INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION
Casanovas-Massana *et al.*, *Int J Syst Evol Microbiol* 2020;70:1450–1456
DOI 10.1099/ijsem.0.003480

MICROBIOLOGY SOCIETY

Leptospira yasudae sp. nov. and *Leptospira stimsonii* sp. nov., two new species of the pathogenic group isolated from environmental sources

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Abstract

Four spirochetes (F1^T, B21, Yale^T and AMB6-RJ) were isolated from environmental sources: F1^T and B21 from soils of an urban slum community in Salvador (Brazil), Yale^T from river water in New Haven, Connecticut (USA) and AMB6-RJ from a pond in a horse farm in Rio de Janeiro (Brazil). Isolates were helix-shaped, aerobic, highly motile and non-virulent in a hamster model of infection. Draft genomes of the strains were obtained and analysed to determine the relatedness to other species of the genus *Leptospira*. The analysis of 498 core genes showed that strains F1^T/B21 and Yale^T/AMB6-RJ formed two distinct phylogenetic clades within the 'Pathogens' group (group I). The average nucleotide identity (ANI) values of strains F1^T/B21 and Yale^T/AMB6-RJ to other previously described *Leptospira* species were below <84 % and <82 %, respectively, which confirmed that these isolates should be classified as representatives of two novel species. Therefore, we propose *Leptospira yasudae* sp. nov. and *Leptospira stimsonii* sp. nov. as new species in the genus *Leptospira*. The type strains are F1^T (=ATCC-TSD-163=KIT0259=CLEP00287) and Yale^T (=ATCC-TDS-162=KIT0258=CLEP00288), respectively.

Leptospirosis is a globally distributed zoonotic disease that has its highest burden among vulnerable populations in both rural and urban slum environments in tropical developing countries [1, 2]. Leptospirosis is caused by spirochetes belonging to the genus *Leptospira*. This genus is divided into three distinct phylogenetic groups [3, 4]: group I or 'Pathogens' that contain virulent strains able to cause severe disease in humans and animals; group II or 'Intermediates' with species that can cause disease in certain circumstances; and group III or 'Saprophytes' which are free-living environmental micro-organisms not known to cause disease.

Recent studies by Thibeaux *et al.* have reported the isolation of 12 novel *Leptospira* species in soils from New Caledonia (French Polynesia) [5, 6]. Currently, the genus *Leptospira* comprises 35 different species: 13 belonging to group I 'Pathogens' (*Leptospira adleri*, *Leptospira alexanderi*, *Leptospira alstonii*, *Leptospira barantonii*, *Leptospira borgpetersenii*, *Leptospira ellisii*, *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira kmetyi*, *Leptospira mayottensis*, *Leptospira noguchii*, *Leptospira santarosai* and *Leptospira weilii*), 11 to group II 'Intermediate' (*Leptospira broomii*, *Leptospira fainei*, *Leptospira haakeii*, *Leptospira hartskeerlii*, *Leptospira inadai*, *Leptospira licerasiae*, *Leptospira perolatii*, *Leptospira neocaledonica*, *Leptospira saintgironsiae*, *Leptospira venezuelensis* and *Leptospira wolffii*), and 11 to group III 'Saprophytes' (*Leptospira biflexa*, *Leptospira brenneri*, *Leptospira harrisiae*, *Leptospira idonii*, *Leptospira levettii*, *Leptospira macculloughii*, *Leptospira meyeri*, *Leptospira terpstrae*, *Leptospira vanthielii*, *Leptospira wolbachii* and *Leptospira yanagawae*). A recent study identified 30 new species from environmental sources in France, Algeria, Japan, Malaysia, Mayotte and New Caledonia, including pathogenic species similar to the isolates described here, revealing a massive diversity within genus *Leptospira* [7]. Here, we report the description of two novel pathogenic species of the genus

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Keywords: Leptospira; leptospirosis; pathogenic; whole-genome sequencing; soil; water.
Abbreviations: ANI, averagenucleotide identity; EMJH, Ellinghausen–McCullough–Johnson–Harris; STAFF, sulfamethoxazole,trimethoprim, amphotericin B, fosfomycin and 5-fluorouracil.
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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains F1^T and Yale^T are MK070913 and MK070914 respectively, and the genome sequence accession numbers are QHCU00000000 and QHCT00000000, respectively.

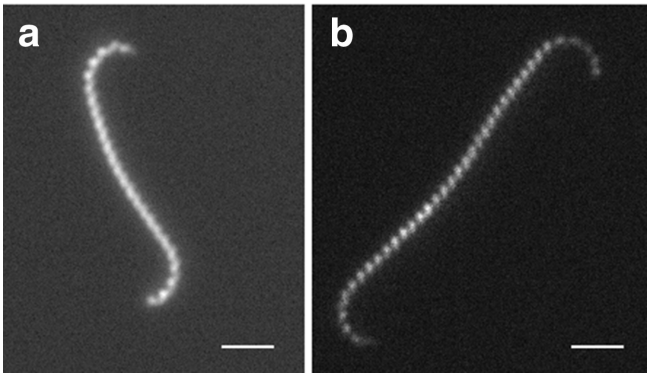

Fig. 1. Dark-field photomicrographs of representative cells of strains F1^T (a) and Yale^T (b) showing a typical leptospiral morphology. Bars, 2 µm.

Leptospira by using phenotypic characterization and whole-genome sequencing.

We isolated four leptospira-like strains in different sampling campaigns performed in Brazil and the USA. Strains F1^T and B21 were obtained from soil samples collected in the community of Pau da Lima, an urban slum community located in the city of Salvador, Bahia, Brazil. This community has high leptospirosis infection rates (37.8 per 1000 individuals per year) [8, 9] and pathogenic *Leptospira* species have been detected in its surface waters and soils [10, 11]. Briefly, during March 2013 and October 2015, 100 g samples of subsurface soil were collected in sterile containers from various sites at the bottom of one of the valleys that form this community. One millilitre of sterile double-distilled water was added to 1 g soil subsamples, vortexed for 1 min and the supernatant (~0.8 ml) added to 1 ml of 2×concentrated Ellinghausen–McCullough–Johnson–Harris (EMJH) medium supplemented with 0.2 ml of 10×concentrated STAFF (sulfamethoxazole, trimethoprim, amphotericin B, fosfomycin and 5-fluorouracil) antibiotic cocktail [12]. Strain Yale^T was isolated from the Mills River in New Haven, Connecticut, USA. A 1 L sample of freshwater was collected in February 2016, filtered through 0.22 µm SteriCup filter (Millipore) and 1 ml subsamples inoculated in EMJH tubes. Finally, strain AMB6-RJ was isolated from a pond located in a horse farm in Iguaba, Rio de Janeiro, Brazil. A sample of 10 ml was collected and 100 µl was inoculated in EMJH MEDIU medium with an STAFF cocktail. After inoculation into EMJH medium, all samples were incubated at 30 °C and checked weekly by dark-field microscopy. When cultures were positive, samples were diluted and plated onto EMJH plates (agar 1 % w/v) and incubated at 30 °C until subsurface colonies appeared (10–15 days). Single colonies were selected, transferred to fresh EMJH liquid medium and incubated at 30 °C for further testing.

The isolated strains F1^T, B21, Yale^T and AMB6-RJ showed a similar morphology and motility to other members of the genus *Leptospira* under dark-field microscopy (Fig. 1). Cells of strain F1^T/B21 were helix-shaped, 14.2±2.5 µm long, ~0.2 µm in diameter and had a wavelength of ~0.6 µm. Cells of strain Yale^T/AMB6-RJ were 17.5±2.7 µm long, ~0.2 µm in diameter and had a wavelength of ~0.6 µm. Phenotypic characterization of strains F1^T and B21 was performed by assessing their growth in EMJH medium at varying temperatures (13 and 37 °C, and at 30 °C in the presence of 8-azaguanine) [13]. Strain F1^T/B21 grew in EMJH at 13 and 37 °C, and showed a slight growth at 30 °C with the presence of 8-azaguanine. Strains Yale^T/AMB6-RJ grew in EMJH at 13 °C and at 30 °C with the presence of 8-azaguanine. Strain AMB6-RJ, but not Yale^T, grew in EMJH at 37 °C.

The whole-genome sequences of strains F1^T, B21, Yale^T and AMB6-RJ were obtained at the Yale Centre for Genomic Analysis. Genomic DNA was prepared by centrifugation of 10 ml exponential-phase cultures and extraction with the Quick-DNA Universal Kit (Zymo). Libraries were reconstructed with the KAPA Hyper Prep Kit (Kapa Biosystems) and sequenced with a HighSeq 4000 Illumina platform (pair-end reads of 150 bp). Reads were pre-processed using BayesHamer [14] and *de novo* assembled with SPAdes 3.10.0 [15]. The quality of the final assemblies was analysed with QUAST [16] and then annotated with the RAST tool kit (RASTtk) [17] in PATRIC [18] (Table 1). Sequencing data generated in this work was deposited at GenBank/EMBL/DDBJ under the accession numbers QHCU00000000, QHCR00000000, QHCT00000000 and QHCS00000000 for strains F1^T, B21, Yale^T and AMB6-RJ, respectively.

The full-length 16S rRNA gene sequences of strains F1^T, B21, Yale^T and AMB6-RJ and those of other *Leptospira* species were extracted from the genomes using Barrnap [19] in GALAXY [20]. Sequences were aligned with MUSCLE [21] and a maximum-likelihood tree reconstructed with PhyML 3.0 [22] using the GTR substitution model and 1000 bootstrap

Table 1. Genomic statistics of the isolated *Leptospira* strains

Strain	GenBank accession	Size (Mbp)	G+C content (mol %)	No. of contigs	Average coverage (×)	Contig N50	No. of genes	No. of proteins
Leptospira stimsonii Yale^T	QHCT00000000	4.837	42.60	46	396	589 757	4815	4682
Leptospira stimsonii AMB6-RJ	QHCS00000000	4.745	42.60	23	161	1 099 403	4747	4599
Leptospira yasudae F1^T	QHCU00000000	4.445	45.50	25	190	623 593	4286	4162
Leptospira yasudae B21	QHCR00000000	4.458	45.50	28	345	472 231	4263	4127

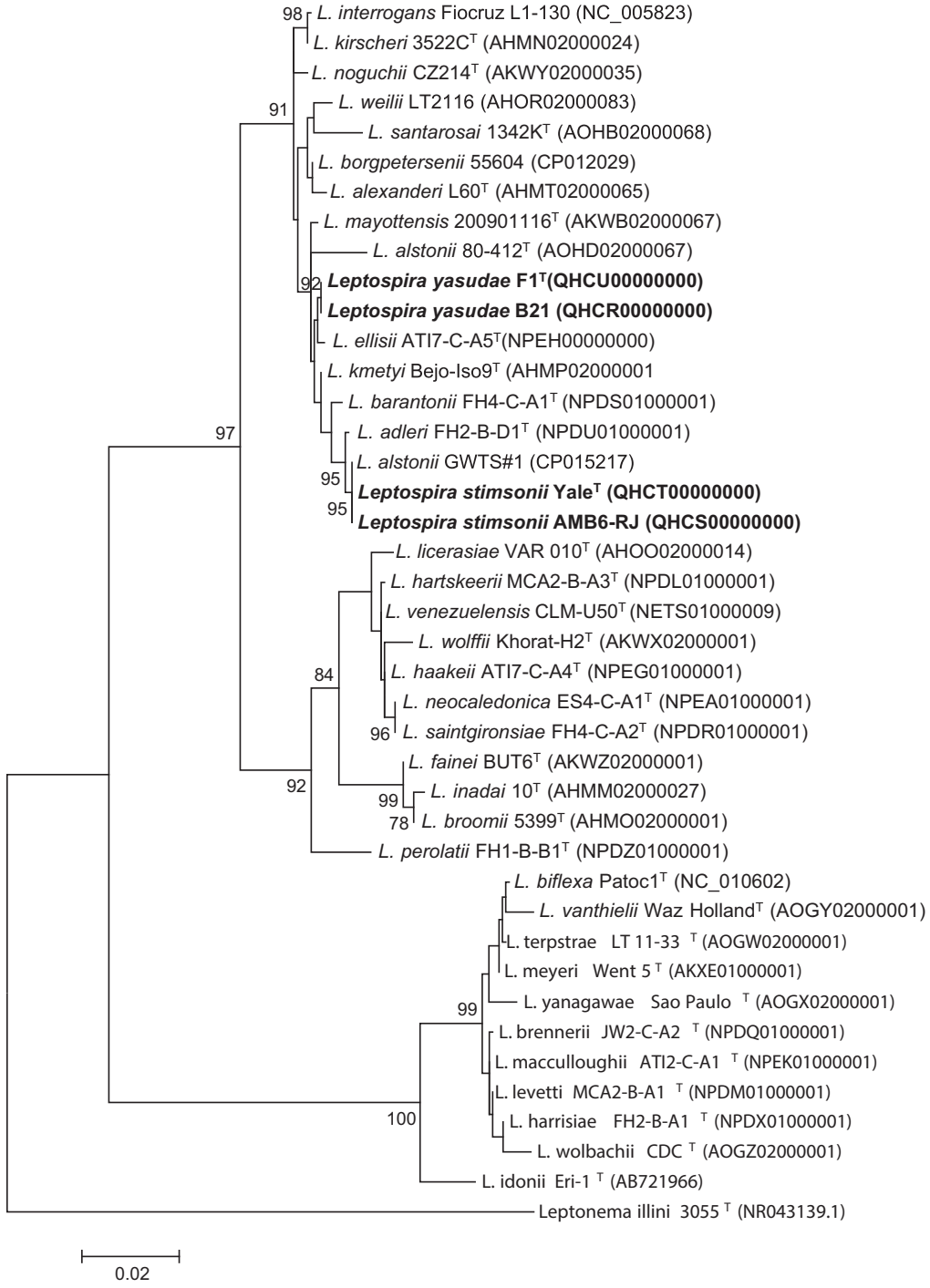

Fig. 2. Phylogenetic tree built with 16S rRNA gene full-length sequences using the maximum-likelihood method. Values at the nodes denote bootstrap support higher than 70 % based on 1000 resampling events. The bar indicates the proportion of nucleotide substitutions. *Leptonema illini* 3055^T was used as the outgroup.

replicates with *Leptonema illini* DSM 21528 as the outgroup. The 16S rRNA phylogenetic tree showed that strains F1^T and B21, and Yale^T and AMB6-RJ formed two well-supported clusters within the 'Pathogens' species (Fig. 2). The 16S rRNA gene sequence similarity between strains F1^T and B21 was 100 % and their closest species was *L. ellisii*

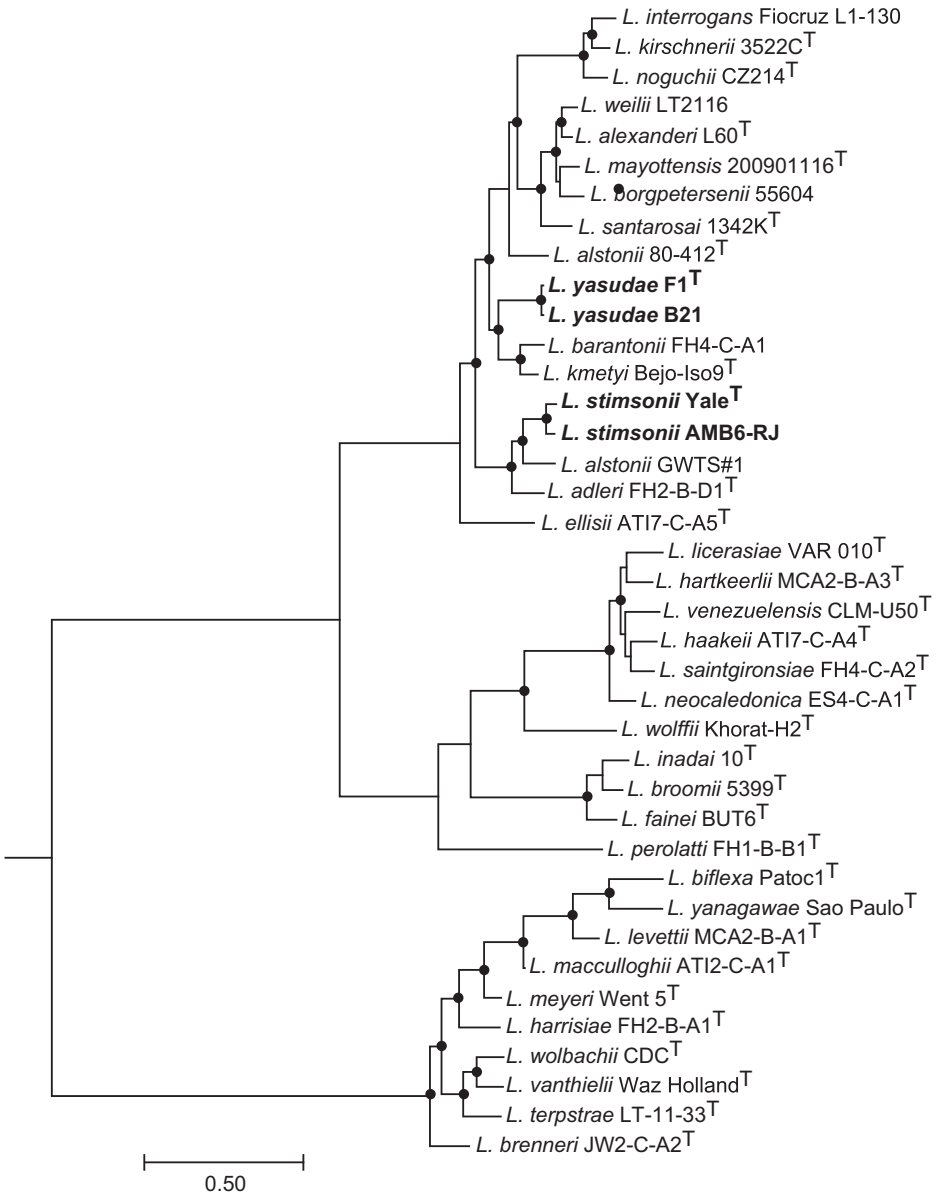

Fig. 3. Core gene phylogeny. The tree was built with a concatenated alignment of 498 single copy genes present in all the analysed genomes, using RAxML with GTR substitution model. Nodes with a black circle denote 100 % bootstrap support based on 100 resampling events.

(99.8 %). Strains Yale^T and AMB6-RJ also showed 100 % similarity between their 16S rRNA sequences and to *Leptospira alstonii* GWTS#1.

Due to the low variability of the 16S rRNA gene in the genus *Leptospira* [23, 24], we screened the genomes of strains F1^T, B21, Yale^T and AMB6-RJ, and all of available genomes of described *Leptospira* species and retrieved a set of highly conserved genes using Roary [25]. A total of 498 single copy genes were identified as the core genome, defined as those genes present in >95 % of the species with an identity higher than 55 %. The sequences were concatenated, aligned using MAFFT [26] (total alignment length was 505 301 bp) and phylogenetic reconstruction was performed using RAxML [27] with the GTR substitution model and 100 bootstrap replicates. The phylogeny showed that strains F1^T and B21, and Yale^T and AMB6-RJ formed two separate groups within the 'Pathogens' clade clearly distinct from the other species of the genus (Fig. 3).

Table 2. ANI values obtained by pairwise comparison of the genomes of strains F1^T, B21, Yale^T and AMB6-RJ and all the described pathogenic *Leptospira* species (group I) using BLAST+ in JspeciesWS

Strain	*Leptospira yasudae* F1^T	*Leptospira yasudae* B21	*Leptospira stimsonii* Yale^T	*Leptospira stimsonii* AMB6-RJ
***Leptospira yasudae* F1^T**	100	–	–	–
***Leptospira yasudae* B21**	98.7	100	–	–
***Leptospira stimsonii* Yale^T**	77.6	77.5	100	–
***Leptospira stimsonii* AMB6-RJ**	77.5	77.5	95.2	100
Leptospira alexanderi L60^T	79.2	79.2	77.0	76.7
Leptospira adleri FH2-B-D1^T	77.9	78.0	83.9	83.9
Leptospira alstonii 80-412^T	80.6	80.5	77.8	77.6
Leptospira alstonii GWTS#1	77.9	77.8	84.7	84.2
Leptospira barantonii FH4-C-A1^T	81.5	81.4	77.7	77.7
Leptospira borgpetersenii 56 604	79.2	79.2	76.9	76.7
Leptospira ellisii ATI7-C-A5^T	76.9	76.9	75.4	75.4
Leptospira interrogans L1-130	77.2	77.2	75.9	76.0
Leptospira kirschneri 3522^T	77.8	77.7	76.2	76.3
Leptospira kmetyi Bejo-Iso9^T	81.9	81.8	77.9	77.8
Leptospira mayottensis 200901116^T	79.1	79.0	76.7	76.5
Leptospira noguchii CZ214^T	77.3	77.3	76.0	76.0
Leptospira santarosai 1342K^T	79.5	79.4	77.0	77.1
Leptospira weilii LT2116	79.6	79.6	77.2	77.0

To conclusively determine if strains F1^T/B21 and Yale^T/AMB6-RJ constituted novel *Leptospira* species, we calculated their genomic relatedness to other *Leptospira* genomes using the average nucleotide identity (ANI) value [28] with JspeciesWS [29]. The ANI value between strains F1^T and B21 was 98.72 %. In contrast, the ANI values between these strains and their closest related species, *L. kmetyi*, was below <82 %. Similarly, strains Yale^T and AMB6-RJ had an ANI value of 95.18 %, with their closest relative, *L. alstonii* GWTS#1 below 85 %. In both cases, the ANI values of the closest species fell clearly below the 94–95 % threshold recommended for species delineation [30] (Tables 2 and S1, available in the online version of this article), confirming that those strains represented two novel *Leptospira* species.

Because of the phylogenetic location of these novel species within the 'Pathogens' group, we screened the genomes for previously characterized virulence-associated genes in *Leptospira. interrogans* Fiocruz L1-130 [3] using BLAST +blastp [31] implemented in GALAXY [32]. Strains F1^T/B21 and Yale^T/AMB6-RJ shared high homologies with *lipl32*/LIC11352 (94.1 and 93.4 %, respectively) and catalase *katE*/LIC12032 (86.9 and 86.3 %, respectively). We also found homologs to *Leptospira* virulence regulator genes *lvrA*/LIC11709 (74.2 % for F1^T/B21 and 62.3 % for Yale^T/AMB6-RJ) and *lvrB*/LIC11708 (84.3 and 74.3 %) which are only found in the pathogenic group [33].

To evaluate the pathogenicity of strains F1^T, B21, Yale^T and AMB6-RJ, we intraperitoneally infected groups of four 3 week old male Golden Syrian hamsters with 10^8 leptospires of each strain. As a control, infections were performed similarly using *L. interrogans* serovar Copenhageni strain L1-130 [34, 35]. Hamsters were monitored daily up to 21 days post-challenge for clinical signs of disease. Surviving animals at the end of the experiment or moribund animals presenting with difficulty moving, breathing or signs of bleeding or seizure were immediately sacrificed by inhalation of CO_2. Kidneys were aseptically removed at day 21, and DNA was extracted [36] and analysed by qPCR targeting the 16S rRNA gene [37]. Hamster protocols were approved by the Yale Institutional Animal Care and Use Committee guidelines (protocol #2017–11424). In contrast with hamsters infected with *L. interrogans* L1-130, euthanized after 4–5 days post-infection due to evident signs of disease, hamsters infected with strains F1^T, B21, Yale^T and AMB6-RJ showed no signs of acute disease and no DNA was detected in the kidneys 21 days post-challenge, indicating the lack of virulence in a hamster model of infection. Similar results were recently reported by Thibeaux *et al.* for three novel pathogenic species isolated from soil (*L. barantonii*, *L. adleri* and *L. ellisii*) [5]. These species are phylogenetically close to the two novel species described here (Fig. 3) and belong to the proposed 'low-virulence pathogens' group [5]. Further studies should be performed to identify other potential animal reservoirs and characterize the role that these species may play in animal and human leptospirosis.

In conclusion, the results of the phenotypic, genotypic and genomic analyses strongly support the hypothesis that strains F1^T/B21 and Yale^T/AMB6-RJ represent two novel species in the 'Pathogens' group of the genus *Leptospira*, for

which the names *Leptospira yasudae* ($F1^T$ and B21) and *Leptospira stimsonii* ($Yale^T$ and AMB6-RJ) are proposed.

DESCRIPTION OF *LEPTOSPIRA YASUDAE* SP. NOV.

Leptospira yasudae (ya.su′dae N.L. gen masc. n. *yasudae*, of Yasuda, named after Dr. Paulo H. Yasuda, a Brazilian microbiologist who made important contributions to the taxonomy of the genus *Leptospira*).

Cells are 14.2±2.5 µm long, ~0.2 µm in diameter, with a wavelength of ~0.6 µm under dark-field microscopy. Cells are aerobic, highly motile and grow well in EMJH medium at 13, 30 and 37 °C, but only slightly in EMJH supplemented with 8-azaguanine. This strain could not induce acute disease or chronic carriage in a hamster model of infection. The type strain, $F1^T$ (=ATCC-TSD-163=KIT0259=CLEP00287), was isolated from topsoil sampled at the urban slum community of Pau da Lima in the city of Salvador (Brazil) in 2015. The G+C content of the genomic DNA of the type strain is 45.50 mol%.

DESCRIPTION OF *LEPTOSPIRA STIMSONII* SP. NOV.

Leptospira stimsonii (stim.so′ni.i. N.L. gen masc. n. *stimsonii*, of Stimson, named after Dr. A. M. Stimson, an American physician who in 1907 observed *Leptospira interrogans* for the first time in kidney tissue slices of a leptospirosis victim diagnosed as having died of yellow fever).

Cells are 17.5±2.7 µm long and ~0.2 µm in diameter, with a wavelength of ~0.6 µm under dark-field microscopy. Cells are aerobic, highly motile and grow well in EMJH medium at 13, 30 and 30 °C with 8-azaguanine. This strain could not induce acute disease or chronic carriage in a hamster model of infection. The type strain, $Yale^T$ (=ATCC-TDS-162=KIT0258=CLEP00288), was isolated from the Mills River in New Haven, Connecticut, USA, in 2016. The G+C content of the genomic DNA of the type strain is 42.60 mol%.

Funding information
This research was supported by the National Institutes of Health research grants R01 AI052473, U01 AI088752, R25 TW009338, R01 TW009504 and R01 AI121207.

Acknowledgements
The authors thank the joint collaborative effort of the resident associations, community leaders and residents, which constitute the Urban Health Council of Pau da Lima as well as the members of the Leptospirosis Group from Gonçalo Moniz Institute, Salvador, Brazil.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Animal experiments were revised and approved by the Yale Institutional Animal Care and Use Committee guidelines (protocol #2017–11424).

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