JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2006, p. 3510–3516 Vol. 44, No. 10
0095-1137/06/$08.00+0 doi:10.1128/JCM.00670-06

Species-Specific Identification of *Leptospiraceae* by 16S rRNA Gene Sequencing

Roger E. Morey, Renee L. Galloway, Sandra L. Bragg, Arnold G. Steigerwalt, Leonard W. Mayer, and Paul N. Levett*

Meningitis and Special Pathogens Branch, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia

Received 29 March 2006/Returned for modification 10 April 2006/Accepted 20 July 2006

The genus *Leptospira* is classified into 13 named species and 4 genomospecies based upon DNA-DNA reassociation studies. Phenotypic tests are unable to distinguish between species of *Leptospira*, and there is a need for a simplified molecular approach to the identification of leptospires. 16S rRNA gene sequences are potentially useful for species identification of *Leptospira*, but there are a large number of sequences of various lengths and quality in the public databases. 16S rRNA gene sequences of near full length and bidirectional high redundancy were determined for all type strains of the species of the *Leptospiraceae*. Three clades were identified within the genus *Leptospira*, composed of pathogenic species, nonpathogenic species, and another clade of undetermined pathogenicity with intermediate 16S rRNA gene sequence relatedness. All type strains could be identified by 16S rRNA gene sequences, but within both pathogenic and nonpathogenic clades as few as two or three base pairs separated some species. Sequences within the nonpathogenic clade were more similar, and in most cases ≤10 bp distinguished these species. These sequences provide a reference standard for identification of *Leptospira* species and confirm previously established relationships within the genus. 16S rRNA gene sequencing is a powerful method for identification in the clinical laboratory and offers a simplified approach to the identification of *Leptospira* species.

Leptospirosis is an acute febrile disease caused by pathogenic spirochetes of the genus *Leptospira*. The disease is maintained in nature by chronic renal infection of carrier animals and acquired by direct or indirect contact with urine or tissues from infected animals. Traditionally, several hundred serovars of *Leptospira* were classified into two species, *Leptospira interrogans* and *L. biflexa* (13), which contained pathogenic and saprophytic strains, respectively. These species were differentiated by several phenotypic characteristics, including growth in the presence of 8-azaguanine and growth at 13°C (14). Based upon DNA-DNA hybridization data, the genus is now classified into 17 species (1, 20, 22, 25, 32), several of which contain both pathogenic and nonpathogenic serovars. Phenotypic characteristics previously used to differentiate *L. interrogans* sensu lato and *L. biflexa* sensu lato (13) are no longer useful in the current classification.

There is a need for a method of identification for *Leptospira* species that is more widely available than DNA-DNA hybridization but which will yield accurate identification to the species level. Analysis of 16S rRNA gene sequence is now widely used for identification of fastidious bacteria (3), including *Leptospira* species (10, 23). Leptospire genomes contain two 16S rRNA genes, which are not closely linked but are on chromosome I (21, 26). Numerous 16S rRNA sequences from *Leptospira* serovars have been deposited in GenBank, but in many cases these are only partial-length sequences. Even though not all species have been sequenced, such sequences are potentially useful for species identification. In the present study, we determined nearly full-length 16S rRNA gene sequences of approximately 1,430 bp from well-characterized type strains and representative serovars of *Leptospira* species, *Turneriella parva*, and *Leptonema illini* to evaluate the use of 16S rRNA gene sequencing for species identification of leptospires.

* Corresponding author. Mailing address: Saskatchewan Health, Provincial Laboratory, 3211 Albert Street, Regina, Saskatchewan S4S 5W6, Canada. Phone: (306) 787-3135. Fax: (306) 787-1525. E-mail: plevett@health.gov.sk.ca.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Leptospira* (n = 39 strains), *Leptonema* (n = 2), and *Turneriella* (n = 2) strains from the Centers for Disease Control and Prevention collection (Table 1) were maintained in semisolid PLM-5 medium (Serologicals Corp., Norcross, GA) containing 1.5% agar (Difco, Sparks, MD) at room temperature. Subcultures in liquid PLM-5 medium were incubated at 30°C for 7 days. The strains were chosen to represent important species and serovars causing human disease and include the type strain of all described species of *Leptospiraceae*.

DNA extraction and 16S rRNA gene sequencing. DNA was extracted from cultures of 43 strains of *Leptospiraceae* using QIAamp DNA minikits according to the manufacturer's directions (QIAGEN, Valencia, CA). The 16S rRNA genes were amplified from the purified DNA by using the Expand High-Fidelity PCR system (Roche Diagnostics Corp., Indianapolis, IN). Briefly, each 50-μl reaction contained approximately 10 ng of DNA, 2.5 U of polymerase, 1.5 mM $MgCl_2$, 5% (vol/vol) dimethyl sulfoxide, 200 μM deoxynucleoside triphosphates, and 100 nM concentrations of primers fD1 and rP2 corresponding to positions 8 and 1492, respectively, of the *Escherichia coli* 16S rRNA gene J01695 (Table 2). Amplification was performed on an AB 9700 thermocycler (Applied Biosystems, Foster City, CA) using 94°C for 5 min, followed by 35 cycles of 94°C for 15 s, 50°C for 5 s, and 72°C for 90 s, with a final single extension of 72°C for 5 min, and then held at 4°C. Amplified products were characterized by electrophoresis of 5 μl of each reaction on a 1.2% agarose gel for 30 min at 85 V. Excess nucleotides and primers were inactivated with the ExoSAP method (USB Corp., Cleveland, OH). Cycle sequencing was performed by standard protocols (27), including the use of a 45°C annealing temperature and the 16 sequencing primers listed in Table 2. Primers F785 and R802 were designed in the present study, but the majority are primers from the European rRNA database (31). Sequencing reaction products were purified with magnetic carboxylate beads (Agencourt Bioscience, Beverly MA). Reactions were sequenced on an AB 3100 (Applied Biosystems). Chro-

TABLE 1. Strains studied and their 16S rRNA gene GenBank accession numbers

Clade	Species	Serovar	Strain	GenBank accession no.	Diff[a]	Gaps[b]
Pathogenic	*Leptospira interrogans*	Icterohaemorrhagiae	RGA^T ATCC 43642^T	AY631894	R	
	Leptospira interrogans	Australis	Ballico	AY996794	1	−1
	Leptospira interrogans	Autumnalis	Akiyami A	AY996791	1	−1
	Leptospira interrogans	Bulgarica	Mallika	AY996792	6	+1, −1
	Leptospira interrogans	Canicola	Hond Utrecht IV	AY996798	1	−1
	Leptospira interrogans	Copenhageni	M 20	AY996790	0	0
	Leptospira interrogans	Hardjo	Hardjoprajitno	AY996796	1	−1
	Leptospira interrogans	Hardjo	Lepto-0184	AY996797	1	−1
	Leptospira interrogans	Pomona	Pomona	AY996800	2	−1
	Leptospira interrogans	Pyrogenes	Salinem	AY996793	0	−1
	Leptospira alexanderi	Manhao 3	L60^T ATCC 700520^T	AY631880	13	+1, −1
	Leptospira alexanderi	Manzhuang	A23	AY996803	15	+3, −1
	Leptospira alexanderi	Nanding	M 6901	AY996804	12	+2, −1
	Leptospira borgpetersenii	Javanica	Veldrat Batavia 46^T ATCC 43292^T	AY887899	10	−1
	Leptospira borgpetersenii	Ballum	Mus 127	AY631884	10	−1
	Leptospira kirschneri	Cynopteri	3522 C^T ATCC 49945^T	AY631895	1	−1
	Leptospira kirschneri	Bim	1051	AY996802	1	−2
	Leptospira kirschneri	Bim	PUO 1247	AY996801	1	−1
	Leptospira noguchii	Panama	CZ 214^T ATCC 43288^T	AY631886	9	−1
	Leptospira santarosai	Shermani	LT 821^T ATCC 43286^T	AY631883	17	−1
	Leptospira santarosai	Georgia	LT 117	AY996805	15	−1
	Leptospira weilii	Celledoni	Celledoni^T ATCC 43285^T	AY631877	13	−1
	Leptospira genomospecies 1	Sichuan	79601^T ATCC 700521^T	AY631881	13	−1
Intermediate	*Leptospira inadai*	Lyme	10^T ATCC 43289^T	AY631896	R	
	Leptospira inadai	Aguaruna	MW 4	AY631891	0	0
	Leptospira inadai	Kaup	LT 64-68	AY631887	1	0
	Leptospira broomii	Not designated	5399^T ATCC BAA-1107^T	AY796065	3	0
	Leptospira fainei	Hurstbridge	BUT 6^T ATCC BAA-1109^T	AY631885	7	0
	Leptospira fainei	Hurstbridge	BKID 6	AY996789	7	0
Nonpathogenic	*Leptospira biflexa*	Patoc	Patoc I^T ATCC 23582^T	AY631876	R	
	Leptospira biflexa	Andamana	CH 11	AY631893	1	0
	Leptospira meyeri	Ranarum	Iowa City Frog^T ATCC 43287^T	AY631878	10	0
	Leptospira meyeri	Hardjo	Went 5	AY631889	10	0
	Leptospira meyeri	Semaranga	Veldrat Semarang	AY631892	8	0
	Leptospira wolbachii	Codice	CDC^T ATCC 43284^T	AY631879	6	0
	Leptospira wolbachii	Gent	Wa Gent	AY631890	6	0
	Leptospira genomospecies 3	Holland	WaZ Holland^T ATCC 700522^T	AY631897	4	0
	Leptospira genomospecies 4	Hualin	LT 11-33^T ATCC 700639^T	AY631888	3	0
	Leptospira genomospecies 5	Saopaulo	Sao Paulo^T ATCC 700523^T	AY631882	5	0
Other	*Leptonema illini*	Illini	3055^T	AY714984	R	
	Leptonema illini	Habaki	Habaki	AY996806	10	0
	Turneriella parva	Parva	H^T NCTC 11395^T	AY293856	R	
	Turneriella parva	Parva	S-308-81	AY398688	0	0

[a] Diff, number of base pairs differing from the prototype strain in that clade. R, prototype strain for that clade.
[b] Gaps, insertion or deletions compared to the prototype strain.

matograms were assembled and analyzed in Seqmerge (Wisconsin Package version 10.3; Accelrys, Inc., San Diego, CA) (6).

Phylogenetic analysis. Sequences were aligned with CLUSTAL X (29) in Mega 3.1 (15) and trimmed to consensus, and a neighbor joining tree was created. Gaps in the aligned sequences were replaced by Ns in BioEdit (Ibis Therapeutics, Carlsbad CA). Evolutionary distances were estimated by using the Jukes and Cantor model in GCG (6).

Nucleotide sequence accession numbers. DNA sequences have been deposited in the GenBank database with the accession numbers shown in Table 1.

RESULTS

The 16S rRNA gene sequences of 43 strains, representing all 17 species of *Leptospira*, as well as *T. parva* and *Leptonema illini*, were determined. The length of the sequences ranged from 1,422 to 1,432 bp for leptospires and 1,428 to 1,440 bp for *T. parva* and *Leptonema illini*. These sequences were compared to each other by alignment and in dendrograms. Different serovars of the same species showed highly similar (only an average of 0.2 bases different of about 1,430) or identical 16S rRNA gene sequences (data not shown). Phylogenetic analysis of 16S rRNA gene sequences confirmed previous reports (10, 22) that the *Leptospiraceae* form five main clusters of species (Fig. 1). The pathogenic species, exemplified by *L. interrogans*, form one clade distinct from the nonpathogenic species, exemplified by *L. biflexa*. A third clade, comprising *L. inadai*, *L. fainei*, and *L. broomii*, was clearly separate from the pathogenic and nonpathogenic clades. These groupings are distinct from

TABLE 2. Primers used to determine 16S rRNA gene sequences in *Leptospiraceae*

Primer	Sequence	Use[a]	Directionality[b]	Source or reference
fD1	CCGAATTCGTCGACAACAGA GTTTGATCCTGGCTCAG	P	F	30
rP2	CCCGGGATCCAAGCTTACGG CTACCTTGTTACGACTT	P	R	30
fD1-5p	CCGAATTCGTCGACAACAG	S	F	This study
BSF8	AGAGTTTGATCCTGGCTCAG	S	F	31
F785e	GGATTAGATACCCTGGTA	S	F	This study
BSR357	CTGCTGCCTCCCGTA	S	R	31
R802e	TACCAGGGTATCTAATCC	S	R	This study
BSF343	TACGGGAGGCAGCAG	S	F	31
R536e	GTATTACCGCGGCTGCTG	S	R	17
R536	GWATTACCGCGGCKGCTG	S	R	17
F519e	CAGCAGCCGCGGTAATAC	S	F	17
BSF917	GAATTGACGGGGRCCC	S	F	31
BSR926	CCGTCAATTYYTTTRAGTTT	S	R	31
BSF1099	GYAACGAGCGCAACCC	S	F	31
BSR1114	GGTTGCGCTCGTTRC	S	R	31
BSF1391	TGTACACACCGCCCGTC	S	F	31
BSR1407	GACGGGCGGTGTGTRC	S	R	31
rP2-5p	CCCGGGATCCAAGCTTAC	S	R	This study

[a] P, PCR; S, sequencing.
[b] F, forward; R, reverse.

the remaining two clades, which include the species *T. parva* (12, 19) and *Leptonema illini* (11).

Within the pathogenic clade, the eight species were readily identified based on consistent differences in 16S rRNA gene sequences (Fig. 2). This clade included *L. alexanderi*, *L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. santarosai*, *L. weilii*, and *Leptospira* genomospecies 1. The percentage similarity of sequences within this clade was high, ≥98.6% or 2 to 20/1,431 bp different (i.e., 2 to 20 bp out of 1,1431 bp were different) (Table 3), reaffirming the high degree of species conservation among spirochetes (16, 23).

The positions of insertions or deletions, as well as differing and mixed bases, are shown in Fig. 2, which includes the positions that differentiate the species within the three *Leptospira* clades but omits positions that only differentiate between clades. Note that many of the positions that differentiate the species within a specific clade are quite conserved within the other two clades. A total of 35 dissimilar base positions distinguish the type strain 16S rRNA gene sequences of pathogenic *Leptospira* species, previously identified by DNA-DNA hybridization studies (1) (Fig. 2). The highest 16S rRNA gene sequence similarity between the type strains of species was between those of *L. interrogans* and *L. kirschneri* (2/1,432 bp different). However, *L. kirschneri* serovar Cynopteri strain 3522^T and serovar Bim strain PUO247 were identical based on 16S rRNA sequence and were differentiated from *L. interrogans* serovar Pyrogenes strain Salinem and serovar Bulgarica strain Mallika by a single base difference. Strain RGA of *L. interrogans* serovar Icterohaemorrhagiae differs from the majority of other strains of this species by one base. In addition to strain RGA, only serovar Copenhageni strain M20 shares an extra G at position 784. All other strains of *L. interrogans* have only five Gs in this position.

Among the nonpathogenic species, there were 3 to 12/1,422 bp differences between species (Table 3). This clade comprised *L. biflexa*, *L. meyeri*, *L. wolbachii*, and *Leptospira* genomospecies 3, 4, and 5. The similarity of sequences within this clade was higher than among species of the pathogenic clade (Table 3). The positions of differing and mixed bases are shown in Fig. 2.

The third cluster of *Leptospira* species comprised *L. inadai*, *L. fainei*, and *L. broomii* (Fig. 1). The sequences in this intermediate cluster were more closely related to the pathogenic cluster than to the nonpathogenic cluster, confirming previous reports of genetic relatedness (10, 22). A total of seven dissimilar bases distinguished these three species (Fig. 2). *L. broomii* was comparable to a mosaic of *L. inadai* and *L. fainei*, with positions 143, 144, 154, and 158 identical to *L. inadai* and positions 222, 1077, and 1165 identical to *L. fainei*, suggesting a crossover between positions 158 and 222, but there is no evidence for recombination by horizontal transfer or convergent evolution as a mechanism.

T. parva and *Leptonema illini* were distant from each other and from *Leptospira* species (Fig. 1), confirming their lack of relatedness to the genus *Leptospira* (19). Both species were easily identified and differentiated from *Leptospira* by their 16S rRNA gene sequences.

DISCUSSION

This study was performed to evaluate the use of 16S rRNA gene sequence analysis for the species identification of leptospires. 16S rRNA gene sequencing is rapidly becoming a common technique for the identification of unknown bacterial isolates, especially those of fastidious organisms such as *Leptospira* species (3). The identification of *Leptospira* isolates has been traditionally accomplished by serological methods (7), and the question of species identity was decided by pathogenicity. As a result, all pathogenic serovars were classified as *L. interrogans* in the past (14).

The adoption of a genotypic classification complicated the identification of leptospires because several serovars are found

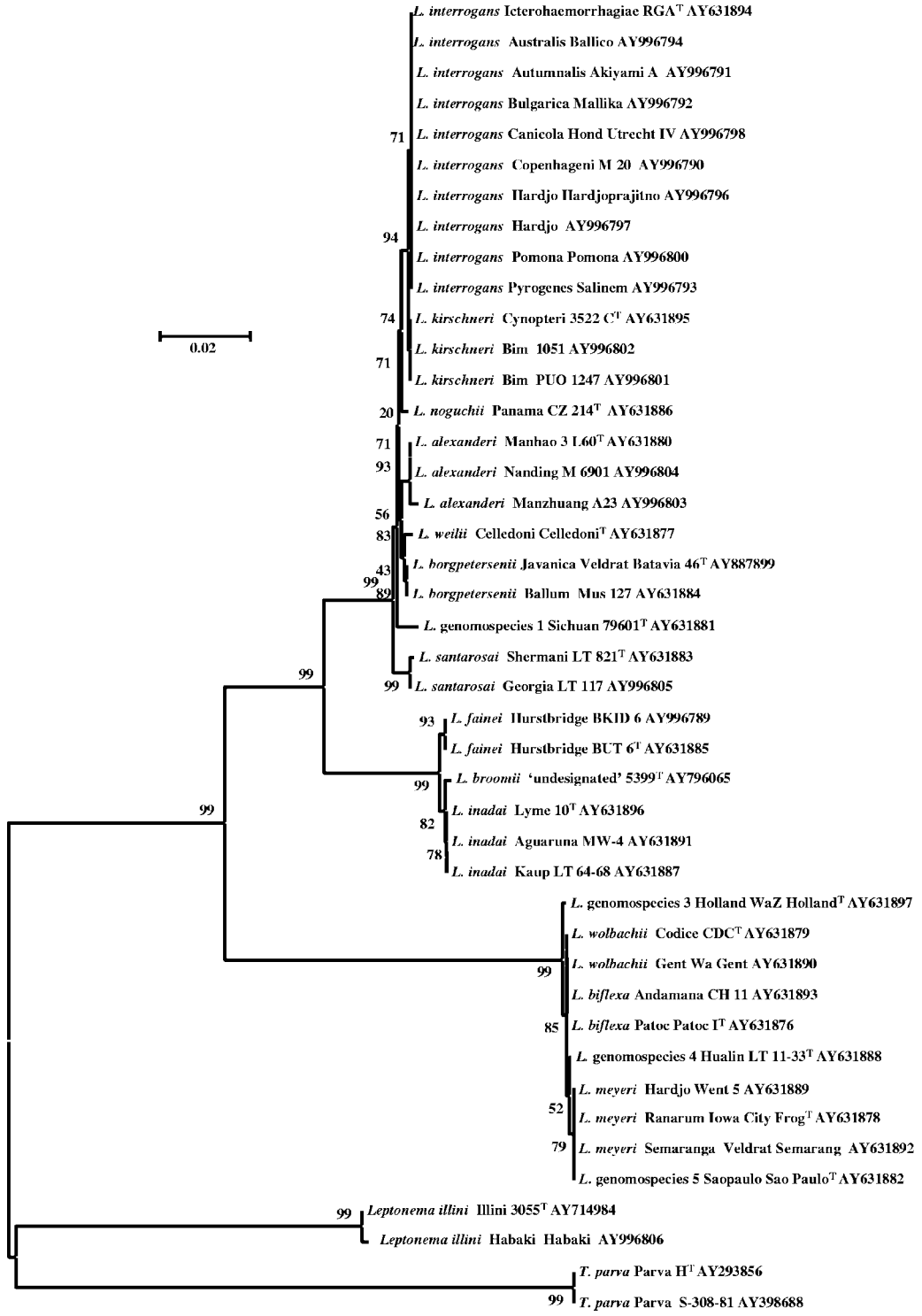

FIG. 1. Unrooted tree of 43 *Leptospiraceae* 16S rRNA gene sequences. The scale bar equals the fraction of base pairs that are different.

in more than one species and some species contain both pathogenic and nonpathogenic serovars (18). The determination of serovar is no longer sufficient to assign an isolate to its correct species. Recently, horizontal transfer of outer membrane protein genes has been shown to occur in *Leptospira* species (9). Horizontal transfer was proposed as the mechanism by which serovar Hardjo antigens are found in strains of both *L. interrogans* and *L. borgpetersenii* (5). Similar horizontal gene transfer probably accounts for at least five serovars (Bulgarica, Grippotyphosa, Mwogolo, Paidjan, and Valbuzzi) being shared between *L. interrogans* and *L. kirschneri* (18), the two most closely related species based on DNA-based methods and their

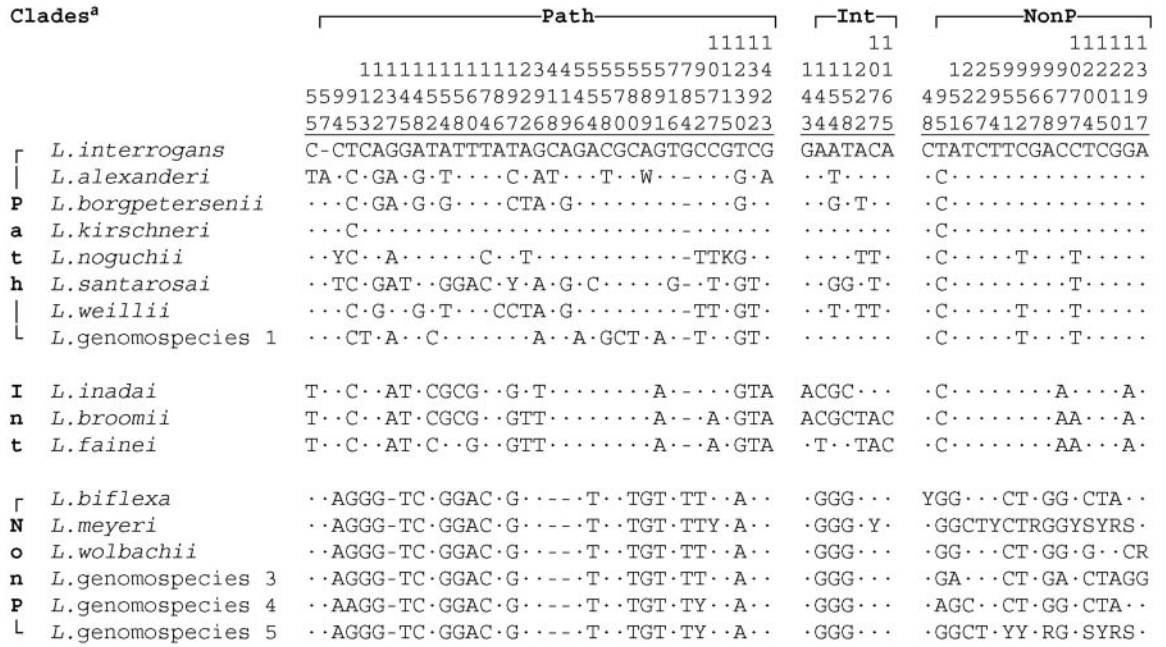

FIG. 2. Base differences in the 16S rRNA genes of type strains formatted to emphasize the differences within each of the three clades of *Leptospira*. Path, pathogenic clade; Int, intermediate clade; NonP, nonpathogenic clade. Vertical numbers show the relative positions in *L. interrogans* AY631894. Center dots indicate the same base as *L. interrogans*; a dash indicates a gap. The A at position 57 in *L. alexanderi* would be inserted between 56 and 57 in the *L. interrogans* AY631894 sequence. The sequences indicate differences within each clade but do not include all differences between clades. Note that positions 95, 154, 158, 222, and 952 appear twice, and position 1077 appears in all three clades. There are >160 other positions that can be used to differentiate between *Leptospira* clades.

propensity to cause human disease. There is no reason to suggest a relationship between serovar and species in pathogenic leptospires. In theory, all combinations of pathogenic serovar and species are possible, but the search for all combinations has not been exhaustive as yet. The mosaic-like 16S rRNA gene of *L. broomii* (compared to those of *L. fainei* and *L. inadai*) suggests that there may also have been horizontal transfer of ribosomal genes between leptospires, as has been shown for outer membrane proteins (9) and an intervening sequence (24), but there is no evidence to explain a mechanism.

We sequenced 1,422 to 1,440 bp of the 16S rRNA gene from type strains of *Leptospira* species, *T. parva*, and *Leptonema illini*, in addition to a number of strains representing common serovars. The near-full-length 16S rRNA gene sequences resolved in the present study allow for identification of all *Leptospira* species. Sequence analysis of 16S rRNA genes is a valuable tool for species identification of isolates (3), but DNA-DNA hybridization is recognized as the definitive methodology for species definition (2, 28). The value of the sequences derived in the present study is greatly enhanced due to the previous characterization of these strains by DNA-DNA hybridization (1, 32). The phylogenetic tree (Fig. 1) confirms the previously described genetic relationships between leptospires, with distinct clades comprised of pathogenic, nonpathogenic, and intermediate species (10, 22).

Only an insertion/deletion and a single base differentiate the type strains of *L. interrogans* and *L. kirschneri* (Fig. 2). These species may be confused with each other because they share some serological properties and are frequently encountered as causes of human disease. The close phylogenetic relationship between these two species and between them and *L. noguchii* was discussed by Haake et al. (9). It is doubtful that these species would have been distinguished by relying on 16S rRNA gene sequence analysis alone without the previous hybridization studies (1, 8, 25). Over-reliance on 16S rRNA gene sequences to choose candidates for DNA hybridization could have also resulted in misidentification of either of these species.

Redundant bidirectional sequencing utilizing 16 primer extension reactions per isolate allowed a greater consistency in resolution of mixed bases or insertion/deletions, as found in *L. alexanderi* serovars Manhao 3, Manzhuang, and Nanding; *L. interrogans* serovar Bulgarica; and *L. kirschneri* serovar Bim. Sequencing the first 500 bases or ignoring mixed bases, while effective for some of the genus *Leptospira*, would not allow the differentiation between the nonpathogens *Leptospira* genomospecies 5 and *L. meyeri* serovar Hardjo or Ranarum (data not shown). Because just one dissimilar base in 1,432 (0.07%) at position 94 and an insertion/deletion at position 784 differentiate the type strains of *L. kirschneri* and *L. interrogans*, sequences encompassing at least these positions are required in order to assure correct identification and differentiation of these two species.

Intraspecies distances sometimes exceed the interspecies distances for the 16S rRNA genes of *Leptospira*. A single base difference differentiated many strains of *L. interrogans* and *L. kirschneri*, so phylogenetic representation may be less meaningful than sequence identity over the variable positions. Searching for matching sequences may be performed with pub-

TABLE 3. Number of base pair differences in the 16S rRNA genes of type strains of *Leptospira*[a]

Clade[a]	Species	No. of bp differences																
		L. interrogans	*L. alexanderi*	*L. borgpetersenii*	*L. kirschneri*	*L. noguchii*	*L. santarosai*	*L. weilii*	*Leptospira* genomospecies 1	*L. inadai*	*L. broomii*	*L. fainei*	*L. biflexa*	*L. meyeri*	*L. wolbachii*	*Leptospira* genomospecies 3	*Leptospira* genomospecies 4	*Leptospira* genomospecies 5
Path	*L. interrogans*	0	15	11	2	10	18	14	14	72	76	72	205	213	205	207	207	209
	L. alexanderi		0	9	13	17	19	13	18	72	76	73	210	218	210	212	212	214
	L. borgpetersenii			0	9	11	12	6	15	71	72	71	208	215	208	209	209	212
	L. kirschneri				0	8	16	12	12	70	73	70	205	213	205	207	207	209
	L. noguchii					0	15	12	14	74	74	71	209	215	209	210	210	214
	L. santarosai						0	14	20	72	74	74	212	218	212	213	213	215
	L. weilii							0	16	76	76	73	209	215	209	210	210	214
	Leptospira genomospecies 1								0	71	74	71	210	218	210	212	212	215
Int	*L. inadai*									0	3	7	221	227	220	221	222	224
	L. broomii										0	4	225	230	224	225	226	227
	L. fainei											0	221	226	220	221	222	224
NonP	*L. biflexa*												0	10	6	4	3	10
	L. meyeri													0	10	12	9	6
	L. wolbachii														0	7	7	10
	Leptospira genomospecies 3															0	5	12
	Leptospira genomospecies 4																0	9
	Leptospira genomospecies 5																	0

[a] Path, pathogenic; Int, intermediate; NonP, nonpathogenic. Insertion/deletions or mixed bases are considered mismatches.

lic databases by using BLAST or with the specialized 16S database RDPII using Seqmatch (4). BLAST gives higher scores for sequences that are equal in length or shorter than the reference but identical across the region. Seqmatch gives higher scores using mixed bases from sequences of PCR amplification products of multiple operons with a genome and is probably the preferred approach for the identification of unknown isolates.

We have shown that all recognized species of the *Leptospiraceae* can be identified by using a standardized 16S rRNA gene sequencing approach (27) with universal primers. Early *Leptospira* sequences in the public databases were frequently short (often stopping at positions where indels occur), were sometimes of poor quality due to early sequencing methods, or were derived from incompletely characterized isolates. With the addition of high-quality sequences, 16S rRNA gene sequencing has become an accurate tool for identification of all described leptospires.

Identification and characterization of leptospiral isolates is based upon polyphasic analysis, with both serological and molecular characterization being essential. For public health purposes it has become essential to identify not only the serovar but also the species of isolates in order to accurately track the transmission of leptospires during outbreaks. An effective vaccine against leptospirosis will probably be composed of serovar- and species-specific antigens. An accurate determination of the burden of disease will depend on both species identification and serovar determination to aid in vaccine development. In combination with the use of a standardized pulsed-field gel electrophoresis approach for serovar identification (R. Galloway and P. N. Levett, Abstr. Int. Conf. Emerg. Infect. Dis. 2004, abstr. 214, 2004), a transition from a serological to molecular identification and characterization of leptospires is now possible.

REFERENCES

1. **Brenner, D. J., A. F. Kaufmann, K. R. Sulzer, A. G. Steigerwalt, F. C. Rogers, and R. S. Weyant.** 1999. Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. Int. J. Syst. Bacteriol. **49:**839–858.
2. **Busse, H. J., E. B. Denner, and W. Lubitz.** 1996. Classification and identification of bacteria: current approaches to an old problem. J. Biotechnol. **47:**3–38.
3. **Clarridge, J. E.** 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. **17:**840–862.
4. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje.** 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. **33:**D294–D296.
5. **de la Peña-Moctezuma, A., D. M. Bulach, T. Kalambaheti, and B. Adler.** 1999. Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjobovis. FEMS Microbiol. Lett. **177:** 319–326.
6. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
7. **Dikken, H., and E. Kmety.** 1978. Serological typing methods of leptospires, p. 259–307. *In* T. Bergan and J. R. Norris (ed.), Methods in microbiology, vol. 11. Academic Press, London, England.
8. **Fox, G. E., J. D. Wisotzkey, and P. Jurtshuk.** 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. **42:**166–170.
9. **Haake, D. A., M. A. Suchard, M. M. Kelley, M. Dundoo, D. P. Alt, and R. L. Zuerner.** 2004. Molecular evolution and mosaicism of leptospiral outer

membrane proteins involves horizontal DNA transfer. J. Bacteriol. **186:** 2818–2828.
10. **Hookey, J. V., J. Bryden, and L. Gatehouse.** 1993. The use of 16S rDNA sequence analysis to investigate the phylogeny of *Leptospiraceae* and related spirochaetes. J. Gen. Microbiol. **139:**2585–2590.
11. **Hovind-Hougen, K.** 1979. *Leptospiraceae*, a new family to include *Leptospira* Noguchi 1917 and *Leptonema* gen. nov. Int. J. Syst. Bacteriol. **29:**245–251.
12. **Hovind-Hougen, K., W. A. Ellis, and A. Birch-Andersen.** 1981. *Leptospira parva* sp. nov.: some morphological and biological characters. Zentbl. Bakteriol. Mikrobiol. Hyg. A **250:**343–354.
13. **Johnson, R. C., and S. Faine.** 1984. *Leptospira*, p. 62–67. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore, Md.
14. **Kmety, E., and H. Dikken.** 1993. Classification of the species *Leptospira interrogans* and history of its serovars. University Press Groningen, Groningen, The Netherlands.
15. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5:**150–163.
16. **Kunin, V., D. Ahren, L. Goldovsky, P. Janssen, and C. A. Ouzounis.** 2005. Measuring genome conservation across taxa: divided strains and united kingdoms. Nucleic Acids Res. **33:**616–621.
17. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S rRNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82:**6955–6959.
18. **Levett, P. N.** 2001. Leptospirosis. Clin. Microbiol. Rev. **14:**296–326.
19. **Levett, P. N., R. E. Morey, R. Galloway, A. G. Steigerwalt, and W. A. Ellis.** 2005. Reclassification of *Leptospira parva* Hovind-Hougen et al., 1982 as *Turneriella parva* gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. **55:**1497–1499.
20. **Levett, P. N., R. E. Morey, R. L. Galloway, and A. G. Steigerwalt.** 2006. *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. Int. J. Syst. Evol. Microbiol. **56:**671–673.
21. **Nascimento, A. L., A. I. Ko, E. A. Martins, C. B. Monteiro-Vitorello, P. L. Ho, D. A. Haake, S. Verjovski-Almeida, R. A. Hartskeerl, M. V. Marques, M. C. Oliveira, C. F. Menck, L. C. Leite, H. Carrer, L. L. Coutinho, W. M. Degrave, O. A. Dellagostin, H. El-Dorry, E. S. Ferro, M. I. Ferro, L. R. Furlan, M. Gamberini, E. A. Giglioti, A. Goes-Neto, G. H. Goldman, M. H. Goldman, R. Harakava, S. M. Jeronimo, I. L. Junqueira-De-Azevedo, E. T. Kimura, E. E. Kuramae, E. G. Lemos, M. V. Lemos, C. L. Marino, L. R. Nunes, R. C. De Oliveira, G. G. Pereira, M. S. Reis, A. Schriefer, W. J. Siqueira, P. Sommer, S. M. Tsai, A. J. Simpson, J. A. Ferro, L. E. Camargo, J. P. Kitajima, J. C. Setubal, and M. A. Van Sluys.** 2004. Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. J. Bacteriol. **186:**2164–2172.
22. **Pérolat, P., R. J. Chappel, B. Adler, G. Baranton, D. M. Bulach, M. L. Billinghurst, M. Letocart, F. Merien, and M. S. Serrano.** 1998. *Leptospira fainei* sp. nov., isolated from pigs in Australia. Int. J. Syst. Bacteriol. **48:**851–858.
23. **Postic, D., N. Riquelme-Sertour, F. Merien, P. Pérolat, and G. Baranton.** 2000. Interest of partial 16S rDNA gene sequences to resolve heterogeneities between *Leptospira* collections: application to *L. meyeri*. Res. Microbiol. **151:**333–341.
24. **Ralph, D., and M. McClelland.** 1994. Phylogenetic evidence for horizontal transfer of an intervening sequence between species in a spirochete genus. J. Bacteriol. **176:**5982–5987.
25. **Ramadass, P., B. D. W. Jarvis, R. J. Corner, D. Penny, and R. B. Marshall.** 1992. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. Int. J. Syst. Bacteriol. **42:**215–219.
26. **Ren, S. X., G. Fu, X. G. Jiang, R. Zeng, Y. G. Miao, H. Xu, Y. X. Zhang, H. Xiong, G. Lu, L. F. Lu, H. Q. Jiang, J. Jia, Y. F. Tu, J. X. Jiang, W. Y. Gu, Y. Q. Zhang, Z. Cai, H. H. Sheng, H. F. Yin, Y. Zhang, G. F. Zhu, M. Wan, H. L. Huang, Z. Qian, S. Y. Wang, W. Ma, Z. J. Yao, Y. Shen, B. Q. Qiang, Q. C. Xia, X. K. Guo, A. Danchin, I. Saint Girons, R. L. Somerville, Y. M. Wen, M. H. Shi, Z. Chen, J. G. Xu, and G. P. Zhao.** 2003. Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. Nature **422:**888–893.
27. **Sacchi, C. T., A. M. Whitney, M. W. Reeves, L. W. Mayer, and T. Popovic.** 2002. Sequence diversity of *Neisseria meningitidis* 16S rRNA genes and use of 16S rRNA gene sequencing as a molecular subtyping tool. J. Clin. Microbiol. **40:**4520–4527.
28. **Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. Grimont, P. Kampfer, M. C. Maiden, X. Nesme, R. Rossello-Mora, J. Swings, H. G. Truper, L. Vauterin, A. C. Ward, and W. B. Whitman.** 2002. Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. Int. J. Syst. Evol. Microbiol. **52:**1043–1047.
29. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
30. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
31. **Wuyts, J., Y. Van de Peer, T. Winkelmans, and R. De Wachter.** 2002. The European database on small subunit rRNA. Nucleic Acids Res. **30:**183–185.
32. **Yasuda, P. H., A. G. Steigerwalt, K. R. Sulzer, A. F. Kaufmann, F. Rogers, and D. J. Brenner.** 1987. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. Int. J. Syst. Bacteriol. **37:**407–415.