or *L. borgpetersenii* (D) four weeks after infection. Arrows indicate areas of interstitial fibrosis are indicated. Scale bars: 150  $\mu\text{m}$ .



**Figure 5. Lung structure from control and infected hamsters.** Microscopic observations of lung sections stained with hematoxylin and eosin obtained from control animals (A) and animals infected with *L. interrogans* (B), *L. mayottensis* (C) or *L. borgpetersenii* (D) four weeks after infection. Scale bars = 150 μm.



**Figure 6. Lung structure from *L. mayottensis*-infected hamsters sacrificed at intermediate timepoints.** Macroscopic examination of lungs (A, B) and lung sections stained with hematoxylin and eosin (C, D) from *L. mayottensis*-infected hamsters sacrificed at 10 (A, C) or 17 dpi (B, D). Scale bars = 150  $\mu\text{m}$ .

#### IV. Main results

This study reveals differences in the virulence of three *Leptospira* isolates from western Indian Ocean, all belonging to the pathogenic group. Clearly, the *L. interrogans* isolate was the most virulent, causing the death of all but one infected hamsters together with significant tissue damage. All hamsters challenged with either the *L. mayottensis* or the *L. borgpetersenii* isolates survived the infection and exhibited no clinical signs of disease. However macroscopic observations and histological data obtained from necropsies performed at intermediate time points indicate that the *L. mayottensis* isolate induced more damage, and hence may be more pathogenic than the *L. borgpetersenii* isolate. The *L. mayottensis* isolate being identical to an isolate from human clinical cases, these results provides a first potential explanation for the differential epidemiology of leptospirosis among the western Indian Ocean islands. More generally, endemic *Leptospira* lineages may display attenuated virulence as compared to cosmopolitan/introduced lineages such as the *L. interrogans* isolate used herein, which causes severe disease among humans, including death.

## CHAPTER 2

# Host specificity of pathogenic *Leptospira* investigated through experimental infection

## I. Structure of *Leptospira* lineages in the SWIO region

Leptospire are highly diverse on the western Indian Ocean islands. This diversity is composed of *Leptospira* for which genotypes have been identified worldwide together with *Leptospira* with genetic profiles that are, as yet, original and unique to the Malagasy region. In this region, the genus *Leptospira* is associated with a wide diversity of mammalian reservoir hosts. Hosts are generally mammalian and can be either introduced or regionally endemic mammals. Introduced mammals include domestic animals (dogs, cats), livestock (cattle, pigs) and wild or synanthropic mammals (e.g. *Rattus rattus*, *Mus musculus*, *Suncus murinus*). Endemic mammalian hosts include the Tenrecidae (morphologically diverse small mammals), Nesomyinae (endemic rodents) and several families of Chiroptera (bats).

A previous study conducted in Madagascar, which shelters a high diversity of mostly endemic bats, has highlighted the association of original *Leptospira* lineages, mostly related to *L. borgpetersenii*, with specific bat families, suggesting a strong pattern of host specificity (Gomard et al., 2016). Moreover, *L. mayottensis* seem to be associated with the Tenrecidae family (Dietrich et al., 2018; Lagadec et al., 2016). By contrast, globally-distributed leptospire do not seem to be host-specific. For instance, *L. interrogans* strains reported on La Réunion are hosted by two distinct introduced mammalian reservoirs, namely rats and domestic dogs (Guernier et al., 2016).

Does the host-specificity pattern observed for endemic *Leptospira* result from genetic traits (such as the adaptation of specific reservoir molecules) or ecological features (such as a lack of contact between different reservoir host species)? The aim of this chapter is to shed light on this problematic.

We addressed this host specificity pattern experimentally by forcing the infection of a renown reservoir of leptospirosis, *Rattus norvegicus*, with the *Leptospira* isolates used in the previous chapter and isolated from different hosts (**Figure 8**). This methodology allowed us to exclude the ecological constraints and directly test the compatibility between the three different *Leptospira* isolates and a specific mammalian host. A positive control consisted of the infection of *R. norvegicus* with an isolate of *L. interrogans* obtained from *R. rattus* on La Réunion and also shed by *R. norvegicus* in the Seychelles and La Réunion (Biscornet et al., 2017; Guernier et al., 2016). The urinary shedding of the different *Leptospira* isolates was analyzed through qPCR for *Leptospira* DNA quantification, and culture for the observation of viable leptospire. The kidneys were analyzed by qPCR for the detection and quantification of *Leptospira* DNA and by histological methods to observe any modifications that could have been induced by the infection.

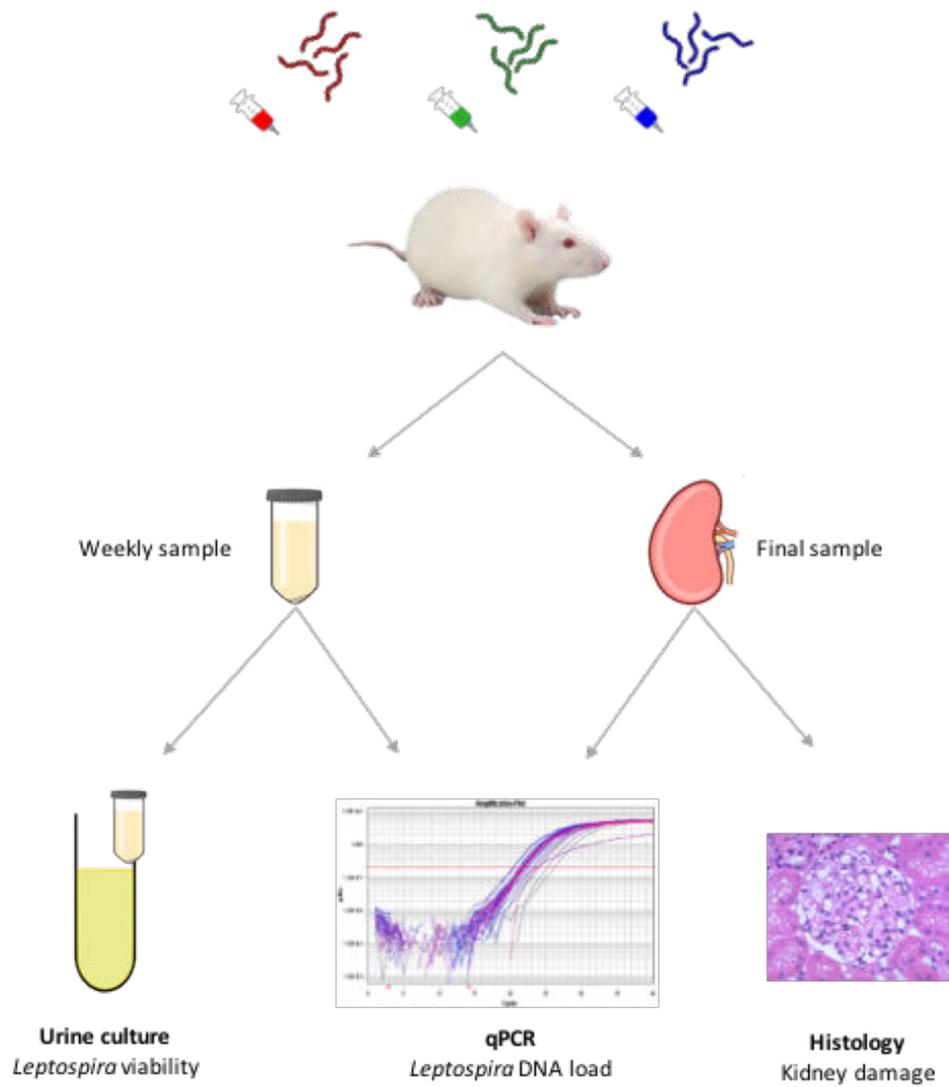


Figure 8. Experimental infection of rats to investigate the host specificity of *Leptospira* isolates

II. Article 2: Pathogenic *Leptospira* and their animal reservoirs: testing host specificity through experimental infection (in preparation)

1           **Pathogenic *Leptospira* and their animal reservoirs: testing host**  
2                           **specificity through experimental infection**

3  
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25

26 **Abstract (199 words)**

27 Leptospirosis is among the most prevalent bacterial zoonosis worldwide. The disease is caused by  
28 infection with pathogenic *Leptospira* which are maintained and shed by mammals including rodents  
29 renown as prominent reservoirs. Although these bacteria have often been considered as displaying  
30 low host specificity, investigations carried out in the leading biodiversity hotspot of Madagascar  
31 have revealed an astonishing diversity of *Leptospira* displaying tight specificity towards their  
32 endemic small volant and terrestrial mammalian hosts. In this study, we tested by experimental  
33 infection whether this apparent host specificity is caused by bacterial genetic determinants or  
34 alternatively by ecological constraints such as a lack of contacts between mammalian species  
35 harboring distinct *Leptospira*. For this, we experimentally infected *Rattus norvegicus* with isolates  
36 obtained from rat, bat or tenrec specimens, belonging to distinct species of *Leptospira*. Results  
37 show that *R. norvegicus* is not chronically infected with *Leptospira* isolated from tenrec and bat  
38 specimens while 2 out of the 7 animals challenged with *L. interrogans* isolated from a rat could  
39 experience chronic infection throughout the 3 months of experimental infection. These data suggest  
40 that the host specificity of pathogenic *Leptospira* species/lineages from this biodiversity hotspot is  
41 driven by genetic determinants likely resulting from long-term co-diversification processes.

## 42 1 Introduction

43 Leptospirosis is a bacterial zoonosis of global importance that is transmitted either by direct contact  
44 with infected animals or most commonly by indirect contact through an environment contaminated  
45 with the urine shed by infected mammalian reservoirs. Pathogenic *Leptospira* are highly diversified  
46 spirochetes currently composed of ten identified species, namely *Leptospira alexanderi*, *Leptospira*  
47 *alstoni*, *Leptospira borgpetersenii*, *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira*  
48 *kmetyi*, *Leptospira mayottensis*, *Leptospira noguchii*, *Leptospira santarosai*, and *Leptospira weilii*  
49 (Adler, 2015; Bourhy et al., 2014). However, diversity of *Leptospira* is likely overlooked (Thibeaux  
50 et al., 2018) and full genome sequencing of environmental samples will surely increase the number  
51 of recognized pathogenic species in the next future. Rodents are often considered as the main  
52 reservoirs, although recent investigations carried out in Seychelles and Reunion have shown that  
53 rats are involved in a portion of human cases only (Biscornet et al., 2017; Guernier et al., 2016).  
54 Molecular typing of *Leptospira* sheltered by rats from these two islands revealed the dominance of  
55 a single species, *L. interrogans*, represented by four Sequence Types (STs), two of them being  
56 reported in different locations worldwide (Biscornet et al., 2017; Guernier et al., 2016)□. The  
57 absence of diversity of these rat-borne leptospires together with the cosmopolitan distribution of  
58 these lineages support a recent introduction of *L. interrogans* in these geographically isolated  
59 islands, possibly associated with the introduction of rats. Beside rodents, a wide range of mammals  
60 can actually host *Leptospira* spp., as confirmed by a thorough investigation of wild mammals from  
61 southwestern Indian Ocean islands (SWIO) (Dietrich et al., 2018)□.

62 Volant and terrestrial small mammals have been investigated in Madagascar and surrounding  
63 islands where bacterial isolation and molecular investigations have uncovered a remarkable  
64 diversity of mostly original *Leptospira* lineages (Allan et al., 2018; Bourhy et al., 2010, 2012, 2014;  
65 Dietrich et al., 2014; Gomard et al., 2016; Lagadec et al., 2012, 2016). Noteworthy, and contrary  
66 to studies highlighting the lack of host specificity and a possible geographic structure among  
67 *Leptospira* spp. (Ballados-González et al., 2018; Lei and Olival, 2014)□, Malagasy *Leptospira*  
68 show a marked host specificity towards their hosts, as best exemplified by the exploration of tenrec  
69 and bat reservoirs (Dietrich et al., 2014; Gomard et al., 2016)□. Indeed, each bat family or genus  
70 shed unique *Leptospira* lineages that have co-diversified with their hosts and can hence be  
71 considered as endemic to Madagascar (Gomard et al., 2016). Similarly, *L. mayottensis* has been  
72 reported in several tenrec species, including in the neighboring island of Mayotte where this  
73 bacterial species is of public health concern (Bourhy et al., 2010, 2014; Lagadec et al., 2016)□.

74 Noteworthy, introduced rats in Mayotte are in turn infected with a handful of *Leptospira* species,  
75 suggesting that rats can shed a wider diversity of *Leptospira* than endemic mammals (Gomard et al.,  
76 2016)□.

77 However, the ability of rats to harbor endemic *Leptospira* lineages is not clear. Indeed, in 2016, a  
78 study conducted in Mayotte did not report *L. mayottensis* infection in any of the 289 screened rats  
79 (Lagadec et al., 2016)□. This leptospiral species was found only in Tenrecidae in Mayotte and  
80 Madagascar (Dietrich et al., 2014; Lagadec et al., 2016), suggesting a strong affinity of *L.*  
81 *mayottensis* for this mammalian family. Nonetheless, the presence of *L. mayottensis* in rats has been  
82 reported on two occasions in Mayotte and Madagascar. In Mayotte, *L. mayottensis* has been  
83 identified in rats by using partial sequences of the 16S rRNA gene (Desvars et al., 2012), a genetic  
84 marker with poor discriminant power. In Madagascar, this genomospecies was identified in a small  
85 percentage of infected *Rattus* through the development of a sophisticated screening strategy  
86 allowing the investigation of multiple infections (Moseley et al., 2018)□. Of note, *L. mayottensis*

87 was found in *Rattus* as co-infections only. Authors claimed that rats may have acquired *L.*  
88 *mayottensis* infection through direct or indirect contact with tenrecs. These observations question  
89 the role of rats in the transmission of endemic strains of *Leptospira* in humans and the ability of  
90 strains, likely host-specific, to be sheltered by introduced reservoirs such as rats. Beside these  
91 observations, endemic *Leptospira* seem to exhibit a strong specificity towards their hosts that may  
92 result from ecological drivers such as the absence of contact between endemic and introduced  
93 mammal hosts impeding trans-contamination of rats by endemic *Leptospira*. Alternatively, co-  
94 evolutionary processes taking place during the extraordinary radiation of tenrecs, rodents and bats  
95 in Madagascar during the last 20-30My (Poux et al., 2008; Samonds et al., 2013) may have led to  
96 specific host-parasite interactions driving strong host specificity (Gomard et al., 2016).  
97 *Rattus norvegicus* is a renown reservoir of leptospirosis in SWIO and has been proven as a good  
98 experimental model to study chronic infection by *Leptospira* (Athanzio et al., 2008; Nally et al.,  
99 2015; Tucunduva de Faria et al., 2007)□. To test whether the tight host specificity observed on the  
100 field may be due to either ecological or genetic constraints, we challenged *R. norvegicus* with two  
101 *Leptospira* isolates considered endemic to SWIO region, namely *L. borgpetersenii* and *L.*  
102 *mayottensis* isolates obtained from bat and tenrec specimens, respectively. An isolate of *L.*  
103 *interrogans* harboring a worldwide distributed ST, obtained from a rat and associated with several  
104 human cases in the region was used as a positive control.

105

## 106 **2 Materials and methods**

### 107 **Animal experiments and ethical statement**

108 Experiments were conducted following the guidelines of the Office Laboratory of Animal Care at  
109 the CYROI platform. Seven- to eight-week-old female Wistar rats (Janvier Labs, Le Genest,  
110 France) were housed by 4 in enriched cages at the CYROI platform's animal facility. The  
111 appropriate number of animals was calculated using the Resource equation method (Festing, 2006).  
112 All animal procedures were performed in accordance with the European Union legislation for the  
113 protection of animals used for scientific purposes (Directive 2010/63/EU) and approved by the  
114 French Ministry of Sciences and Superior Education under the number APAFIS#8773-  
115 2016111615105111 v2.

116

### 117 **Bacterial isolates**

118 *Leptospira* isolates were obtained from the kidneys of volant and terrestrial small mammals trapped  
119 in the field. *Leptospira interrogans* was isolated from *Rattus rattus* on Reunion Island (2013RR  
120 GLM983) (Guernier et al., 2016)□, *L. mayottensis* was isolated from *Tenrec ecaudatus* on Mayotte  
121 (2014TE MDI222) (Lagadec et al., 2016), and *L. borgpetersenii* was isolated from an insectivorous  
122 bat, *Triaenops menamena*, endemic to Madagascar (2014TM FMNH228863) (Lebarbenchon et al.,  
123 2017). All isolates were previously genotyped through Multi Locus Sequence Typing (scheme 3).  
124 The *L. interrogans* and *L. mayottensis* isolates were selected for being associated with acute  
125 leptospirosis human cases on Reunion Island and Mayotte, respectively. The *L. borgpetersenii*  
126 isolate exhibited an original ST that has never been identified in any human leptospirosis case.  
127 *Leptospira* isolates were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid  
128 medium (Difco, Detroit, MI, USA) supplemented with albumin fatty acid supplement (AFAS)  
129 (purchased at AO/OIE and National Collaborating Centre for Reference and Research on  
130 Leptospirosis Academic Medical Center, Department of Medical Microbiology, Amsterdam) and 5-  
131 fluorouracil (5-Fu) at 28°C (for detailed protocol, see

132 <http://dx.doi.org/10.17504/protocols.io.ifccbiw>). The isolates were stored at -80°C or in liquid  
133 nitrogen for three to four years and their virulence was restored by two serial passages in hamsters.  
134 Restored *Leptospira* were then stored at -80°C and passaged less than 5 times before the beginning  
135 of the experiments (Cordonin et al., 2019).

136

### 137 **Infection and sample collection**

138 Challenged rats were inoculated intraperitoneally (i.p.) with  $1.5 \times 10^8$  cells of *L. interrogans* (n=7),  
139 *L. mayottensis* (n=8) or *L. borgpetersenii* (n=7) resuspended in 500 µl sterile phosphate buffered  
140 saline (PBS) 1X and control animals (n=8) were injected i.p. with 500 µl sterile PBS 1X. Animals  
141 were monitored for three months and urine samples were collected once a week during the whole  
142 experiment. After three months, animals were anesthetized with 50 mg/kg pentobarbital injected i.p.  
143 and subsequently euthanized by cardiac puncture. The animals were then transcardially perfused  
144 with PBS 1X to flush out the blood. The kidneys were then collected and processed for further  
145 experiments or immediately frozen at -80°C.

146

### 147 **Bacterial load measurement**

148 A piece of up to 25 mg of frozen kidney was lysed in ATL buffer (Qiagen, Germany) for 3 hours  
149 minimum. DNA from frozen kidneys and from urine samples (30 to 100 µl) was extracted using  
150 DNeasy Blood & Tissue Kit (Qiagen, Germany). The pathogenic *Leptospira* DNA amounts were  
151 then determined in each sample by a probe-specific real-time PCR using Quantinova probe PCR  
152 mix (Qiagen, Germany). All samples were triplicated and samples with at least two replicates  
153 leading to a PCR amplification at Ct < 45 were considered as positive. Results are expressed as  
154 genome copies per milligram of tissue for kidney samples or as genome copies per microliter for  
155 urine samples. The genome size of *L. interrogans* strain Fiocruz L1-130 was used to calculate the  
156 number of genome copies (Nascimento et al., 2004).

157

### 158 **Viability of excreted leptospire**

159 2 ml of EMJH liquid medium supplemented with AFAS and 5-Fu were inoculated with 30 to 50 µl  
160 urine. Cultures were observed regularly for three months to check the presence of cultivable  
161 leptospire under a dark-field microscope (Axio Lab.A1, Zeiss France).

162

### 163 **Histological studies**

164 For each animal, a whole kidney was fixed in 4% paraformaldehyde for 24 to 48 hours. Kidneys  
165 were then serially dehydrated using a spin tissue processor STP120 (Myr, Spain) as follows: three  
166 baths of Ottix Shaper at room temperature (10 minutes, 1 hour and 1.5 hours) were followed by 6  
167 baths of Ottix Plus at room temperature (1 hour, 1.5 hour twice, 2 hours and 2.5 hours), and 2  
168 paraffin baths at 62°C (1.5 hour and 2.5 hours). Kidney tissues were then embedded in paraffin.  
169 Four- to six-µm-thick paraffin kidney sections were deparaffinized in three successive xylene baths,  
170 10 minutes each, rehydrated in 3 successive ethanol baths (100%, 70% and 50%, 2 minutes each)  
171 and in distilled water bath, 2 minutes each. Rehydrated sections were eventually stained with  
172 hematoxylin and eosin, and observed using a digital slide scanner (NanoZoomer S60, Hamamatsu,  
173 France). Altogether, a minimum of three distinct animals were examined for each *Leptospira*  
174 infection and control.

175

## 176 **Statistics**

177 Non-parametric Kruskal-Wallis test was used on RStudio 1.0.153 (RStudio Team, 2015) to compare  
178 mean body weight percentage.

179

## 180 **3 Results**

### 181 **Experimentally infected rats show no clinical signs**

182 Animals infected with either *L. interrogans*, *L. borgpetersenii* or *L. mayottensis* isolates were  
183 monitored to evaluate a possible pathogenic effect of the different isolates. Groups of 6- to 8-week-  
184 old rats (n = 7-8 per group) inoculated intraperitoneally (i.p.) with  $1.5 \times 10^8$  leptospire or injected  
185 with PBS 1X were monitored for 3 months. None of the challenged rats showed any clinical sign of  
186 disease and all animals survived to the whole experiment. The weight gain of challenged rats was  
187 not significantly distinct from that of the control rat group at the end of the experiment (from  
188 37.93% to 41.70% of increase, p-value = 0.9, Fig 1).

189

### 190 **Differential *Leptospira* urinary shedding from rats**

191 Urinary shedding in experimentally infected rats was addressed through *Leptospira* culture and  
192 DNA detection on urine samples collected every week throughout the 3 months experiment. As  
193 shown in Table 1, no live leptospire were observable in culture medium inoculated with urine from  
194 any of the *L. mayottensis*- and *L. borgpetersenii*-infected rats. By contrast, live leptospire could be  
195 observed in culture medium inoculated with urine from 2 out of 7 *L. interrogans*-infected rats,  
196 starting from 4 weeks post-infection and until the end of the experiment. The shedding of  
197 *Leptospira* was transitory as cultivable leptospire could not be obtained at weeks 8 and 10 after the  
198 infection.

199 *Leptospira* DNA was detected in the urine of all *L. interrogans* infected rats, although  
200 discontinuously and with distinct concentration (Table 1, Fig 2). *Leptospira* DNA was detected in  
201 urine samples from 2 out of 7 *L. interrogans*-infected rats during 10 and 12 consecutive weeks with  
202 amounts ranging from 7,303.0 to 94,599.8 genome copies/ $\mu$ l. Urine samples of 1 additional rat  
203 tested positive during the first 3 weeks of the experiment. On the other hand, the detection of  
204 *Leptospira* DNA in the 4 remaining rats was sporadic and ranged from 230.2 to 100,746.5 genome  
205 copies/ $\mu$ l.

206 *Leptospira* DNA was detected less often and at lower levels in the urine of rats infected with *L.*  
207 *mayottensis* and *L. borgpetersenii* in comparison to individuals infected with *L. interrogans*.

208 *Leptospira* DNA was sporadically detected in the urine of all *L. mayottensis*-infected rats with  
209 amounts ranging from 168.4 to 34,816.1 genome copies/ $\mu$ l.

210 Six out of seven *L. borgpetersenii*-infected rats shed *Leptospira* DNA in their urine. The detection  
211 was continuous in the first three weeks of the experiment for 2 rats and sporadic for the 4 remaining  
212 rats. Continuous DNA excretion ranged from 132.9 to 11,625.2 genome copies/ $\mu$ l while sporadic  
213 excretion ranged from 19.9 to 4,323.3 genome copies/ $\mu$ l.

214

### 215 ***Leptospira* spp. infection does not necessarily induce chronic renal carriage and histological 216 damage**

217 Following euthanasia, the detection of *Leptospira* DNA in kidneys through qPCR was negative for  
218 all rats infected with either *L. borgpetersenii* or *L. mayottensis*. Only those two rats chronically  
219 infected with *L. interrogans*, as shown by qPCR on urine, tested positive with loads of  $108,670.8 \pm$   
220  $8178.1$  and  $201,132.3 \pm 1077.7$  copies/mg of kidney tissue. The kidneys from rats experimentally

221 infected with all three *Leptospira* species were used for histological studies through hematoxylin-  
222 eosin staining. Kidney sections showed no particular renal damage induced by the infection of rats  
223 with any of the three *Leptospira* isolates, including the two rats that were chronically shedding *L.*  
224 *interrogans*. Glomeruli and renal tubules of infected rats harbored the same phenotype as the  
225 control rats (Fig 3).

226

## 227 **Discussion**

228 In this study, we tested the ability of *Leptospira* isolates obtained from bat and tenrec reservoirs  
229 endemic to SWIO islands to chronically infect rats, a renown *Leptospira* reservoir shedding *L.*  
230 *interrogans* worldwide including in several islands of SWIO (Biscornet et al., 2017; Guernier et al.,  
231 2016; Lagadec et al., 2016; Rahelinirina et al., 2010)□. First of all and as expected, our data  
232 indicate that rats are not susceptible to any of the three *Leptospira* isolates used in this study as  
233 shown by the lack of clinical signs in any of the infected rats. The tenrec-borne *L. mayottensis* and  
234 the bat-borne *L. borgpetersenii* isolates were unable to colonize the kidneys of *R. norvegicus*  
235 contrary to the rat-borne *L. interrogans* isolate. Indeed, *Leptospira* DNA was detected during 10 to  
236 12 consecutive weeks in the kidneys of two out of seven *L. interrogans*-infected rats while more  
237 sporadically and for not more than 3 consecutive weeks in rats infected with either *L. mayottensis*  
238 or *L. borgpetersenii*. Moreover, live leptospires were observed in cultures of urine samples obtained  
239 from the two aforementioned *L. interrogans*-infected rats during several consecutive weeks while  
240 urine samples obtained from *L. mayottensis*- and *L. borgpetersenii*-infected rats did not lead to any  
241 detectable *Leptospira* growth.

242 The renal colonization of rats by the *L. interrogans* isolate was less successful in this study  
243 compared to previous comparable studies using a rat model as a reservoir of *L. interrogans*.  
244 Although i.p. inoculation of leptospires has been shown to be efficient to induce chronic *Leptospira*  
245 infection in rats (Athanzio et al., 2008; Tucunduva de Faria et al., 2007; Zilber et al., 2016), only  
246 two out of seven i.p. infected rats supported renal chronic infection. Hence the *L. interrogans* strain  
247 used herein and isolated from a wild rat may be less invasive as compared to previously described  
248 *L. interrogans* strains that have been experiencing multiple *in vitro* culture passages. Results  
249 obtained by qPCR indicate that the bacteria reached the kidneys of two rats that supported renal  
250 infection for 2 to 3 weeks after inoculation, and urine samples of these rats led to positive cultures  
251 either from 4 or 5 weeks post-infection. By contrast, previous studies reported kidney colonization  
252 as soon as 1 week after infection with a lower amount of inoculated bacterial cells (Athanzio et al.,  
253 2008). Of note, the two rats harboring chronic infection with *L. interrogans* were housed in the  
254 same cage with 2 other rats seemingly not chronically infected with *Leptospira*. The two  
255 chronically infected rats shed bacteria with comparable kinetics throughout the experiment (Figure  
256 2). Hence direct or indirect horizontal contamination was likely not involved in renal colonization  
257 in our experiment.

258 The tenrec-borne *L. mayottensis* and the bat-borne *L. borgpetersenii* isolates were not able to induce  
259 chronic infection in any of the infected rats as shown by qPCR and culture experiments. Although  
260 *Leptospira* DNA could be sporadically detected in the urine of some rats, these two isolates were  
261 not able to induce a persistent renal colonization as shown by the absence of both live leptospires in  
262 urine cultures and *Leptospira* DNA in the kidneys of infected rats at the end of the experiment.  
263 No particular lesions were observed in the kidneys of infected rats following infection. This is not  
264 unexpected as rats are resistant to *Leptospira* infection and previous studies have already reported  
265 the absence of renal lesions in experimentally *L. interrogans*-infected rats (Nally et al., 2005,

266 2018)□. However, nephritis and cellular infiltrates have been previously reported in rats harboring  
267 chronic renal carriage of *L. interrogans* (Nally et al., 2015; Tucunduva de Faria et al., 2007)□.  
268 Despite a limited renal colonization ability of the *L. interrogans* isolate used in our experiment, two  
269 out of seven rats managed to become reservoir of this strain while none of the rats infected with *L.*  
270 *mayottensis* or *L. borgpetersenii* isolates supported chronic carriage of leptospires in their kidneys.  
271 The inability of those latter isolates to colonize rats' kidneys may be linked to an attenuated  
272 virulence of these isolates. Indeed, the *L. interrogans* isolate used in this study has been shown to be  
273 (i) significantly more virulent than *L. mayottensis* and *L. borgpetersenii* isolates and (ii) able to  
274 colonize kidneys of experimentally infected hamsters more efficiently than did the endemic  
275 *Leptospira* (Cordonin et al., 2019). In addition, the absence of renal colonization in rats by the  
276 endemic strains could result from a genetic incompatibility between the bacterium and this  
277 alternative vertebrate host. The *L. mayottensis* and *L. borgpetersenii* isolates may not be able to  
278 adhere to host cells thus contributing to the decreased infection success of both strains observed in  
279 the rat and hamster models (Baumler and Fang, 2013; Cordonin et al., 2019). Altogether, our results  
280 suggest that the strong host specificity of *Leptospira* species/lineages endemic to SWIO islands  
281 towards their mammalian hosts (Dietrich et al., 2018) is not driven by ecological constraints but  
282 rather results from co-evolutionary processes.

283 Noteworthy, *L. mayottensis* has been recently reported in rats from Madagascar, indicating that  
284 rats are indeed able to shelter *L. mayottensis* (Moseley et al., 2018) and that genetic factors alone  
285 cannot explain the tight association between endemic *Leptospira* and hosts, such as *L. mayottensis*  
286 and Tenrecidae (Dietrich et al., 2014, 2018; Lagadec et al., 2016)□. However, the aforementioned  
287 study did not report *L. mayottensis* in rats as single infections but in co-infections only, and hence a  
288 primary infection with *L. interrogans* in rats may be a prerequisite for a secondary infection with *L.*  
289 *mayottensis*. This could be tested through experimental infections such as those presented herein.  
290 The study of virulence evolution of the Friend virus has shown that viral fitness could be impaired  
291 if several passages of the virus are proceeded through hosts of different genotypes. By contrast,  
292 several passages of the pathogen through hosts of identical genotypes led to an increase in pathogen  
293 fitness (Kubinak et al., 2014). Thus, in our study, the fitness of the *Leptospira* isolates obtained  
294 from bat and tenrec specimens may have a decreased fitness in rats hence impairing chronic  
295 infection. Further investigations are thus needed to test whether serial passages of a specific  
296 *Leptospira* lineage through a novel host (such as rats) can allow the adaptation of those bacterial  
297 lineages to an alternative reservoir.

298 In conclusion, the *Leptospira* isolates obtained from small volant and terrestrial mammals endemic  
299 to western Indian Ocean were not able to colonize rats' kidneys, which is in keeping with the host  
300 specificity observed in the field (Dietrich et al., 2018; Lagadec et al., 2016). Such results support  
301 the existence of genetic determinants of host/parasite specificity. It would be however interesting to  
302 carry out symmetrical experiments, *i.e.* challenging bats and tenrecs with all three isolates.  
303 Unfortunately, such experiments require the availability of lab colonies of bats and tenrecs, which  
304 are very few worldwide. Although host specificity may be driven by genetic determinants between  
305 both host and parasite, as suggested by our results, other biological features such as attenuated  
306 virulence (Cordonin et al., 2019)□ or bacterial growth rate, could impair the ability of both *L.*  
307 *mayottensis* and *L. borgpetersenii* to chronically infect another reservoir host. Further work is  
308 therefore needed to explore the bacterial genetic determinants and characterize the host factors that  
309 may be at play in such interactions.

310

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413

#### 414 **Author contributions**

415 Conceived and designed the experiments: CC, KD, PT, MR, PM. Performed the experiments: CC,  
416 MR, PT, MT, MB. Contributed resources/reagents/materials/analysis tools: CC, PT, PM, MR, MB,  
417 JB. Wrote the paper: CC. Revised the paper: all co-authors

418

#### 419 **Competing interests.**

420 The authors declare no competing interests.

421

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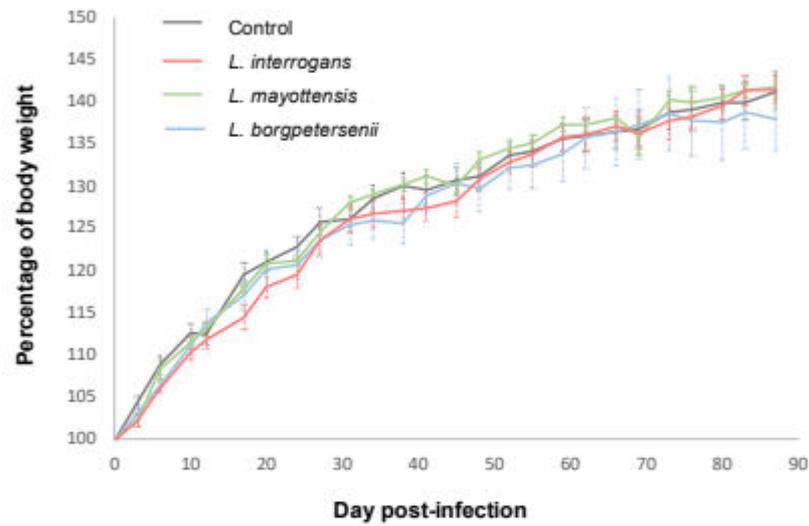
426 **Tables**

427 **Table 1.** Rate of *Leptospira*-positive samples using either qPCR or cultures on urine of  
 428 experimentally infected rats.

429 wpi: week post-infection; *Li*: *Leptospira interrogans*; *Lm*: *L. mayottensis*; *Lb*: *L. borgpetersenii*

wpi	qPCR <i>Leptospira</i> -positive urine samples (%)			<i>Leptospira</i> -positive cultures (%)		
	<i>Li</i>	<i>Lm</i>	<i>Lb</i>	<i>Li</i>	<i>Lm</i>	<i>Lb</i>
1	1/7 (14.3)	0/8 (0.0)	2/7 (28.6)	0/7 (0.0)	0/8 (0.0)	0/7 (0.0)
2	3/7 (42.9)	6/8 (75.0)	4/7 (57.1)	0/7 (0.0)	0/8 (0.0)	0/7 (0.0)
3	5/7 (71.4)	1/8 (12.5)	4/7 (57.1)	0/7 (0.0)	0/8 (0.0)	0/7 (0.0)
4	2/7 (28.6)	0/8 (0.0)	1/7 (14.3)	1/7 (14.3)	0/8 (0.0)	0/7 (0.0)
5	2/7 (28.6)	1/8 (12.5)	0/7 (0.0)	2/7 (28.6)	0/8 (0.0)	0/7 (0.0)
6	1/7 (14.3)	1/8 (12.5)	0/7 (0.0)	2/7 (28.6)	0/8 (0.0)	0/7 (0.0)
7	2/7 (28.6)	1/8 (12.5)	0/7 (0.0)	2/7 (28.6)	0/8 (0.0)	0/7 (0.0)
8	1/7 (14.3)	1/8 (12.5)	0/7 (0.0)	0/7 (0.0)	0/8 (0.0)	0/7 (0.0)
9	1/7 (14.3)	0/8 (0.0)	1/7 (14.3)	2/7 (14.3)	0/8 (0.0)	0/7 (0.0)
10	2/7 (28.6)	0/8 (0.0)	0/7 (0.0)	0/7 (0.0)	0/8 (0.0)	0/7 (0.0)
11	2/7 (28.6)	0/8 (0.0)	0/7 (0.0)	2/7 (28.6)	0/8 (0.0)	0/7 (0.0)
12	1/7 (14.3)	1/8 (12.5)	0/7 (0.0)	2/7 (28.6)	0/8 (0.0)	0/7 (0.0)
13	1/7 (14.3)	0/8 (0.0)	0/7 (0.0)	1/7 (14.3)	0/8 (0.0)	0/7 (0.0)

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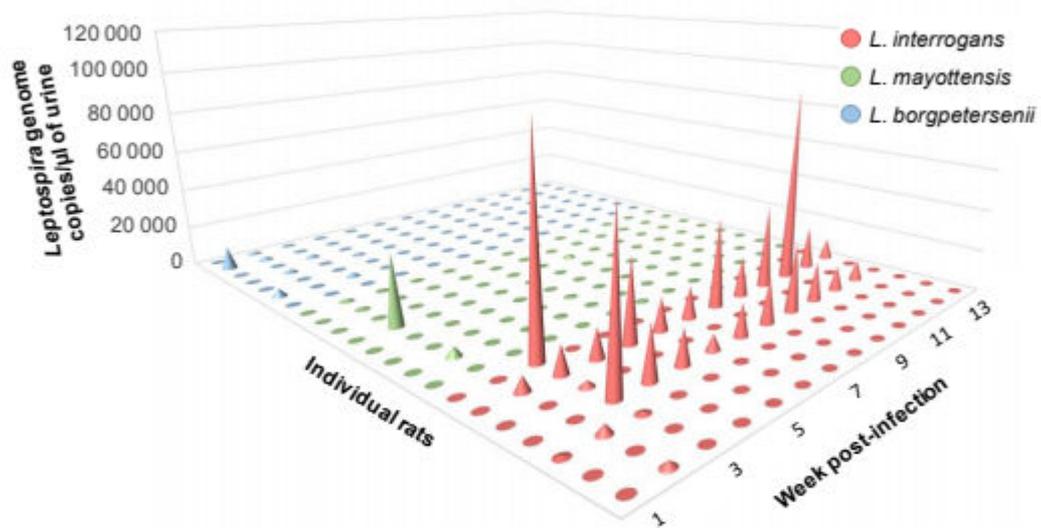


432

433

434 **Figure 1.** Rats monitoring. Growth curves of control rats (black) and rats infected with either *L.*  
435 *interrogans* (red), *L. mayottensis* (green) or *L. borgpetersenii* (blue). Results are expressed as means  
436  $\pm$  SEM.

437

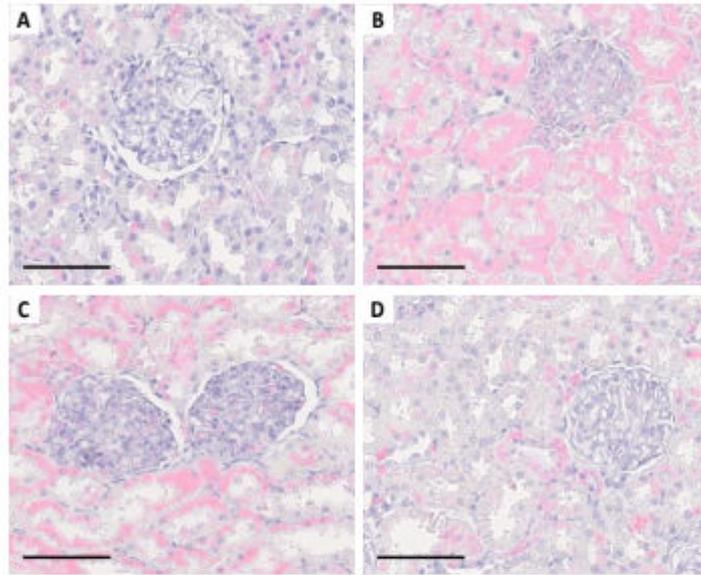


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439

440 **Figure 2.** Urinary shedding of *Leptospira* from rats infected with either *L. interrogans* (red), *L.*  
 441 *mayottensis* (green) or *L. borgpetersenii* (blue). Results are expressed for each individual rat as  
 442 genome copies/μl of urine.

443



444  
445

446 **Figure 3.** Microscopic observations of kidney sections stained with hematoxylin-eosin from control  
447 rats (**A**) and rats infected with either *L. interrogans* (**B**), *L. borgpetersenii* (**C**) or *L. mayottensis* (**D**).  
448 Scale bars = 60  $\mu$ m

### **III. Main results**

This chapter highlights that *L. mayottensis* and *L. borgpetersenii* isolates could not establish a chronic renal colonization in *R. norvegicus* as shown by urine analysis of the infected rats. However, these results should be interpreted with caution as the rate of renal colonization achieved in *R. norvegicus* by the *L. interrogans* isolate was low compared to other studies.

## CHAPTER 3

### Exploring *Leptospira* diversity through comparative genomics

## I. *Leptospira* whole-genome sequencing

*Leptospira* genomes are composed of two circular chromosomes and a variable number of replicons. The total genome length is between 3.8 and 4.7 Mb (Picardeau, 2017).

In recent years, the classification of *Leptospira* strains based on their genetic features has begun to overtake classical techniques of classification based on antigenic properties as the method of choice for *Leptospira* typing. Perhaps the most commonly used genetic classification scheme for *Leptospira* is that of Multi-Locus Sequence Typing (MLST). MLST generally relies on the PCR-amplification and Sanger sequencing of 6-7 gene loci. Sequences obtained from these specific loci are then used to attribute allele and sequence type definitions for an isolate, and sequences are shared globally on the PubMLST website (Jolley et al., 2018) so that standardized typing information can be compared between laboratories and countries. Currently, three MLST schemes are available (<https://pubmlst.org/leptospira/>) for the identification and classification of *Leptospira* strains. However, the highly divergent sequences of *Leptospira* means that standard primers do not always work for PCR-based typing, and none of the three MLST schemes are designed to work for the entire *Leptospira* genus (Boonsilp et al., 2013). Consequently, and as a result of the increase in accessibility to next generation sequencing technology, more and more *Leptospira* isolates are identified and classified using whole genome sequencing (WGS).

The analysis of the *Leptospira* whole genome is, by definition, the most precise method for the taxonomic classification of *Leptospira* strains. However, genomic data are also used in many other research contexts. Whole genomes have been studied and compared to identify characteristics that differ among *Leptospira* groups and/or species *Leptospira* (Barragan et al., 2011; Bulach et al., 2006) and to study the evolution of pathogenicity for the genus (Ricaldi et al., 2012; Thibeaux et al., 2018).

Whole genomes are not always easily obtained. Genomic data must be generated from pure *Leptospira* cultures, and many variants of *Leptospira* are difficult to isolate. Most commonly, genomes are sequenced using short-read technology such as that offered by the Illumina platform. This results in genomic assemblies being fragmented (draft genomes). Although draft genomes contain a lot of information, the gene set obtained may be incomplete due to missing data and some important features of *Leptospira* strains may therefore be lost. Hence, wherever possible it is important to obtain the complete genome sequences of strains, thus providing the most complete information possible for each isolate. Although complete genome sequencing (as opposed to draft genome sequencing) is prohibitively expensive for many research projects, the additional accuracy of fully assembled genomic data allows for better comparisons between isolates, and the eventual

identification of genetic features that may be specific to *Leptospira* with clinically relevant traits (Chou et al., 2014). For example, the comparison of whole genome of *L. interrogans* and *L. borgpetersenii* strains led to the observation of a genome reduction in *L. borgpetersenii* and a lower number of genes involved in signal transduction compared to *L. interrogans*. These features were associated with a lower ability of *L. borgpetersenii* to survive outside a host, promoting a transmission through direct contact between different hosts (Bulach et al., 2006).

The quality of a genome assembly depends on the sequencing method used. Second (SGS) and third (TGS) generation sequencing platforms are available for sequencing whole *Leptospira* genomes. The main used SGS platform, namely Illumina, produces accurate short read lengths up to 600 bp (Weirather et al., 2017). However, given that *Leptospira* genomes contains numerous long repeated sequences, the assembly of reads obtained from Illumina sequencing may lead to incomplete draft genomes with a lack of genetic information. By contrast, TGS platforms such as Pacific Biosciences (PacBio) produce long fragments, over 1,000 bp, and may be more suitable to generate whole genomes (Weirather et al., 2017). However, the error rates of TGS platforms are higher than SGS platforms although an improvement of read quality has been observed (Schloss et al., 2016; Wagner et al., 2016).

In this chapter, we first provided whole genomes of three strains of *L. mayottensis* which have been sequenced using the PacBio platform. *Leptospira mayottensis* was recognized as a novel *Leptospira* species a few years ago (Bourhy et al., 2014) and thus only a few genomes of this species are available in international databases. The three assembled genomes of *L. mayottensis* presented here contribute to the global *Leptospira* genome database and will allow more accurate *Leptospira* genome comparisons.

Secondly, we analyzed a large set of *Leptospira* genomes including the three genomes of *L. mayottensis* obtained from PacBio sequencing, 8 assembled draft genomes of *Leptospira* strains isolated on SWIO islands obtained from Illumina Miseq sequencing and all available genomic sequences from public data repositories. We classify the *Leptospira* strains that were isolated in SWIO, and go on to identify genes that are specific to each of the three groups of *Leptospira*, namely pathogenic, intermediate, and saprophytic *Leptospira*. A second genomic comparison was also performed using published genomes and genomes of *Leptospira* isolates from SWIO belonging to each of the species *L. interrogans*, *L. mayottensis* and *L. borgpetersenii*.

## **II. Genome announcement: Complete genome sequences of three *Leptospira mayottensis* strains from tenrecs that are endemic to the Malagasy region**



# Complete Genome Sequences of Three *Leptospira mayottensis* Strains from Tenrecs That Are Endemic in the Malagasy Region

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**ABSTRACT** Leptospirosis is a zoonosis caused by *Leptospira*, a diversified genus containing more than 10 pathogenic species. Tenrecs are small terrestrial mammals endemic in the Malagasy region and are known to be reservoirs of the recently described species *Leptospira mayottensis*. We report the complete genome sequences of three *L. mayottensis* strains isolated from two tenrec species.

Leptospirosis is an environmental infectious disease caused by spirochetal bacteria belonging to the genus *Leptospira*. Humans usually get infected during recreational or work-related outdoor activities through contact with urine excreted by animal reservoirs. This zoonosis is estimated to cause more than one million human cases and 58,900 deaths each year (1). Disease incidence is highest on tropical islands, notably in the southwestern Indian Ocean, where investigations carried out under a One Health framework have revealed distinct transmission chains in the different islands (2–5), including the occurrence of *Leptospira mayottensis*, a pathogenic *Leptospira* species recently recognized as new to science (6). Representatives of *L. mayottensis* (formerly known as *Leptospira borgpetersenii* group B) were originally isolated from human acute cases (7) and later identified in tenrecs (2, 5, 8), a diversified family of mammals endemic to Madagascar (9, 10).

Three *L. mayottensis* strains were isolated in the field from two tenrec species, namely, *Tenrec ecaudatus* on Mayotte (MDI222 and MDI272) (2) and *Microgale dobsoni* on Madagascar (VS2413) (5). Frozen isolates were thawed in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium containing albumin fatty acid supplements (AFAS) (3), and they were further subcultured in EMJH supplemented with AFAS, 1% fetal calf serum, and 8% rabbit serum. DNA was extracted with the EZ1 virus minikit v.2.0 (Qiagen, Germany), and sequencing was performed on PacBio platforms. The sequence for VS2413 was assembled *de novo* with SMRT Analysis HGAP.2, those for MDI222 and MDI272 were assembled *de novo* using Canu 1.6, and contigs were circularized using AMOS v.3.1 (11).

Overall, genome sizes and structures (see Table 1) are close to those of previously published ones, with comparably low ( $\leq 40\%$ ) GC contents, two chromosomes, and, in the case of *T. ecaudatus*-borne strains, an additional plasmid (12).

Automatic annotation was performed with the NCBI Prokaryotic Genome Annotation Pipeline (13), which revealed that the VS2413, MDI272, and MDI222 genomes are composed of 3,893, 3,917, and 4,136 coding sequences, respectively.

Human leptospirosis on Mayotte is peculiar because it is associated with four distinct bacterial species, including *L. mayottensis* (14), and characterized by a relatively low fatality rate (0.9%) (15). The epidemiology is clearly distinct in the neighboring Seychelles and on Reunion Island, where *L. interrogans* is overwhelmingly dominant and fatality rates are higher (11.8% in the Seychelles and 3 to 5% on Reunion Island) (3, 4, 16). An attenuated virulence of *Leptospira* species prevailing on Mayotte, including *L. mayottensis*, may be at

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C.C. and C.T. contributed equally to this work.

**TABLE 1** Genome architecture of *L. mayottensis* isolates<sup>a</sup>

Isolate	Source host	Sampling location	Chromosome length (bp)		Plasmid length (bp)	No. of reads	N <sub>50</sub> length (bp)
			I	II			
MDI222	<i>Tenrec ecaudatus</i>	Mayotte	3,934,331 (not circularized)		111,328	44,976	20,779
MDI272	<i>Tenrec ecaudatus</i>	Mayotte	3,861,885	332,647	52,509	52,101	17,832
VS2413	<i>Microgale dobsoni</i>	Madagascar	3,787,562	300,219		55,595	21,656

<sup>a</sup>All but one replicon were successfully circularized.

least in part responsible for this contrasted regional epidemiology. The genomes presented herein will accelerate comparative genomic approaches aimed at delineating the main genomic features involved in *Leptospira* virulence.

**Data availability.** Genome sequences were deposited in DDBJ/EMBL/GenBank under the accession numbers CP030142 and CP030143, CP030144 to CP030146, and CP030147 to CP030149 for strains VS2413, MDI222, and MDI272, respectively, as well as BioProject number PRJNA477299. Raw sequence data are available from the NCBI Sequence Read Archive (accession number SRP154442).

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### III. Comparative genomics: methodology

#### 1. Introductory statement

In leptospirosis research, there are two main goals when sequencing the genomes of *Leptospira* isolates:

- 1) To provide precise taxonomic classifications of all sequenced isolates
- 2) To identify genetic elements that are associated with specific traits or taxa.

Both of these purposes employ the comparison of multiple genomes, often requiring computationally demanding analytical techniques to analyze large datasets. Different methodologies are often used for these purposes due to some of the common technical challenges of comparative genomics.

Taxonomic classification, which is often (but not necessarily) achieved by using phylogenetic techniques, relies on the identification of mutations in regions that are present in all analyzed genomes (conserved, or “core” regions). Individual mutations, or single nucleotide polymorphisms (SNPs), are then enumerated or analyzed using substitution models to describe the similarity between isolates. The resulting similarity matrix allows isolates to be assigned to a taxonomic group. As an organism’s full genome provides the maximum possible amount of information related to its evolutionary history, genomic taxonomic classifications are considered extremely precise. The result is that isolates sharing very recent common ancestors (with respect to the mutation rate of the organism) can be identified, and in an epidemiological context we may infer that extremely similar isolates are likely to have originated from a common source.

Here, for taxonomic classification we identify conserved genomic regions using a technique called sequence “mapping”. Data from one genome are broken into short sequences, each being compared to the sequence of a reference genome. SNPs are identified in regions where the query and reference sequence can be aligned with certainty. Sequences cannot be aligned with certainty if they are highly divergent, thus mapping-based SNP estimates are generally constrained to species-level or subspecies-level comparisons. Additionally, it is impossible to map sequences with certainty to regions that are repeated within the same genome. This second point is one of the main complicating factors in *Leptospira* genomics, as *Leptospira* genomes contain large numbers of repeated regions and duplicated genes.

In the presented work, we successfully define SNPs from members of each genomospecies of *Leptospira* for which genomic data was generated as part of this project. Using this approach, a large proportion of each genome is mapped successfully to the chosen reference data, meaning that isolate similarities are estimated with high genomic coverage. We use these data to provide accurate

classifications of each of our isolates relative to all publicly available reference data worldwide. We then examine the overall genetic diversity of each genomospecies and discuss the implications of these observations.

The study of associations between genetic content and isolate traits is generally performed at the gene level and these types of analysis are commonly referred to as “Genome Wide Association Studies” (GWAS). Bacteria have the capacity to acquire genetic material from their environments, meaning that the total genome content of the studied organism is dynamic over time and between taxa. Traits such as virulence or antibiotic resistance are often the result of the acquisition of single genes, sets of genes of mobile genetic elements such as phage or plasmids. Therefore we use comparative genomics to address the question “which genes are found (uniquely) in the isolates with phenotype X?”, in the hope that the identification of characteristic gene markers will lead to the development of new drug targets, detection schemes or help to better understand gene function.

Thus to study associations, we predict the location of genes in genomic datasets and use comparison techniques to identify those genes that are not conserved between all genomes (“accessory” genes). This is performed by computer-intensive algorithms that identify clusters of corresponding genes between genomes based on the similarities between all genes from all genomes (the “pangenome”). All genes belonging to a single cluster are termed “orthologous genes” or “orthologues”. When multiple copies of orthologous genes are present in the same isolate, these are termed “paralogues”. Due to the computational time required to estimate clusters of orthologous groups (COGs), here we opt for a two-stage method where COGs are defined for the pangenome of a subset of available *Leptospira* genomes, representative of the total diversity of the *Leptospira* genus. Then a less computationally demanding algorithm is used to determine the presence/absence of this set of defined core and accessory genes in all other genomes.

Using the outlined approach, we successfully define the *Leptospira* pangenome and identify gene components that are linked with i) the classification of *Leptospira* as “pathogenic, intermediate or saprophytic” and ii) the membership to each genomospecies.

## **2. Methodology**

### ***Taxonomic classification of Leptospira genomes***

Sequence mapping and SNP-derived phylogenies were used to classify each *Leptospira* genome generated in this study relative to all publicly available genomes belonging to the same species (i.e. *L. interrogans*, *L. borgpetersenii* and *L. mayottensis*).

Publicly available reference genome data were downloaded using the ncbi-genome-download tool (Blai), specifying taxon (species) names with the argument “-g”.

### **Reference data**

A single reference sequence was chosen based on an assessment of the assembly statistics of all genomic contigs. Assembly statistics were extracted using seqkit (Shen et al., 2016), and for each species the complete genome with the largest genome size was chosen as the reference against which all other sequences were mapped. Genomes were considered complete if the number of contigs in the assembly was below 5. Complete genomes were available for each species.

All genomes were assigned to an MLST-type using the program mlst (Jolley et al., 2018; Seemann) to query *Leptospira* MLST schemes 1, 2 and 3.

### **Sequence Mapping**

The program Snippy version 4.0.1 (Seemann) was used to map genomic sequences to the reference for each species. The program was used in “contigs” mode (specified by the call “-ctgs”) as raw read data were not available for all reference isolates. In contigs mode, Snippy shreds each queried genome into overlapping sequences of 250bp in length, generating artificial “raw data” at a sequencing depth of 20x. These short sequences are then mapped to the reference based on minimum coverage and identity criteria. We used Snippy’s default settings for both minimum coverage and identity.

The core genome alignment containing all SNP data was generated using snippy-core (part of Snippy), and snp-sites version 2.4.1 (Page et al., 2019), using both -b and -c parameters to remove ambiguities and retain invariant sites. SNP coverage was defined as the proportion of each genome that was retained in the core genome alignment, and was calculated as the alignment length divided by the total number of bases present in each genome. Genetic distances between isolates were calculated using the R package ‘ape’, and were measured as the proportion of variant sites in pairwise comparisons between each genome after removing missing data (gaps).

### **Phylogenetics**

Gubbins (Croucher et al., 2015) (Genealogies Unbiased By recomBINations In Nucleotide Sequences) version 2.3.4 was used under its default settings to generate a maximum likelihood phylogeny from the output of snp-sites. Gubbins works by simultaneously analyzing mutation rates within a sliding window along the DNA alignment and assessing phylogenetic structure. In this way, it removes “hotspots” of mutation from the alignment that are likely to result from historical recombination events. In doing this, the phylogeny generated is suggested to be more representative of the clonal frame of the organism. The resulting phylogenies were manipulated and displayed using the R package ‘ggtree’ (Yu et al., 2016).

## **Pangenome Analysis**

In addition to the genomes sequenced as part of this project, a random selection of 32 high quality draft genome assemblies spanning the full taxonomic spectrum of the *Leptospira* genus were chosen for the primary definition of the organism's pangenome. The genomes chosen were from the species *L. alexanderi* (n=3), *L. alstoni* (n=1), *L. biflexa* (n=2), *L. borgpetersenii* (n=3), *L. broomii* (n=1), *L. fainei* (n=1), *L. inadai* (n=2), *L. interrogans* (n=2), *L. kirschneri* (n=3), *L. kmetyi* (n=1), *L. licerasiae* (n=3), *L. meyeri* (n=2), *L. noguchi* (n=2), *L. santarosai* (n=3), *L. terpstrae* (n=1), *L. weilii* (n=1) and *L. yanagawae* (n=1).

Clusters of orthologous groups were defined using the R package 'pewit' (Thibeaux et al., 2018). Briefly, pewit defines orthologues primarily based on similar protein domain architectures, established by comparison to the Pfam database (Finn et al., 2016). Genes that contain no identifiable protein domains are grouped based on sequence-similarity using OrthoMCL (Li et al., 2003). Tree-pruning methods are then used to assess in- and out- paralogue members for each orthologous group; in-paralogues are collapsed and treated as multiple copies of the same orthologous gene, whereas out-paralogues are split to redefine new orthologous groups. This approach is highly advantageous for pangenome definitions of *Leptospira* as elevated levels of sequence diversity mean that clustering techniques that are entirely based on sequence-similarity between genes underperform for this genus (Wilkinson DA, personal communication).

The pangenome data was extracted from pewit as a numeric matrix indicating the number of copies of each gene orthologue present in each genome. This matrix was used to extract the presented pangenome statistics, such as the number of core and accessory genes. Rarefaction analysis was performed using pewit to examine the open/closed nature of the defined pangenome.

## **Analysing gene content for all genomes**

In order to obtain robust definitions of gene associations based on the maximum possible amount of data, the presence or absence of all gene orthologues defined in the above pangenome protocol was assessed for all publicly available *Leptospira* genomes. As above, reference genome assemblies were downloaded from the NCBI database using the ncbi-genome-download tool, and the parameter "-g Leptospira".

Representative gene sequences were obtained for each orthologous group defined by pewit. These sequences were exported in "fasta" format, and then used to generate a BLAST database. Using in-house scripts, blastn (Altschul et al., 1990) was then run iteratively to test for the presence of each orthologous group in each reference genome. Genes were considered as present within a genome if sequences with greater than 75 % pairwise identity to the representative sequence of each orthologous group were identifiable. This process was run for a total of 511

genomes from 34 different species of *Leptospira* (Tables 3, 4). The majority of available *Leptospira* genomes came from the species *L. interrogans* (n=283).

The final presence/absence matrix for all genes in all genomes was exported and used for all downstream analyses of gene associations.

**Table 3.** *Leptospira* genomes publicly available used in comparative genomics

SPECIES	TYPE	COUNT
<i>Leptospira adleri</i>	pathogen	2
<i>Leptospira alexanderi</i>	pathogen	6
<i>Leptospira alstonii</i>	pathogen	6
<i>Leptospira barantonii</i>	pathogen	1
<i>Leptospira borgpetersenii</i>	pathogen	42
<i>Leptospira ellisii</i>	pathogen	2
<i>Leptospira interrogans</i>	pathogen	283
<i>Leptospira kirschneri</i>	pathogen	31
<i>Leptospira kmetyi</i>	pathogen	4
<i>Leptospira mayottensis</i>	pathogen	9
<i>Leptospira noguchii</i>	pathogen	16
<i>Leptospira santarosai</i>	pathogen	38
<i>Leptospira weilii</i>	pathogen	18
<i>Leptospira broomi</i>	intermediate	1
<i>Leptospira fainei</i>	intermediate	1
<i>Leptospira haakei</i>	intermediate	2
<i>Leptospira hartskeerli</i>	intermediate	2
<i>Leptospira inadai</i>	intermediate	2
<i>Leptospira licerasiae</i>	intermediate	4
<i>Leptospira neocaledonica</i>	intermediate	1
<i>Leptospira perolatii</i>	intermediate	2
<i>Leptospira saintgironsiae</i>	intermediate	1
<i>Leptospira venezuelensis</i>	intermediate	3
<i>Leptospira wolffi</i>	intermediate	2
<i>Leptospira biflexa</i>	saprophyte	2
<i>Leptospira brenneri</i>	saprophyte	1
<i>Leptospira harrisiae</i>	saprophyte	2
<i>Leptospira levetti</i>	saprophyte	3
<i>Leptospira mcculloughi</i>	saprophyte	1
<i>Leptospira meyeri</i>	saprophyte	5
<i>Leptospira terpstrae</i>	saprophyte	1
<i>Leptospira vanthielli</i>	saprophyte	1
<i>Leptospira wolbachii</i>	saprophyte	1
<i>Leptospira yanagawae</i>	saprophyte	1

**Table 4.** *Leptospira* genomes obtained in this study and used in comparative genomics

Sample ID	<i>Leptospira</i> sp.	Host species	Origin
bn_li	<i>L. interrogans</i>	<i>Homo sapiens</i>	Reunion
clone1_li	<i>L. interrogans</i>	<i>Rattus rattus</i>	Mayotte
clone9_li	<i>L. interrogans</i>	<i>Rattus rattus</i>	Reunion
clone14_li	<i>L. interrogans</i>	<i>Rattus norvegicus</i>	Seychelles
glm956_li	<i>L. interrogans</i>	<i>Rattus rattus</i>	Reunion
glm983_li	<i>L. interrogans</i>	<i>Rattus rattus</i>	Reunion
sey504_li	<i>L. interrogans</i>	<i>Rattus rattus</i>	Seychelles
mdi247_lb	<i>L. borgpetersenii</i>	<i>Rattus rattus</i>	Mayotte
mdi260_lb	<i>L. borgpetersenii</i>	<i>Rattus rattus</i>	Mayotte
UR_L_may_mdi272_tenrec	<i>L. mayottensis</i>	<i>Tenrec ecaudatus</i>	Mayotte
UR_L_may_mdi222_tenrec	<i>L. mayottensis</i>	<i>Tenrec ecaudatus</i>	Mayotte
UR_L_may_vs2413_microgale	<i>L. mayottensis</i>	<i>Microgale dobsoni</i>	Madagascar

### **Discriminant analysis**

We examined gene associations in an attempt to identify specific genetic markers for two different groupings of *Leptospira* genomes:

- Pathogens vs. Intermediates vs. Saprophytes
- *L. interrogans* vs. *L. borgpetersenii* vs. *L. mayottensis*

In each instance, a Discriminant Analysis of Principal Components (DAPC) was used to identify genes whose presence/absence profiles allowed the discrimination of the defined groups. DAPCs were implemented in R package “adegenet” (Jombart, 2008). We limited the list of discriminant genes to those that contributed the most to the observed clustering in the DAPC by identifying the minimum number of principle component axes required to achieve robust group predictions (using cross validation) and subsampling genes based on their principle component axis loadings. Loadings values for each principle component axis were transformed to normal distributions using the “bestNormalize” R package (Peterson), and then a list of genes with loadings more than two standard deviations from any mean were selected.

The associations between the list of identified discriminant genes and each group category were then assessed. Genes that were representative markers of each group were defined as those discriminant genes that were present in greater than 90 % of the genomes assigned to each group.

### **Identification of protein function**

Predictions of gene functionality were performed using the EGGNog-mapper (Huerta-Cepas et al.) tool. Briefly, gene sequences of all the representative sequences chosen for each orthologous group were translated in-frame to generate a single protein sequence fasta file. This file was uploaded to the EGGNog server, which compares protein sequences to those contained within a

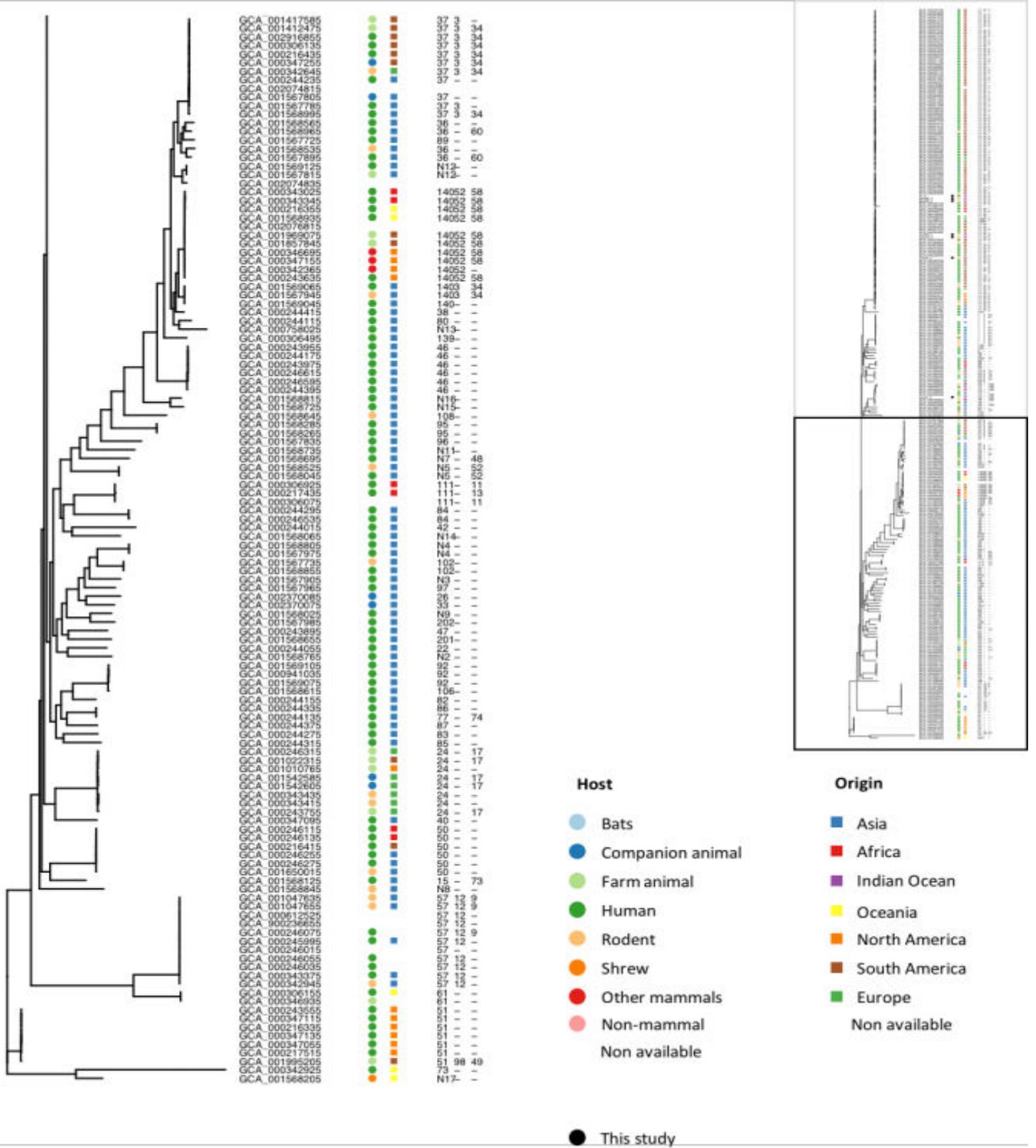
clustered database. Standard functional categories defined for COGs were thus assigned to all orthologous groups. The COG functional categories provide a rough categorization of each protein's function, which is designed to allow an overview of potential functional changes with this form of large dataset.

#### **IV. Comparative genomics: results and discussion**

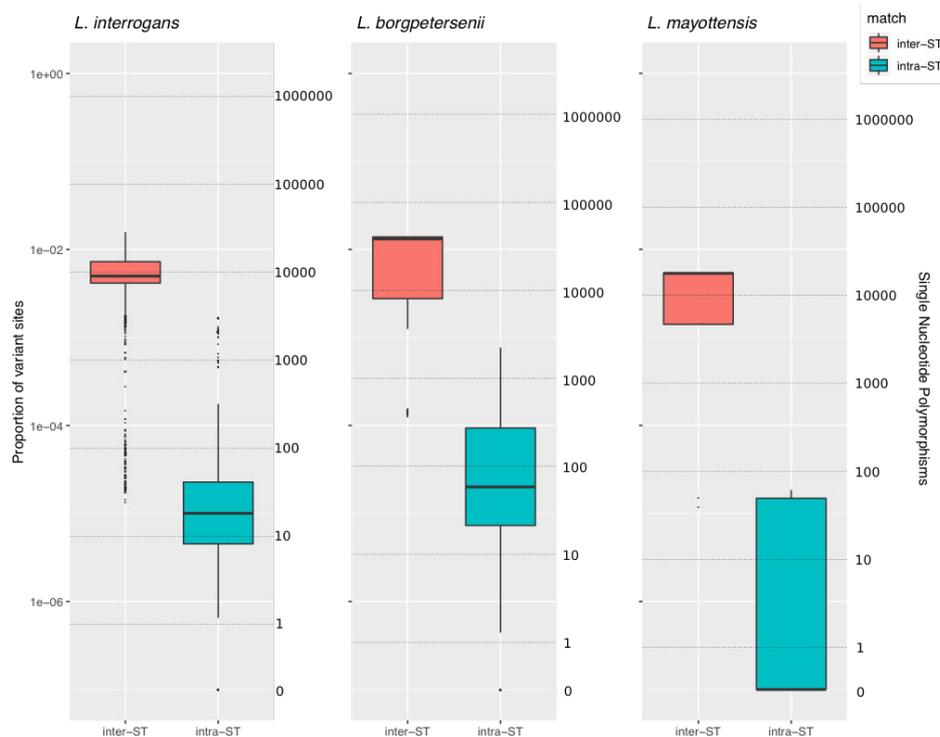
##### **1. Taxonomic classification**

Six of our samples were identified as *L. interrogans* and five of them, originating either from La Réunion or Seychelles, have the same MLST profile as a large number of *L. interrogans* sequences globally distributed and essentially isolated from rodents and humans (**Figure 9**). This highlights the important role of rodents in the transmission of ST17 (MLST scheme 1) all around the world. The last sample of *L. interrogans* originates from rodents of Mayotte and shares a common ST with *Leptospira* sequences from African and Asian rodents as well as Asian human patients (ST49, MLST scheme 1). The analysis of SNPs from *Leptospira* whole-genome reveals low intra-ST diversity (**Figure 10**). When observing conserved elements by this methodology, whole genomes of *L. interrogans* within a specific ST commonly had as few as 10 identifiable SNPs between them in pairwise comparisons. For most intents and purposes, *L. interrogans* identified as ST17 according to MLST scheme 1, despite being distributed all around the world, share identical genomes, no matter their origin. Thus, genomic sequences provide little or no additional resolution for epidemiological classification beyond that of MLST-typing for this ST.





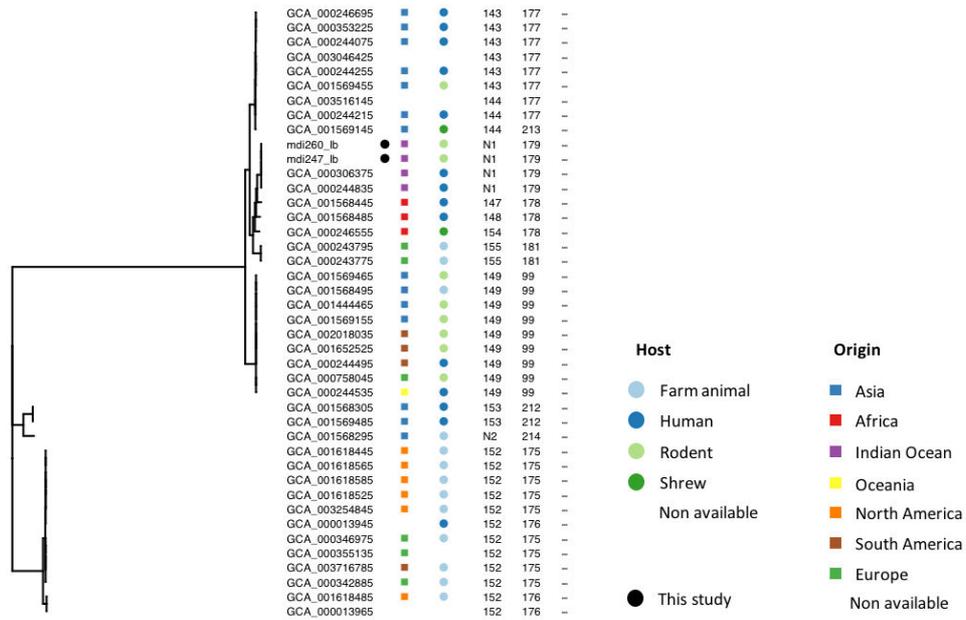
**Figure 9.** Phylogeny including all *L. interrogans* genomes publicly available and *L. interrogans* obtained in this study and isolated in the SWIO region. The ST of each genome are indicated according to MLST scheme 1 (column 1), scheme 2 (column 2) or scheme 3 (column 3). Genomes assembled in this study are indicated with a black dot.



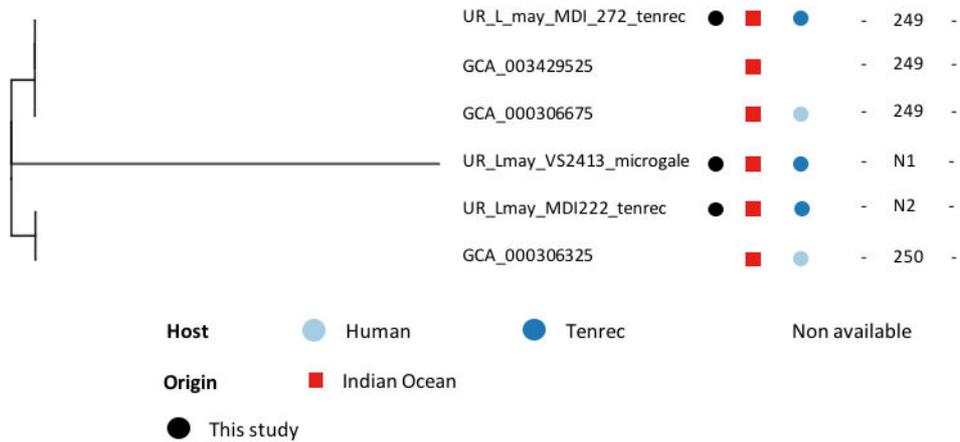
**Figure 10.** SNPs diversity among strains or within STs of *L. interrogans*, *L. borgpetersenii* and *L. mayottensis*

Two of our samples were identified as *L. borgpetersenii* and have an ST not yet identified according to MLST scheme 1, but identical to an ST found in human patients from Mayotte (**Figure 11**). Again, this highlights the important role of rodents in human disease on Mayotte and the uniqueness of *Leptospira* of medical importance on this island. The diversity of *L. borgpetersenii* sequences within each ST is quite low, although higher than for *L. interrogans* (**Figure 10**), suggesting that some level of differentiation may be possible for epidemiologically independent lineages of some *L. borgpetersenii* strains.

Finally, three samples were identified as *L. mayottensis*. These samples include three isolates and two clones obtained from two of the isolates used. All three isolates have a different ST. Two of these isolates (and their respective clones), MDI222 and MDI272, obtained from *Tenrec ecaudatus* cluster with sequences obtained from human patients in Mayotte (**Figure 12**), supporting the role of tenrecs in human disease in Mayotte. Again, the variability of genome sequences within an ST is quite low (**Figure 10**), but this observation has to be interpreted cautiously given the low number of *L. mayottensis* sequences available from public databases.



**Figure 11.** Phylogeny including all *L. borgpetersenii* genomes publicly available and *L. borgpetersenii* obtained in this study and isolated in the SWIO region. The ST of each genome are indicated according to MLST scheme 1 (column 1), scheme 2 (column 2) or scheme 3 (column 3). Genomes assembled in this study are indicated with a black dot.



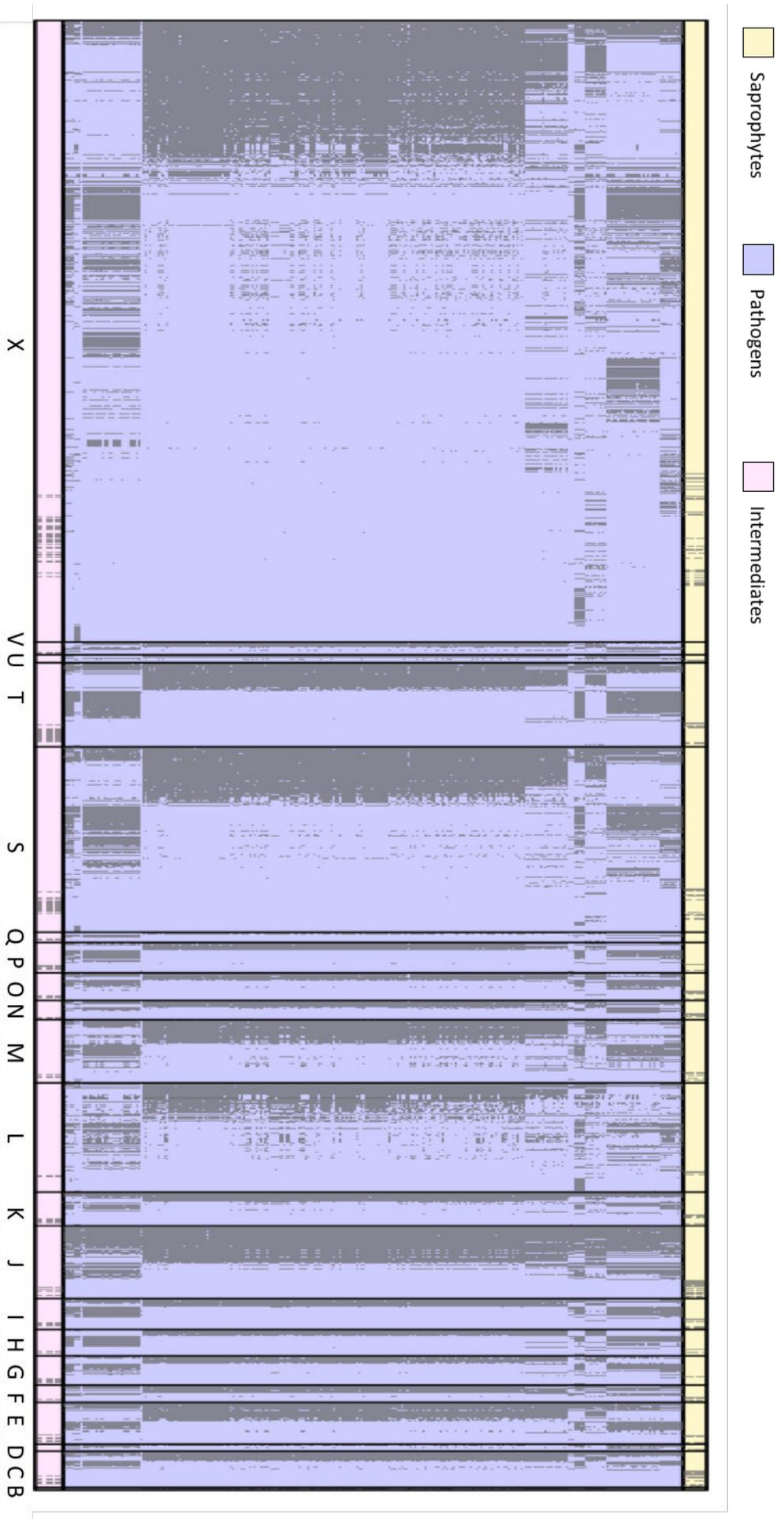
**Figure 12.** Phylogeny including all *L. mayottensis* genomes publicly available and *L. mayottensis* genomes obtained in this study and isolated in the SWIO region. The ST of each genome are indicated according to MLST scheme 1 (column 1), scheme 2 (column 2) or scheme 3 (column 3). Genomes assembled in this study are indicated with a black dot.

These results, obtained from whole genome sequences, confirm that the *L. interrogans* isolates from La Réunion and Seychelles are similar to *L. interrogans* found worldwide while the ST49 from Mayotte has not been reported from many other locations. On the other hand, the STs of *L.*

*borgpetersenii* and *L. mayottensis* clustered with sequences originating from Mayotte only, indicating the uniqueness and potential regional importance of these STs. Lastly, phylogenetic associations support the involvement of rats and tenrecs in disease transmission in the SWIO region.

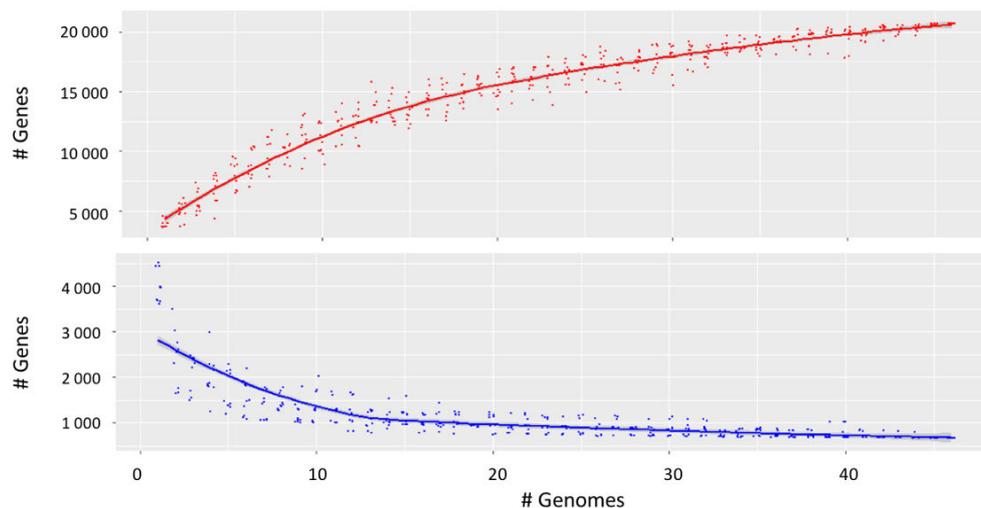
## 2. Pangenome characteristics

The pangenome determined from pewan analyses of a subset of 46 *Leptospira* genomes includes previously published sequences and sequences obtained from our *Leptospira* isolates. A total of 20,689 clusters of orthologous groups (COGs) were identified in the pangenome, including 13,128 accessory genes and 6,316 singletons (genes that were only observed in a single genome). The core genome is composed of genes present in all 46 genomes and includes 672 genes while the soft-core genome is composed of genes present in over 95% of the genomes and contains 1,245 genes (**Figure 13**).



**Figure 13.** Pangenome matrix using all *Leptospira* genomes publicly available and *Leptospira* genomes obtained in this study and isolated in the SWIO region

As shown in **Figure 14**, the number of genes in the pangenome increases with the number of genomes and does not reach a plateau, meaning that the pangenome is open. The open pangenome of *Leptospira* has previously been reported by using a set of 18 genomes of pathogenic *Leptospira* (Xu et al., 2016). This characteristic is shared by bacteria which adapt to multiple environments (Medini et al., 2005), which is consistent with the ability of *Leptospira* to colonize different host species or abiotic environments. On the other hand, the number of genes in the core genome decreases with the number of genomes and also does not reach a plateau, meaning that the more genomes considered, the smaller the core gene set becomes.

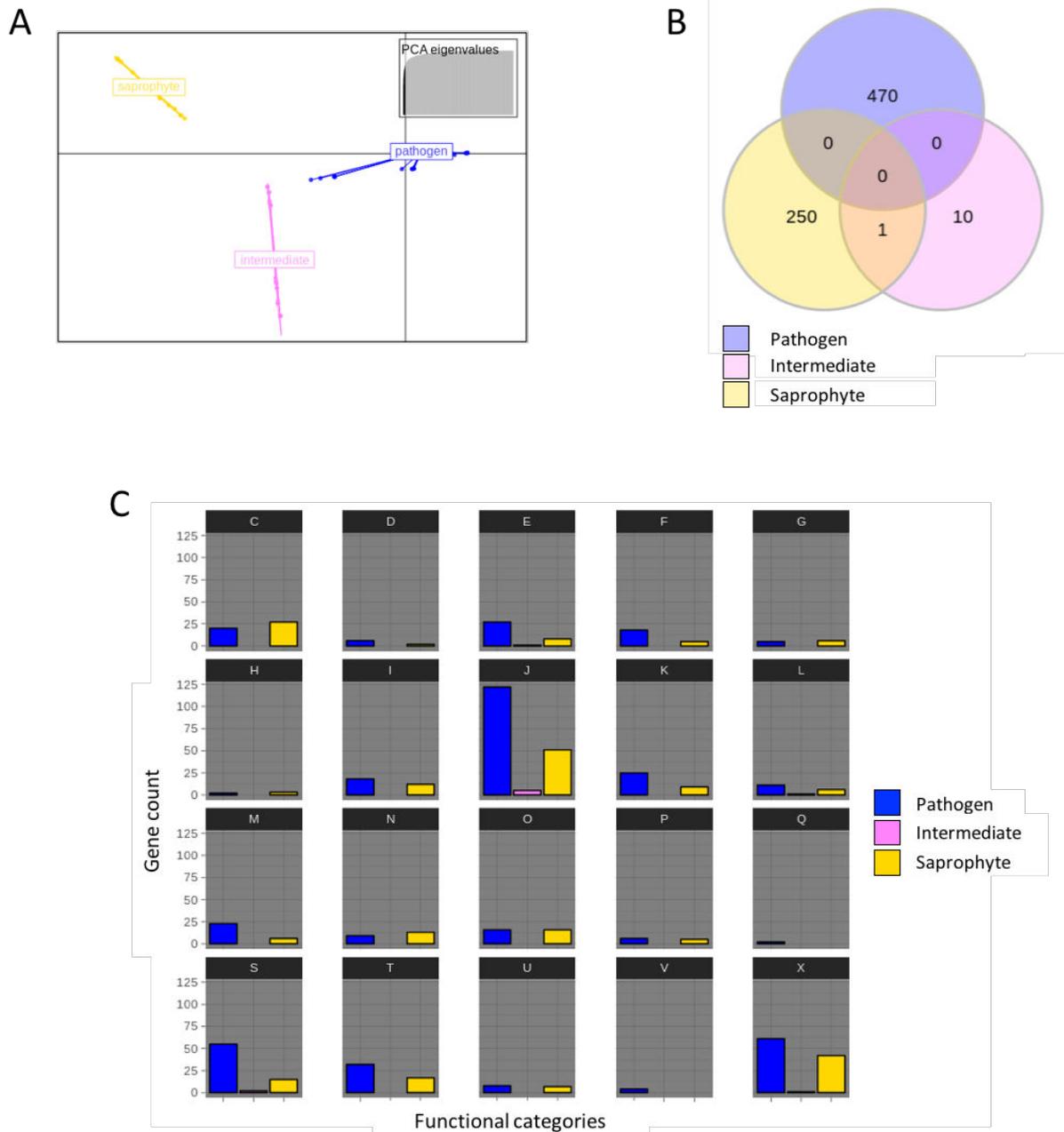


**Figure 14.** Rarefaction curves of the pangenome (red curve) and the core-genome (blue curve) of 46 *Leptospira* genomes including *Leptospira* genomes obtained in this study.

### 3. Genome-Wide Association Study (GWAS)

#### **Types analysis (Pathogens vs. Intermediates vs. Saprophytes)**

The DAPC analysis of the gene presence/absence profiles from each genome allowed the successful discrimination of different groups of *Leptospira*, namely pathogens, intermediate and saprophytes (**Figure 15A**). To determine what genes may distinguish the different groups, a comparative genomic analysis of a large set of genomes was performed. Genes belonging to at least 90% genomes belonging to an individual group were considered to be conserved within that group. As shown in **Figure 15B**, 470 genes are conserved and specific to the pathogenic group, 250 to the saprophytic group and only 10 to the intermediate group. None of these genes are shared between the pathogenic group and the two other groups while one gene is shared between the intermediate and the saprophytic group.



**Figure 15.** Analysis of discriminatory accessory genes of the different types of *Leptospira*. **(A)** Discriminant Analysis of Principal Components performed using the first ten discriminant genes. **(B)** Venn diagram showing the number discriminatory genes specific to each *Leptospira* type. **(C)** Barplots showing the number of discriminant genes assigned to each COG functional category in each cluster.

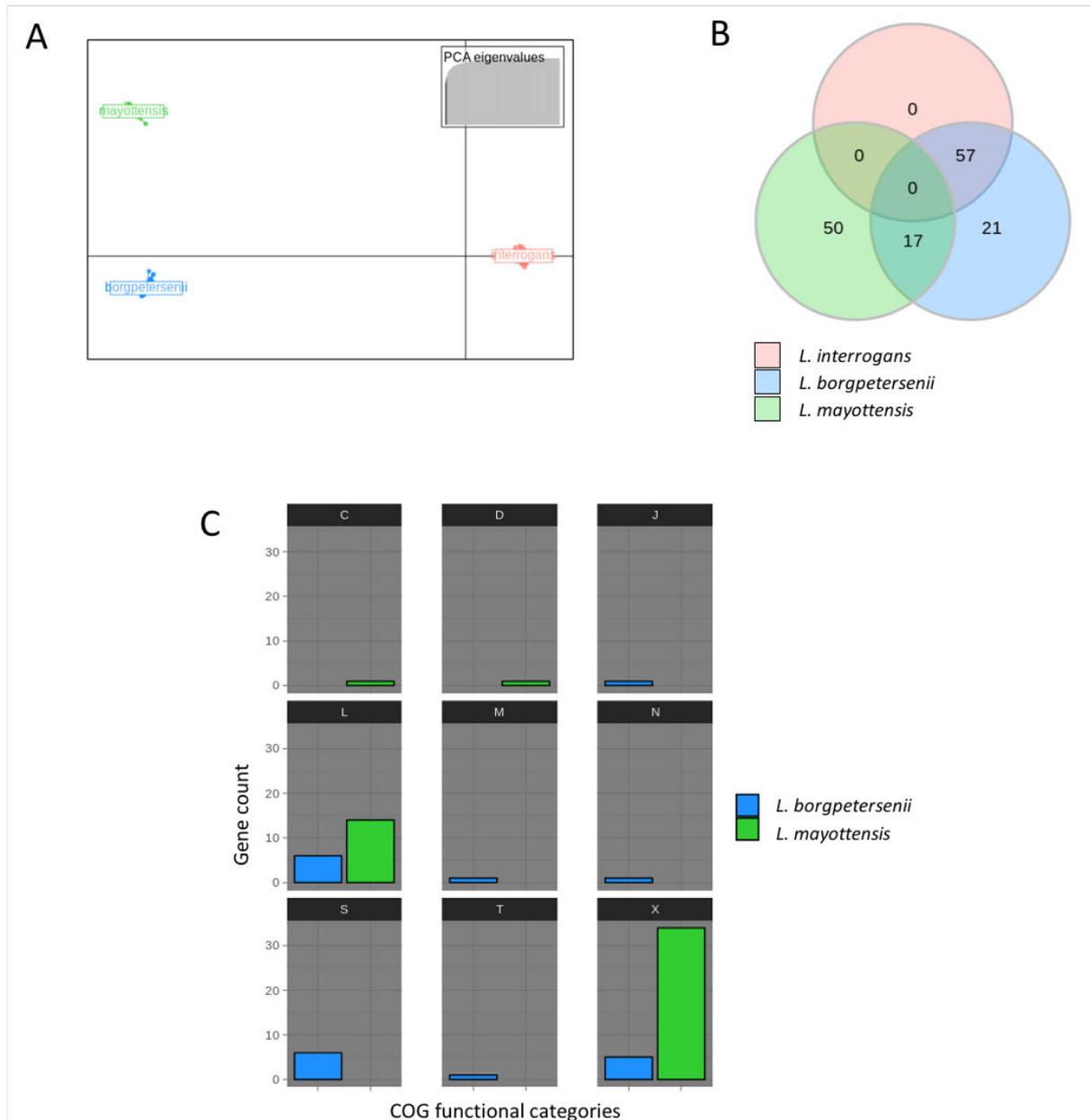
More than 100 genes specific to the pathogenic group are of unknown function (category S) or have not been identified yet (assigned to category X), suggesting the presence of virulence mechanisms specific to the genus *Leptospira* (Picardeau et al., 2008). Another set of over 100 genes specific to the pathogenic group belongs to the category J which includes genes involved in translation and ribosomal structure and proteins (**Figure 15C**). The pathogenic group has a higher number of specific genes than the intermediate and the saprophytic groups which belong to the

functional categories E, F, K, M and T. Genes belonging to these categories are involved in amino-acid and nucleotide metabolism (E and F, respectively), transcription (K), biogenesis of structural elements (M) and signal transduction mechanisms (T). In 2018, Thibeaux et al. distinguished three *Leptospira* groups with differential virulence including 2 subgroups in the pathogenic group (virulent pathogens and low-virulence pathogens) and the intermediate group. As shown by their analysis of discriminatory accessory genes, the virulent pathogens harbor a higher number of genes in functional category M, suggesting an important role of the latter in the virulence of *Leptospira* (Thibeaux et al., 2018). On the contrary, genes included in category E and K may have a less significant role in the virulence of *Leptospira* given their lower number in virulent pathogens compared to low-virulence pathogens and intermediates (Thibeaux et al., 2018). The genes of category K that are specific to the pathogenic group include several genes encoding RNA polymerase and transcriptional regulators. The latter are essential in the virulence but also in adaptation and survival of bacteria (Liu et al., 2006). It appears that only the pathogenic group harbors specific genes belonging to categories Q and V which correspond to secondary metabolism and defense mechanisms, respectively. As for the pathogenic group, the saprophytic group has the highest portion of its specific genes in the category J. This group also has a higher number of specific genes in the category C than the pathogenic and the intermediate groups. Genes in this category are involved in energy production and conversion.

### **Species analysis (*L. interrogans* vs. *L. borgpetersenii* vs. *L. mayottensis*)**

As we experimentally tested the pathogenicity and the host specificity of three *Leptospira* isolates belonging to either of the three pathogenic species *L. interrogans*, *L. mayottensis* and *L. borgpetersenii*, the same aforementioned analysis was performed on the available set of genomes belonging to these three species. Again, the analysis of the pangenome composition allowed us to distinguish the three species based on their gene presence/absence profiles **Figure 16A**. As shown in **Figure 16B**, none of the most discriminatory genes are conserved and specific to *L. interrogans* but 57 are shared between *L. interrogans* and *L. borgpetersenii*. Fifty genes are specific to *L. mayottensis* while 21 are specific to *L. borgpetersenii* and 17 are shared between those two groups. Both groups have specific genes belonging to functional category L (**Figure 16C**) which includes genes involved in replication, recombination and repair. Genes related to phage activity such as transposases and integrases are found in this category indicating that *L. borgpetersenii* and *L. mayottensis* may undergo more genetic rearrangements than *L. interrogans* (Chou et al., 2014). Only *L. borgpetersenii* have specific genes in categories J, M, N and T, which correspond to functions in translation (J) and cellular processes and signaling (M, N, T), or specific genes of unknown function. *Leptospira mayottensis* is the only species with a specific dehydrogenase in category C and D (which corresponds to energy

production/conversion and cell cycle control). As for the distinction of the different *Leptospira* groups, a large portion of the discriminatory genes, including more than 40 genes specific to either of the three species, are of unknown function or previously unreported genes.



**Figure 16.** Analysis of discriminatory accessory genes of three *Leptospira* species. **(A)** Discriminant Analysis of Principal Components performed using the first three discriminant genes. **(B)** Venn diagram showing the number discriminatory genes specific to each *Leptospira* species. **(C)** Barplots showing the number of discriminant genes assigned to each COG functional category in each *Leptospira* species.

## V. Conclusion

The comparative analysis of *Leptospira* genomes allowed us to accurately classify the *Leptospira* isolates that have been isolated in the SWIO region. Sequences of *L. interrogans* isolates

clustered with globally distributed *L. interrogans* isolates of the same ST (ST17). By contrast, the *L. borgpetersenii* and *L. mayottensis* isolates obtained in Mayotte clustered only with isolates from human cases on Mayotte, indicating the endemic nature of those isolates. The very low diversity among sequences within an ST indicates that WGS may not provide more epidemiological information than MLST does.

The open pangenome of *Leptospira* and the decreased number of core genes when adding genomes indicate a lack of conservation of the *Leptospira* genome, which may be consistent with their ability to colonize different environments. The comparative analysis of the discriminant accessory genome of the different *Leptospira* group reveals a higher number of genes from different functional categories in the pathogenic group that may have an important role in the virulence. The same analysis performed on three *Leptospira* species, namely *L. interrogans*, *L. mayottensis*, and *L. borgpetersenii*, revealed specific genes related to phage activities in *L. borgpetersenii* and *L. mayottensis* which may indicate a higher phage activity in those species. By contrast, no specific gene was identified in *L. interrogans*. Overall, these observations suggest that complex polyvalent changes in genomic content are associated with the differentiation of *Leptospira* taxa and pathogen categories. Therefore no single genomic element is likely to be responsible for important epidemiological characteristic of *Leptospira* lineages, such as their virulence or host-association traits.

## **DISCUSSION and PERSPECTIVES**

The investigations on leptospirosis carried out in the western Indian Ocean region in the last decade have revealed a high diversity of *Leptospira* lineages shed by a highly diversified mammalian host community. Most *Leptospira* lineages found in the western Indian Ocean region have novel MLST profiles and express a strong affinity towards their mammalian hosts (Dietrich et al., 2014b; Gomard et al., 2016). This pattern of host specificity is suggestive of a co-diversification between *Leptospira* and their specific hosts. This thesis firstly contributed to provide additional genetic data on *Leptospira* lineages from the eastern African continent. The obtained data confirm the previous observations regarding the structure of *Leptospira* diversity in the western Indian Ocean region: *Leptospira* lineages do not cluster according to their geographic origin but according to their hosts. More specifically, *Leptospira* lineages shed by bats from continental Africa cluster with lineages found in Madagascar and sheltered by closely related bat species. This strongly suggests that ancestral African bat populations that migrated to Madagascar brought *Leptospira* with them. *Leptospira* and their hosts may have then experienced long-term co-diversification processes contributing to the highly diversified *Leptospira* community currently reported in the Malagasy region.

These biogeographical features are associated with a distinct burden of human disease in the different islands of the western Indian Ocean, which is particularly obvious on the two French islands of Mayotte and La Réunion where human leptospirosis is surveyed by the same Public Health Agency (Agence Régionale de Santé-Océan Indien). Epidemiological data reveal differences in severity of human disease on each island. This pattern may result from the presence of different *Leptospira* lineages on each island, resulting in turn from different compositions of the local mammalian reservoir community. To disentangle the contrasted epidemiology of leptospirosis on the islands of western Indian Ocean, a better knowledge of the different *Leptospira* lineages at play is essential. This thesis aimed at exploring relevant features of leptospirosis epidemiology through *in vivo* and *in silico* approaches. For this purpose, three *Leptospira* isolates from the Malagasy region were selected: a rat-borne isolate of *L. interrogans*, a tenrec-borne strain of *L. mayottensis* and a bat-borne strain of *L. borgpetersenii*. The first strain is present worldwide while the two latter have only been described in the Malagasy region and are considered as endemic to the region. In chapters 1 and 2, we aimed at testing the virulence and the host specificity of the aforementioned *Leptospira* isolates *in vivo*, respectively. In the third chapter, we explored genomic features possibly involved in virulence and/or host-specificity by comparing the genomes of a set of *Leptospira* strains with available whole-genome or draft genomes sequences.

## I. Virulence features of *Leptospira* isolates from the Malagasy region

The virulence of the three *Leptospira* isolates was tested through experimental infection of hamsters, an animal model mimicking acute leptospirosis and commonly used to characterize pathogenic *Leptospira* strains. This chapter reveals a higher virulence of the cosmopolitan rat-borne isolate compared to the isolates endemic to the Malagasy region as shown by the higher bacterial load and more severe damage observed in *L. interrogans*-infected hamsters' tissues. Both endemic isolates (*L. mayottensis* and *L. borgpetersenii* isolates) induced no clinical symptoms in hamsters although the *L. mayottensis* isolate harbored a genetic profile identical to isolates obtained from human acute cases.

In a previous study conducted in Mayotte, the authors managed to obtain 94 *Leptospira* isolates representing 48% of autochthonous leptospirosis human cases from 2007 to 2010 (suppl. Material in Bourhy et al., 2012). These isolates were distributed in four species, namely *L. interrogans*, *L. borgpetersenii*, *L. kirschneri* and *L. mayottensis* (previously named *L. borgpetersenii* group B) representing 8%, 53%, 22% and 16% of obtained autochthonous isolates, respectively. Ten patients infected by one of the three former species and from which *Leptospira* isolates could be obtained, were admitted in intensive care unit. Two fatal issues resulted from infection by *L. borgpetersenii* and one by *L. kirschneri*, respectively. On the other hand, *L. mayottensis*-induced cases did not lead to any admission in ICU and no death. Thus, there is a correspondance between the virulence profiles obtained in our study and those observed in human disease in the region. However, this set of data does not include all leptospirosis patients and some observations may be missing to conclude on the pathogenicity level of *L. mayottensis*. It would be interesting to describe the symptoms observed in all human cases of leptospirosis and specify the species/strains responsible for the differential pathologies observed among human clinical cases.

It is possible that isolates of *L. mayottensis* and *L. borgpetersenii* may have induced a mild, self-resolving illness in hamsters preventing the detection of clinical symptoms during one month of infection. Nonetheless, the necropsy of two *L. mayottensis*-infected hamsters at intermediate time points reveals lung tissue damage that could be self-resolving. To investigate this further, it would be interesting to repeat the experiment by performing necropsy of hamsters at regular intervals after the infection and assess possible tissue damage that could be induced by less pathogenic isolates even though no clinical signs are observable.

It has been previously observed that male patients tend to be more susceptible to severe symptoms of leptospirosis than female patients (Jansen et al., 2007). Moreover, a recent study highlighted that male hamsters were more susceptible to leptospirosis than females at a lower dose of inoculation ( $10^3$  leptospores) than the one used in our study (Gomes et al., 2018; Tomizawa et al.,

2017). Given the lack of clinical symptoms induced by the endemic *Leptospira* strains, it can be supposed that the use of female hamsters in this study did not allow the observation of possible symptoms induced by the endemic isolates although a high dose of leptospires was used ( $2 \times 10^8$  leptospires). Thus, repeating this experiment by using male hamsters may provide more information on the pathogenic potential of the studied strains of *Leptospira*.

*Leptospira* infection can induce a strong immune response that may be responsible for tissue damage. Indeed, high levels of cytokine expression have been reported in severe disease (Cagliero et al., 2018; Reis et al., 2013; Tomizawa et al., 2017). Investigating the balance of pro- and anti-inflammatory cytokines using all three studied strains may shed light on the role of the host immune response on the infection.

## II. Infectivity of endemic and host-specific *Leptospira* isolates in new hosts

Our results show that *L. borgpetersenii* and *L. mayottensis* isolates, considered endemic and specific to their respective hosts, failed to induce renal colonization in *R. norvegicus* as shown by the absence or low amounts of *Leptospira* DNA in the urine of rats infected by either one of these two isolates. Hence, the rat may not be a reservoir for both endemic *Leptospira* isolates.

The ability/inability of *Leptospira* isolates to colonize a host genetically different to their original host may depend on both host and pathogen genotypes, or the interaction between them (Bono et al., 2017). Previous studies of the Friend virus complex reported that when the virus is inoculated into a host with an unknown genotype compared to their natural host, its fitness is reduced as well as its virulence (Kubinak et al., 2013). Given the lower infectivity of the endemic isolates as shown by the absence of clinical symptoms in hamsters, which are susceptible to *Leptospira*, the adaptation of these leptospires to a new host may be too costly an impede sufficient multiplication to allow them establishing renal colonization of a new host (i.e. the rat in this study). These costly adaptations may involve the escape of an immune response of the new host which is unfamiliar to the endemic leptospires and may induce decreased fitness (Kubinak et al., 2012). In a similar way, pathogens that are highly specialized to their hosts undergo virulence tradeoffs when invading novel hosts (Kubinak et al., 2012), which could explain the low virulence of the *L. borgpetersenii* and *L. mayottensis* isolates when infecting hamsters. Thus, the specialization of *Leptospira* strains towards specific hosts may prevent the expression of a virulence phenotype, decreasing the pathogenic effects of these strains in susceptible hosts and their ability to cross the host species barrier and colonize novel hosts.

Whether the endemic strains used here are able to infect other animals still remains unknown but the inability to establish renal colonization in synanthropic rats limits their dispersal

potential in the environment and hence likely lowers the risk of human infection. The risk of human infection with strains specific to their hosts will depend on the physical contacts that could be established between these reservoirs and humans. If the distribution of leptospires is driven by a host specificity pattern, this may constrain several pathogenic strains to specific environments with low human influence, thus limiting the medical importance of those bacterial strains.

Previous studies have shown that serial passages of a pathogen through an unfamiliar resistant host may enhance the fitness of the pathogen within this new host (Kubinak et al., 2014; Kubinak and Potts, 2013). It may be possible that several passages of endemic *Leptospira* into rats are necessary so they can achieve renal colonization in this unfamiliar host. The host specificity of *Leptospira* observed in the field may be due to genetic factors of the host and/or the pathogen but also to a lack of contact between their natural hosts and another reservoir such as rats, impeding the adaptation of these endemic leptospires to new hosts. This hypothesis can be supported by the host switch observed within some *Leptospira* lineages and their bat hosts which share a common habitat, allowing frequent physical contact between individuals from different species and *Leptospira* adaptation to a novel host (Gomard et al., 2016).

Nonetheless, the results obtained in this study must be interpreted with caution. The cosmopolitan rat-borne *L. interrogans* managed to induce renal colonization but the rate of renal colonization was low (28.6%) compared to other studies investigating the infection of *R. norvegicus* by *L. interrogans* strains. The strain of *L. interrogans* used in this study was originally isolated from *R. rattus* while the rats used in our study were *R. norvegicus*. The *L. interrogans* isolate may present features that complicate renal colonization in *R. norvegicus*. This isolate could also be less invasive than previously studied strains of *L. interrogans*.

Our observations support a pattern of host-specificity for endemic *Leptospira* lineages although only one potential reservoir was challenged in our experiments. It would be interesting to carry out symmetrical experiments in which the endemic hosts of both endemic *Leptospira* lineages are used as experimental models and verify that they are hosts to their specific *Leptospira*. Such experiments may in addition allow testing if these reservoirs (i) support chronic infection with the cosmopolitan *L. interrogans* isolate, (ii) are susceptible to the *L. interrogans* isolate and develop acute disease or (iii) are able to clear this infection. Nonetheless, lab colonies of bats and tenrecs are necessary to perform such experiments but these are uncommon in the field of animal experimentation. Establishing a colony of *Triaenops* bats looks unrealistic. However, we are aware of a *T. ecaudatus* colony established in Nevada. The establishment of other colonies is forecasted in Madagascar as this tropical animal displays unique physiological features that are of great interest to physiologists and space scientists, i.e. their ability to undergo hibernation without interbout arousal

(Lovegrove et al., 2014). If such colonies were settled in Madagascar, that would provide an interesting setup to further explore the virulence and host specificity of pathogenic *Leptospira* endemic to Malagasy region.

### III. Genomic features of *Leptospira*

The analysis of the bacterial pangenome is useful to better understand the evolution of bacteria. The pangenome may inform on the diversification status of the bacteria. Open pangenomes are associated with bacteria that occupy diverse ecological niches and need to adapt to different environments (Medini et al., 2005). By contrast, closed pangenomes are associated with highly conserved bacteria that occupy specific and isolated ecological niches and thus do not undergo lateral gene transfer from their environment. In this thesis, the pangenome of a subset of 46 *Leptospira* strains revealed an open pangenome. The overlap of an open pangenome with a decreased number of core genes when adding genomes suggests that *Leptospira* lineages undergo relatively frequent gene loss and gain, which is consistent with the high diversity of environments that the different species of *Leptospira* can colonize (Xu et al., 2016).

The core genome informs on the conserved genes within a group of bacteria. By contrast, the accessory genome may shed light on genes involved in phenotypic traits specific to one group of bacteria.

Firstly, we compared the discriminant accessory genome of three commonly referred to groups of *Leptospira*, namely pathogens, intermediates and saprophytes. Through this comparison, we tried to identify genes involved in virulence, given the different virulence phenotypes observed among the three groups. Our results suggest an important role of genes encoding for the biogenesis of structural elements (i.e. cell wall, membrane and envelope) in virulence given their higher number in the pathogenic group and as reported previously (Thibeaux et al., 2018). Genes encoding secondary metabolism and defense mechanisms are also important as suggested by their presence in the discriminant accessory genomes of pathogenic *Leptospira* only. This is consistent with the necessity for *Leptospira* to escape the host immune system to induce infection and renal colonization.

Secondly, we compared the discriminant accessory genome of three *Leptospira* species that include the three isolates we used in the previous chapters. Given the host specificity observed for *L. mayottensis*, this comparison may allow the identification of specific genes that are involved in host adaptation. Most genes specific to *L. mayottensis* may be associated with mobile genetic elements such phage, as indicated by the presence of transposases and integrases. This may suggest that genetic rearrangements are involved in the host specificity of *L. mayottensis*. However, the presence

of genes with similar functions in *L. borgpetersenii*, within which the host specificity is not systematic, imply that these genes may be involved in other phenotype traits specific and common to all strains of *L. borgpetersenii*. Interestingly, no specific discriminant genes were associated with *L. interrogans*. The set of *L. interrogans* genomes include mostly sequences obtained from human cases, indicating the virulent feature of this group. The absence of specific genes for *L. interrogans* may involve that virulence in this group is mediated by gene loss. This would be consistent with a previous study reporting an increase fitness of *Leptospira* mutants lacking some specific genes (Lourdault et al., 2016).

The analysis of the most discriminant accessory genes is useful for the identification of associations between specific genes and specific phenotypic traits attributable to pre-defined groups. The definition of the groups to be compared is thus a critical step in the analysis of the discriminant accessory genome. Indeed, the accessory genome contains genes that may be specific to a limited number of strains within a group (Lehmann et al., 2014). If groups are highly diverse, the discriminant accessory genome may lack important information on phenotypic traits specific to undefined subgroups. For instance, in the first comparative analysis, we used the three renown groups of *Leptospira* to identify genes specific to virulence. In a previous study, the pathogenic group was subdivided into two clusters including virulent pathogens and low-virulence pathogens. This subdivision applied to our results highlighted differential importance of genes included in different functional categories in the virulence of *Leptospira* while our analysis alone inferred the same importance of different functional categories. To more accurately identify genes involved in virulence and host adaptation, groups with higher resolution have to be defined and compared. Nonetheless, the open pangenome associated with the presence of a large amount of unreported genes, suggesting mechanisms specific to *Leptospira* (Picardeau et al., 2008), make the identification of such genes more difficult.

Spirochetes diverged from other bacteria during early evolutionary history (Saier, 2000), which is surely related to the numerous unique cell structures typical of this order and to the abundance of genes of unknown function including in *Leptospira*. This makes the investigation of these slow growing bacteria even more challenging but should not temper our enthusiasm to study a prototypical zoonotic pathogen of global medical importance.

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## Other communications

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RESEARCH ARTICLE

# Human Leptospirosis on Reunion Island, Indian Ocean: Are Rodents the (Only) Ones to Blame?

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## Abstract

### Background

Although leptospirosis is a zoonosis of major concern on tropical islands, the molecular epidemiology of the disease aiming at linking human cases to specific animal reservoirs has been rarely explored within these peculiar ecosystems.

### Methodology/Principal Findings

Five species of wild small mammals ( $n = 995$ ) as well as domestic animals ( $n = 101$ ) were screened for *Leptospira* infection on Reunion Island; positive samples were subsequently genotyped and compared to *Leptospira* from clinical cases diagnosed in 2012–2013 ( $n = 66$ ), using MLST analysis. We identified two pathogenic species in human cases, namely *Leptospira interrogans* and *Leptospira borgpetersenii*. *Leptospira interrogans* was by far dominant both in clinical samples (96.6%) and in infected animal samples (95.8%), with *Rattus spp* and dogs being its exclusive carriers. The genetic diversity within *L. interrogans* was apparently limited to two sequence types (STs): ST02, identified among most clinical samples and in all rats with complete MLST, and ST34, identified in six humans, but not in rats. Noteworthy, *L. interrogans* detected in two stray dogs partially matched with ST02 and ST34. *Leptospira borgpetersenii* was identified in two clinical samples only (3.4%), as well as in cows and mice; four haplotypes were identified, of which two seemingly identical in clinical and animal samples. *Leptospira borgpetersenii* haplotypes detected in human cases were clearly distinct from the lineage detected so far in the endemic bat species

**Competing Interests:** The authors have declared that no competing interests exist.

*Mormopterus francoismoutoui*, thus excluding a role for this volant mammal in the local human epidemiology of the disease.

## Conclusions/Significance

Our data confirm rats as a major reservoir of *Leptospira* on Reunion Island, but also pinpoint a possible role of dogs, cows and mice in the local epidemiology of human leptospirosis. This study shows that a comprehensive molecular characterization of pathogenic *Leptospira* in both clinical and animal samples helps to gaining insight into leptospirosis epidemiology within a specific environmental setting.

## Author Summary

Leptospirosis is a zoonosis caused by infection with pathogenic *Leptospira* species. A broad range of animals, including rodents, pets and livestock, are maintenance hosts for leptospires. However, assessing the relative importance of each host in the contamination of the environment and, *in fine*, in the infection of humans, has rarely been performed. In this study, we surveyed various wild and domestic animal species and their *Leptospira* carriage in Reunion Island, where human leptospirosis is endemic. We determined and compared the *Leptospira* genetic diversity at the species and infra-species levels in laboratory-confirmed human cases and in infected animals. The two *Leptospira* species infecting humans, *Leptospira interrogans* and *Leptospira borgpetersenii*, could be traced back to different animal species: rats and dogs for the former species, cows and mice for the latter. The *Leptospira* infecting the single bat species endemic to the island was not found to be involved in human leptospirosis. Aside from rats, which were expected to play a role in the local epidemiology of the disease, the putative role of dogs, cattle and mice in human epidemiology on Reunion Island, pinpointed by our data, deserves a specific investigation. These results have strong implications in terms of local control actions aimed at reducing the burden of human leptospirosis.

## Introduction

Leptospirosis is a bacterial systemic infection, occasionally fatal, caused by the pathogenic spirochetes of the genus *Leptospira*. Though claimed as the most widespread zoonosis in the world [1,2], the disease is considered as emerging in many parts of the world. Leptospirosis is most prevalent in tropical and subtropical countries [3,4], presumably because the survival of the bacterium outside the host requires humid and warm conditions that are typical of tropical areas [5]. Rodents are recognized as the main reservoir of pathogenic *Leptospira* although several animal species are also capable of sustaining biofilm colonization of the renal tubules and shedding the bacteria in their urine [6]. Humans usually get infected through indirect exposure with water or soil contaminated with urine, but direct transmission has also been suggested as important for some species [7].

Traditionally, *Leptospira* have been classified serologically into 25 serogroups and over 300 serovars using Microscopic Agglutination Test (MAT) and Cross agglutination absorption test (CAAT) analysis, respectively [1,8]. More recently, a genetic classification based on DNA-DNA hybridization complemented by molecular methods and experimental studies have confirmed the existence of at least 22 *Leptospira* species [9]. The congruence between serological and

molecular classifications is poor [1]: one serogroup can be linked to several *Leptospira* species while serovars can vary within a given clone or lineage, an observation considered as probably resulting from horizontal gene transfer [10]. Among the various molecular tools currently used to genotype *Leptospira*, multilocus sequence typing (MLST) has emerged as a method of choice as it provides data produced at a local scale that can be further compared to genotypes obtained all over the world and made freely accessible to the scientific community through public databases.

Leptospirosis is endemic in several islands of the southwestern Indian Ocean (SWIO) including the two French overseas departments of Mayotte [11,12] and Reunion Island [13,14] together with the island state of Seychelles [14–16], the latter recording the highest human incidence reported worldwide [17]. On Madagascar, serological evidence of exposure to leptospirosis has been reported in the human community of Moramanga [18], while to our knowledge, a single case of acute leptospirosis infection has been PCR-confirmed on a traveller returning from Madagascar [19]. Only serological evidence of human exposure to pathogenic *Leptospira* has been reported on the three neighboring islands of Union of the Comoros [20], while no or only scarce data is available for Mauritius [21] and Rodrigues Islands. On Mayotte, improved diagnostic procedures allowed to significantly increase the number of confirmed cases, with about 100 new human cases reported each year since 2009 [12,22]. The MLST analysis of *Leptospira* isolated from human incident cases on the island revealed a large bacterial diversity of clinical isolates, represented by at least 17 different “sequence types” (STs) and four distinct *Leptospira* species, namely *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira kirschneri*, and the newly described *Leptospira mayottensis* [12,23].

Comparatively, information is more abundant on pathogenic *Leptospira* infecting wild animals from the SWIO. On Mayotte, sequencing of a portion of the 16S rRNA (*rrs*) gene of leptospires infecting twenty black rats *Rattus rattus* has identified the same four *Leptospira* species previously reported in clinical cases from the same island, with a strict identity between the sequences of *Leptospira* infecting *R. rattus* and humans [11], designating this rodent species as the probable major reservoir and transmission source of *Leptospira* to humans on Mayotte [11]. On Comoros and Madagascar, sequencing of the nearly complete 16S rRNA gene of *Leptospira* infecting kidneys from six bat species ( $n = 7$ ) has shown the carriage of *L. interrogans* (Comoros), *L. borgpetersenii* (Comoros and Madagascar) and other thus far unknown *Leptospira* species [24]. On Madagascar, partial sequencing of *rrs* locus has identified a single *L. interrogans* haplotype among 70 samples from introduced small mammals (rats, shrews and mice) [25]. Only one full five gene-based MLST analysis has been reported so far from the SWIO region, i.e. on Madagascar, where authors identified *L. borgpetersenii*, *L. mayottensis* and *L. kirschneri* from endemic small mammals and bats [26]. This study revealed distinct clustering associated with host type, with no overlap between *Leptospira* species infecting endemic small mammals versus those infecting introduced ones, a feature which certainly deserves further investigation.

On Reunion Island, human leptospirosis was first reported in 1953 but the first outbreak most likely occurred in 1868 [27]. Since 1953, several studies have been conducted to assess the burden of leptospirosis on the island. From 2008 to 2012, the mean annual incidence was estimated at 8.2 cases per 100,000 inhabitants with a fatality rate around 4%, and Icterohaemorrhagiae identified as the major serogroup in severe forms [13]. Lower prevalences of serogroups Canicola, Grippityphosa and Australis have also been reported [28]. Pagès and colleagues [13] have highlighted the following population groups as being at highest risk: farmers and green space workers, people under 20 years old and participating in aquatic activities, people between 20 and 30 years old that fish, and people between 50 and 60 year old gardening at home. The seroprevalence of infection and/or *Leptospira* carriage in potential reservoirs, have

been explored in wild small mammals (rodents, shrews, tenrecs and bats) and domestic animals (dogs, cats, cattle, goats, swine, rusa deers and horses) [14,29–32]. Since then, the black rat, *Rattus rattus*, abundant in most regions and biotopes of the island, has been considered to be the primary reservoir and transmitter of *Leptospira* spp., a conclusion mainly based on the observation that Icterohaemorrhagiae is by far the main serogroup found in rats and clinical cases [29], though the same serogroup has also been identified in dogs, pigs, rusa deer and tenrecs [29]. Recently, the first molecular study from Reunion Island identified that patients with acute leptospirosis (n = 42) were all infected by *L. interrogans* [33]. Although the typing method, High Resolution Melting (HRM) analysis of two VNTR sequences, allows a rapid diagnosis on clinical samples together with the characterization at the serovar and species levels, it is likely not resolute enough as to trace the source(s) leading to human infection and illness.

The aim of our study was to determine the wild and/or domestic animal species that not only serve as reservoir hosts for pathogenic *Leptospira* species, but also are primary sources for human infection on Reunion Island, an area considered as endemic for human leptospirosis.

## Materials and Methods

### Human samples

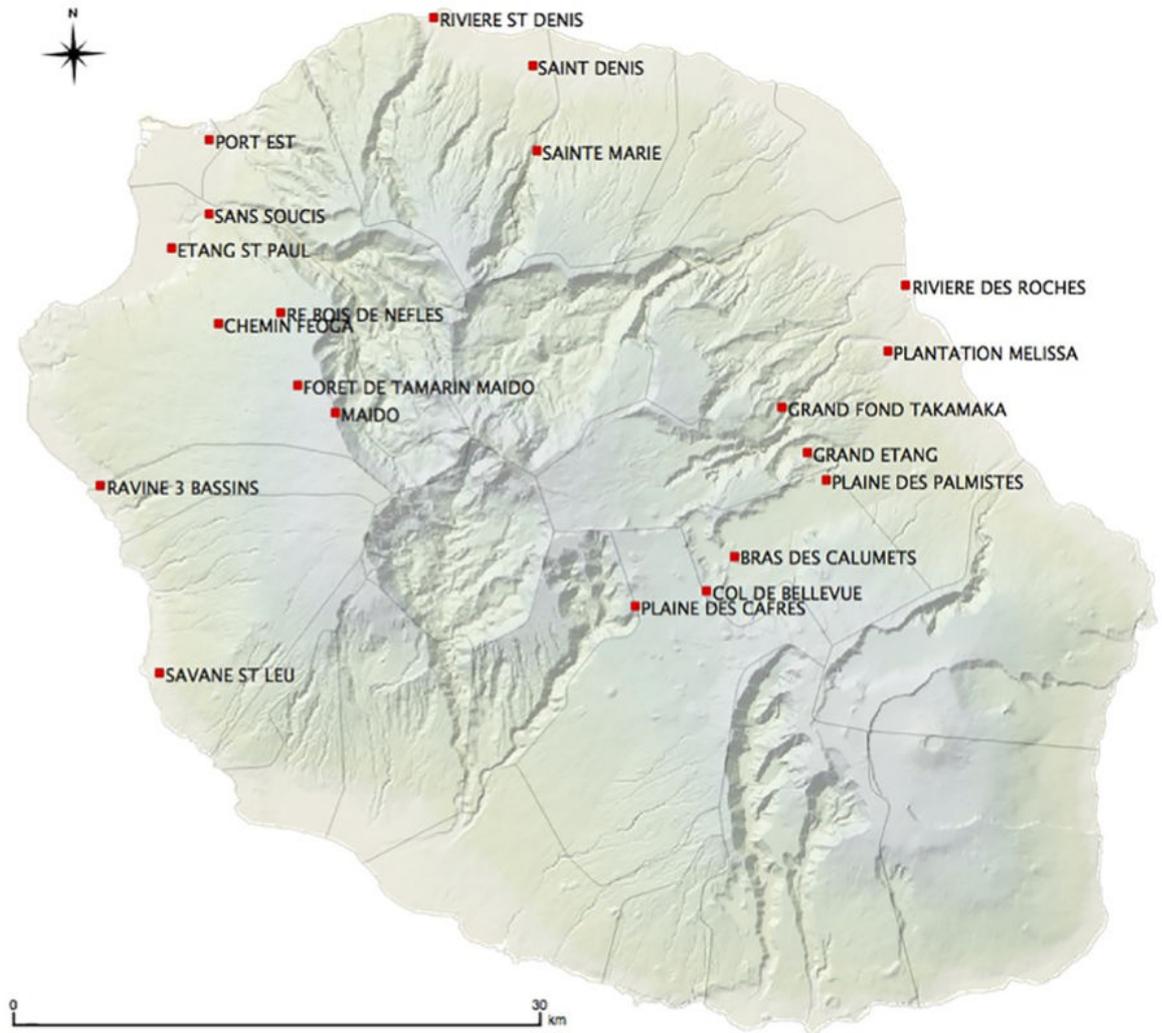
Sixty-six patients whose blood samples tested positive for *Leptospira* (see details hereafter) were included in the study. Sera were collected for diagnostic purpose from patients originated from all four sanitary regions recognized on the island and admitted for acute febrile syndrome in 2012–2013 to either of the two main University hospitals of La Reunion (northern and southern University hospitals, hereafter referred to as NUH and SUH respectively). All patients included in the study were considered as autochthonous leptospirosis cases as none of them reported travel history during the month preceding the onset of symptoms.

### Animal samples

We trapped 799 rodents (562 *Rattus rattus*, 170 *Rattus norvegicus*, 67 *Mus musculus domesticus*), 171 shrews (*Suncus murinus*) and 25 tenrecs (*Tenrec ecaudatus*), those species representing all terrestrial small mammal diversity occurring on Reunion Island. Trapping occurred in 2012–2014 during dry and rainy seasons on twenty sampling sites, fifteen of them selected along two altitudinal transects on wet windward and dry leeward coasts (Fig 1). Details on trapping of wild animals and study sites have been published elsewhere (see [34]). Kidneys from cows (n = 33) and pigs (n = 22) were directly collected at the unique slaughterhouse of the island, which was not able to provide information regarding the geographic origin of the samples. Kidneys from stray dogs (n = 45) were collected just after the animals were euthanized at the community pound of Saint André (eastern coast). The only cat sample included in the study was urine collected by a veterinarian.

### Ethics statement

The ethical terms of the research protocol were approved by the CYROI institutional ethical committee (Comité d’Ethique du CYROI n°114, IACUC certified by Ministry of Higher Education and Research) and by the Ministry of higher education and research under accreditation 03387.01 (LeptOI). All animal procedures carried out in our study were performed in accordance with the European Union legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU). The stray dogs were euthanized by lethal injection administered by a veterinarian in the frame of population control measures implemented by the local



**Fig 1. Sampling sites along the two altitudinal transects on western and eastern coasts, together with additional sampling sites in the north and west coast of Reunion Island.**

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authorities. Residual sera from anonymized clinical samples collected for diagnostic purposes and laboratory-confirmed as leptospirosis cases were obtained from the clinical laboratories of NUH and SUH.

### PCR detection for diagnosis

Genomic DNA was extracted from human sera at hospital laboratories using NucliSENS easy-MAG system (BioMérieux, Marcy l’Etoile, France) (NUH) or Dneasy Blood and Tissue Kit (Qiagen, Courtaboeuf, France) (SUH) according to the manufacturer’s instructions. The presence of *Leptospira* was assessed with a probe-specific real-time PCR targeting either (i) the 23S rRNA gene [35] in NUH or (ii) LA0322 locus (LFB1 primers) [36] in SUH.

Extraction of total nucleic acids from animal samples was performed from a pool of kidney, lung and spleen tissues (for wild animals) or from kidneys only (for domestic animals) using the Biorobot EZ1 and EZ1 Virus Mini Kit version 2.0 (QIAGEN, Les Ulis, France). A reverse transcription step was then performed with GoScript reverse transcriptase (Promega,

Charbonnières-les-Bains, France); generated cDNA was used as template for *Leptospira* detection through a previously described probe-specific real-time PCR targeting the *rrs* (16S rRNA) locus [24,37]. Animal samples leading to a PCR amplification at Ct < 42 were considered as positive.

### *Leptospira* isolation

Twenty-four animal samples were randomly selected in order to attempt *Leptospira* isolation from kidney cultures, i.e. 15 *R. rattus*, two *R. norvegicus*, two *M. musculus*, three *S. murinus* and two *T. ecaudatus*. A small piece of the freshly sampled kidney was crushed under sterile conditions and used to inoculate three distinct culture media: (i) Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco, Detroit, MI, USA) supplemented with Albumin Fatty Acid Supplement (AFAS; Royal Tropical Institute, Amsterdam, Netherlands) [38,39]; (ii) EMJH liquid medium supplemented with AFAS, rabbit serum and foetal calf serum (1% each); and (iii) semisolid Fletcher medium (Difco, Detroit, MI, USA) supplemented with rabbit serum (8%). All media were supplemented with 5-fluorouracil (5-FU) at a final concentration of 200 µg.mL<sup>-1</sup>. Cultures were incubated at 28°C, visually checked for the presence of *Leptospira* using a dark field microscope once a week for four months, and positive cultures were further sub-cultured in fresh EMJH liquid medium deprived of 5-FU. DNA was extracted from 1 mL of each positive culture using the EZ1 Biorobot with Qiagen EZ1 DNA Tissue kits (Qiagen, Les Ulis, France).

### *Leptospira* genotyping

Three major MLST schemes exist for *Leptospira* spp. typing worldwide, all supported by the website database <http://pubmlst.org/leptospira/>. In our study, scheme #3 [40] was preferred to the other two [10,41] as several molecular data from the SWIO have been made freely available using this same scheme, hence allowing comparison of local STs with those from SWIO islands [12,26]. MLST was attempted for *adk*, *icdA*, *lipL32*, *rrs2*, *secY*, and *lipL41* loci as previously described [40] and recently optimized [26]. All positive domestic animal samples were first submitted to *secY* sequencing, revealing a nearly clonal population among infected rats (see Results). Hence, a randomly selected subgroup of positive wild animal samples was selected from eleven sites (n = 17; Table 1) and submitted to full MLST genotyping.

For species identification of other positive wild animal samples, including two culture isolates, the *secY* gene was amplified with the primers *secY* F/R [40] or alternatively G1/G2 [42]. When *secY* PCR amplification failed, a 16S rRNA gene sequencing was alternatively used with previously published primers, i.e. LA/LB [43] or LA/R2 [44].

Obtained amplicons were directly sent for direct Sanger sequencing on both strands (Genoscreen, Lille, France), or alternatively cloned into the pGEM-T easy vector (Promega, Madison, WI, USA) when the quantity was insufficient for direct sequencing. Obtained clones (five per sample) were subsequently sequenced using M13 universal primers for Sanger sequencing. The overall analysis strategy is presented in Fig 2.

### Phylogenetic analysis

Consensus sequence for each sample and multiple alignments between sequences were obtained using Geneious Pro version 5.4 [45]. In order to provide a comparison with *Leptospira* strains occurring worldwide, our dataset included sequences from two human clinical isolates from Mayotte [12] and 118 human and animal isolates from all over the world [2]. Sequence alignments were constructed separately for all six considered loci. A five-loci concatenate was generated using SEQMATRIX v1.7.8 [46], excluding *icdA* locus that was unavailable

**Table 1. Leptospirosis testing results by host species.** *Leptospira* prevalence after real-time PCR diagnosis; species identification, either by 16S rRNA or *secY* sequencing and rate of success; and *secY* or MLST amplification and sequencing success.

	<i>Leptospira</i> prevalence (%)	Identification success (%)	Infecting <i>Leptospira</i>	<i>secY</i> amplification <sup>a</sup>	6-loci (or 5 <sup>b</sup> ) MLST
Cattle	14/33 (42.4)	8/14 (57.1)	<i>Lb</i> (n = 8)	5	0/5 (0)
Dog	7/45 (15.6)	2/7 (28.6)	<i>Li</i> (n = 2)	2	0/2 (0)
Pig	0/22 (0)	-	-	-	-
Cat	1/1 (100)	0/1 (0)	-	-	-
Domestic animals	22/101 (21.8)	10/22 (45.4)	<i>Lb</i> (n = 8), <i>Li</i> (n = 2)	7	0/7 (0)
<i>RR</i>	214/562 (38.5)	201/214 (93.9)	<i>Li</i> (n = 201)	164	4/11 (9)
<i>RN</i>	52/170 (30.6)	45/52 (86.5)	<i>Li</i> (n = 45)	27	1/6 (2)
<i>SM</i>	6/171 (3.5)	3/6 (50)	<i>Li</i> (n = 3)	-	-
<i>MM</i>	6/67 (9.0)	3/6 (50)	<i>Lb</i> (n = 1), <i>Lk</i> (n = 2)	-	-
<i>TE</i>	0/25 (0)	-	-	-	-
Wild animals	278/995 (27.9)	252/278 (90.6)	<i>Lb</i> (n = 1), <i>Li</i> (n = 249), <i>Lk</i> (n = 2)	191	5/17 (11)
Human	-	59/66 (89.4)	<i>Li</i> (n = 57), <i>Lb</i> (n = 2)	48-	44/48 (44)-

RR: *Rattus rattus*; RN: *Rattus norvegicus*; SM: *Suncus murinus*; MM: *Mus musculus*; TE: *Tenrec ecaudatus*. Li: *Leptospira interrogans*; Lb: *Leptospira borgpetersenii*; Lk: *Leptospira kirschneri*.

<sup>a</sup> This excludes partial *secY* amplification (< 501 bp) of four clinical samples and one cow sample;

<sup>b</sup> Amplification and sequencing success for the 5-loci MLST scheme excluding the locus *icdA*.

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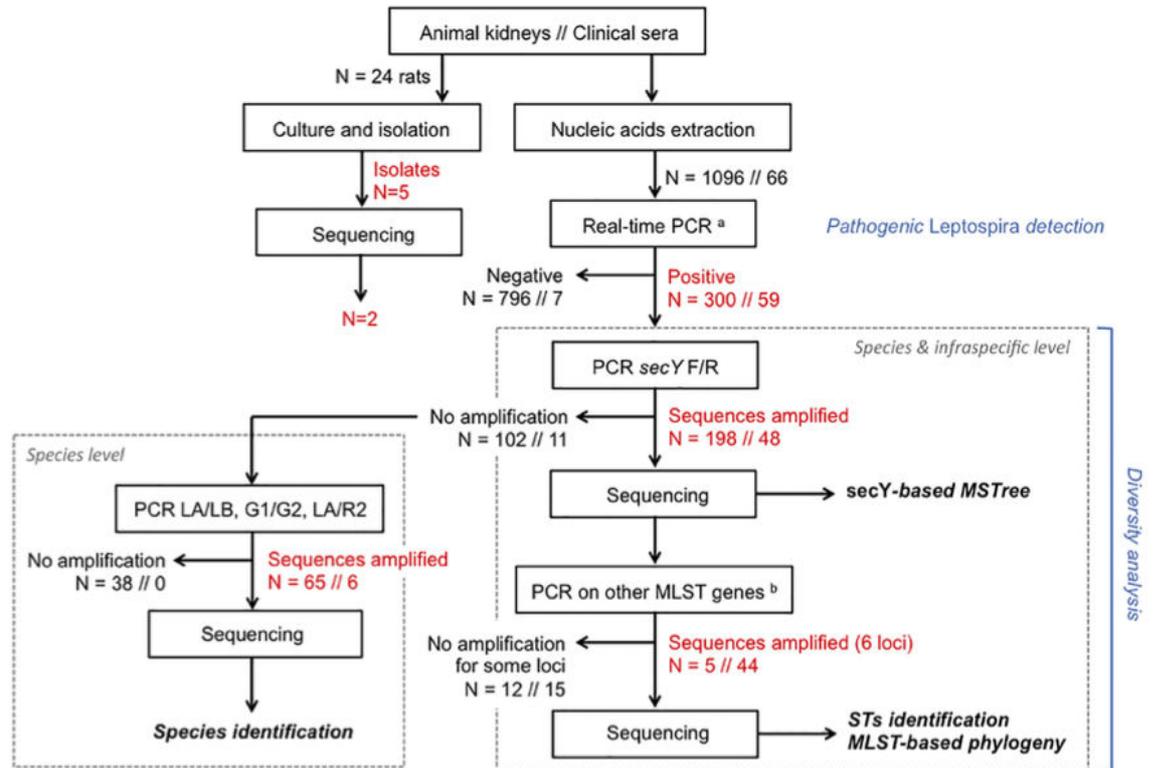
from the Mayotte clinical dataset and for some of our samples. Bayesian analyses were performed to infer phylogenetic relationships between *Leptospira* species. The best-fitting model and associated parameters were selected using jModelTest [47] and phylogenies were constructed by Bayesian inference. We performed two independent runs of Metropolis-coupled Markov chain Monte Carlo (MCMCMC) analyses in MrBayes v3.2.1 [48] of all loci independently and of concatenated sequences. Each run included 20,000,000 generations, and trees were sampled every 100 generations. The initial 20,000 trees were discarded as a conservative "burn-in" and the harmonic mean of the likelihood was calculated by combining the two independent runs. The 50% majority-rule consensus tree was then computed from the sampled trees under the best model. Neighbor-joining trees were constructed using Seaview v4.3 (Kimura's 2-parameter distances, 500 replicates). Trees were visualized in FigTree v1.3.1 (<http://tree.bio.ecd.ac.uk/>). GenBank accession numbers of the sequences produced in the frame of the present study are provided as additional table 1 (S1 Table).

Unique allele identifiers for all six loci were assigned, and corresponding allelic profiles (or sequence types STs) were defined using the established *Leptospira* MLST website (<http://pubmlst.org>), focusing on MLST scheme #3. *Leptospira*-positive samples with incomplete *Leptospira* MLST cannot be assigned to a ST. In order to determine the DNA relatedness among *Leptospira* carried in the human or animal specimens, we drew a minimum-spanning tree (MST) based on a 501 bp *secY* gene fragment, using the goeBURST Full MST algorithm ([goeBURST.phyloviz.net/](http://goeBURST.phyloviz.net/)) [49].

## Results

### *Leptospira* diversity within clinical samples

For 44 of the 66 clinical samples, we successfully amplified all six MLST loci (Table 1). For twelve clinical samples, only one to five MLST loci could be successfully amplified, even when



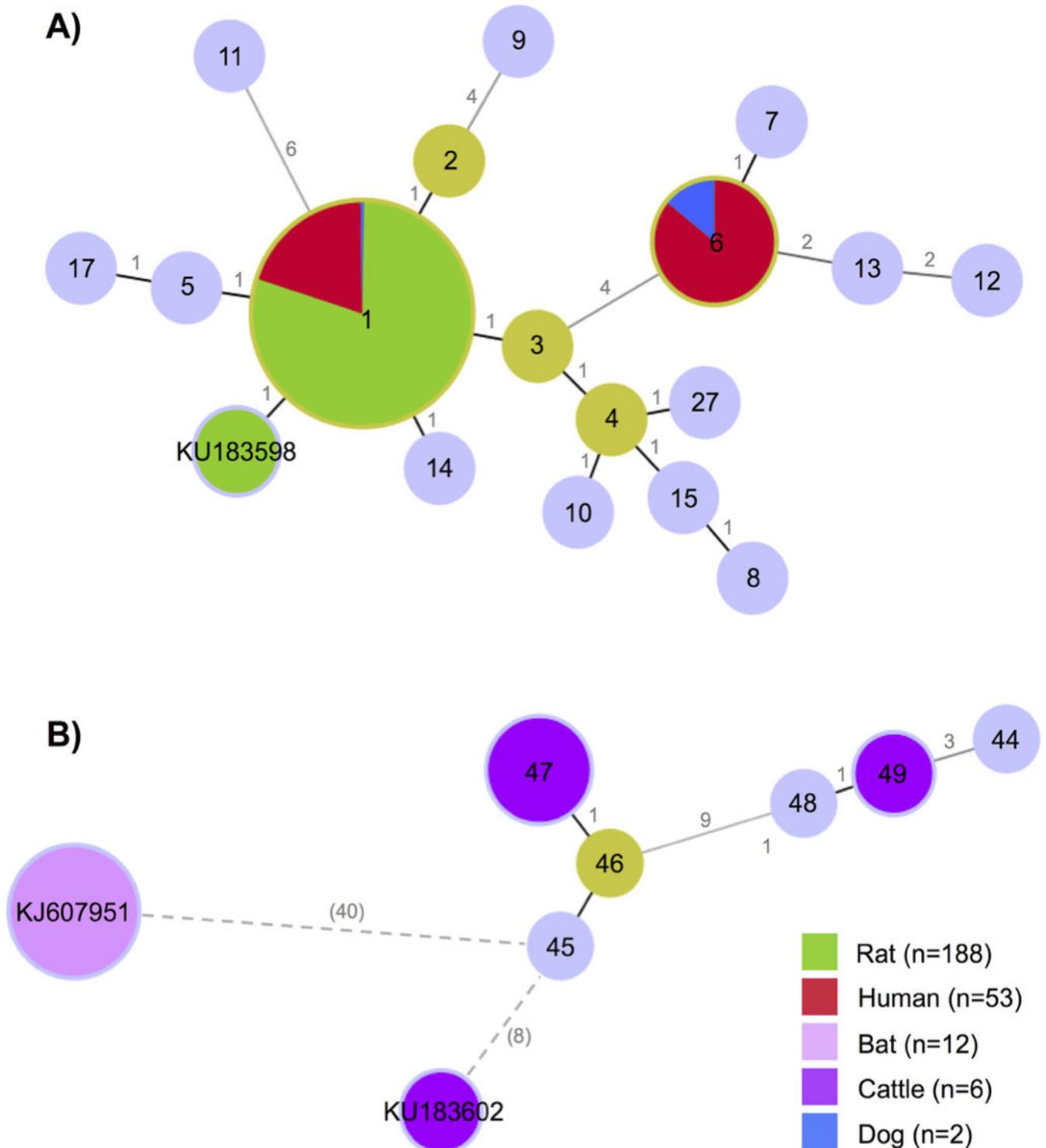
**Fig 2. Flow chart of the molecular methods performed to detect and identify *Leptospira* species and haplotypes infecting samples from Reunion Island.** At each step, the number (N) of tested animal samples // clinical samples is indicated. <sup>a</sup> The real time PCR diagnosis was attempted on either DNA extracted from clinical samples, or cDNA obtained after a reverse transcription step for animal samples. <sup>b</sup> Six-loci MLST was attempted only for a selected subgroup of the 198 animal samples successfully amplified on *secY* locus using MLST scheme #3, i.e. eight domestic animals and 17 randomly selected wild small mammals (all *Rattus* spp).

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using degenerated primers [26] (see S2 Table for details). For the last seven clinical samples, we failed to obtain successful PCR amplification at any of the six MLST loci. When these seven samples were further re-tested through an alternative real time PCR [37], only two samples tested positive at Ct values exceeding 42, whereas five sera provided negative amplification, suggesting that these were either false positive samples or positive samples that degraded during transport or conservation. Those seven human negative samples were discarded from the analysis and were no longer considered (Fig 2). The twelve partially sequenced samples allowed only identification of the infecting *Leptospira* at the species level; samples for which *secY* PCR product could be amplified were included in the MST analyses. *In fine*, of the 59 clinical samples successfully amplified at one or more locus, 57 were assigned to *L. interrogans* and two to *L. borgpetersenii* (Table 1). The *secY*-based MST (Fig 3), the neighbor-joining trees based on *secY* and *rrs2* (Fig 4), and the MLST analysis all showed two *L. interrogans* clusters. As the two *L. borgpetersenii*-positive clinical samples allowed PCR amplification on two or three loci only (i.e. *lipL32* and *rrs2* for both samples, *lipL41* for one sample only) but not on *secY* locus, they do not appear in the *secY*-based MST or the MLST-deduced phylogeny.

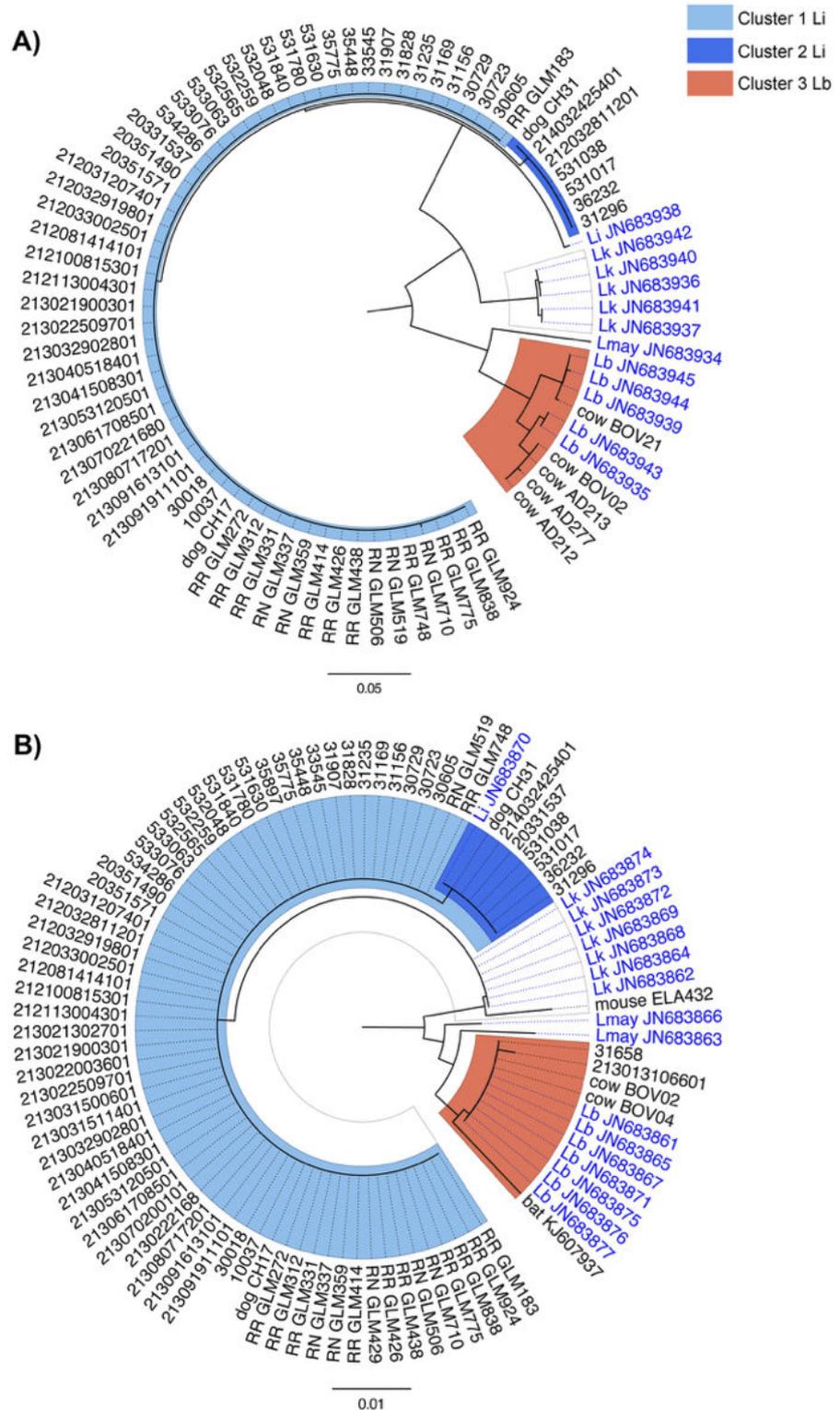
### *Leptospira* detection and identification in animal samples

Out of 995 wild terrestrial small mammals trapped on Reunion Island, 278 animals (27.9%) tested positive for *Leptospira*. Out of 24 wild terrestrial small mammals for which culture was



**Fig 3. Minimum spanning trees of *Leptospira* based on *secY* gene (501 bp sequence), including our sequences from Reunion Island and twelve bat sequences previously published [46].** A) Minimum spanning tree of *Leptospira interrogans* (n = 257) and B) *Leptospira borgpetersenii* (n = 22). We used the goeBURST Full MST algorithm. The alleles are identified by a number (*secY*-1 to *secY*-54, see <http://www.pubmlst.org/leptospira/>) or an identifier; the circle size reflects their abundance in the data set. Group founders are in light green and common nodes are in light blue, except when overlapping with alleles from our sample, with specific colours referring to hosts (see legend). Links between the elements uses a grayscale, with lighter gray links showing more differences; the number of differences is indicated on the links. For two incomplete *secY* sequences (< 501 bp), the numbers of differences, shown in parentheses, were calculated on the overlapping nucleotides only, i.e. 443 for KU183602, and 491 for KJ607951.

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**Fig 4. Neighbour-joining phylogenetic trees (Kimura's 2-parameter distances, 500 replicates) inferred from (A) *secY* (501 bp sequence) and (B) *rrs2* (450 bp sequence) genes.** Clinical and animal samples from Reunion Island are written in black, using GenBank accession numbers (for one published bat sequence) or identifiers accompanied by the host name in the case of animal samples (RR: *Rattus rattus*; RN: *Rattus norvegicus*). Corresponding GenBank accession are reported on S1 Table (supporting

information). Clinical samples from Mayotte are written in blue, using GenBank accession numbers and corresponding *Leptospira* species. Li: *Leptospira interrogans*; Lb: *Leptospira borgpetersenii*; Lk: *Leptospira kirschneri*; Lmay: *Leptospira mayottensis*.

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attempted, five were tested positive for *Leptospira*, among which two were successfully cultured (both *R. rattus*). Positive samples amplified and sequenced on *secY* locus provided sequences for 191/278 (68.7%) individuals, all of them being *Rattus* spp. and including the two successfully cultured samples. The remaining 87 positive samples were further tested for each of the other five MLST loci, as well as for small portions of the 16S region. This effort allowed identifying the *Leptospira* species infecting 61 additional samples from wild animals. However, because of the lack of either *secY* sequence or full MLST genotyping, these samples were excluded from the diversity analyses at the infra-specific level. Altogether, 249 out of the 252 *Leptospira* sequences that were obtained from wild mammal samples were identified as *L. interrogans* (98.8%). The three remaining sequences, all from mouse tissue samples, were identified either as *L. borgpetersenii* (n = 1) or *L. kirschneri* (n = 2); the *L. borgpetersenii* sample allowed *lipL32* amplification while *rrs2* amplification was obtained from one of the two *L. kirschneri* samples. The last *L. kirschneri* sample did not lead to any PCR amplification at any of the six MLST loci but was identified at the species level using LA/LB primers. Noteworthy, all *T. ecaudatus* tested PCR negative. These results are summarized in Table 1. Interestingly, when aligning the 191 available *secY* sequences, all from rat tissue samples, it appeared that a single *L. interrogans secY* allele (*secY*-1) was present in all rats but two. The two exceptions (both *R. norvegicus*) had different single substitutions at the *secY* sequence. The amino acid translation showed an internal stop codon in one of the two sequences, suggesting a sequencing error, and this sequence was later excluded. Thus, just one undescribed allele remained (KU183598; see Fig 3).

The 101 domestic animals screened in our study provided the following positivity rates for *Leptospira*: 42.4% in cows, 15.6% in dogs and 0% in pigs (Table 1). The single sample from a cat was urine tested positive for *Leptospira*. Sequences on different loci were obtained for ten animals (S1 Table). *Leptospira* infecting eight cows were identified as *L. borgpetersenii*, whereas *Leptospira* infecting two dogs were identified as *L. interrogans*, each dog showing a different *secY* allele, i.e. *secY*-6 (also identified in six clinical samples) and *secY*-1 (highly dominant in both clinical and rat samples) (Fig 3).

### ***Leptospira borgpetersenii* diversity**

*Leptospira* samples that were identified as *L. borgpetersenii* (see Table 1) did not allow PCR amplification at most MLST loci, even when using recently described degenerated primers [26]. For cows (n = 8), we could amplify either *adk* (n = 4), *lipL32* (n = 1), *rrs2* (n = 3), or *secY* locus (n = 6), depending on samples (see S1 Table), whereas only *lipL32* locus was successfully amplified for the single *L. borgpetersenii* positive mouse. As for clinical samples (n = 2), we could amplify either *lipL32* (n = 2), *lipL41* (n = 1) or *rrs2* (n = 2). Thus, the only locus that allowed a comparison between human and animal samples was a 450 bp *lipL32* PCR fragment amplified from two clinical samples, one mouse and one cow (S1 Table). Alignments showed 100% nucleotide identity between *L. borgpetersenii* infecting the first clinical sample and the only mouse positive for *L. borgpetersenii*, and between *L. borgpetersenii* infecting the second clinical sample and one cow sample (based on a partial 434 bp amplification for the cow).

The *secY* locus amplification and sequencing provided sequences for six cow samples, revealing alleles *secY*-47 (n = 4), *secY*-49 (n = 1), and a third not yet described allele in one sample (KU183602, see Fig 3). The allele *secY*-47 is closely related to *secY*-48 identified in clinical

samples from Mayotte, whereas *secY*-49 has been identified in *Leptospira*-positive Tanzanian rodent samples and in a clinical sample from China (comparison with sequences from [2]). The third undescribed *secY* allele was identified in the cow sample showing a *lipL32* sequence common to those from one clinical sample. Noteworthy, twelve *secY* *Leptospira* sequences from the insectivorous bat *Mormopterus francoismoutoui* endemic to Reunion Island (GenBank accession numbers KJ607946 to KJ607957) [50] were not related to any of these alleles (Fig 3).

### Genetic diversity using MLST analysis

For 44 clinical samples and eleven rats, five to six loci of the MLST scheme [40] were successfully amplified, sequenced and concatenated for subsequent analyses (Fig 2, Table 1 and S1 Table). The 44 clinical samples which allowed successful amplification at all six MLST loci were identified as *L. interrogans* and fell into two clusters corresponding to previously described sequence types (STs): one predominant, ST02 (38/44 = 86.4%) and one minor, ST34 (6/44 = 13.6%). Including samples with successful amplification at 5 MLST loci only, we identified two clonal complexes, CC02 and CC34, including human and rats for the former, and human and dogs for the later (see S1 Fig).

Among the 17 randomly selected *Leptospira*-positive wild animal samples, six rat samples were successfully amplified on five loci only (excluding *icdA*, which PCR failed) and five rat samples were successfully amplified on all six loci; they were all identified as *L. interrogans* CC02 or ST02. Of note, we failed to amplify DNA at *icdA*, *lipL32* and *lipL41* loci for the rat sample that showed an alternative *secY* allele.

Among the 22 *Leptospira*-positive domestic animals, none were successfully amplified on all six MLST loci. However, produced sequences revealed a perfect match between *L. interrogans* genotyped from two dogs and from clinical samples. As for the first dog, the six-loci concatenated sequence showed close identity with ST34 but a 14 nucleotides long sequence was missing on *LipL41* locus, while for the second dog, the sequencing of four loci revealed relatedness to ST02 (four alleles in common; *adk* and *LipL41* non sequenced, see S2 Table).

As highlighted above, the *L. borgpetersenii*-infected samples were hardly detectable by PCR and could not be fully genotyped, likely because of low bacterial loads; thus the phylogenetic tree based on the concatenated sequences of the MLST loci included *L. interrogans* samples only (S1 Fig).

### Discussion

Although the mean prevalence of renal carriage among rats on Reunion Island (36.3%) was close to that reported in *R. rattus* on Mayotte (29.8%) [11], the genetic data reported in the present study reveal a striking contrast between the two islands: the rich *Leptospira* genetic diversity (at least 4 species with rather balanced representation) reported in rats from Mayotte, also found in human cases, contrasts with the low *Leptospira* genetic diversity reported herein in humans, rats and shrews on Reunion Island: though we identified three *Leptospira* species, *L. interrogans* represents 96.6% of clinical samples, 95.8% of positive animal samples and 100% of rat samples.

Other studies have reported a low genetic diversity among locally circulating *Leptospira* infecting local rat populations [25,51–53]. In contrast to earlier studies in New Caledonia [54], New Zealand [55] or Argentina [56], showing the carriage of *L. interrogans* or *L. borgpetersenii* in black rats (*R. rattus*), and the absence of *L. borgpetersenii* in Norway rats (*R. norvegicus*), *L. borgpetersenii* was absent in rats or shrews in our large sample of wild small mammals but was identified in a single mouse. The role of mice as maintenance hosts for *L.*

*borgpetersenii* serogroup Ballum is largely recognized [1,3]. The perfect identity between *lipL32* fragments from a single mouse and one of the two clinical samples positive for *L. borgpetersenii* highlights mice as a possible reservoir of pathogenic *Leptospira* at risk for humans on Reunion Island. However, this result should be interpreted cautiously as a single locus might not be resolute enough as to infer *Leptospira* species; indeed, *LipL32* sequences of *Leptospira borgpetersenii* and *Leptospira weilii* have been shown to be indistinguishable [57]. Further investigations targeting other loci with higher nucleotide polymorphism are needed to ascertain this point.

A different *L. borgpetersenii* infecting the second clinical case suggested the existence of a second potential reservoir host. A bat species endemic to Reunion Island, *Mormopterus francoismoutoui*, has been demonstrated as a *L. borgpetersenii* carrier in urine [50]. However, the comparison of *rrs2* sequences from our two clinical samples infected with *L. borgpetersenii* to GenBank sequences obtained from this endemic bat species ( $n = 12$ ) showed a low degree of genetic relatedness (Fig 3), thus excluding bats as a source of contamination leading to overt clinical leptospirosis in humans. A serological survey conducted in 2009 showed that up to 32% of cattle were seropositive for leptospirosis in Reunion Island, with Sejroe reported as the main circulating serogroup [30]. Our investigation finally identified cattle as a potential reservoir of *L. borgpetersenii* at risk for humans, as one *L. borgpetersenii* infecting a cow was found related to the *L. borgpetersenii* infecting a clinical case (perfect identity between 434 bp *lipL32* fragments). As for mice, further investigation is needed in order to verify a potential transmission between cattle and human, possibly through an environmental maintenance of pathogenic leptospires [58].

Apart from the two *L. borgpetersenii* alleles common to human cases of leptospirosis and one cow or one mouse, at least two additional *L. borgpetersenii* haplotypes were identified in cows only, indicating a higher *Leptospira* diversity in cattle than in any other animal investigated on the island, which was already evidenced in other settings [59]. Our experience and previous published work [60,61] have highlighted recurrent failure to detect and amplify specifically *L. borgpetersenii* strains. Consequently, acute clinical cases related to this species might be underestimated.

The *L. kirschneri* found in two mice was not identified in any of our clinical samples on Reunion Island, whereas this species has been reported in clinical infection in Mayotte [11,12] as well as in other countries [2]. The reasons that may account for this difference are unknown. As mild infections not requiring hospitalisation or spontaneously healing cases are probably being underdiagnosed and underreported, it would be worth exploring whether these cases are associated with infection with other *Leptospira* species.

At an infraspecific level, we identified two clusters of *L. interrogans*, referring to sequence types ST02 and ST34 (<http://www.pubmlst.org/leptospira/>). International isolates previously published on the pubmlst.org database showed ST02 to be isolated from Belgium and Brazil, while ST34 was isolated from Jamaica and India. The second (minor) cluster, ST34, identified in six clinical samples, could not be detected in any of the five wild small mammal species targeted by our study. Additional sampling of domestic animals interestingly highlighted stray dogs as possible carriers of both *L. interrogans* STs found in human acute cases. Dogs have been repeatedly pinpointed as involved directly or indirectly in human epidemiology (contamination from dogs to humans, or from a common environmental source) [62–66]. For instance, a serosurvey conducted on Reunion Island in 2009 identified Canicola and Icterohaemorrhagiae as the major serogroups (43.5% and 21.7% respectively) in seropositive stray dogs, while Sejroe, Panama, Tarassovi and Ballum serogroups were also identified [29]. On the other hand, up to 17% of clinical cases were found to be infected with serogroup Canicola [28], suggesting that dogs, as the reservoir for this serogroup, are the source of contamination on Reunion

Island. The importance of dogs in the epidemiology of the disease, if confirmed on Reunion Island, might be related to the lack of mammals diversity locally, as suggested on the island of Barbados [67].

An epidemiological survey conducted by CIRE-OI (Regional Office of French Institute for Public Health Surveillance in Indian Ocean, see [13]) indicates that, regarding the six patients infected with *L. interrogans* ST34, (i) five were actually living in the eastern, windward humid coast of the island and (ii) for those five cases, the disease was associated to leisure activities in the eastern side rivers and waterfalls. Noteworthy, of 56 *R. rattus* positive for *Leptospira* trapped in sites near rivers in the eastern transect (Fig 1), none of the 53 available sequences corresponded to *L. interrogans* ST34. Hence, it seems very unlikely that *R. rattus* acts as the reservoir of *L. interrogans* ST34.

In the end and as expected, our analyses stress black and Norway rats as reservoirs of *Leptospira* lineages of medical importance on the island. However, we were able to identify other animal species likely playing a role in the local epidemiology of human leptospirosis: mice, cattle and stray dogs. Noteworthy, the very low infra-specific diversity of *L. interrogans* infecting our infected samples did not enable us to assess the respective contribution of rats versus dogs to acute human infection with *L. interrogans*. *Leptospira* isolation from animals and humans would help to better assess the epidemiological links between humans and animals. Our conclusions are weakened by PCR and/or sequencing failures, likely resulting from different primer annealing efficiencies depending on the *Leptospira* species used as template (supported by our incomplete results on cattle and mice samples infected with *L. borgpetersenii*), or from low renal carriage, as suggested by high *Ct* values (e.g. *Ct* >30 in rats or >37 in dogs with undetermined infecting *Leptospira*, see S3 Table). Although time-consuming and fastidious, *Leptospira* culture needs to be carried out systematically, whenever possible, as previously suggested by other authors [68].

The high infection prevalence of cows with *L. borgpetersenii* raises another issue: the available human vaccine against leptospirosis currently proposed on the island target only *L. interrogans* species serogroup Icterohaemorrhagiae, while Sejroe was reported to be the main serogroup circulating in cattle in Reunion Island in 2009 [29]. This means that the vaccination of the ranchers' population, at risk for leptospirosis for being in close contact with cows, might be inefficient against any other serogroup likely carried by livestock, in particular the one associated with *L. borgpetersenii*.

Pigs are another potential reservoir that deserves attention. Leptospirosis is a common disease of swine throughout the world and can be a significant cause of reproductive loss [69]. None of the 22 pig samples included in the study tested positive to *Leptospira*, while a serological survey conducted in 2009 on Reunion Island showed that up to 47.2% of pigs were seropositive for leptospirosis [29]. These two observations are not contradictory. First, the acute infection of an animal with leptospires does not prejudice its ability to develop a bacterial biofilm in the renal tubules and to shed the bacteria into the environment. Hence, serological data and PCR data on kidneys may be discrepant. Second, the kidney samples used in our study were collected from growing-finishing pigs under one year old, a potential age bias that might have affected our findings. Indeed, a proper assessment of pigs as reservoirs of leptospires at risk for human health requires the screening of older animals sampled from different farms as well as from family breedings throughout the entire island.

It is hypothesized that *Leptospira* were introduced onto islands with their animal hosts, and that a variable number of introduced strains have adapted to the new local environment and available hosts [14]. On Reunion Island, the fact that *L. interrogans* is almost clonal in rats is in favour of a recent leptospiral introduction on the island concomitant with the introduction of rats, back to the 16<sup>th</sup> century. Leptospires detected in dogs, mice and cows show higher genetic

diversity, which could result from multiple introduction events of infected animals of different species (for cows, there has been multiple importations from France of both Holstein Frisonne and Montbéliarde until 2008) or from distinct geographical origin, carrying different *Leptospira* lineages. We suggest that in contrast to Mayotte where the high diversity of *Leptospira* in humans and animal reservoirs is most likely related to (older) introduction events of infected hosts, potentially from countries with high host endemicity associated with *Leptospira* diversity [26], on Reunion Island, the narrow diversity of *Leptospira* might reflect more recent and/or fewer host introduction events. This is partly supported by the fact that fifteen of the seventeen STs from Mayotte strains have not been previously identified [12], contrary to STs from Reunion Island clinical cases that have already been identified worldwide. Previous studies have suggested that *Leptospira* from Mayotte and Madagascar are genetically closely related, and that *Leptospira* identified in Mayotte are probably mainly derived from *Leptospira* from Madagascar, possibly introduced in Mayotte via their hosts [14]. Although no genetic information is available so far on pathogenic *Leptospira* prevalent on Comoros, the serological profiles in humans from Comoros are comparable to those depicted in Mayotte, and noteworthy, antibodies from the serogroup Icterohaemorrhagiae are not detectable [20]. These findings contrast with those from human leptospirosis in Reunion Island and the Seychelles, where the Icterohaemorrhagiae serogroup is most common [70]. Hence there is compelling evidence that leptospirosis epidemiology might be similar along the Comoros, Mayotte and Madagascar axis and associated to indigenous or endemic *Leptospira* lineages, but different from that prevalent on Reunion Island and potentially Seychelles and Mauritius, where Icterohaemorrhagiae serogroup is dominant in clinical cases [15,16,21]. The near clonality reported herein in rat-borne *Leptospira* on Reunion Island is highly suggestive of recent pathogen introduction. This has to be addressed in the future by similar molecular analyses of *Leptospira* isolates from the other SWIO islands for which almost no molecular data are available so far, i.e. Mauritius and Seychelles.

## Supporting Information

**S1 Table. GenBank accession numbers of the nucleotide sequences used in this study.** (XLSX)

**S2 Table. List of samples investigated with successfully amplified MLST loci and non-successfully amplified (NA).** For each sample, the table provide information on the host species, the infecting *Leptospira* species, the allele identifiers (numbers assigned to unique sequences) for each locus and the corresponding allelic profile or sequence type (ST) when available. \* Nearest match for the sequence type considering the available loci. (XLSX)

**S3 Table. List of *Leptospira*-positive animal samples.** For each sample, the table provide Ct values obtained by real-time PCR diagnosis, the sample site, the host species and the infecting *Leptospira* species. RR: *Rattus rattus*; RN: *Rattus norvegicus*; SM: *Suncus murinus*; MM: *Mus musculus*; TE: *Tenrec ecaudatus*. (XLS)

**S1 Fig. Phylogeny of Reunion Island samples and reference strains using 5 MLST loci. *Leptospira interrogans* phylogeny based on a concatenated 2292 bp sequence.** Black legends indicate reference strains, red legends indicate samples from Reunion Island, blue legends indicate a clinical sample from Mayotte. GenBank accession numbers are provided in S2 Table. CC: clonal complex (sequence type [ST] and their single locus variants). (TIF)

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## Author Contributions

Conceived and designed the experiments: VG PT KD. Performed the experiments: VG PT EL CC GLM. Analyzed the data: VG PT YG KD. Contributed reagents/materials/analysis tools: VG PT EL CC GLM. Wrote the paper: VG PT EL CC GLM YG FP MCJB AM KD. Obtained permission and issuance of permits for animal sampling: EL GLM.

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RESEARCH ARTICLE

# Identification of *Tenrec ecaudatus*, a Wild Mammal Introduced to Mayotte Island, as a Reservoir of the Newly Identified Human Pathogenic *Leptospira mayottensis*

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## Abstract

Leptospirosis is a bacterial zoonosis of major concern on tropical islands. Human populations on western Indian Ocean islands are strongly affected by the disease although each archipelago shows contrasting epidemiology. For instance, Mayotte, part of the Comoros Archipelago, differs from the other neighbouring islands by a high diversity of *Leptospira* species infecting humans that includes *Leptospira mayottensis*, a species thought to be unique to this island. Using bacterial culture, molecular detection and typing, the present study explored the wild and domestic local mammalian fauna for renal carriage of leptospires and addressed the genetic relationships of the infecting strains with local isolates obtained from acute human cases and with *Leptospira* strains hosted by mammal species endemic to nearby Madagascar. Tenrec (*Tenrec ecaudatus*, Family Tenrecidae), a terrestrial mammal introduced from Madagascar, is identified as a reservoir of *L. mayottensis*. All isolated *L. mayottensis* sequence types form a monophyletic clade that includes *Leptospira* strains infecting humans and tenrecs on Mayotte, as well as two other Malagasy endemic tenrecid species of the genus *Microgale*. The lower diversity of *L. mayottensis* in tenrecs from Mayotte, compared to that occurring in Madagascar, suggests that *L. mayottensis* has indeed a Malagasy origin. This study also showed that introduced rats (*Rattus rattus*) and dogs are probably the main reservoirs of *Leptospira borgpetersenii* and *Leptospira kirschneri*, both bacteria being prevalent in local clinical cases. Data emphasize the epidemiological link between the two neighbouring islands and the role of introduced small mammals in shaping the local epidemiology of leptospirosis.

design, data collection and analysis, decision to publish, or preparation of the manuscript.

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## Author Summary

Islands are exceptionally prone to species introduction, including pathogens with detrimental public health consequences for which the invasive alien species could act as reservoirs. Our study shows how the local non-native mammal fauna of Mayotte Island is associated with the introduction and epidemiology of human leptospirosis, a zoonosis strongly affecting tropical islands. Data presented herein identify *Tenrec ecaudatus* (Family Tenrecidae), an omnivorous species introduced from neighbouring Madagascar, as a reservoir of the recently named *Leptospira mayottensis*. Further, we suggest a Malagasy origin of *L. mayottensis*, which occurs naturally within the Tenrecidae radiation endemic to Madagascar. Finally, we provide evidence that dogs and rats are reservoirs for other *Leptospira* lineages of medical importance on Mayotte. Altogether, our study highlights the impact of species introduction on human health and further suggests that the biogeography of microorganisms in insular ecosystems, including pathogenic endemic lineages, should be considered from evolutionary and medical perspectives.

## Introduction

Leptospirosis is a bacterial zoonosis caused by pathogenic spirochetes of the genus *Leptospira*. Human infection is common in tropical countries where warm and humid conditions favour the survival of *Leptospira* spp. in water and soil. Furthermore, human populations in these countries often live in rural areas or in impoverished urban zones with inadequate sanitation and can be at higher risk of exposure to the infection via the contaminated environment. A wide variety of mammal species can be infected by *Leptospira* spp. but not all act as reservoirs. Animal reservoirs support the chronic colonization of their renal tubules by a biofilm of leptospires and thus, release over prolonged periods the bacteria into the environment via their urine. Rodents and particularly rats, especially *Rattus* spp., are considered as the principal reservoir of these pathogenic bacteria and the main source of human infection, a role likely favoured by the worldwide distribution and commensal behaviour of some invasive species in this genus.

Two methods are currently used to identify leptospires: a phenotypic classification based on the microscopic agglutination test (MAT) that recognizes over 25 serogroups and 300 serovars [1] and a more recently introduced genetic classification based on bacterial DNA sequences that allows the identification of 20 *Leptospira* spp., nine of which are pathogenic [2]. Genotyping through Multiple Locus Sequence Typing (MLST) based on a selected set of housekeeping genes [3] has become a method of choice to identify *Leptospira* at the species and infra-species levels. Indeed, molecular typing allows deciphering the molecular epidemiology of the disease, sharing data among laboratories investigating distant geographic regions and analyzing the evolution of host-parasite relationships using robust molecular characters [4].

The incidence of human leptospirosis has been reported to be highest on tropical islands and this observation holds true for the southwestern Indian Ocean (SWIO) region [5]. Poorly documented on Mauritius and Madagascar, the incidence of the disease in the Seychelles ranks first worldwide among surveyed countries [5], while on Reunion Island, a French overseas department, the rate of human leptospirosis has been reported to be nearly 20 times higher than in continental France [6].

Mayotte, the most southern island of the Comoros Archipelago is a French overseas department of about 375 km<sup>2</sup>, located in the northern entrance of the Mozambique Channel about 300 km off the northwestern coast of Madagascar. Human leptospirosis is highly prevalent on

the island with an annual incidence in 2013 estimated at 35 per 100,000 [6]. Most interestingly, *Leptospira* strains isolated from patients, hereafter referred to clinical isolates, showed a much larger species diversity on Mayotte than on Reunion Island: 16 different sequence types (STs) were identified by MLST on Mayotte [7], while only three on Reunion Island [8]. Further, pathogenic leptospires causing acute human infections on Mayotte were identified as *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira kirschneri* and members of a previously undefined phylogroup that has recently been named as *Leptospira mayottensis* [7,9]. By contrast, only *L. interrogans* and *L. borgpetersenii* have been reported on Reunion Island [8]. The high diversity of leptospires on Mayotte, unique so far within the SWIO region, likely reflects local eco-epidemiologic specificities of animal reservoirs. With the exception of bats, no native small mammals occur on Mayotte [7]. Although one study has previously investigated animal reservoirs on Mayotte [7], it targeted *Rattus rattus*, the only rat species known on the island, for kidney carriage of pathogenic leptospires, whereas other species of the local wild and domestic fauna were screened by MAT, a serological test that provides evidence of previous infections but does not assess the actual carrier state of the investigated animals.

Through the investigation of the local wild and domestic fauna, the present study aimed at identifying animal reservoirs of leptospires on Mayotte, with a particular focus on *L. mayottensis*. This leptospiral species has been recently detected in wild small mammals endemic to neighbouring Madagascar [4] and, hence, it can be hypothesized that the geographic proximity and various socio-economic exchanges between these two islands could have facilitated the introduction of this pathogenic bacteria to Mayotte.

## Methods

### Ethics statement

All animal procedures carried out in this study were performed in accordance with the European Union legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU). The ethical terms of the research protocol were approved by the CYROI Institutional Animal Care and Use Committee (Comité d'Ethique du CYROI n° 114, IACUC certified by the French Ministry of Higher Education and Research) under accreditation 03387 (LeptOI) and 03584 (BatMan).

### Animal sampling

Mammals were trapped on Mayotte during three field sessions carried out from July 2012 to December 2014. *Rattus rattus* and *Tenrec ecaudatus* were captured using live traps baited with grilled coconut at eight and five localities, respectively. Sampling sites were selected in order to maximize the species diversity and geographic distribution of mammals in our sample. Trapping localities included a variety of different ecological settings across the island (see S1 Fig). In a local laboratory, *R. rattus* were sacrificed by cervical dislocation and *T. ecaudatus* by percussive blow to the head. For each individual, tissue was quickly collected from kidney, lung and spleen, pooled together and immediately stored in dry vials at -80°C. Insectivorous bats, *Chaerephon* sp. (Family Molossidae), were captured at dusk or at night using mist nets placed either in the vicinity of synanthropic roost sites or across flyways, while frugivorous bats, *Pteropus seychellensis comorensis* (Family Pteropodidae), were captured at night using mist nets set around fruiting trees. Captured bats were individually placed in clean cloth bags and brought back to a local laboratory. In most cases, animals spontaneously urinated after their removal from holding bags allowing the collection of urine. Bats were subsequently released at dusk at the initial capture site. Dogs (*Canis lupus familiaris*) were only manipulated and sampled by a local veterinary doctor; kidneys were obtained from animals that were euthanized for medical

purposes, when possible urine collected from these same animals and immediately kept in dry vials at  $-80^{\circ}\text{C}$ . In addition, urine samples were obtained from non-euthanized dogs through aseptic urethral catheterization. Kidneys from zebus (*Bos indica*) were purchased at the slaughterhouse of Mayotte (Kaweni) or obtained during traditional butchering. All samples were sent in liquid nitrogen to Reunion Island for analyses.

### *Leptospira* spp. isolation

In addition to the sampling and storage at  $-80^{\circ}\text{C}$  of organs and urine mentioned above, a few urine droplets and/or a small piece of freshly sampled kidney (crushed under sterile conditions) were used individually to inoculate three distinct culture media: (i) Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco, Detroit, MI, USA) supplemented with Albumin Fatty Acid Supplement (AFAS; Royal Tropical Institute, Amsterdam, Netherlands) [10] (ii) EMJH liquid medium supplemented with AFAS, rabbit serum and fetal calf serum (1% each); and (iii) semisolid Fletcher medium (Difco, Detroit, MI, USA) supplemented with rabbit serum (8%). All media were supplemented with 5-fluorouracil (5-FU) at a final concentration of  $200\ \mu\text{g}\cdot\text{mL}^{-1}$ . Cultures were incubated at  $28^{\circ}\text{C}$ , visually checked for the presence of leptospires using a dark field microscope once a week for four months and positive cultures were further sub-cultured in fresh EMJH liquid medium deprived of 5-FU. DNA was extracted from 1 mL of each positive culture using the EZ1 Biorobot with Qiagen EZ1 DNA Tissue kits (Qiagen, Les Ulis, France).

### Detection of pathogenic leptospires

Approximately  $1\ \text{mm}^3$  of pooled organs (kidney, lung and spleen) from each *R. rattus* and *T. ecaudatus* specimen and only kidney from dogs and zebus were dissected on sterile ice and further processed as previously described [11]. Thirty microliters of urine from bats and dogs were combined with  $120\ \mu\text{L}$  of Dulbecco's modified medium (GIBCO, Grand Island, NY, USA) and  $50\ \mu\text{L}$  of ATL buffer (Qiagen, Les Ulis, France). Subsequently, total nucleic acids were extracted from urine or homogenized tissues by using an EZ1 extraction robot and the EZ1 Virus Mini Kit version 2.0. A reverse transcription step was performed on total nucleic acids with GoScript Reverse Transcriptase (Promega, Madison, WI, USA) to obtain cDNA. Detection of a portion of the 16S rRNA gene of pathogenic *Leptospira* spp. was then carried out on  $5\ \mu\text{L}$  of cDNA using a probe-specific real-time Polymerase Chain Reaction system (RT-PCR) [12].

### *Leptospira* spp. genotyping

*Leptospira* spp. in samples testing positive by RT-PCR and/or culture were genotyped using a previously described MLST scheme encompassing six genes: *secY*, *adk*, *rrs2*, *icdA*, *lipL32* and *lipL41* [13], and recently optimized to improve the amplification of SWIO lineages [4]. RT-PCR is more sensitive than any MLST PCR (likely because of the very short length of RT-PCR amplicon), and some samples that were positive by the former detection system were MLST negative. In an attempt to further characterize *Leptospira* spp. from these particular samples, we used an alternative primer set (LA-LB) targeting a shorter piece of *rrs2* [14]. The amplification of each marker was realized with GoTaq Hot Start Green Master Mix 2X (Promega, Madison, WI, USA) and further sequenced on both strands by direct Sanger sequencing (Genoscreen, Lille, France) using the same amplification primer sets. All sequences were deposited on GenBank under the following accession numbers: KT338823-KT338942, KT725237-KT725243 and KX427207-KX427227.

## Phylogenetic analyses and genetic diversity

Accessible sequences from clinical *Leptospira* isolates from Mayotte were included in the study [15]. Since *icdA* sequences were not provided for these clinical isolates, we used five out of the six markers of the MLST scheme in our analyses. In addition, we added bacterial sequences obtained from endemic terrestrial small mammals from Madagascar, *Microgale cowani*, *Microgale dobsoni*, *Microgale majori*, *Microgale longicaudata* and *Microgale principula* (Family Tenrecidae, Subfamily Oryzorictinae). The complete MLST of these *Leptospira* strains were sequenced in a previous study [4] and from cultures realized during field missions carried out in the Central Highlands of Madagascar, Réserve Spéciale d'Ambohitantely, in March and October 2014 [16].

Phylogenetic analyses were performed on each gene separately and subsequently on concatenated sequences using the best model of sequence evolution determined by jModelTest v.0.1.1 [17] for each dataset. Bayesian analyses were performed with MrBayes 3.1.2 [18] and 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 10,000,000 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 from each run were discarded as burn-in and the consensus phylogeny along with posterior probabilities were obtained from the remaining trees. Bayesian trees were visualized and rooted to midpoint with FigTree v.1.3.1 (Andrew Rambaut, Institute of Evolutionary Biology, University of Edinburgh, 2006 to 2009; <http://tree.bio.ed.ac.uk/>).

Genetic diversity was compared between the identified clades in the multilocus phylogeny by estimating the nucleotide diversity ( $\pi$ ) within each clade from the concatenated sequences using DNASP v.5.10.01 [19].

## Results

Altogether, 486 samples of tissue and/or urine collected on Mayotte from 289 *Rattus rattus*, 37 *Tenrec ecaudatus*, 53 *Canis lupus familiaris*, 18 *Bos indica*, 69 *Chaerephon* sp. and 20 *Pteropus seychellensis comorensis* were screened for the presence of *Leptospira* spp. by RT-PCR and/or culture. The results are summarized on Table 1.

### *Leptospira* spp. detection by RT-PCR and culture

Pathogenic *Leptospira* spp. were detected by RT-PCR in 27.0% of *T. ecaudatus*, 15.9% of *R. rattus*, 13.2% of dogs, 10.0% of *P. seychellensis comorensis* and 5.6% of zebus. All urine samples from *Chaerephon* sp. tested negative by RT-PCR. Bacterial cultures were attempted using 117 freshly sampled kidney and 94 urine samples. Overall, leptospire culture was successful for samples testing positive through RT-PCR only. Among the 81 *R. rattus* tested by culture inoculated with kidney, 14 were positive by RT-PCR, of which eight allowed positive cultures (57.1%). Similarly, eight of the 10 RT-PCR-positive samples (80.0%) from *T. ecaudatus* allowed isolation of leptospire by culture. Two of them (MDI295 and MDI321) yielded positive cultures from both urine and kidney samples. Lastly, no isolate grew in urine cultures from RT-PCR positive *P. seychellensis comorensis* samples. No culture was attempted when sampling dogs or zebus.

### *Leptospira* species diversity in wild and domestic mammals

We were able to PCR amplify all six loci of the MLST scheme using DNA extracted from all 16 *Leptospira* cultures, obtained from eight *R. rattus* and eight *T. ecaudatus* samples. Interestingly,

**Table 1. Animals from the wild and domestic fauna of Mayotte tested for carriage of *Leptospira* spp. by real-time PCR, culture, and genotyping.**

Host species	Number of animals	Number of animals	Number of <i>Leptospira</i>	Number of typed	<i>Leptospira</i> spp.			
	tested by RT-PCR	tested by culture	spp. positive animals	<i>Leptospira</i> strains	<i>Lb</i>	<i>Lm</i>	<i>Li</i>	<i>Lk</i>
	(OP, K, U)*	(K, U)*	(%)					
<i>Rattus rattus</i>	289	81	46 (15.9)	21	13	0	3	5
	(OP = 289)	(K = 81)	(38PCR+/Culture-8PCR+/Culture+)					
<i>Tenrec ecaudatus</i>	37 (OP = 37)	36 (K = 36, U = 17, 17†)	10 (27.0) (2PCR+/Culture-8PCR+/Culture+)	8	0	8	0	0
<i>Bos indica</i>	18 (K = 18)	0	1 PCR+ (5.6)	0	0	0	0	0
<i>Canis lupus familiaris</i>	53 (K = 38, U = 45, 30†)	0	7 PCR+ (13.2)	6	3	0	0	3
<i>Chaerephon sp.</i>	69 (U = 69)	69 (U = 69)	0	0	0	0	0	0
<i>Pteropus seychellensis comorensis</i>	20 (U = 20)	20 (U = 20)	2 PCR+ (10.0)	1	0	0	0	1

*Lb*: *L. borgpetersenii*, *Lm*: *L. mayottensis*, *Li*: *L. interrogans*, *Lk*: *L. kirschneri*.

\* OP: organ pool, K: kidney, U: urine.

† Number of animals with paired sampling (OP/K+U)

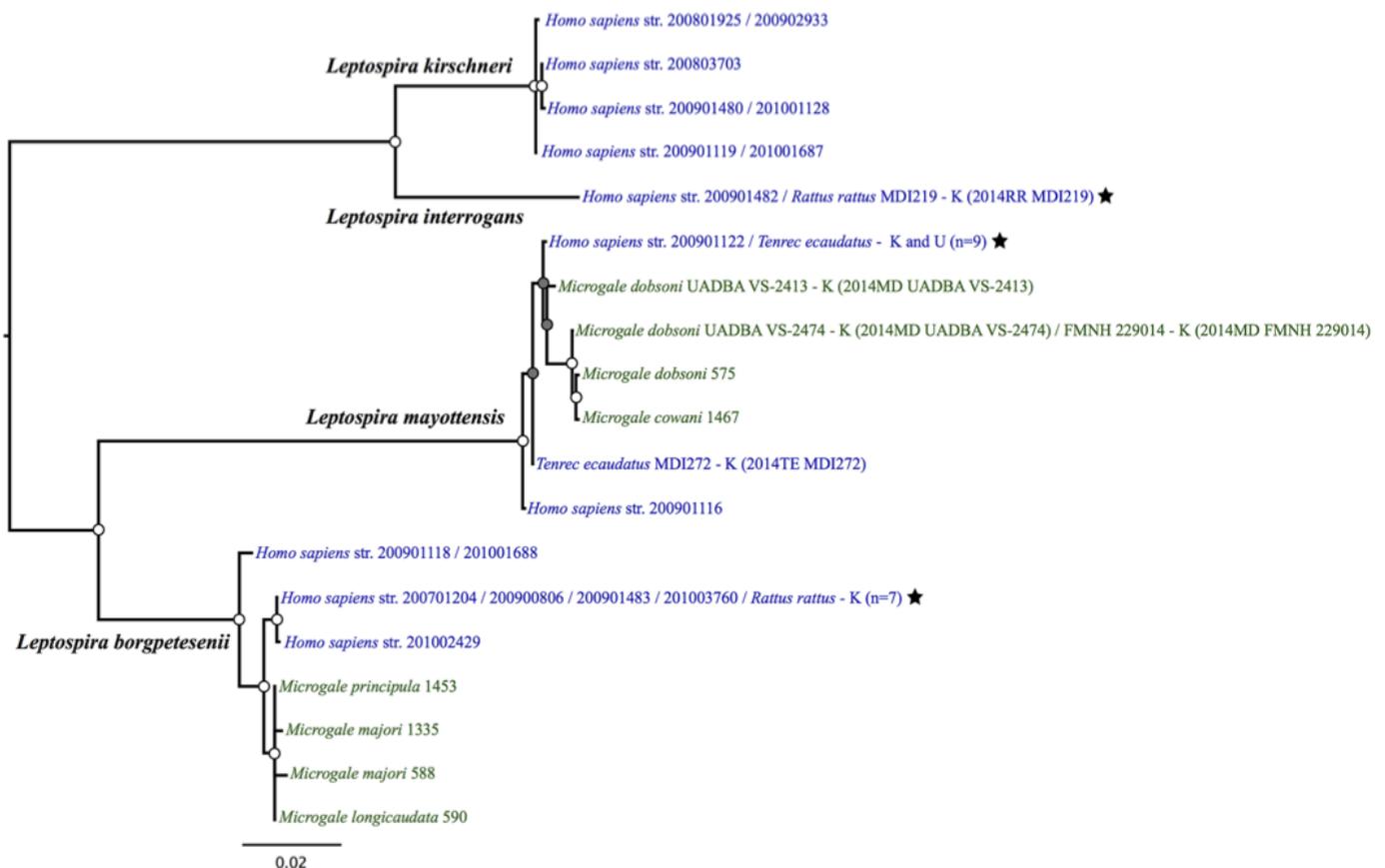
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for *T. ecaudatus*, we failed to amplify the *lipL41* gene with the conventional primers from Ahmed *et al.*[13], and used alternative primers designed by Dietrich *et al.* [4]. As expected, PCR targeting MLST loci were more arduous when using DNA extracted from tissue and urine of PCR-positive samples for which culture had failed. For instance, no successful PCR targeting MLST loci was recorded for the two RT-PCR positive *T. ecaudatus* failing to yield positive culture. Using the MLST scheme, only *rrs2* locus was amplified from four PCR-positive *R. rattus*, while two loci (*secY* and *rrs2*) were amplified from two other *R. rattus*. Similarly, only *rrs2* was amplified from six out of the seven RT-PCR-positive dog samples and from one out of the two RT-PCR-positive *P. seychellensis comorensis* samples. Lastly, no sequence could be obtained from the single zebu sample testing positive by RT-PCR. In addition, for seven PCR-positive *R. rattus* samples failing to produce any data using the MLST scheme, we successfully amplified and sequenced the *rrs2* gene using an alternative primer set (LA-LB). Altogether, we could identify leptospiral diversity at the species level for 21 *R. rattus*, eight *T. ecaudatus*, six dogs and one frugivorous bat from Mayotte (see Table 1). *Rattus rattus*-borne *Leptospira* spp. appeared genetically diverse as *L. borgpetersenii*, *L. kirschneri* and *L. interrogans* were identified for thirteen, 5 and 3 samples, respectively. The sequencing of *rrs2* locus in dog samples identified the infecting *Leptospira* spp. as *L. borgpetersenii* (n = 3) and *L. kirschneri* (n = 3). We also identified one of the two PCR-positive frugivorous bats as infected by *L. kirschneri*. Most importantly, all *Leptospira* strains isolated and/or genotyped from *T. ecaudatus* samples were identified as *L. mayottensis*.

### Phylogenetic analyses of occurring *Leptospira* spp

We constructed a concatenated phylogeny (2215 bp) by using bacterial sequences from the 16 animal *Leptospira* isolates from Mayotte of the present study, 17 previously described clinical

isolates from Mayotte [20] and nine *Leptospira* strains infecting Malagasy endemic Tenrecidae (six previously published [4] and three obtained from three distinct animals sampled in Réserve Spéciale d'Ambohitantely). The phylogeny presented on Fig 1 shows that all bacterial sequences obtained from *T. ecaudatus* clustered into the monophyletic *L. mayottensis* clade that includes two clinical *L. mayottensis* isolates (str. 200901116 and str. 200901122). This clade also contains *Leptospira* sequence types (STs) obtained from *Microgale cowani* and *M. dobsoni*, these latter species being tenrecids endemic to Madagascar. Out of the eight sequences obtained from *T. ecaudatus* in Mayotte, seven were identical to each others and similar to clinical *L. mayottensis* str. 200901122 (100% of pairwise identity based on 2215 bp) and the last one (MDI272) was closely related to the clinical *L. mayottensis* str. 200901116 (99.70% of pairwise identity based on 2215 bp). Of note, the couple of kidney/urine cultures obtained from two *T. ecaudatus* (MDI295 and MDI321) yielded identical sequence. *Leptospira mayottensis* infecting



**Fig 1. Bayesian phylogenetic tree of pathogenic *Leptospira* species from Mayotte (blue) and Madagascar (green) based on concatenated sequences of five genes (*secY*, *adk*, *lipL32*, *lipL41* and *rrs2*, total size: 2215 bp).** The analysis was realized under the GTR+G substitution model. At the nodes, grey and white circles indicate posterior probabilities superior to 0.70 and 0.90, respectively. Strain numbers of cultures produced herein are indicated in parentheses, "K" and "U" designating the sequences obtained from kidney or urine, respectively. Stars indicate lineages common to humans and animals (*Rattus rattus* or *Tenrec ecaudatus*). For *Homo sapiens* str. 200901122, the same sequence type was found in nine cultures from *T. ecaudatus* (2014TE MDI222, 2014TE MDI224, 2014TE MDI294U, 2014TE MDI295, 2014TE MDI295U, 2014TE MDI306, 2014TE MD308, 2014TE MDI321 and 2014TE MDI321U). For *H. sapiens* str. 200701204, 200900806, 200901483 and 201003760, the same sequence type found was in seven cultures from *R. rattus* (2014RR MDI247, 2014RR MDI250, 2014RR MDI251, 2014RR MDI259, 2014RR MDI260, 2014RR MDI284 and 2014RR MDI291). Specimen system: MDI = CRVOI specimen catalogue during field trips to Mayotte; FMNH = Field Museum of Natural History, Chicago; UADBA = Université d'Antananarivo, Département de Biologie Animale, Madagascar; for the other bacterial sequences from *H. sapiens* and *Microgale* spp. see Bourhy *et al.* 2012 [15] and Dietrich *et al.* 2014 [4]. Museum numbers for *Microgale* spp.: 575 = UADBA 30869; 588 = UADBA 30289; 590 = UADBA 30291; 1335 = UADBA 32122; 1453 = UADBA 32125; 1467 = UADBA 32101.

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Malagasy *Microgale* spp. displayed a 27-fold higher nucleotide diversity ( $\pi = 0.00298$ ) compared to that infecting *T. ecaudatus* from Mayotte in four different sampling sites ( $\pi = 0.00011$ , S1 Fig). The phylogeny confirms *R. rattus* as carriers of *L. interrogans* and *L. borgpetersenii*. For *L. interrogans*, the same ST is shared by one *R. rattus* (MDI219) and the *L. interrogans* clinical isolate str. 200901482 (100% of pairwise identity based on 2215 bp). For *L. borgpetersenii*, one clade of this bacterial species included seven sequences obtained from *R. rattus* that were almost identical (99.99% pairwise identity, 2215 bp) to five previously described clinical *L. borgpetersenii* isolates. Noteworthy, sequences embedded in this clade were clearly distinct from *L. borgpetersenii* sequences obtained from Malagasy tenrecids. The nucleotide diversity of *L. borgpetersenii* from Malagasy tenrecids ( $\pi = 0.00181$ ) was higher than that found in *R. rattus* from Mayotte ( $\pi = 0$ , one single ST).

Although full MLST was not successful for bats, dogs and for some of the *R. rattus*, we still used *rrs2* sequences alone in order to disentangle the role of these animals in human leptospirosis on Mayotte. For instance, the single leptospiral *rrs2* (obtained using MLST scheme primers) sequence obtained from a frugivorous bat (*P. seychellensis comorensis*) was genetically related to clinical *L. kirschneri* isolates (98.70% pairwise identity, based on 452 bp) although nucleotide divergence at this locus together with PCR failure on all other loci suggested that *L. kirschneri* detected in frugivorous bats was actually divergent from clinical samples (see S2 Fig). Sequences obtained from dogs using the same primers revealed perfect identity with *L. borgpetersenii* ( $n = 3$ ) and *L. kirschneri* ( $n = 2$ ) clinical isolates from Mayotte. Lastly, as these *rrs2* primers failed to produce amplification on some rat samples, we used an alternative primer set (LA-LB) that allowed amplification of a shorter *rrs2* sequence, revealing five identical *L. kirschneri* sequences that, importantly, diverged from clinical isolates (3 mismatches out of 245 bp), thus in favour of a predominant role of dogs in *L. kirschneri* transmission to humans.

## Discussion

Recent reports of human leptospirosis in the SWIO have stressed specificities singularizing Mayotte from the other islands of the region. *Leptospira* spp. infecting humans on Mayotte are diverse and belong to four bacterial species of which one, *Leptospira mayottensis*, was recently elevated to the rank of new species [9]. This situation clearly contrasts from that occurring on Reunion Island where *Leptospira interrogans* and *Leptospira borgpetersenii* are the only two species reported in human cases [8,21].

Our screening of Mayotte fauna allowed identifying 36 *Leptospira* strains as *L. interrogans*, *L. borgpetersenii*, *L. mayottensis* and *L. kirschneri*. We revealed either perfect or nearly perfect identity between (i) the single *L. interrogans* ST obtained from patients and *Rattus rattus* (100% identity), (ii) the prevailing *L. borgpetersenii* ST obtained from patients and *R. rattus* (100% identity), and (iii) both *L. mayottensis* STs obtained from patients and *Tenrec ecaudatus* (100% identity and 99.7% identity). The sequencing of *rrs2* locus suggests that *L. kirschneri* reported in clinical cases originates from dogs although this phylogeny, based on the single *rrs2* locus, known to display low polymorphism [22], is clearly weaker than the phylogeny based on full MLST. Finally, although a previous study reported a seroprevalence of 10% in *Pteropus seychellensis comorensis* in Mayotte [7], here all urine samples from bats tested negative for *Leptospira* sp. Although we cannot exclude that we missed positive samples due to our limited number of samples and the dynamic nature of leptospires excretion in bats over time [23], the single *L. kirschneri rrs2* sequence obtained from a *P. seychellensis comorensis* specimen was clearly distinct from the lineages involved in human disease, suggesting that local bat species do not appear to play a significant epidemiological role in the transmission of leptospirosis. Considering bats being the only indigenous mammal species occurring on Mayotte, and dogs,

*R. rattus* and *T. ecaudatus* the more widely represented mammal species in the environment [7], these results highlight the role of introduced wild and domestic small mammal species in the epidemiology of leptospirosis on Mayotte, and are specifically discussed hereafter.

Firstly and most importantly, our results suggest that *T. ecaudatus* is a main reservoir of *L. mayottensis* as this species was detected only in this host (infection rate: 27.0%) and not in the 289 *R. rattus* investigated herein. Although a previous study reported the detection of *L. mayottensis* in two out of 20 positive *R. rattus* [7], our results on a much larger sampling suggests that this species is not a significant reservoir of *L. mayottensis*. Moreover, all *Leptospira* strains infecting *T. ecaudatus* on Mayotte were strictly typed as *L. mayottensis*, although a larger sample size would be needed to confirm that *L. mayottensis* is the only *Leptospira* species infecting *T. ecaudatus*.

Secondly, the genetic analyses suggest that *L. mayottensis* likely originates from neighbouring Madagascar. Indeed, all *L. mayottensis* sequences cluster into a monophyletic well-supported clade including sequences obtained from three terrestrial wild small mammal species, namely *T. ecaudatus*, *Microgale dobsoni* and *Microgale cowani*, all belonging to the highly diversified Tenrecidae family representing an adaptive radiation of at least 32 endemic species, which colonized Madagascar some 25–30 million years ago [24]. This introduction scenario is further supported by the narrow genetic diversity of *L. mayottensis* in *T. ecaudatus* from Mayotte, based on positive samples from four different geographical sites. However, additional sampling of *T. ecaudatus* in Madagascar is necessary to confirm this hypothesis.

Thirdly, *R. rattus* is a major reservoir of pathogenic leptospires on Mayotte as also reported worldwide. Unexpectedly, the screening of a large number of rat samples ( $n = 289$ ) from different localities ( $n = 8$ ) shows that *R. rattus* on Mayotte is only marginally infected by *L. interrogans*. This feature contrasts with many investigations reported worldwide, including on the neighbouring islands of Madagascar and Reunion Island where rats are only infected with *L. interrogans* [8,25]. This peculiarity of Mayotte supports a massive contamination of the environment on this island by a diversity of *Leptospira* species, notably *L. borgpetersenii* detected in 62.0% of analysed *R. rattus*. Noteworthy, all *L. borgpetersenii* and *L. interrogans* obtained from *R. rattus*, showed perfect identity with human isolates based on the multilocus analysis. Although we identified *L. kirschneri* in five *R. rattus*, sequence analyses (*rrs2*) are rather supportive of a predominant role of dogs as reservoirs of *L. kirschneri* lineages of local medical importance. However, the absence of culture did not allow full genotyping of dog-hosted leptospires and their implication in human transmission needs further investigations.

Fourthly, *L. borgpetersenii* STs isolated from acute human cases on Mayotte group with two clearly distinct clades. The first lineage can be traced to *R. rattus* and is interestingly also closely related to a clade previously described as associated with endemic Malagasy *Microgale* species [4], indicating a possible host shift from endemic (Tenrecidae) to introduced (rats) mammals. The second *L. borgpetersenii* lineage, represented by two human isolates (str. 200901118 and 201001688), appears related to *L. borgpetersenii* lineages reported worldwide, e.g. Denmark, China, and Slovakia [4], but not in any wild and domestic animals sampled herein. This cosmopolite lineage may have been introduced through a reservoir, which is yet to be identified.

Finally, the remarkably high success rate to grow in culture *L. mayottensis* from *T. ecaudatus* (8/10, 80.0%) and *L. borgpetersenii* from *R. rattus* (7/13, 53.8%) indicates some specific biological peculiarities of these strains on Mayotte. Indeed, using the same protocol, we could grow in culture on Madagascar only three strains of *L. mayottensis* (obtained from kidney extracts from *M. dobsoni*, included on Fig 1), while other *Leptospira* species were found in terrestrial Malagasy wild animals [4,25]. Hence, this property to readily grow on liquid culture medium may reflect some adaptive advantage to survive and/or multiply in the environment.

Interestingly, *L. mayottensis* was reported to grow over a wide range of temperature including 11°C and 37°C in contrast to other pathogenic strains [9].

Islands are considered as exceptionally prone to the successful introduction and subsequent invasive nature of exotic animals and plants [26] and have been studied in detail because of adaptive features of species in insular contexts. Our data document that a pathogen most certainly originally endemic to Madagascar [4] has been introduced to the small island of Mayotte where it expanded to become a significant emerging human pathogen. From an evolutionary perspective, our study highlights that beside macro-organism diversity, the associated microbial diversity, including endemic microbial lineages, deserves to be included in studies of invasive biology in the context of island biogeography. For instance, although we cannot exclude that other animal species may excrete *L. mayottensis* on Mayotte Island, the absence of *L. mayottensis* in other animal species together with the monophyly of *L. mayottensis* clade, composed of lineages strictly infested to tenrecids, support that this new *Leptospira* species has actually coevolved during the Malagasy radiation of tenrecs, as recently proposed for bat-borne *Leptospira* lineages in Madagascar [22].

## Supporting Information

**S1 Fig. Distribution of small mammals sampling sites on Mayotte.** Numbers correspond to the 18 sampling sites where *Rattus rattus* and *Tenrec ecaudatus* (white) and bats (orange) were trapped. Map was created with QGIS 2.8.1 (QGIS Development Team, 2016, QGIS Geographic Information System, Open Source Geospatial Foundation Project). Photography of Mayotte: BD Topo IGN, 2008. (TIFF)

**S2 Fig. The figures A and B display Bayesian phylogenetic trees of pathogenic *Leptospira* from Mayotte (blue) and Madagascar (green) based on 452 bp (57 taxa, HKY+I+G) (A) and 245 bp (64 taxa, K80+I) (B) of the *rrs2* gene.** At the nodes, the black numbers indicate posterior probabilities. The sequences highlighted in grey (A) and red (B) represent PCR positive samples for which only the *rrs2* gene was obtained. Strain numbers of cultures produced herein are indicated in parentheses, “K” and “U” designating sequences obtained from kidney or urine, respectively. Specimen system: MDI and MAY = CRVOI specimen catalogue during field trips to Mayotte; all *Canis lupus familiaris* were sampled during field trips to Mayotte; FMNH = Field Museum of Natural History, Chicago; UADBA = Université d’Antananarivo, Département de Biologie Animale, Madagascar; for the other bacterial sequences from *Homo sapiens* and *Microgale* spp. see Bourhy *et al.* 2012 [15] and Dietrich *et al.* 2014 [4]. Museum numbers for *Microgale* spp.: 575 = UADBA 30869; 588 = UADBA 30289; 590 = UADBA 30291; 1335 = UADBA 32122; 1453 = UADBA 32125; 1467 = UADBA 32101. (TIFF)

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## Author Contributions

**Conceived and designed the experiments:** EL SMG PT KD.

**Performed the experiments:** EL YG GLM CC BR.

**Analyzed the data:** EL YG CC MD PT KD.

**Contributed reagents/materials/analysis tools:** EC PT KD.

**Wrote the paper:** EL YG MD PT KD.

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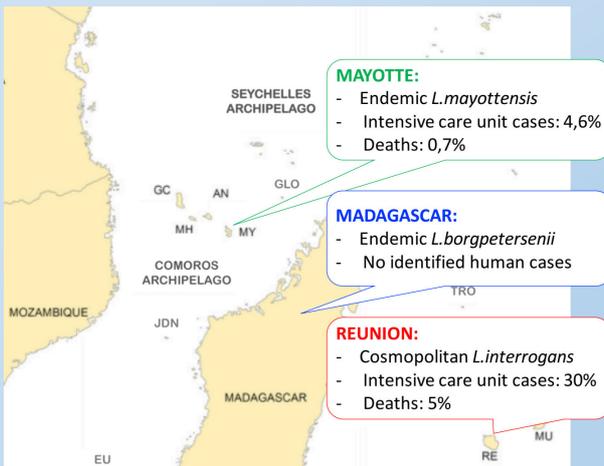
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# Pathogenicity of *Leptospira* strains in Southwestern Indian Ocean (SWIO)

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## Background

- Leptospirosis incidence is maximal in tropical islands for unknown reasons.
- South Western Indian Ocean (SWIO) islands are home to introduced and endemic *Leptospira*.
- Epidemiological data suggest a lower pathogenicity of endemic *Leptospira* strains



## Aim of the study

To compare the pathogenicity of endemic vs. introduced *Leptospira*

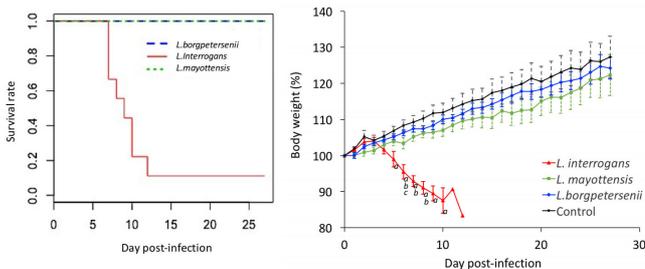
## Methods



- Intraperitoneal infection of hamsters with  $2.10^8$  *L.interrogans* (n=9), *L.mayottensis* (n=7) or *L.borgpetersenii* (n=7)
- Monitoring for 27 days
- Quantification of leptospiral loads in urine and distinct organs
- Observation of tissue damage

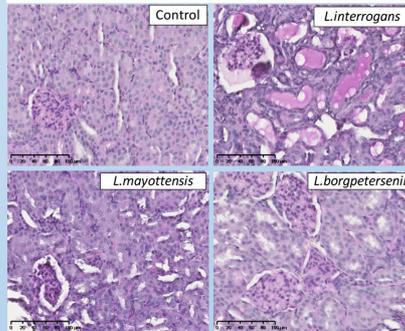
## Results

### Hamsters monitoring



**Figure 1.** Survival curve and evolution of weight percentage of hamsters infected with different strains of *Leptospira*. Weight percentages are expressed as mean  $\pm$  SEM. The letters indicate significant difference with (a) control, (b) *L.mayottensis* and (c) *L.borgpetersenii*.

## Histology



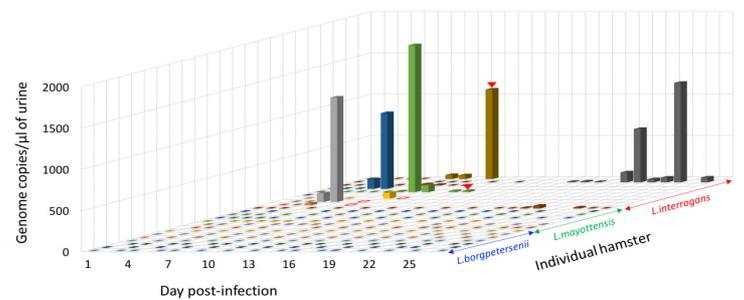
**Figure 2.** Microscopic examination of control and infected kidneys

- Kidneys infected with *L.interrogans*
- Retracted glomeruli
  - Strongly dilated tubules
  - Presence of hyaline casts

- Kidneys infected with *L.mayottensis*
- Glomeruli slightly retracted
  - Tubules slightly dilated

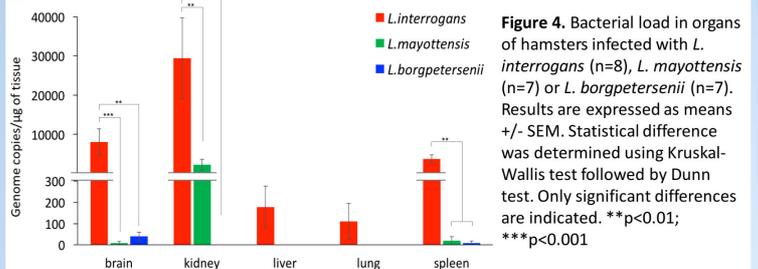
- Kidneys infected with *L.borgpetersenii*
- Glomeruli and tubules of regular shape (comparable with control)

## Urinary excretion of *Leptospira* sp.



**Figure 3.** Quantification of the excretion of *Leptospira* sp. in the urine of infected hamsters. Individual data are represented. Symbols  $\square$  and  $\blacktriangledown$  represent days when euthanasia was necessary for hamsters infected with *L.interrogans*.

## *Leptospira* sp loads in organs



**Figure 4.** Bacterial load in organs of hamsters infected with *L.interrogans* (n=8), *L.mayottensis* (n=7) or *L.borgpetersenii* (n=7). Results are expressed as means  $\pm$  SEM. Statistical difference was determined using Kruskal-Wallis test followed by Dunn test. Only significant differences are indicated. \*\*p<0.01; \*\*\*p<0.001

## Conclusion

- Both tested endemic strains *L.mayottensis* and *L.borgpetersenii* are less pathogenic than introduced *Leptospira interrogans*.
- Compared to *L.interrogans*, endemic strains spread less efficiently in the organism.
- Endemic strains are poorly excreted in urine.

## Acknowledgements



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