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Genus-wide *Leptospira* core genome multilocus sequence typing for strain taxonomy and global surveillance

Julien Guglielmini1*, Pascale Bourhy2*, Olivier Schiettekatte2,3, Farida Zinini2, Sylvain Brisse4‡*, Mathieu Picardeau2‡*

1 Institut Pasteur, Bioinformatics and Biostatistics Hub, C3BI, USR 3756 IP CNRS, Paris, France, 2 Institut Pasteur, Biology of Spirochetes unit, National Reference Center for Leptospirosis, Paris, France, 3 Université Paris Diderot, Ecole Doctorale BioSPC, Paris, France, 4 Institut Pasteur, Biodiversity and Epidemiology of Bacterial Pathogens, Paris, France

* These authors contributed equally to this work.
‡ These authors are joint senior authors on this work.
* sylvain.brisse@pasteur.fr (SB); mathieu.picardeau@pasteur.fr (MP)

Abstract

*Leptospira* is a highly heterogeneous bacterial genus that can be divided into three evolutionary lineages and >300 serovars. The causative agents of leptospirosis are responsible of an emerging zoonotic disease worldwide. To advance our understanding of the biodiversity of *Leptospira* strains at the global level, we evaluated the performance of whole-genome sequencing (WGS) as a genus-wide strain classification and genotyping tool. Herein we propose a set of 545 highly conserved loci as a core genome MLST (cgMLST) genotyping scheme applicable to the entire *Leptospira* genus, including non-pathogenic species. Evaluation of cgMLST genotyping was undertaken with 509 genomes, including 327 newly sequenced genomes, from diverse species, sources and geographical locations. Phylogenetic analysis showed that cgMLST defines species, clades, subclades, clonal groups and cgMLST sequence types (cgST), with high precision and robustness to missing data. Novel *Leptospira* species, including a novel subclade named S2 (saprophytes 2), were identified. We defined clonal groups (CG) optimally using a single-linkage clustering threshold of 40 allelic mismatches. While some CGs such as *L. interrogans* CG6 (serogroup Icterohaemorrhagiae) are globally distributed, others are geographically restricted. cgMLST was congruent with classical MLST schemes, but had greatly improved resolution and broader applicability. Single nucleotide polymorphisms within single cgST groups was limited to <30 SNPs, underlining a potential role for cgMLST in epidemiological surveillance. Finally, the proposed cgMLST strategy allows high-resolution genotyping of *Leptospira* isolates across the phylogenetic breadth of the genus. The unified genomic taxonomy of *Leptospira* strains, available publicly at http://bigsdb.pasteur.fr/leptospira, will facilitate global harmonization of *Leptospira* genotyping, strain emergence follow-up and novel collaborative studies of the epidemiology and evolution of this emerging pathogen.
Introduction

Spirochetes constitute an evolutionarily and morphologically unique group of bacteria [1]. Pathogenic members of this phylum are the causative agents of several important diseases including leptospirosis, an emerging zoonotic disease with more than one million severe cases and 60,000 deaths every year worldwide, mostly in the tropical countries [2]. Pathogenic *Leptospira* species can cause a wide range of diseases in human, ranging from mild flu-like symptoms to severe complications, such as Weil’s disease and pulmonary hemorrhagic syndrome, in which the case fatality rate can reach 40% [3]. Leptospirosis is expected to become more prominent worldwide due to climate change and the growing urban population living in slums. In addition, infections with pathogenic species can lead to major economic losses in livestock, as animal infections include e.g., abortion and loss of milk production [4].

The high public health and economic importance of *Leptospira* calls for better control of the infections the bacteria cause to both humans and animals. However, the control of *Leptospira* transmission is challenging for several reasons. First, the life cycle of pathogenic *Leptospira* is complex. Pathogenic leptospires are excreted through the urine of a wide range of animals including rodents which are asymptomatic reservoirs and livestock. Transmission to susceptible hosts usually occurs through contact with water contaminated with the urine of infected animals [5]. Therefore, multiple environmental sources of exposures, linked to multiple animal species, must be considered as possibilities.

Further complicated matters, the genus *Leptospira* is genetically highly heterogeneous and knowledge of its biodiversity remains largely incomplete. Taxonomically, the genus is currently subdivided into 35 species [6]. These species are ordered into three major evolutionary clades named according to their virulence status: pathogens, intermediates and saprophytes [1]. The agents of leptospirosis belong to two subclades, the pathogens (13 species) and the intermediates (11 species). The pathogenic species are responsible of the most severe infections in both human and animals, yet we know little about which component of the spirochete are critical for virulence. The species of the intermediates subclade are widely distributed in the environment [6–10] and they may be responsible for mild infections in both human and animals [11–19]. Intermediates possess most of the virulence factors found in the pathogens [1, 20]. In turn, the saprophytes form a single clade containing eleven species that are regarded as non-pathogenic environmental bacteria [1]. Saprophytes are relatively fast-growing in *vitro* when compared to the pathogens and lack the virulence factors described in infectious strains [1]. Classification into the three main clades has been typically performed using housekeeping and 16S rRNA genes sequencing [20].
Yet another barrier against leptospirosis control is the difficulty in isolating and cultivating *Leptospira*, which hinders optimal diagnostics of infections as well as laboratory identification, and hampers the constitution and maintenance of strain culture collections that are needed for microbiological studies and diagnostic or vaccine development purposes.

Finally, there is a lack of efficient strain typing methods that would allow tracking *Leptospira* strains (i) from their environmental or animal sources to their infected hosts and (ii) as they spread across time and space. Serotyping, which relies on the use of specific monoclonal antibodies, has led to the distinction of >300 serovars based on the structural heterogeneity of the surface-exposed lipopolysaccharides (LPS). This method has demonstrated an association of serovars with some animal reservoir hosts [21], even though the mechanisms that have allowed the adaptation of pathogenic *Leptospira* to various hosts are still unknown. However, serovar identification is currently performed by only two reference laboratories worldwide and is fastidious and time-consuming [22]. Furthermore, correlation between serotypes and genomic background is not always accurate, as the LPS biosynthetic locus (*rfb*) can be horizontally transferred between *Leptospira* species [23–25].

Molecular typing methods include pulsed-field gel electrophoresis (PFGE) [26, 27], and multilocus variable-number tandem-repeat analysis (MLVA) [28], but both methods have important practical limitations. Thus, PFGE [26] is not widely used and laborious, and only the most common serovars are typeable. More recently, multilocus sequence typing (MLST) was developed [29–31], but unfortunately three distinct MLST schemes have been proposed and applied to distinct collections of isolates, resulting in fragmentation of *Leptospira* epidemiological knowledge. Further, given the heterogeneity of *Leptospira*, the above methods are not universally applicable to all clades and species. In particular, MLST schemes are mainly focused on pathogens. As a consequence, current knowledge on the biodiversity and epidemiology of *Leptospira* is limited, and there is a critical need for a consensus *Leptospira* genotyping method that would be inclusive for its entire biodiversity, would facilitate fine-level strain discrimination for epidemiological purposes, and would reach high standardization allowing comparison of data from laboratories globally.

Whole-genome sequencing (WGS) has emerged as a powerful tool for bacterial strain classification and epidemiological typing [32]. The core genome MLST (cgMLST) approach, which extends the MLST concepts to the core genome, was demonstrated to be a useful high-resolution typing method in other bacterial species [33–36].

Taking advantage of the unique strain collection of the Reference Center for Leptospirosis in charge of the leptospirosis surveillance in mainland France and French overseas territories, our objectives were (i) First, to define based on genomic sequencing, the phylogenetic diversity of *Leptospira*, and its links with ecology and geography. In particular, our purpose was to shed light on the saprophyte and intermediate clusters, which have been scarcely studied thus far, and to include potentially novel species in this analysis. (ii) Second, we aimed to devise a genomic sequence-based genotyping method that is simultaneously universally applicable across the entire *Leptospira* genus and highly discriminatory at the strain level, and to propose a genomic taxonomy of *Leptospira* strains.

**Methods**

**Leptospira strains**

We sequenced 327 genomes from the collection of the National Reference Centre for Leptospirosis (Institut Pasteur, Paris, France), which is a globally representative strain collection of isolates from environmental, animal, and human samples gathered in the last 50 years. All strains and genome sequences used here are listed in S1 Table. *Leptospira* strains were grown at 30°C.
in liquid Ellinghausen, McCullough, Johnson and Harris (EMJH) medium. Species identification and serovar typing were performed at the National Reference Centre for Leptospirosis (Institut Pasteur, Paris, France) as previously described [37–39].

**Ethics statement**

Collection of the strains was conducted according to the Declaration of Helsinki. A written informed consent from patients was not required as the study was conducted as part of routine surveillance of the national reference center and no additional clinical specimens were collected for the purpose of the study. Cultures originating from human samples were anonymized. Approval for bacterial isolation from soil and water was not required as the study was conducted as part of investigations into leptospirosis outbreaks. For New Caledonia, approval for bacterial isolation from the natural environment was obtained from the South Province (reference 1689–2017) and North Province (reference 60912-2002-2017).

**Whole-genome sequencing and assembly**

Bacterial genomic DNA was purified using MagNA Pure 96 Instrument (Roche). Next-generation sequencing was performed by the Mutualized Platform for Microbiology (P2M) at Institut Pasteur, using the Nextera XT DNA Library Preparation kit (Illumina), the NextSeq 500 sequencing systems (Illumina), and the CLC Genomics Workbench 9 software (Qiagen) for analysis. Draft genomes with 50x minimum coverage, a total size < 5.3 Mb, and a minimum N50 of 10,000 nt were used for subsequent analysis. All raw reads generated and/or contig sequences were submitted to NCBI under the project number PRJEB29877 and are available under genome accession numbers ERR3047203 to ERR3047514.

We also downloaded 182 assembled genome sequences from the NCBI and PATRIC (www.patricbrc.org) databases, including reference strains of previously described species [20] and representative isolates for each clade (S1 Table).

**cgMLST scheme definition, phylogenetic analysis and comparison with MLST schemes**

To determine a core gene set, 103 high-quality genome sequences of *Leptospira* covering the whole diversity of the *Leptospira* genus, i.e., representative isolates from the three clusters (50% from the pathogens, 12% from the intermediates, and 38% from the saprophytes) were selected (S1 Table); 50% of the genomes were downloaded from NCBI, the others were sequenced as described above.

From this set we inferred the genus core genome using the CoreGeneBuilder pipeline [40] and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (GCF_000007685) as a reference. The pipeline’s first step relies on the eCAMBer software [41], which consists of a de novo annotation of the genomes (except the reference) using Prodigal [42] and the harmonization of the positions of the stop and start codons. In the next step, the core genome is inferred with a bidirectional best hits (BBH) approach as previously described by Touchon et al. [43]. We used CoreGeneBuilder default settings except for the synteny parameters (options–R and–S) both of which were set to 1. A gene was considered as part of the core genome if found in at least 90% of our genomes. Genes were not requested to be present in all genomes, as this stringent definition of a core genome would have resulted in too few genes given the diversity of *Leptospira*. Instead, the set of genes defined using the relaxed requirement of 90% presence can be viewed as a “soft core genome”. This resulted in an initial core genome containing 764 genes. We then filtered out some genes based on the following criteria. (i) First, we removed potential paralogs. Indeed, the presence of paralogs inside a typing scheme can lead to
ambiguities, as a candidate gene might be attributable to two different core gene loci. To detect those potential paralogs, we compared each allele of each locus against all the alleles of all the other loci using the software BLAT [44]. If a single hit was found between two different loci (more than 70% protein identity between two alleles), we removed both. (ii) Second, we also removed genes that belong to one of the 3 existing Leptospira MLST schemes [29, 45, 46] and the ribosomal genes, so that they can be analyzed independently. (iii) Third, we also removed loci whose length varies too much among alleles, which is useful in reducing ambiguities during the genotyping process. We aligned the protein sequences and removed those for which the alignment contained more than 10% of gaps (total number of gaps compared to the total number of characters). (iv) We removed loci containing ambiguous characters. (v) Finally, to avoid redundancy in the information contained within the cgMLST scheme, we removed loci that were overlapping in the reference genome using the definition of Prodigal [42]: a minimum of 60 bp of overlap if genes are on the same strand, and of 200 bp if genes are on different strands. The analysis resulted in the selection of 545 core genes listed in S2 Table and this cgMLST scheme was then used to analyze the presence of genes and to call alleles in 509 genomes (S1 Table), including the 103 genomes used for core genome definition. The allele and profiles definitions of the Leptospira cgMLST scheme were made publicly available through an Internet-accessible genotyping platform at https://bigdb.pasteur.fr/leptospira/.

To derive a phylogenetic tree based on cgMLST gene loci, the allelic sequences of each locus were extracted and aligned as protein sequences using MAFFT v7 [47]. The concatenation of all loci yielded to a supermatrix of characters. IQ-TREE v1.5.4 [48] was used to infer a phylogenetic tree from this supermatrix of characters with an LG+G evolutionary model. Branch supports were assessed with both bootstrap (1,000 replicates) and aLRT-SH methods [49]. All trees were drawn using the iTOL webserver [50].

To evaluate classical MLST against the newly defined cgMLST scheme, all available Leptospira STs were downloaded from the Oxford University MLST database at https://pubmlst.org/leptospira/ [51], which comprises schemes 1, 2, and 3 developed by Boonsilp et al. [45], Varni et al. [46] and Ahmed et al. [29], respectively (S1 Table). MLST alleles derived from our WGS data were compared to the MLST database to determine the ST of our genome assemblies. Simpson index of discrimination and Wallace or Rand indices of concordance among partitions were computed using the web site http://www.comparingpartitions.info [52, 53].

Results

Leptospira isolates and genomes

A total of 327 Leptospira isolates were sequenced, covering the diversity of the Leptospira genus. A complementary set of 182 genome sequences of Leptospira strains, mostly reference strains from the Leptospira Genome project [20], was downloaded from GenBank and PATRIC (S1 Table). The total set of 509 genomes contained representatives of most Leptospira species currently described. The clusters of pathogens, intermediates and saprophytes were represented by 402, 31, and 76 genomes, respectively. Geographically, the dataset was highly diverse: strains were isolated from different geographical areas (Africa: 19, East Asia: 17, Caribbean: 13, Central America: 7, Europe: 73, Indian Ocean: 123, Middle East: 4, North America: 24, Oceania: 11, Pacific Ocean: 14, South America: 101, Southeast Asia: 97). The ecological sources of the strains were also diverse: 111 were from the environment, 226 were from humans, while the remaining isolates were from various animal hosts, such as rodents, cows, dogs, and pigs (S1 Table). The strains corresponded to 42 species including 15 novel species isolated from the environment in Japan, Mayotte, France, Malaysia, Algeria, and New Caledonia [54]. There were 26 serogroups and 73 serovars in the dataset (S1 Table). The strains
selected for this study are therefore highly diverse geographically, ecologically and taxonomically.

The general features of the 509 genomes are reported in S1 Table and summarized in S1 Fig. Genomic assembly sizes ranged from 3,450,639 to 5,267,227 base pairs. Pathogens had a heterogeneous genome size, which was larger on average than the genome size of intermediates, which in turn had a larger genome than saprophytes (p < 0.001 for both comparisons). The genomic assemblies of pathogens were more fragmented (average contig number, 222) than those of the two other clusters (52 and 47 for the saprophytes and intermediates, respectively), which may reflect the high number of mobile elements in the pathogens [55]. The guanine+cytosine content (G+C%) of genomes was higher in the intermediates (42.39%) than in the saprophytes (38.27%, p < 1e-7) and in the pathogens (38.83%, p < 1e-7). Saprophytes were more homogeneous in their G+C% content than the two other clusters (S1 Fig).

**Genome-based phylogeny of the genus *Leptospira***

To define the phylogenetic diversity of the dataset, 545 selected genes (see Methods, section cgMLST definition) were translated, aligned and then concatenated (S2 Table). The resulting phylogenetic tree is shown in S2 Fig. ANI analysis [54] revealed 42 species defined using the 95% ANI cutoff [56, 57], including 15 novel species for which a formal description was proposed elsewhere [54]. The phylogenetic tree with representatives of each species (Fig 1) is consistent with previous data [1] showing two major clades, the “saprophytes” containing species isolated in the natural environment and not responsible for infections and “pathogens” containing all the species responsible for infections in both humans and animals, plus environmental species for which the virulence status is not clearly established. This latter clade is further subdivided in two subclades that we named P1 (formerly described as the pathogen group) and P2 (formerly described as the intermediate group). Note that two strains previously assigned to the saprophytes (strains 201400974 and E30 isolated from the natural environment in Algeria and Japan, respectively) were clearly distinct from the other saprophytes and represent new species, named *L. ilyitchenensis* and *L. kobayashii*, of a novel subclade within the clade of saprophytes. We named this new subclade S2 for convenience, in comparison to S1 which is constituted by species formerly described as the saprophyte group [54]. The basal position of the saprophyte clade with respect to P1 and P2 subclades is concordant with previous studies [58, 59]. The mean genetic distances among the three main subclades S1, P1 and P2 (S3 Fig) ranged between 0.33 substitutions per site (pathogens P1- intermediates P2) and 0.47 substitutions per site (intermediates P2- saprophytes S1), underlining the fact that these subclades are separated by large evolutionary distances. In contrast, mean intra-subclade genetic distances were 0.13 (saprophytes S1), 0.12 (pathogens P1) and 0.17 substitutions per site (intermediates P2), reflecting the higher heterogeneity and deeper phylogenetic branching of the intermediates P2 subclade. The distance between the new subclade S2 and saprophytes S1 was 0.29, showing that it lies close the P1-P2 inter-subclade distance.

We found that all species were monophyletic (S2 and S4 Fig). Furthermore, as expected, the intra-species distances were much lower than the inter-species. For example, *L. borgpetersenii* isolates formed a tight cluster with a maximum genetic divergence of 0.179 substitutions per site. Similarly, *L. interrogans* isolates showed high genetic relatedness, with a maximum distance of 0.033. This is remarkable given that both species are distributed worldwide (Fig 2). *L. mayottensis*, which is confined to the islands of Mayotte and Madagascar, showed a level of diversity of 0.008.

The phylogenetic analysis (Fig 1) revealed some structuration and led us to recognize several subgroups of species within subclades. Regarding the subclade P1, species *L. interrogans*,
Fig 1. Phylogeny of representative strains of 42 *Leptospira* species. The phylogenetic tree was obtained from the concatenation of 545 amino-acid sequence alignments using IQ-TREE [48] and the maximum likelihood criterion. In addition to previously described *Leptospira* species, new species (S1 Table) from subclades S2 (*L. iiyamaensis* and *L. kobayashii*), S1 (*L. bouyoucaniensis*, *L. kemamanensis*, *L. jelkensis*, *L. bandrabouensis*, *L. mtsangambouensis*, *L. perakensis*, *L. congkakensis*), P2 (*L. sarikeiensis*, *L. dzoumogneensis*, *L. selangorensis*), and P1 (*L. putramalaysiae* and *L. dzianensis*) are included. Species are grouped into subgroups (numbers within blocks in the column on the right) within each subclade (colors of blocks; see key).

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L. noguchi and L. kirschneri clustered into one subgroup (P1-1), whereas L. borgpeterseni, L. alexanderi, L. weilii, L. mayottensis, and L. santarosai formed a second subgroup (P1-2). Two other subgroups are constituted by L. alstonii (P1-3) and L. kmetyi, L. barantonii from New Caledonia [60] and L. dzianensis isolated from the environment in Mayotte (P1-4). Finally, subgroup P1-5 comprised L. adleri, L. putramalaysiae from the environment in Malaysia and L. typperaryensis. These subgroups are consistent with previous studies [20, 59, 61, 62].

To improve resolution, separate trees were constructed for the saprophytes S1 and the intermediates P2 (S4 Fig), showing the high level of genetic diversity among environmental isolates. The saprophytes were grouped into two subgroups. Subgroup 1 (S1-1) comprised L. vanthielli, L. brenneri, L. wolbachii; two new species: L. perdikensis and L. congkakensis from Malaysia; L. meyeri, L. harrisiae and the new species L. mtsangambouensis and L. bandrabouensis isolated from Mayotte. Subgroup 2 (S1-2) comprised L. biflexa and three new species, L. bouyoujniensis, L. kemamanensis, and L. jelokensis, isolated from Mayotte and Malaysia; and L. levetti and the new species L. ellinghausenii isolated from soil in Japan.

Among the intermediates P2, three subgroups were recognizable: subgroup P2-1 with L. fainei, L. broomi, and L. inadai; subgroup P2-2 with L. wolffii; and subgroup P2-3 with L. venezuelensis, L. licerasiae, L. saintgironsiae and four new species, named L. dzoumognensis, L. johnsonii, L. selangorensis, and L. sarikeiensis, isolated from soils in Malaysia, Japan, and Mayotte (Figs 1, S2 and S4). A scheme for classifying Leptospira strains is proposed in S3 Table.

The phylogenetic structuration reflects a strong contrast between inter- and intra-species distances, which makes it possible to assign isolates at the species level based on their genome sequence-derived phylogenetic position. This led us to re-identify some isolates. For example,
strain GWTS assigned to pathogen *L. alstonii* based on the 16S rRNA and *secY* genes [63, 64] did not cluster with the *L. alstonii* reference strain and formed a distinct branch in our phylogenetic tree (S2 Fig). Based on ANI values with representative species, including new species described in this study, it represents a new pathogenic species that we named *L. tipperaryensis* (S1 Table) [54]. Similarly, strains of serovar Rushan were previously identified as belonging to *L. noguchi* [65] but were phylogenetically clustered with *L. alstonii* (Figs 1 and S2) and had ANI values of 99.29% compared with the type strain of *L. alstonii*. These strains therefore appear to be new members of *L. alstonii*. Interestingly, the *L. alstonii* reference strain, of serovar Sichuan, was isolated from a frog [66], as were the strains from serovar Rushan, suggesting a tropism of this species for frogs.

**Geographical distribution of *Leptospira* species and *L. interrogans* sublineages**

Species of the saprophytes and intermediates subclades were represented by few strains. In contrast, some species of pathogens subclade P1 were represented by multiple isolates (e.g., 160 for *L. interrogans*, 76 for *L. borgpetersenii*, 52 for *L. kirschneri*, 27 for *L. santarosai*, 27 for *L. noguchi* and 23 for *L. mayottensis*). Based on the present sample of *Leptospira* genomes, the geographic distribution of these species showed clear differences (Fig 2). *L. interrogans*, *L. borgpetersenii* and *L. kirschneri* were found in all world regions, even though *L. kirschneri* appeared more rarely in Asian and American samples than in Europe and Mayotte. In contrast, in our dataset, *L. santarosai* was only sampled from the American continent and the Caribbean islands and *L. noguchi* was found predominantly in the Americas and rarely in Asia. So far, *L. mayottensis* has been only isolated from the Indian Ocean islands (Fig 2).

We analyzed in more details the geographic distribution of the diversity of *L. interrogans*, the most common *Leptospira* species from human infections around the world, and which was the most represented in our dataset. S5 Fig presents a phylogenetic tree of the 152 *L. interrogans* isolates for which the geographic source was known; these were from 32 countries in all world regions. The data reveal extensive geographical spread of *L. interrogans* sublineages. Although some sublineages were sampled in a single world region (e.g., the sublineage containing serovars Szwajizak, Wewak, and Hawain originated in Oceania), it is clear that most sublineages are geographically widespread (S5 Fig). This is true even for genetically homogenous subgroups, which have limited phylogenetic depth and have therefore emerged recently. These data demonstrate the rapid spread of *L. interrogans* sublineages over large geographic distances.

**High-resolution cgMLST genotyping for *Leptospira* isolates**

To develop a standardized subtyping strategy for *Leptospira*, we analyzed genome sequences using a gene-by-gene approach [34], based on the 545 genes that were highly conserved across the genus (S1 and S2 Tables). We define this set of gene loci as a core genome MLST (cgMLST) scheme [33, 34] for *Leptospira*; note that due to occasional absence of a few genes in some genomes, strictly speaking this set of genes is a ‘soft core genome’. The majority of cgMLST genes (527 loci per isolate on average, 96.7%) were called successfully (i.e., an allele was defined), including in the saprophytes S1 and intermediates P2 (S1 Table). The number of successfully called alleles per isolate ranged from 436 to 545 depending on the gene (S1 Table). Hence, this cgMLST scheme allows genotyping of all *Leptospira* genomes, with only a few missing data points.

For high-resolution subtyping, we defined cgMLST sequence types (cgST) as groups of cgMLST allelic profiles that are identical at all loci except for missing data, which are ignored...
in pairwise comparisons of allelic profiles (S1 Table). Considering the 509 genomes, there were 463 distinct profiles (defined by their cgST identifier, S1 Table), i.e., most genomes could be identified by a unique allelic profile. The discriminatory power of cgST classification (Simpson’s index) was 99.9%, much higher than that of MLST: for genomes that were typeable by cgMLST and the three MLST schemes [29, 45, 46], the Simpson indices of discrimination were 0.999 (confidence interval: 0.998–1.000), 0.793 (0.735–0.851), 0.787 (0.730–0.845) and 0.787 (0.730–0.845) for cgST, MLST1, MLST2 and MLST3, respectively. Hence, as expected, the use of 545 genes instead of 7 cgMLST largely improves our ability to distinguish among Leptospira isolates.

To assess the reproducibility and stability of cgST subtyping, sequencing replicates were performed for three isolates: L. licerasiae strain VAR010, L. meyeri strain Veldrat, and L. interrogans strain L495. The two replicates of the same isolate shared the same cgST, indicating high reproducibility of cgST classification. We next analyzed a culture-attenuated strain of L. interrogans serovar Lai that had accumulated mutations (insertions, deletions, and single-nucleotide variations) in 101 genes after serial in vitro passages over several years [67]. The derived strain (cgST20) was clearly distinct from the virulent parental strain (cgST23, differing by 15 loci). Nevertheless, these subcultures were grouped together in the phylogenetic tree (S2 Fig). Similarly, a virulence-attenuated isolate of L. interrogans serovar Manilae passed 67 times was sequenced [68] and compared with the corresponding parental virulent culture. The cgMLST analysis classified the 2 cultures as cgST31 and cgST32, differing by only 2 alleles out of 545 genes. These results illustrate the high resolutive power of cgMLST, which can distinguish genomes of isolates that evolved in-vitro over several generations.

To evaluate the genetic diversity among isolates classified into the same cgST (or groups of cgSTs differing only by missing data in some isolates), we analyzed the three most numerous ones (highlighted with colors in S1 Table, column cgST) using a whole-genome single nucleotide polymorphisms (SNP) approach. First, cgST128 and its related cgST123 and cgST308 comprised eight L. borgpetersenii isolates from Mayotte. These differed by a maximum of 16 SNPs, and five isolates had only up to 2 SNPs among themselves. Second, cgST262 and related cgSTs (cgST130, cgST321 and cgST396) comprised 11 isolates, also of L. borgpetersenii from Mayotte. These isolates differed among themselves by up to 23 SNPs. Finally, cgST482 and related cgST484 comprised seven L. interrogans isolates from cows in Uruguay; all these isolates were identical (no SNP) except one, which differed by only three SNPs from the others. These results show that isolates sharing the same cgST, or cgSTs that are identical except for missing data, are very closely related also based on whole-genome SNPs, and include levels of whole-genome SNPs that are compatible with the isolates being part of recent chains of transmissions [69, 70].

A genomic taxonomy for Leptospira strains

To define groups of Leptospira strains based on cgMLST, we first explored the distribution of pairwise distances among all cgMLST profiles (S6 Fig). We also evaluated the quality of clustering, using the Silhouette index [71], resulting from the use of all possible threshold values (from 1 to 544) in single-linkage clustering (S7 Fig), revealing a plateau of maximal clustering quality between 40 and 300 allelic mismatches. Based on the above analyses, a threshold of 40 allelic differences was chosen as the cut-off value to define clonal groups (CG). In other words, a CG is defined as a group of cgMLST allelic profiles differing by no more than 40 allelic mismatches, out of 545 gene loci, from at least one other member of the group. This definition resulted in the identification of 237 CGs (S1 Table). To evaluate this choice as compared to alternative thresholds, we compared using the adjusted rand coefficient [72] the partitions (i.e.,
groups of isolates classified into the same CG) obtained using thresholds of 20, 30, 50, 60, 150, 200 and 300 mismatches (S7 Fig). Interestingly, confidence intervals overlapped with those of threshold 40 within a wide range of possible cutoff values (20 to 150). Hence, a choice of alternative thresholds in that range would have a limited impact on the resulting clusters. Finally, the effect of missing data (uncalled cgMLST alleles) on the clustering results was evaluated in silico by introducing increasing amounts of missing data and assessing the resulting clusters of isolates as compared to their initial cluster (S8 Fig). This simulation showed that cluster assignment is robust to even high amounts of missing data (affecting up to 400 loci out of 545).

The clusters created at the 40-mismatch level represent a potentially useful genome-based taxonomy of Leptospira strains. To evaluate this classification system in comparison with previous Leptospira strain classifications, we first compared them to the 6- or 7-gene MLST classifications currently in use [29, 45, 46]. The three MLST classifications (S1 Table) were mapped onto the phylogenetic tree and their concordance with cgMLST was analyzed (Fig 3). A total of 260, 106, and 143 Leptospira STs are currently defined for MLST schemes 1, 2, and 3, respectively (April 2018; https://pubmlst.org/leptospira/). These MLST schemes were developed for strain typing of the main pathogenic Leptospira species but not for the saprophytes and intermediates [29, 30, 45, 46, 73]. As expected, saprophytes and most intermediates were not typeable by the three classical MLST schemes, whereas by design, all strains were typeable by cgMLST (Fig 3). Therefore, the typeability of the proposed cgMLST scheme appears greatly enhanced as compared with classical MLST.

We also assessed the concordance among assignments produced by the three MLST schemes and the cgMLST clustering into CGs, using Sankey diagrams (S9 Fig) and adjusted Rand and Wallace coefficients [72]. The adjusted Rand index of concordance of MLST with cgMLST was 0.86, 0.89 and 0.89 for MLST1 [45], MLST2 [46] and MLST3 [29], respectively. Wallace indices are not symmetrical, and thus produce two values: one for the comparison of MLST vs cgMLST clustering (i.e., how well MLST identity predicts CG identity), and one for the reciprocal comparison. The results were 0.86 and 0.86 for MLST1, 0.82 and 0.97 for MLST2, and 0.83 and 0.96 for MLST3. Hence, the CG accurately predicts with high accuracy the STs of MLST2 and MLST3. Only 4, 1 and 2 cgMLST clusters matched more than one MLST ST for scheme 1, 2 and 3, respectively (S9 Fig). Reciprocally, 26, 9 and 13 STs for MLST1, MLST2 and MLST3, respectively, were subdivided into more than one CG. In other words, despite accepting 40 mismatches within members of the groups, CG classification is still more discriminatory than each of the classical MLST systems. Note that although the low- and high-passage strains (see above) of L. interrogans serovar Lai and L. interrogans serovar Manilae were distinguishable at the level of cgST subtypes, they were classified into the same CG (CG16 and CG23, respectively), consistent with their recent evolutionary link.

To provide access to the cgMLST allele and profiles nomenclature, allowing for comparison and sharing of typing results among laboratories worldwide, a database was set up and was made publicly accessible online (https://bigsdb.pasteur.fr/leptospira/). This database is based on the software framework Bacterial Isolate Genome Sequence Database (BIGSdb) [33, 34, 74].

**Phylogenetic distribution of serovars and correspondence with clonal groups**

The distribution of serovars and serogroups along the phylogeny showed that most serogroups had a polyphyletic distribution. The fact that phylogenies can be in disagreement with serotyping was previously reported, and some serovars or related serovars from a same serogroup may belong to different species [21]. Thus, isolates from the same serogroup can be distributed
in different species or sublineages within species. For example, *L. interrogans* strains of serogroup Australis or of serogroup Pyrogenes did not all cluster together in the phylogenetic tree (S2 Fig).

We investigated the correspondence of cgMLST groups with serovars. Serogroups (sg) were usually sub-divided into several CGs (S1 Table). For example, the 29 isolates of sg Australis...
were subdivided into 14 CGs, the 42 isolates of sg Grippotyphosa fell into 16 CGs, and the 20 isolates of sg Pyrogenes were grouped into 12 CGs. At the serovar level, highly related strains belonged to the same clonal group (S1 Table). This was the case for the 19 isolates from serovars Copenhageni and Icterohaemorrhagiae, which were clustered together in CG6, and for serovars Ratnupura and Vanderhoedeni (CG185, *L. kirschneri* sg Grippotyphosa) and Bajan and Barbudensis (CG179, *L. noguchii* sg Australis). However, some serovars were genetically more heterogeneous and were themselves sub-divided into different cgMLST clonal groups (e.g. *L. kirschneri* and *L. interrogans* sv Grippotyphosa: 6 CGs; *L. interrogans* sv Lai, 3 CGs; *L. interrogans* sv Pyrogenes: 8 CGs) (S1 Table). Therefore, cgMLST groups represent a useful classification system that is genome sequence-based and is complementary to serogroup and serovar classification, which are based on surface antigens.

**cgMLST and *Leptospira* epidemiology**

To explore the links between cgMLST classifications and the epidemiology of *Leptospira* strains, we first analyzed the correspondence of cgMLST groups with hosts. It is well established that serovars are usually associated with a specific animal reservoir; for example, rats usually carry serovars of the Icterohaemorrhagiae serogroup; and serovar Canicola is associated with dogs [21]. Here, the most frequent cgMLST clonal groups of subclade P1 contained isolates obtained from both human and animals (except in Mayotte where few isolates have been isolated from animals). Thus, isolates of *L. interrogans* sg Pyrogenes (CG23), *L. borgpeterensis* sg Ballum (CG15) and *L. borgpetersenii* sg Javanica (CG25), associated with human leptospirosis, were clustered by cgMLST with rodent isolates, suggesting that these serogroups are maintained in rodents and that these animals represent reservoirs of human infections (S1 Table). Similarly, CG19 corresponding to serovar Sejroe comprised human and cattle isolates (S1 Table). Some CGs were found in an even larger range of hosts. For example, the 37 isolates belonging to CG5 (serovar Pomona) were obtained from humans, dogs and cows from seven countries. Likewise, CG28 contained isolates from dogs, rodents, pigs, and humans, indicating that some CGs or serotypes are not always restricted to specific hosts and may have a more generalist ecology. The environmental strains from our study were usually not grouped with animal or human isolates, as they formed distinct CGs.

We next analyzed 90 clinical isolates collected in the island of Mayotte (Indian Ocean) over a period of 10 years (2007–2017). cgMLST separated them into 10 CGs, which were highly congruent with their serotypes and species (S1 Table). Serogroup Mini was predominant (60%) and subdivided into five CGs, which agreed with their species assignments (CG63, CG83 and CG84 for *L. kirschneri*, CG78 for *L. borgpetersenii*, and CG79 for *L. mayottensis*). The most frequent CG was CG78, corresponding to 39 isolates, which were distributed into 25 cgSTs and were isolated over the 10-year period. Isolates belonging to *L. mayottensis* were subdivided into two CGs, CG79 (*n* = 7, 5 cgSTs) and CG82 (*n* = 16, 14 cgSTs) (S1 Table). These two groups were previously recognized by PFGE, MLST and serotyping [37, 75]. Isolates from the island of Mayotte belonged to cgMLST groups that were not found in other world regions, consistent with the unique epidemiology of leptospirosis in this insular ecosystem [37, 39]. In contrast, multiple CGs were observed in different geographical locations around the world (S1 Table). The wide geographic distribution of CGs indicates that geographic spread of *Leptospira* strains is faster than their genetic evolution into distinct CGs.

We next analyzed the geographic distribution of the high-resolution cgMLST types (cgST). One of the most represented cgSTs (cgST482) in our dataset is constituted by *L. interrogans* serovar Pomona strains (*n* = 6) isolated from cattle in Uruguay [76] (S1 Table). Although five out of six of these strains have been isolated from the same farm and were undistinguishable
by SNP analysis, one isolate from another region of the country differed from the group of five isolates by 3 SNPs. This shows that cgST classification could possibly inform on the epidemiological links among Leptospira isolates.

Discussion

Until now, a consensus approach to characterize and compare Leptospira isolates has been lacking, limiting our understanding of the biology and epidemiology of strains within this important genus and impeding progress in establishing appropriate control and prevention measures. Advanced knowledge on the diversity and distribution of Leptospira strains is also essential for the design and evaluation of the efficacy of new vaccines and diagnostic tools.

This study lays a foundation for a comprehensive understanding of the biodiversity of Leptospira and for the epidemiological surveillance of medically important Leptospira pathogens. The availability of high-throughput sequencing technologies and the reduction of their costs makes genome sequencing a viable option as the new gold standard for Leptospira genotyping and taxonomy. Recently, 14 new species were identified based on genomic comparisons and a high degree of biodiversity of Leptospira species in soils and water was recently uncovered [6, 17, 75]. Besides, there is growing evidence that “intermediate” species are responsible for mild infections in humans [6, 8, 11–17, 19, 77, 78]. Novel genotyping methods should therefore encompass the entire genus, including both potentially pathogenic and non-pathogenic strains, in order to provide universal Leptospira strain characterization systems.

The classical MLST schemes were developed using six or seven genes with a focus on pathogenic Leptospira species [29, 45, 73]. More recently, a new MLST scheme was proposed and applied to a wider collection of strains, including a few intermediate species [46, 62]. However, none of these MLST methods enables the inclusion of all major Leptospira lineages, including saprophytic strains. Here we sought to develop a cgMLST strategy, which is an extension of conventional MLST at genome scale [34]. Our comparative genome analyses resulted in the identification of 764 genus-wide core genes, including 545 that were deemed suitable for use in cgMLST genotyping. This is in accordance with previous estimates of 700 to 1,000 Leptospira core genes [6, 20, 59]. Importantly, our cgMLST scheme was developed using genomes representing the entire breadth of the phylogenetic diversity of the genus and was validated using Leptospira strains from diverse sources and geographical locations.

The cgMLST scheme was used to construct amino-acid sequence-based phylogenetic trees that were consistent with previous work and current species designations. In addition, this work revealed the existence of novel Leptospira species isolated from soils and water across a wide geographic range (Algeria, Mayotte, Japan, New Caledonia and Malaysia), including species from the new subclade S2 that is phylogenetically related to the previously known saprophytes S1. This work confirms the high diversity of Leptospira species in the natural environment [6, 60], and the novel taxa were described more formally elsewhere [54]. Further, cgMLST-based phylogenetic analysis provides high-level resolution, allowing discrimination among closely related species and strains.

Much like classical MLST data, cgMLST data can be used to devise a classification of isolates using the single linkage algorithm [79]. Here we defined clonal groups based on cgMLST with a 40 allelic mismatches cut-off value. In order to optimize discrimination among groups, this threshold was chosen as the smallest threshold within the range of thresholds that maximized the quality of clustering. We demonstrated the robustness of CG classification to missing data and to threshold choice, and therefore propose that CG identifiers will become a practical and highly stable genomic taxonomy system for Leptospira strains. However, it must be underlined that clonal groups are broad classification categories that are of limited use for transmission
studies, as illustrated by the wide geographical and temporal distribution of isolates from single clonal groups. Isolates belonging to the same clonal group always belonged to the same serogroup. Conversely, strains of a given serogroup can fall into phylogenetically unrelated clonal groups, suggesting that some Leptospira serogroups are derived from multiple independent ancestors. Further, strains belonging to the same serovar were not always clustered together by cgMLST, indicating that serovars can also be polyphyletic. In contrast, genetically related serovars were sometimes conflated by cgMLST clustering. These observations underline the complementarity of cgMLST clonal groups with previous classifications based on serotyping.

cgMLST allows assigning Leptospira isolates both at the species and serogroup levels, and in most cases at the serovar level as well. With the increasing description of novel species and the continuous recording of strain diversity within species by surveillance networks and microbiology laboratories, a precise understanding of the biodiversity of Leptospira strains is needed. cgMLST might represent a useful standard for classification and nomenclature, and would advantageously replace the current classical MLST nomenclatures, which are incomplete, and the serotyping nomenclature, which is complex and does not always reflects phylogenetic relationships, as is the case for other pathogens [80].

Although many CGs were found in distinct geographic regions, the island of Mayotte was a notable exception in that its CGs were endemic. The lack of dissemination of CGs from Mayotte, or of colonization of Mayotte by cosmopolitan CGs such as those of Icterohaemorrhagiae, illustrates the unique ecosystem of this island [81]. However, whether the distribution of species or CGs reported here reflects strong endemicity, or is due to currently limited sampling, will be subject of future studies. As an example of our sampling limitations, L. santarosai is not only found in America as shown in Fig 2 but also in Taiwan where this species is the most frequently encountered species in patients [82]. Isolation of additional strains from both humans and animals will also be required to evaluate whether or not environmental strains belonging to subclades P1 and P2 have the ability to cause infections.

We propose a high-resolution classification of Leptospira strains into cgSTs, which correspond to groups of isolates with total sequence identity at the 545 cgMLST genes, with a tolerance of missing data. We showed that this level of discrimination is able to distinguish among in-vitro evolved cultures. Due to the occurrence of missing data, the cgMLST profiles of some isolates can match several distinct cgSTs. Isolates with identical cgST or belonging to groups of related cgSTs (defined as matching single isolates’ profiles) were shown to differ at the whole-genome scale by less than ~30 SNPs. This level of divergence is indicative that they share a very recent common ancestor and might be part of an ongoing transmission chain [69, 70], even though genomic epidemiology applications to Leptospira remain to be evaluated taking into account its specific mutation rate and transmission dynamics.

L. interrogans and L. borgpetersenii are ubiquitous pathogenic species. This is probably due to the fact that rodents are major reservoir hosts for these species [45]. Thus, L. interrogans strains belonging to serovars Copenhageni and Icterohaemorrhagiae share the same CG regardless of their geographic origin. This limited genetic diversity and broad geographic distribution (S5 Fig) is consistent with recent evolution/expansion following extensive migration of rodents, the main reservoir of serovars Copenhageni and Icterohaemorrhagiae, and multiple introductions due to modern global transport, in particular long-range, ship-based travel and trade. Due to this rapid geographic diffusion, little phylogeographic signal was present in the dataset, rendering challenging the reconstruction of the geographic origins of L. interrogans and its sublineages with confidence. By contrast, species such as L. noguchi, L. kirschneri, and L. mayottensis are not associated with rats and are largely confined in specific geographical areas. The pathogen L. mayottensis may have been introduced into Mayotte from Madagascar via the tenrec, a small terrestrial mammal [83].
This work provides a framework for the definition of *Leptospira* clades, subclades, subgroups, species, as well as strains at two levels of resolution (S3 Table). The possibility for laboratories around the world to identify the same strains using a unified nomenclature and a centralised genotyping database will facilitate the sharing and dissemination of knowledge on circulating *Leptospira* genotypes, worldwide. The cgMLST scheme will also enable early detection of new genotypes being introduced into locations where they are not usually found. The links between genotypes and their pathogenic potential and virulence will be an important subject for future studies. For yet unknown reasons, a limited number of *Leptospira* serovars are much more likely to cause severe disease than others [84–87]. The role of phages, plasmids, and horizontal transfer in the acquisition of virulence factors also remains to be determined. The molecular basis of host specificity is also largely unknown. Future dedicated studies will be needed to characterize the gene content of subclades, species and strains, and their association with the clinical presentation and outcome of *Leptospira* infections.

**Supporting information**

**S1 Table. Summary of the *Leptospira* genomes used in this study.** The characteristics of the 509 *Leptospira* isolates and their genomes are indicated; this set includes the 103 genomes representative of the diversity of *Leptospira* used for core genome definition (flagged with a value of 1 in the third column). Clonal groups (CG) based on single-linkage clustering of cgMLST allelic profiles with the 40 allelic differences threshold are indicated. cgSTs, *i.e.*, profiles that differ by no allele other than for missing data, are given; some cgSTs are expressed as multiple numbers separated by semi-colons given the occurrence of missing data. The ST of the three MLST classifications, when defined, is indicated. The number of called alleles out of the 545 cgMLST loci is also provided.

(XLSX)

**S2 Table. Core genes selected for the cgMLST scheme.** The genes are named after the corresponding locus tag in the reference strain (*L. interrogans* serovar Copenhageni str. Fiocruz L1-130).

(XLSX)

**S3 Table. Genomic classification levels proposed in this study for *Leptospira* strains.**

(XLSX)

**S1 Fig. Genome characteristics of *Leptospira* subclades.** The variation of size, number of contigs and G+C% are indicated in each panel as the median, the first and third quartiles (hinges). The upper whisker extends from the hinge to the largest value no further than 1.5 * IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles). The lower whisker extends from the hinge to the smallest value at most 1.5 * IQR of the hinge. Data beyond the end of the whiskers are outliers.

(TIFF)

**S2 Fig. Maximum-likelihood phylogenetic tree of the 509 *Leptospira* isolates based on the concatenated alignment of 545 protein sequences.** The tree was rooted on the branch separating the pathogens from the saprophyte and intermediates clusters. Three circles surround the tree; the colors of the internal circle represent the species; the colors of the middle circle correspond to the serogroups, and those of the outer circle to the host. Note that the two members of subclade S2 were placed on a long branch within the saprophyte subclade S1, unlike in Fig 1; this placement corresponds to a phylogenetic artefact.

(TIFF)
S3 Fig. Distribution of the intra- and inter-subclade genetic distances. The distances were calculated from the concatenated alignment of the 545 cgMLST loci nucleotidic sequences of the 509 isolates used in this study, under the TN93 mode (https://doi.org/10.1093/oxfordjournals.molbev.a040023) and using the software goalign (https://github.com/fredericlemoine/goalign).

S4 Fig. Maximum-likelihood phylogenetic tree based on the concatenated alignment of the 545 cgMLST loci sequences of the isolates from the subclades S1 (panel 1) and P2 (panel B). Tip labels contain the name of the species when known. Species are grouped into subgroups within each subclade.

S5 Fig. Maximum-likelihood phylogenetic tree of 152 L. interrogans strains. The tree was built from the concatenated alignment of the 545 cgMLST protein sequences, and includes all L. interrogans strains for which the isolation location was known. The country of isolation and serogroup are indicated.

S6 Fig. Distribution of the number of cgMLST allelic differences between pairs of isolates among the 509 genomes. The inner panel corresponds to a zoom on the region between 0 and 100 allelic differences.

S7 Fig. Evolution of the silhouette index in relation to the number of mismatches used to cluster cgMLST profiles by single-linkage. For each possible mismatch threshold (1 to 544), we performed a single-linkage clustering of the cgMLST profiles and calculated the corresponding silhouette index. The better the clustering, the closest the index is to 1. The upper panel represents different adjusted rand indices inferred using the "Comparing partitions" tool (http://www.comparingpartitions.info/?link=Tool) when comparing the clustering at the given mismatch threshold with the clustering at the 40 loci mismatch threshold. Vertical bars correspond to the 95% confidence interval.

S8 Fig. Simulation of the impact of missing data on single-linkage clustering. For each profile (n = 359) belonging to a clonal group with at least 2 isolates, data were removed randomly so that the resulting profiles had from 25 to 525 missing alleles with a step size of 25; this was done 100 times for each profile and missing data point. The resulting profiles were then compared to all other (initial) isolates profiles, and all isolates that differed by less than the threshold (40 mismatches) were recorded. There are three possibilities. (1) If all recorded isolates belonged to the same CG, and the CG was the one of the test isolate, this is counted as a match (reproducible clustering). (2) If no isolate belonged to the CG of the test isolate, this is a mismatch. (3) If at least one isolate belonged to the cluster of the test isolate, and at least one other to another CG, this would lead through single-linkage clustering to the merging of initially separate CGs, and is counted as a fusion. We then computed the proportion of matches, mismatches and fusions in the simulation exercise. The figure presents the proportion of correct associations (matches) for each missing data level.

S9 Fig. Sankey diagram representing the correspondence between MLST and cgMLST clonal groups (CG) identifiers. The three panels correspond to each of the three classical MLST schemes, as indicated on top of the right columns. Connectors indicate the
correspondence of ST and CG; the vertical width of connectors is related to the number of isolates with both identifiers; the numbers connected from left to right denote the CG (left) or the ST (right). Vertical order and colors are arbitrary and were automatically chosen by the software RAWGraphs (https://doi.org/10.1145/3125571.3125585).

(TIFF)

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Author Contributions

**Conceptualization:** Pascale Bourhy, Sylvain Brisse, Mathieu Picardeau.

**Data curation:** Olivier Schiettekatte, Mathieu Picardeau.

**Formal analysis:** Julien Guglielmini, Pascale Bourhy, Olivier Schiettekatte, Mathieu Picardeau.

**Funding acquisition:** Sylvain Brisse, Mathieu Picardeau.

**Investigation:** Julien Guglielmini, Olivier Schiettekatte, Farida Zinini.

**Methodology:** Julien Guglielmini, Pascale Bourhy, Sylvain Brisse.

**Resources:** Sylvain Brisse, Mathieu Picardeau.

**Supervision:** Pascale Bourhy, Sylvain Brisse, Mathieu Picardeau.

**Validation:** Julien Guglielmini, Olivier Schiettekatte, Sylvain Brisse.

**Visualization:** Julien Guglielmini, Olivier Schiettekatte.

**Writing – original draft:** Sylvain Brisse, Mathieu Picardeau.

**Writing – review & editing:** Julien Guglielmini, Pascale Bourhy, Olivier Schiettekatte, Sylvain Brisse, Mathieu Picardeau.

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