Leptospira mayottensis sp. nov., a pathogenic species of the genus Leptospira isolated from humans

Pascale Bourhy,1 Louis Collet,2 Sylvain Brisse3,4 and Mathieu Picardeau1

1Institut Pasteur, Biology of Spirochetes Unit, National Reference Centre and WHO Collaborating Center for Leptospirosis, Paris, France
2Hospital Centre of Mayotte (CHM), Mayotte, France
3Institut Pasteur, Microbial Evolutionary Genomics Unit, Paris, France
4CNRS, UMR 3525, Paris, France

A group of strains representing species of the genus Leptospira, isolated from patients with leptospirosis in Mayotte (Indian Ocean), were previously found to be considerably divergent from other known species of the genus Leptospira. This was inferred from sequence analysis of rrs (16S rRNA) and other genetic loci and suggests that they belong to a novel species. Two strains from each serogroup currently identified within this novel species were studied. Spirochaete, aerobic, motile, helix-shaped strains grew well at 30–37 °C, but not at 13 °C or in the presence of 8-azaguanine. Draft genomes of the strains were also analysed to study the DNA relatedness with other species of the genus Leptospira. The new isolates formed a distinct clade, which was most closely related to Leptospira bor Borgpetersenii, in multilocus sequence analysis using concatenated sequences of the genes rpoB, recA, fusA, gyrB, leuS and sucA. Analysis of average nucleotide identity and genome-to-genome distances, which have recently been proposed as reliable substitutes for classical DNA–DNA hybridization, further confirmed that these isolates should be classified as representatives of a novel species. The G+C content of the genomic DNA was 39.5 mol%. These isolates are considered to represent a novel species, for which the name Leptospira mayottensis sp. nov. is proposed, with 200901116T (=CIP 110703T=DSM 28999T) as the type strain.

The genus Leptospira is classified in the family Spirochaetales, which belongs to the order Spirochaetales. This family was designated by Hovind-Hougen (1979) on the basis of morphological features of bacterial cells observed by microscopy (Hovind-Hougen, 1979). Species of the genus Leptospira are helix-shaped bacteria with two periplasmic flagella (Goldstein & Charon, 1988). Species of the genus Leptospira have a Gram-negative-like cell envelope in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlaid by an outer membrane, which contains surface-exposed lipoproteins and lipopolysaccharides (LPS) (Haake & Matsunaga, 2010). Because of the limited phenotypic differences recognizable within the genus, researchers have utilized antigenic differences in agglutinating antigens as the basis for identification and classification. Members of the genus Leptospira are classified into serovars, which have been defined from the structural heterogeneity in the carbohydrate component of the LPS with over 300 different serovars currently identified (Faine et al., 1999; Kmet & Dikken, 1993).

To date, the genus Leptospira comprises twenty-one different species isolated from various environments. Pathogenic species of the genus Leptospira include nine species (Leptospira interrogans, Leptospira kirschneri, Leptospira borgpetersenii, Leptospira santarosai, Leptospira noguchii, Leptospira weilii, Leptospira alexanderi, Leptospira kmentyi and Leptospira alstonii), which are capable of infecting and causing disease in humans and animals. The five intermediate species of the genus Leptospira (Leptospira inadai, Leptospira brokeri, Leptospira fainei, Leptospira woffii and Leptospira licerasiae) have been isolated from humans and animals and may be the cause of a variety of mild clinical manifestations. Finally, a third subgroup includes six

Abbreviations: ANI, average nucleotide identity; CDS, coding DNA sequence; GGD, genome-to-genome distance; LPS, lipopolysaccharides; MAT, microscopic agglutination test; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis.

The GenBank/EMBL/DDBJ accession numbers for the draft genome sequences of strains 200901116 T and 200901122 are AKWB00000000 and AKWM00000000, respectively.

Three supplementary tables and one supplementary figure are available with the online Supplementary Material.
saprophytic species (Leptospira biflexa, Leptospira wolbachii, Leptospira meyeri, Leptospira vanthieli, Leptospira terpstrae, Leptospira idonii and Leptospira yanagawae), which are environmental bacteria that do not cause disease in humans or animals.

Mayotte is a French overseas department in the Indian Ocean where leptospirosis is endemic. We previously reported a group of 15 strains isolated from the blood of patients with leptospirosis in Mayotte, which were identified as belonging to a novel species based on the analysis of 16S rRNA sequences and multilocus sequence typing (MLST) (Bourhy et al., 2012). This species was also identified in small mammals in Mayotte and Madagascar (Desvars et al., 2012; Dietrich et al., 2014). MLST and serogrouping by microscopic agglutination tests (MAT) with rabbit antiserum against reference serovars of the main serogroups (Australis, Autumnalis, Bataviae, Canicola, Ballum, Cynopteri, Grippotyphosa, Sejroe, Hebdomadis, Icterohaemorrhagiae, Panama, Semaranga, Pomona, Pyrogens, Tarassovi, Celledoni, Djamison, Mini, Sarmin, Shermani, Javanica and Louisiana) revealed two groups within this novel species (Bourhy et al., 2012). Strains from the sequence type ST5 belonged to serogroup Mini, but strains from ST5 did not show any positive agglutination with rabbit antiserum (Bourhy et al., 2012). Strain 200901122 from ST5 was further tested against a panel of monoclonal antibodies, showing an agglutination pattern similar to the one of L. borgpetersenii serovar Kenya strain Nijenga from serogroup Ballum (Bourhy et al., 2012). The reason for these discordant serotyping results is unknown. Whether strain 200901122 belongs to serovar Kenya and serogroup Ballum remains to be confirmed. In the present paper, two representative strains of the two groups, 200901122 and 200900116T, from this novel species, designated Leptospira mayottensis sp. nov., were further characterized. Strain 200901116T, which belongs to serogroup Mini was designated as the type strain.

The two strains can be cultivated in Ellinghausen-McCullough-Johnson-Harris medium (EMJH) (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967), which is an oleic-acid albumin medium containing Tweenes as the source of fatty acids and serum albumin as a detoxifier. Under dark-field microscopy (Olympus BX51) cells were found to show motility and morphology that were similar to those of the genomic DNA was 39.5 mol%, which is within the 35–45 mol% range reported for members of the genus Leptospira (Table 1). Pulsed-field gel electrophoresis (PFGE), which has a high discriminatory power, was used for genotyping strains of species of the genus Leptospira (Galloway & Levett, 2008; Herrmann et al., 1992). The isolates displayed unique PFGE patterns, which differed from the PFGE patterns of the reference strains (Bourhy et al., 2012). Group A (isolates belonging to the serogroup Mini) and B (isolates of an unknown serogroup) isolates demonstrated a high level of diversity by PFGE typing (Fig. S1, available in the online Supplementary Material).

In the past few years, hundreds of Leptospira genomes have been sequenced, including the published genome sequences of the saprophytic L. biflexa, the pathogens L. interrogans, L. borgpetersenii and L. santarosai, and the intermediate L. licerasiae (Bulach et al., 2006; Chou et al., 2012; Nascimento et al., 2004; Picardeau et al., 2008; Ren et al., 2003; Ricaldi et al., 2012). The 4 135 276 and 4 161 553 bp genomes of strains 200900116T and 200901122, respectively, are part of the ‘Leptospira Genomics and Human Health’ project from the J. Craig Venter Institute and the NIAID Genomic Sequencing Centers for Infectious Diseases. All the general aspects of library construction and sequencing performed at the JCVI can be found on the JCVI website (http://gcid.jcvi.org/). The G+C content of the genomic DNA was 39.5 mol%, which is within the 35–45 mol% range reported for members of the genus Leptospira (Tables 1 and S1).

The 16S rRNA sequences of strains 200901116T and 200901122 were amplified with primers rs1 (5’-CGCTGG-GGCGCGGTCTTAAACATGC-3’) and rs2 (5’-AGTTAT-TCACCCGCGGATGC-3’) and the sequences were compared...
Table 1. Phenotypic and genetic characteristics of reference strains of the described species in the genus *Leptospira*

Phenotypic characteristics were determined with standardized methods recommended for detection and characterization of *Leptospira* spp. Taxa: 1: *L. interrogans* RGA T (Yasuda et al., 1987); 2: *L. kirschneri* 3522C T (Ramadass et al., 1992); 3: *L. noguchii* CZ 214 K T (Yasuda et al., 1987); 4: *L. borgpetersenii* M84 (Yasuda et al., 1987); 5: *L. weilii* Celledoni (Yasuda et al., 1987); 6: *L. santarosai* LT821 T (Yasuda et al., 1987); 7: *L. alexanderi* L 60 T (Brenner et al., 1999); 8: *L. alstonii* 79601 T; 9: *L. kneyei* Bejo-Iso 9 T (Slack et al., 2009); 10: *L. mayottensis* 200901161 T; 11: *L. wolffii* Khorat-H2 T (Slack et al., 2008); 12: *L. licerasiae* VAR010 T (Matthias et al., 2008); 13: *L. inadai* LT64-68 T (Yasuda et al., 1987) (Schmid et al., 1986); 14: *L. fainei* BUT6 T (Perolat et al., 1998); 15: *L. broomii* 5399 T (Levett et al., 2006); 16: *L. wolbachii* CDC T (Yasuda et al., 1987); 17: *L. meyeri* Veldrat Semarang 173 T (Yasuda et al., 1987); 18: *L. biflexa* Patoc 1 T (Yasuda et al., 1987); 19: *L. vanhieii* WaZ Holland T (Smythe et al., 2012); 20: *L. terpstrae* LT 11-33 T (Smythe et al., 2012); 21: *L. yanagawae* Sao Paulo T (Smythe et al., 2012); 22: *L. idonii* Eri-1 T (Saito et al., 2012). +, positive; −, negative; N/A, data not available. All *Leptospira* strains are motiles and they grow at 30 °C. G+C content was determined by the thermal-denaturation method (Mandel & Marmur, 1968), except when indicated.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at/in</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>presence of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-13 °C</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>37 °C</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8-aza*</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Virulence</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
<td>N/A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DNA GC content</td>
<td>34.9±0.9</td>
<td>35.9±</td>
<td>36.5±1.2</td>
<td>39.8±0.3</td>
<td>40.0±</td>
<td>40.7±0.6</td>
<td>38.0</td>
<td>39±8</td>
<td>36.2</td>
<td>39.5±</td>
<td>41.8</td>
</tr>
<tr>
<td>(mol%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>12</th>
<th>13</th>
<th>14</th>
<th>15</th>
<th>16</th>
<th>17</th>
<th>18</th>
<th>19</th>
<th>20</th>
<th>21</th>
<th>22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at/in</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>presence of</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-13 °C</td>
<td>N/A</td>
<td>−</td>
<td>+</td>
<td>N/A</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>N/A</td>
</tr>
<tr>
<td>37 °C</td>
<td>N/A</td>
<td>+</td>
<td>N/A</td>
<td>N/A</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>8-aza*</td>
<td>−</td>
<td>−</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Virulence</td>
<td>−</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>−</td>
</tr>
<tr>
<td>DNA GC content</td>
<td>43.9</td>
<td>42.6±0.9</td>
<td>43.5±</td>
<td>42</td>
<td>37.2±0.5</td>
<td>33.5±0.2</td>
<td>36.0±0.3</td>
<td>43±4</td>
<td>38±9</td>
<td>37±9</td>
<td>42.5±0.1</td>
</tr>
<tr>
<td>(mol%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Growth in EMJH liquid medium supplemented with 225 μg ml⁻¹ 8-azaguanine at 30 °C.
†G+C content was determined by genome sequencing.

with sequences from the GenBank database for each of the species of the genus *Leptospira*. Multiple sequence alignments of DNA sequences were performed using MUSCLE (Edgar, 2004). Phylogenetic analysis was performed using PhyML with the GTR model of nucleotide substitution; the proportion of invariable sites and gamma shape parameters were estimated with 4 nt substitution rate categories (Guindon & Gascuel, 2003). Dendrograms generated from the 16S rRNA gene sequences revealed three clades (Matthias et al., 2008; Paster et al., 1991; Schmid et al., 1986). Strains 2009001116T and 200901122 were recovered from the draft genome sequences (see the accession numbers in Table S1) using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the *L. interrogans* sequence as query. They were aligned using MUSCLE, concatenated and analysed with PhyML as described above, except that a uniform rate was used. The two strains exhibited identical sequences for all six housekeeping genes and formed a clade, which was clearly distinct from other pathogenic species of the genus *Leptospira* (Fig. 3). These data confirm the results obtained by MLST with the genes *adk*, *icaA*, *secY*, *lipL32* and *lipL41*, thus indicating that these two strains should be classified as representatives of a novel species (Bourhy et al., 2012).

The genomic relatedness among strains was determined from fully or partially sequenced genomes (Table S1) using the average nucleotide identity (ANI) (Konstantinidis & Tiedje, 2005) and the genome-to-genome distance (GGD)
These algorithms have been used to replace standard DNA–DNA hybridization (DDH) by calculating DNA–DNA relatedness (Auch et al., 2010a, b; Goris et al., 2007; Wolf et al., 2001). Thus, a previous study performed sequence-based comparisons on six phylogenetically distinct groups, including strains of the genera *Bacillus*, *Burkholderia*, *Escherichia/Shigella*, *Pseudomonas*, *Shewanella* and *Streptococcus*, and showed good agreement between GGD and DDH values (Goris et al., 2007). In this study, genome relatedness was calculated using the GGD Calculator, formula 2, performed at http://ggdc.dsmz.de (Meier-Kolthoff et al., 2012). As previously recommended, *in silico* DDH estimates. 70% suggest that strains belong to the same species (Wayne et al., 1987). The GGD was calculated between strain 200901116T and genomes of representative strains of species of the genus *Leptospira* (Table S2). Strain 200901116T showed less than 70% similarity with all the other strains, except strain 200901122. Strains 200901116T and 200901122 showed values of greater than 70% similarity to each other (estimated hybridization 96.70% ± 1.05), suggesting that they belong to the same species. Similarly, strains within the species *L. interrogans*, *L. borgpetersenii* and *L. kirschneri* had GGD values higher than the cut-off value of 70% DDH similarity (Table S2). For example, *L. interrogans* Fiocruz L1-130 and *L. interrogans* strain 56601 belong to the same species (estimated hybridization 93.00% ± 1.73) and *L. interrogans* is phylogenetically related to *L. kirschneri* (estimated hybridization >42%), while other pathogenic, intermediate and saprophytic species are distantly related to *L. interrogans*. These data are phylogenetically consistent and reflect genetic relatedness among species of the genus *Leptospira*.

The pairwise ANI values were determined from the genomic sequences using *j*Species (Richter & Rossello-Móra, 2009) (Table S1). The ANI value between strains 200901116T and 200901122 was nearly 99%. In contrast, the ANI values between these strains and the most closely related pathogenic species were below 92%, thus falling below the threshold of 95% recommended for species delineation (Richter & Rossello-Móra, 2009) (Table S3). GGD and ANI analyses were in good agreement and indicated that *L. mayottensis* sp. nov. does not belong to any of the previously described species of the genus *Leptospira*, further suggesting that *L. mayottensis* sp. nov. should be recognized as a representative of a novel species.

**Fig. 2.** Phylogenetic tree using maximum-likelihood based on the analysis of a ~1300 bp sequence of the 16S rRNA gene of species of the genus *Leptospira*. Bar, proportion of nucleotide substitutions. Accession numbers are indicated under brackets. Values at the nodes denote bootstrap support (in percentage) obtained based on 1000 resampling events.
Comparative genome analysis was performed using the MaGe interface in the SpiroScope database (https://www.genoscope.cns.fr/agc/microscope/home/index.php). Strains 2009001116T and 200901122 share 3501 coding DNA sequences (CDS), with an average pair-wise amino acid identity of higher than 99 %. In comparison, using the same criteria, strain 2009001116T shares only 34 and 192 CDS with L. interrogans Fiocruz L1-130 and L. borgpetersenii strain L550, respectively. Strains 2009001116 T and 200901122 share homologies with leptospiral virulence factors, including lipoproteins LipL32/LIC11352 (94 % identity) and LigB/LIC10464 (64 % identity), collagenase ColA/LIC12760 (>84 % identity) and sphingomyelinase SphC/LIC13198 (76 % identity). The genomes also encode virulence-associated proteins that are absent from the genomes of saprophytic strains, such as catalase KatE (>87.5 % identity with LIC12032), which is involved in the resistance to oxidative stress conditions (Eshghi et al., 2012). This further confirms that these strains belong to a pathogenic species of the genus Leptospira.

Classical DDH, which estimates the overall similarity between the genomes of two strains, is recommended for species delineation, with hybridization values \( \leq 70 \% \) indicating that the tested organisms belong to a different species (Wayne et al., 1987). This technique has been used for the identification of the 21 species of the genus Leptospira with validly published names described to date. Today, the availability of more than 300 whole genome sequences of species of the genus Leptospira in the NCBI and JCVI databases makes genome comparison a viable option as the new gold standard for taxonomy. In the present study, there was a high correlation between the results of ANI and GGD with DNA–DNA relatedness, mimicking wet-lab hybridization results, as shown previously for other bacteria (Goris et al., 2007; Konstantinidis & Tiedje, 2005). In silico DDH values indicate that the tested strains, 2009001116T and 200901122, belong to a species of the genus Leptospira, which is different from those with validly published names described at the time of writing. They also show that there was strong DNA–DNA relatedness between the two strains within the novel species. The DNA G+C contents, and housekeeping and 16S rRNA gene sequences were extracted from genome sequences to verify that the data were phylogenetically consistent; the use of genome sequences provides reusable data and reproducible results. We propose that the genomic sequence of at least the type strain should be established for description of a novel species of the genus Leptospira in the future (Richter & Rossello-Mora, 2009; Tindall et al., 2010).

**Description of Leptospira mayottensis sp. nov.**

*Leptospira mayottensis* (ma.yott.en’sis. N.L. fem adj. mayottensis after the island of Mayotte in the Indian Ocean).

Motility and morphology of the isolates are similar to those of other members of the genus Leptospira. Cells are \( 9 \pm 2.1 \mu m \) long and \( \sim 0.2 \mu m \) in diameter, with a wavelength (\( \lambda \)) of \( \sim 0.5 \mu m \) and an amplitude (\( h \)) of \( \sim 0.5 \mu m \) under dark-field microscopy. The strains grow well in EMJH medium at 30 °C and 37 °C, but not in EMJH media at 13 °C or in EMJH supplemented with 8-azaguanine.
The type strain, 200901116T (=CIP 110703T=DSM 28999T), was isolated from the blood of a leptospirosis patient during the acute phase of illness (fever of 38 °C, accompanied by headache and myalgia) on the island of Mayotte in 2009. The G+C content of the genomic DNA of the type strain is 39.5 mol%.

Acknowledgements

We thank the technicians of the NRC for Leptospirosis (Sylvie Brémont, Annie Landier and Farida Zini) for typing of isolates and Ambrose Lambert for his contribution to microscopy studies. Draft genome sequences used in this study are part of a project which was funded in part with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services under contract number HHSN272200900007C. Authors thank J. Vinetz and D. Fouts for their permission to use the draft genomes. This work was also funded by the Institut Pasteur and the French Ministry of Health (InVS).

References


