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Multilocus Sequence Analysis for Typing *Leptospira interrogans* and *Leptospira kirschneri*▽†

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Fifty-three strains belonging to the pathogenic species *Leptospira interrogans* and *Leptospira kirschneri* were analyzed by multilocus sequence analysis. The species formed two distinct branches. In the *L. interrogans* branch, the phylogenetic tree clustered the strains into three subgroups. Genogroups and serogroups were superimposed but not strictly.

Leptospira spp. belong to the bacterial phylum "*Spirochaetes*." Members of the genus *Leptospira* are generally divided into a pathogenic species, *Leptospira interrogans sensu lato*, and a nonpathogenic species, *Leptospira biflexa sensu lato* (3, 12). Pathogenic members are the causal agents of leptospirosis, a widespread zoonosis that is a major public health dilemma. In the natural reservoirs of the bacteria, such as rodents, infection produces chronic and persistent asymptomatic shedding in the renal tubules, and bacteria are then excreted in urine.

A wide range of molecular methods to type leptospiral isolates, including PCR-restriction endonuclease analysis (4, 19), pulsed-field gel electrophoresis (7), and random amplification (6, 17), have been applied with more or less success. The genomic DNA-DNA hybridization method has been widely used for the determination of phylogenetic relationships between closely related strains (3, 5, 14). Most procedures are time-consuming, and depending on the method, various limitations that include low degrees of reproducibility and high background levels have been pointed out.

The availability of an increasing number of sequenced genomes has favored the application of sequence-based approaches that can yield much deeper information than previous methods about relationships between strains (13). Nucleotide sequence-based methods are more suitable than conventional procedures, as they facilitate direct, unambiguous comparison between isolates typed in different locations (15).

Multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) have been recently proposed as alternative ways for defining species or recognizing distinct strains of named species (8, 20). These techniques require identification of loci that evolve more rapidly than rRNA genes and analyses of multiple genes to provide a buffer against the distorting effects of recombination at a single locus. The diversity and relationship of different isolates across related taxa are then assessed by using an appropriate phylogenetic or cladistic approach. This strategy has been used recently to obtain new perspectives on *Listeria monocytogenes* evolution and to characterize the genomic diversity of *Enterobacter cloacae* (16), *Streptococcus agalactiae* (10), and *Campylobacter jejuni* (11). In this study, we have applied an MLSA typing schema to pathogenic isolates of *Leptospira*.

A total of 51 strains, including 11 reference strains, from different sources and geographical regions were analyzed in this study (Table 1). Species identification was performed by rRNA gene sequencing using LeptoA (5′-GGCGGCGCGTC TTAAACATG-3′) and LeptoB (5′-TTCCCCCCATTGAGCA AGATT-3′) nucleotide primers. In addition, all strains were checked for the presence of the *hap1* fragment (262 bp) by amplification of pathogenic *Leptospira* DNA with the specific primers described previously (2).

For MLSA, 14 housekeeping genes were selected in a preliminary study by comparison between two *Leptospira* genomic sequences available in GenBank (http://www.ncbi.nlm.nih.gov): those of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 chromosome I (accession number AE016823.1) and *L. interrogans* serovar Lai strain 56601 chromosome I (accession number AE010300.1). Since studies by Haake et al. revealed that outer membrane protein sequences have mosaic compositions consistent with horizontal transfer of DNA between related bacterial species (9), genes encoding surface proteins were excluded from the initial choice of gene targets. Primers were evaluated against the collection of reference strains. Based on the performance of primers and the number of alleles at a given locus, seven pairs of primers were selected. The nucleotide primers and the sizes of amplified DNA fragments from internal gene regions are shown in Table 2. PCRs were carried out using the following conditions: initial denaturation at 94°C for 5 min, followed by 45 cycles of 94°C for 30 s, 46°C (for *recF*) or 50°C (for *accA2*, *ccmF*, *czcA*, *gcvP*, *groEL*, and *polA*) for 45 s, and 72°C for 60 s. The samples were maintained at 72°C for another 10 min. Both strands of each fragment were then sequenced.

Sequence data for the 51 study strains, *L. interrogans* serovar

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TABLE 1. Characteristics of *Leptospira* strains

No.	Species	Serogroup	Serovar	Origin of isolate	Geographical area	Source
1	*L. interrogans*	Australis	Bratislava	Animal (*Erinaceus* sp.)	Czechoslovakia	ATCC (ATCC 23578)
2	*L. interrogans*	Australis	Bratislava	Unknown	United States	Queensland Laboratory
3	*L. interrogans*	Australis	ND	Animal	France	National Veterinary School of Nantes (France)
4	*L. interrogans*	Australis	Australis	Environment	Malaysia	ATCC (ATCC 23605)
5	*L. interrogans*	Australis	Muenchen	Animal	France	National Veterinary School of Nantes (France)
6	*L. interrogans*	Australis	Muenchen	Animal (dog)	France	National Veterinary School of Nantes (France)
7	*L. interrogans*	Australis	ND	Human	France	National Veterinary School of Nantes (France)
8	*L. interrogans*	Autumnalis	Autumnalis	Animal	France	Adiagène (France)
9	*L. interrogans*	Autumnalis	Autumnalis	Human	Japan	ATCC (ATCC 23476)
10	*L. interrogans*	Autumnalis	Autumnalis	Human	France	Pasteur Institute (France)
11	*L. interrogans*	Autumnalis	Autumnalis	Animal	France	National Veterinary School of Nantes (France)
12	*L. interrogans*	Bataviae	Bataviae	Environment	Malaysia	ATCC (ATCC 23602)
13	*L. interrogans*	Canicola	Canicola	Animal (dog)	ND	ATCC (ATCC 23606)
14	*L. interrogans*	Canicola	Canicola	Animal	France	Adiagène (France)
15	*L. interrogans*	Canicola	Canicola	Unknown	France	Virbac (France)
16	*L. interrogans*	Canicola	ND	Animal (dog)	France	National Veterinary School of Nantes (France)
17	*L. interrogans*	Icterohaemorrhagiae	Copenhageni	Animal	France	Adiagène (France)
18	*L. interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae	Animal	France	Adiagène (France)
19	*L. interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae	Human	France	Pasteur Institute (France)
20	*L. interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae	Human	France	Pasteur Institute (France)
21	*L. interrogans*	Icterohaemorrhagiae	IH CF1	Unknown	France	Merieux (France)
22	*L. interrogans*	Icterohaemorrhagiae	ND	Environment	France	Mérial (France)
23	*L. interrogans*	Icterohaemorrhagiae	Icterohaemorrhagiae	Human	France	ATCC (ATCC 23581)
24	*L. interrogans*	Icterohaemorrhagiae	Lai	Animal	France	National Veterinary School of Nantes (France)
25	*L. interrogans*	Icterohaemorrhagiae	ND	Animal (*Rattus norvegicus*)	France	National Veterinary School of Nantes (France)
26	*L. interrogans*	Icterohaemorrhagiae	ND	Animal (*Ondatra zibethicus*)	France	National Veterinary School of Nantes (France)
27	*L. interrogans*	Icterohaemorrhagiae	ND	Animal (*Myocastor coypus*)	France	National Veterinary School of Nantes (France)
28	*L. interrogans*	Pomona	Pomona	Human	Australia	ATCC (ATCC 23478)
29	*L. interrogans*	Pomona	Pomona	Animal	France	Adiagène (France)
30	*L. interrogans*	Pyrogenes	Pyrogenes	Human	Indonesia	ATCC (ATCC 23480)
31	*L. interrogans*	Pyrogenes	Pyrogenes	Animal	France	National Veterinary School of Nantes (France)
32	*L. interrogans*	Sejroe	Wolfii	Human	Indonesia	ATCC (ATCC 23482)
33	*L. interrogans*	Unknown	Unknown	Animal (*Myocastor coypus*)	France	Frank Duncombe Laboratory (France)
34	*L. interrogans*	Unknown	Unknown	Human	France	National Veterinary School of Nantes (France)
35	*L. interrogans*	Unknown	Unknown	Animal (*Rattus norvegicus*)	France	National Veterinary School of Nantes (France)
36	*L. interrogans*	Unknown	Unknown	Animal (*Rattus norvegicus*)	France	National Veterinary School of Nantes (France)
37	*L. interrogans*	Unknown	Unknown	Animal (*Mus musculus*)	France (Guadeloupe)	National Veterinary School of Nantes (France)
38	*L. interrogans*	Unknown	Unknown	Animal (*Rattus norvegicus*)	France	National Veterinary School of Nantes (France)
39	*L. interrogans*	Unknown	Unknown	Animal (bovine)	France	National Veterinary School of Nantes (France)
40	*L. kirschneri*	Cynopteri	ND	Animal	France	National Veterinary School of Nantes (France)
41	*L. kirschneri*	Grippotyphosa	Grippotyphosa	Environment	Malaysia	ATCC (ATCC 23604)
42	*L. kirschneri*	Grippotyphosa	Grippotyphosa	Animal	France	Adiagène (France)
43	*L. kirschneri*	Grippotyphosa	Grippotyphosa	Animal	France	Adiagène (France)
44	*L. kirschneri*	Grippotyphosa	Grippotyphosa	Human	France	Pasteur Institute (France)
45	*L. kirschneri*	Grippotyphosa	Grippotyphosa	Human	France	Pasteur Institute (France)
46	*L. kirschneri*	Grippotyphosa	Grippotyphosa	Human	Russia	ATCC (ATCC 23469)
47	ND[a]	Pomona	ND	Animal (pig)	France	New Caledonia Laboratory
48	ND	Pomona	ND	Animal (pig)	France	New Caledonia Laboratory
49	ND	Pomona	ND	Animal (pig)	France	New Caledonia Laboratory
50	ND	Unknown	Unknown	Animal	France	National Veterinary School of Nantes (France)
51	ND	Unknown	Unknown	Animal (*Rattus norvegicus*)	France	National Veterinary School of Nantes (France)

[a] ND, not determined.

TABLE 2. Details of gene loci and corresponding primer sequences used for MLSA

Gene	Product	Locus[a]	Gene size (bp)	Coordinates	Size of analyzed polymorphic sequence (bp)	G+C content (%)	No. of alleles	Primer sequence (5′–3′)[b]
accA2	Acetyl coenzyme A carboxylase α subunit	LIC11517	1,667	1874272–1875939	452	41.59	23	+ TTGATGCTTATGTTTGGGTTCAAT − AGAATCGAATAAATCTAGTTCCT
ccmF	Cytochrome *c* biogenesis factor	LIC10813	2,189	983052–985241	295	40.31	17	+ GATATTCCTGTACAATTCCGG − GGCAGTTTTCTTTTTAATAAC
czcA	Heavy-metal efflux pump	LIC11937	3,320	2340958–2344278	448	41.75	15	+ TCTTTTGGGAGAAATGATCGG − TAAGTTTGAAAGACTACAACG
gcvP	Glycine cleavage system P protein	LIC10309	2,894	350674–353568	275	41.28	14	+ AAGAGCTAAAAGATCTGCAGC − CTGGCCTTGAAATTTCGAACG
groEL	60-kDa chaperonin (Hsp60)	LIC11335	1,640	1646985–1648625	553	42.2	26	+ TCGCCTCGTCTCCATCCA − TGACGCAGAATCCATGGTAG
polA	DNA polymerase I	LIC10586	2,753	711562–714315	432	38.11	12	+ TCATAGTCGTTTGAATCTACGGT AAAA − ATTAAAGATTCCGGTGTAGAA CCAA
recF	DNA replication and repair protein RecF	LIC10003	1,097	5062–6159	400	31.94	22	+ CGGCGGAAAGAAGAAAGTT − CTCGACTTTTCCTTGTCGAA

[a] *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 has been used as a reference.
[b] +, foward primer; −, reverse primer.

Copenhageni strain Fiocruz L1-130, and *L. interrogans* serovar Lai strain 56601 were used to construct a phylogenetic tree (Fig. 1). Sequenced DNA fragments were concatenated in the following order to generate a single 2,855-bp sequence: *accA2*, *ccmF*, *czcA*, *gcvP*, *groEL*, *polA*, and *recF*. Phylogenetic analysis was conducted with MEGA4 (21). The neighbor-joining method (18) was used for sequence comparison. The evolutionary distances were computed using the maximum composite likelihood method (22).

16S RNA sequencing identified 39 strains of various serogroups (Icterohaemorrhagiae, Australis, Autumnalis, Canicola, Pomona, Pyrogenes, and Bataviae) as *L. interrogans* genomospecies and 7 strains (serogroups Grippotyphosa and Cynopteri) as *L. kirschneri* genomospecies. For the five remaining strains, the genomospecies could not be determined because of insufficient quantity of genomic material (Table 1).

The seven loci selected were suitable for MLSA, as they could be amplified from all isolates and sequenced, irrespective of the serogroup or species. Depending on the gene considered, 12 to 26 alleles could be observed, with the *groEL* gene being the most discriminant.

Phylogenetic analysis clustered the sequences into two main clades. The *L. interrogans* strains grouped into one clade, and the *L. kirschneri* strains grouped into the other clade, except for one strain, *L. kirschneri* serogroup Grippotyphosa ATCC 23604, which was classified in the *L. interrogans* clade, in contrast to the other reference strain, *L. kirschneri* serogroup Grippotyphosa ATCC 23469.

The major *L. interrogans* branch was further divided into several subgroups. A major subgroup comprised most Icterohaemorrhagiae serogroup strains ($n = 10$), although it also included one Australis serogroup strain. Eight strains of unknown serogroups belonged to this subgroup. The three remaining Icterohaemorrhagiae serogroup strains were placed into distant branches. Autumnalis ($n = 4$), Canicola ($n = 4$), and Pomona ($n = 5$) serogroup strains were clustered into one group. The other subgroups were more heterogeneous, comprising Australis, Pyrogenes, and Bataviae serogroup strains ($n = 9$). Individual gene analysis (see the figures in the supplemental material) showed that, in general, strains which clustered together in the multilocus analysis shared the same allele for each individual gene.

Noticeably, strain 17 (*L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni) was placed alone on a distinct branch. Individual gene analysis placed this strain in the Icterohaemorrhagiae clade, except for the *recF* allele, which was closely related to that of the Grippotyphosa strains (see the figures in the supplemental material). This finding may be explained by horizontal gene transfer events that are observed in *Leptospira* and lead to mosaic compositions of sequences (9, 25). The positioning of *L. kirschneri* serogroup Grippotyphosa ATCC 23604 in the *L. interrogans* clade was unexplained. In this case, clustering using individual gene analysis was similar to that using MLSA. To exclude the possibility of contamination, we analyzed another sample of the isolate provided by the ATCC and found identical sequences. Possibly this strain was misidentified.

An MLSA technique for *Leptospira* species was developed previously by Ahmed et al. (1). Genotyping was based on DNA sequencing of six genes and allowed differentiation of the major species of *Leptospira*, *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. santarosai*, and *L. borgpetersenii*. The set of primers used permitted analysis of the genetic relationships among species and the respective evolution of the species. However, the clustering was limited to the species level. In contrast, the primers that we have used distinguished clusters at the serogroup level for the pathogenic species *L. interrogans* and *L. kirschneri.*

Recently, an MLST schema based on the sequences of seven loci was used for typing of *Leptospira* isolates from humans and rodents in Thailand (23). Twelve sequence types (ST) were identified among the isolates. ST34 was predominant in human and rodent populations, representing 71 and 88% of isolates, respectively. In contrast, strains from a reference collection

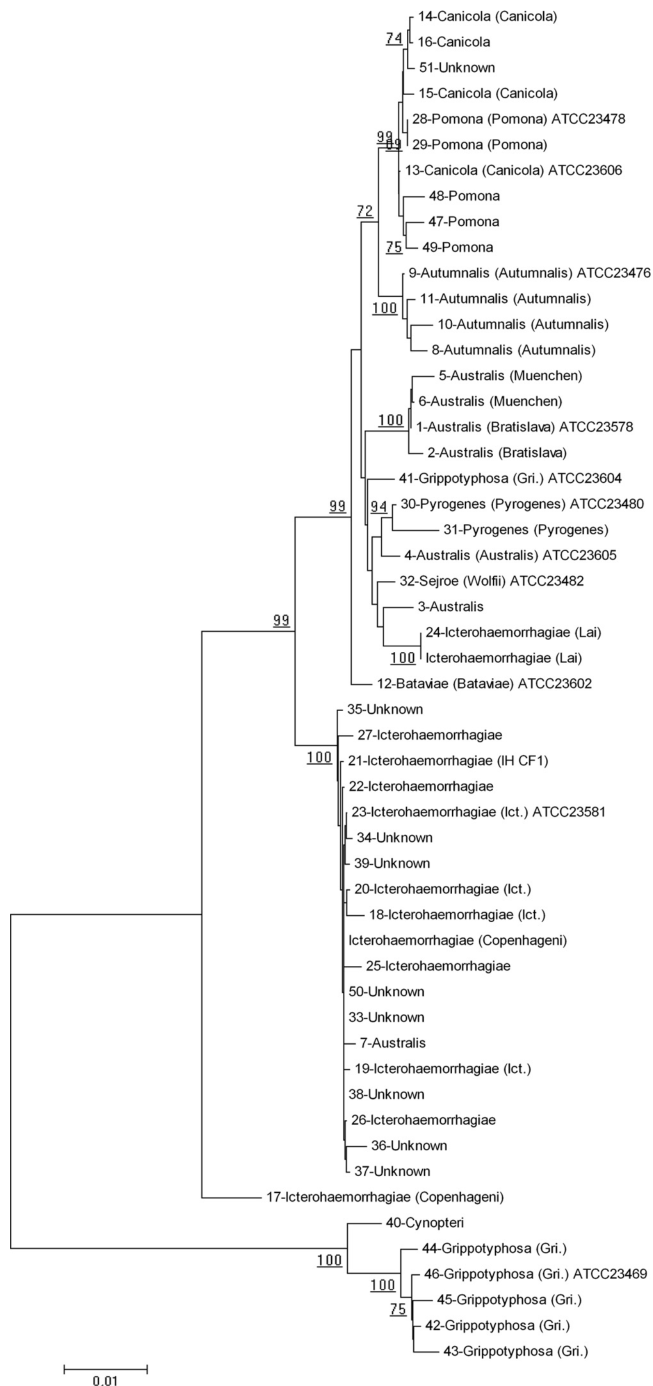

FIG. 1. Phylogenetic tree constructed from concatenated sequences (2,855 bp) of seven housekeeping genes of *Leptospira* strains. The identification numbers of the strains, the serogroups, and the serovars (in parentheses) are indicated. Gri., Grippotyphosa; Ict., Icterohaemorrhagiae. The neighbor-joining method (18) was used for sequence comparison. Phylogenetic analysis was conducted with MEGA4 (21). The evolutionary distances were computed using the maximum composite likelihood method (22). Only bootstrap values greater than 70 are shown.

had various STs. This study showed that ST34 may have a greater propensity than the other clones to cause human diseases in Thailand. Analysis of our sequences by the MLST method (15) showed a great diversity of STs and no clustering (data not shown). This result was not surprising, since our collection was composed of epidemiologically unrelated isolates.

In another study, Victoria et al. (24) showed that the *S10-spc-*α locus, which encodes ribosomal proteins and is highly conserved in *L. interrogans*, is a useful tool to elucidate evolutionary patterns. This analysis provided phylogenetic insights into the genus *Leptospira* at the species level but only limited information on subspecies discrimination.

Development of various schemas for molecular classification of *Leptospira* will probably allow better characterization of subtypes of *Leptospira* with particular epidemicity or propensity to cause diseases in humans or animals. More strains in various ecological niches need to be tested to determine which loci among those proposed in this study and those by Ahmed et al. (1) and Thaipadungpanit et al. (23) are suitable for a definitive typing schema.

Nucleotide sequence accession numbers. Nucleotide sequences determined in this study have been deposited in the GenBank data library under accession numbers GU113162 to GU113532.

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