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

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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 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 200901116^{T} (=CIP 110703^{T}=DSM 28999^{T}) as the type strain.

The genus *Leptospira* is classified in the family *Leptospiraceae*, 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; Kmety & 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 kmetyi* 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 broomii*, *Leptospira fainei*, *Leptospira wolffii* 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 2009001116^{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 vanthielii*, *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 antisera against reference serovars of the main serogroups (Australis, Autumnalis, Bataviae, Canicola, Ballum, Cynopteri, Grippotyphosa, Sejroe, Hebdomadis, Icterohaemorrhagiae, Panama, Semaranga, Pomona, Pyrogenes, Tarassovi, Celledoni, Djamisan, Mini, Sarmin, Shermani, Javanica and Louisiana) revealed two groups within this novel species (Bourhy *et al.*, 2012). Strains from the sequence type ST7 belonged to serogroup Mini, but strains from ST5 did not show any positive agglutination with rabbit antisera (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 200901116^T, from this novel species, designated *Leptospira mayottensis* sp. nov., were further characterized. Strain 200901116^T, 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 Tweens 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 members of the genus *Leptospira*. Cells were helix-shaped with a length of 9 ± 2.1 μm and a diameter of ~0.2 μm, with a wavelength of ~0.5 μm and an amplitude of ~0.5 μm (Fig. 1). Cells grew as subsurface colonies on 1 % agar solid EMJH media after 3 weeks of incubation at 30 °C.

Phenotypic characterization of strains 200901122 and 2009001116^T was performed by assessing their growth at varying temperatures and in the presence of the purine analogue 8-azaguanine (Johnson & Rogers, 1964). Strains 200901116^T and 2009001122 shared characteristics common to pathogenic species of the genus *Leptospira* (Table 1).

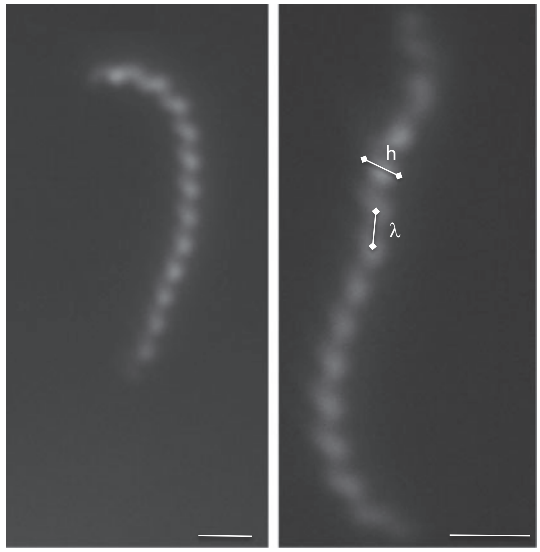

Fig. 1. Dark-field photomicrographs of cells of strain 200901116^T showing typical helical morphology and hooked ends. Cells were 9 ± 2.1 μm long and ~0.2 μm in diameter, with a wavelength (λ) of ~0.5 μm and an amplitude (h) of ~0.5 μm. Bars, 1 μm.

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 saprophyte *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 2009001116^T 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 200901116^T and 200901122 were amplified with primers rrs1 (5′-CGCTGGCGGCGCGTCTTAAACATGC-3′) and rrs2 (5′-ACGTATTCACCGCGGCATGC-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. kmetyi* Bejo-Iso 9^T (Slack *et al.*, 2009); 10: *L. mayottensis* 200901116^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. vanthielii* 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.

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Growth at/in presence of											
11-13 °C	−	−	−	−	−	−	−	−	+	−	−
37 °C	−	−	−	−	−	−	−	−	+	+	+
8-aza*	−	−	−	−	−	−	−	−	+	−	+
Virulence	+	+	+	+	+	+	+	+	N/A	+	N/A
DNA GC content (mol%)	34.9±0.9	35.9†	36.5±1.2	39.8±0.3	40.±0.7	40.7±0.6	38.0	39±8	36.2	39.5†	41.8

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

*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 2009001116^T and 200901122 were recovered in the 'pathogen' clade (Fig. 2). The 16S rRNA sequence similarity of these strains with other pathogenic species was 99 % (11 mismatches over 1314 bp between *L. mayottensis* sp. nov. and *L. borgpetersenii*). Due to the low variability and conservative nature of the 16S rRNA gene in species of the genus *Leptospira* (Morey *et al.*, 2006), the strains were subjected to multilocus sequence analysis utilizing sequences of the housekeeping genes *rpoB*, *recA*, *fusA*, *gyrB*, *leuS* and *sucA* to infer more precise phylogenetic relationships. The sequences for the six housekeeping gene loci were extracted from the draft genome sequences (see the accession numbers in Table S1) with BLASTN (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*, *icdA*, *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)

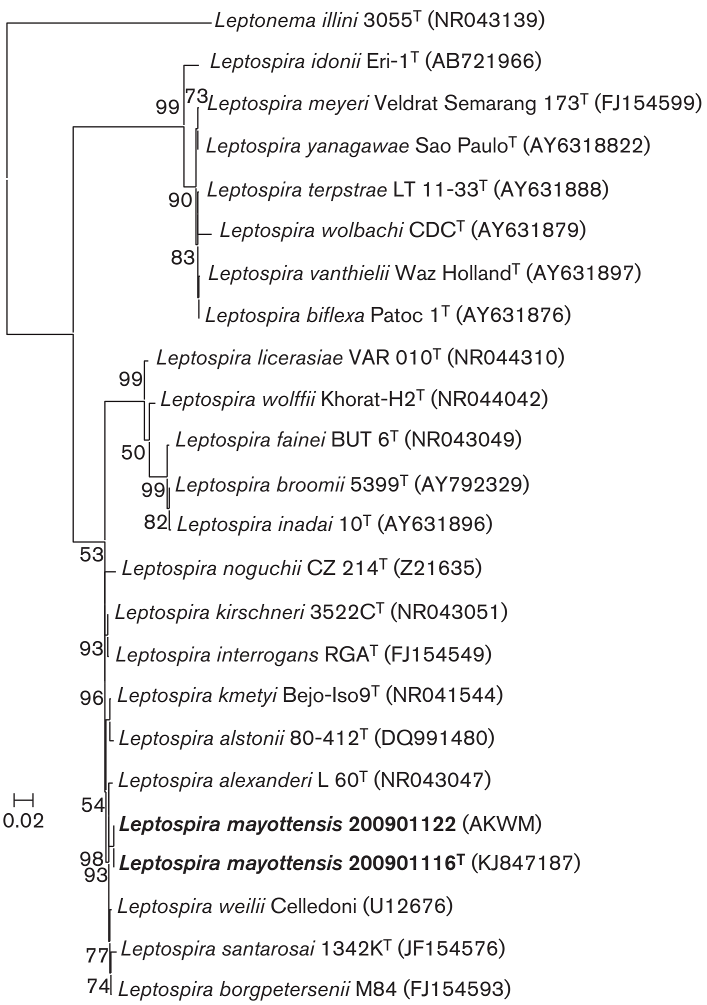

Fig. 2. Phylogenetic tree using maximum-likelihood based on the analysis of a ~1300 bp sequence of the 16S rRNA gene of species of the genus *Leptospira*. Bar, proportion of nucleotide substitutions. Accession numbers are indicated under brackets. Values at the nodes denote bootstrap support (in percentage) obtained based on 1000 resampling events.

calculator (Meier-Kolthoff *et al.*, 2012). 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 200901116^T and genomes of representative strains of species of the genus *Leptospira* (Table S2). Strain 200901116^T showed less than 70 % similarity with all the other strains, except strain 200901122. Strains 200901116^T 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 jSpecies (Richter & Rosselló-Móra, 2009) (Table S1). The ANI value between strains 200901116^T 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 & Rosselló-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.

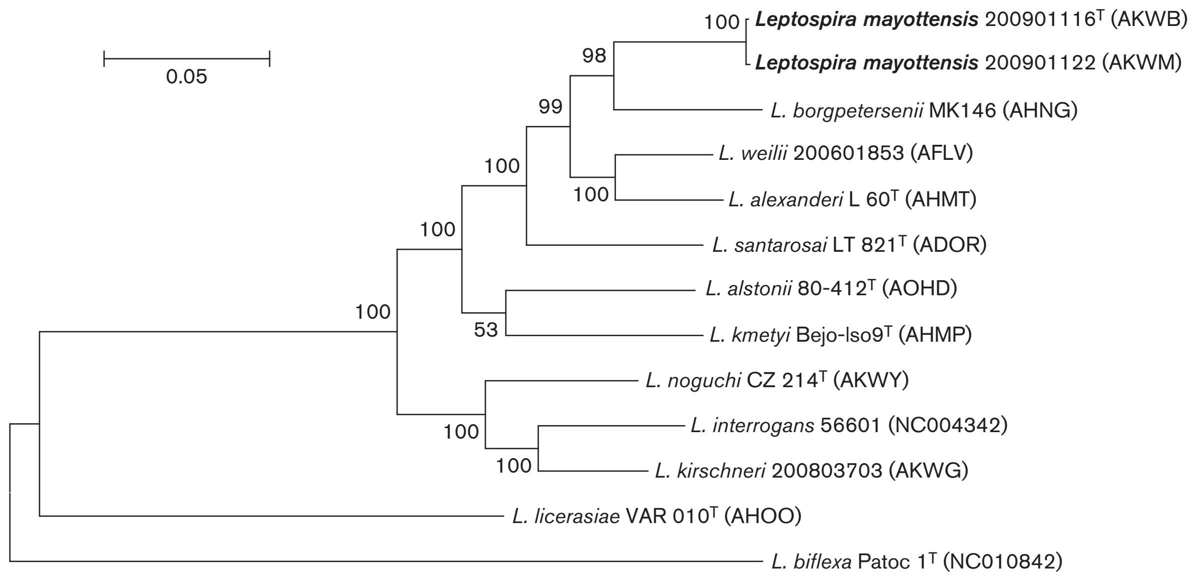

Fig. 3. Phylogenetic tree of concatenated sequences of genes *rpoB*, *recA*, *fusA*, *gyrB*, *leuS* and *sucA* of species of the genus *Leptospira*. Bar, proportion of nucleotide substitutions. Accession numbers are indicated under brackets. Values at the nodes denote bootstrap support (in percentage) 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 2009001116^{T} 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 2009001116^{T} 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 ⩽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, 200901116^{T} 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 & Rosselló-Móra, 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 ± 2.1 µm long and ~0.2 µm in diameter, with a wavelength (λ) of ~0.5 µm and an amplitude (h) of ~0.5 µm 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, 200901116^{T} (=CIP 110703^{T}=DSM 28999^{T}), 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%.

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