The use of 16S rDNA sequence analysis to investigate the phylogeny of *Leptospiraceae* and related spirochaetes

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The 16S rDNA sequences from 15 *Leptospiraceae* were determined by automated PCR-directed cycle sequencing. Nucleotide comparisons, including those from published sequences for *Leptospira canicola* Moulton and *Serpulina* spp., were used to construct phylogenetic trees. *Serpulina hyodysenteriae* and *S. innocens* were related to each other but were distinct from the *Leptospiraceae* comprising *Leptospira parva incertae sedis* (*Turneria parva* H), *Leptonema illini* and *Leptospira* spp. The pathogenic and the saprophytic leptospires were distinct and separated from each other. *Leptospira inadai* occupied an intermediate position between the two forms. The pathogens formed three groups. Group I was represented by *L. interrogans sensu stricto* and *L. kirschneri*, Group II by *L. weilii*, *L. borgpetersenii* and *L. santarosai*, and Group III comprised *L. noguchii* and *L. meyeri*. The saprophytic species, *L. wolbachii* and *L. biflexa sensu stricto* shared about 99% sequence similarity. The freshwater isolates were distinct from the marine isolate *L. biflexa sensu lato ancona* Ancona Porto.

Introduction

The *Leptospiraceae*, comprising *Leptospira* and *Leptonema*, are motile, flexible helical aerobic spirochaetes. Some are pathogenic for man and animals and others are free-living in soil, fresh- and salt-waters (Johnson & Faine, 1984). Their classification remains problematic and two different classifications co-exist, one based on serological affinities and the other on genetic relatedness (Kmety & Dikken, 1988; Yasuda *et al.*, 1987). Traditionally, two species *Leptospira interrogans sensu lato* (the pathogens) and *L. biflexa sensu lato* (the so-called 'saprophytes') could be delineated on the basis of their DNA base composition and a limited number of phenotypic characteristics (Johnson & Faine, 1984), and each in turn was arranged into the basic taxon of serovar; *L. interrogans* has 202 serovars and *L. biflexa* 65 (Kmety & Dikken, 1988). Recently eight new species, *L. borgpetersenii*, *L. inadai*, *L. meyeri*, *L. noguchii*, *L.*

santarosai, *L. weilii*, *L. wolbachii* (Yasuda *et al.*, 1987) and *L. kirschneri* (Ramadass *et al.*, 1992) were described on the basis of DNA:DNA homology studies. Similarly, Ramadass *et al.* (1990) revealed that the *L. biflexa sensu lato* 'complex' comprised at least six DNA homology groups.

Comparative sequencing of small subunit rRNA is now the method of choice for constructing higher level classifications (Woese, 1987). Oligonucleotide cataloguing and, more recently, reverse transcriptase sequencing of 16S rRNA showed that the spirochaetes were a phylogenetically distinct group subdivided into the *Spirochaetaceae* (*Borrelia*, *Cristispira*, *Serpulina*, *Spirochaeta* and *Treponema*) and the *Leptospiraceae* [*Leptospira* and *Leptonema* (Paster *et al.*, 1984, 1991)].

In view of the limited amount of information available with regard to the evolutionary relatedness of leptospires, the intent of this study was to establish more fully the phylogenetic relationships between members of the family *Leptospiraceae* on the basis of 16S rDNA sequence analysis.

Methods

Bacterial strains and culture. Fifteen strains of *Leptospiraceae* (Table 1) were cultured in EMJH broth (Ellinghausen & McCullough, 1965)

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Table 1. *Bacterial strains examined and their rDNA sequence accession numbers*

Species	Serovar Reference Strain*	Source†	Accession No.‡
<i>L. borgpetersenii</i> §	<i>javanica</i> Veldrat Batavia 46 (ATCC 43292)¶		Z21630
<i>L. interrogans sensu stricto</i> §	<i>icterohaemorrhagiae</i> RGA¶	W. J. Terpstra (Royal Tropical Institute, Meibergdreef, Amsterdam, The Netherlands)	Z12817
<i>L. noguchii</i> §	<i>panama</i> CZ 214 (ATCC 43288)¶	W. J. Terpstra	Z21635
<i>L. santarosai</i> §	<i>shermani</i> 1342K (ATCC 43286)¶		Z21649
<i>L. weilli</i> §	<i>celledoni</i> Celledoni (ATCC 43285)¶		Z21637
<i>L. kirschneri</i> §	<i>cynopteri</i> 3522 C¶	W. J. Terpstra	Z21628
<i>L. inadai</i> §	<i>lyme</i> (ATCC 43289)¶	A. F. Kaufmann (Centres for Disease Control, Atlanta, GA, USA)	Z21634
<i>L. biflexa sensu stricto</i> **	<i>patoc</i> Patoc 1¶	B. Cacciapuoti (Istituto Superiore di Sanità, Roma, Italy)	Z12821
<i>L. meyeri</i> **	<i>ranarum</i> ICF (ATCC 43287)¶	W. J. Terpstra	Z21648
<i>L. wolbachii</i> **	<i>cdc Biflexa</i> CDC (ATCC 43284)¶	B. Cacciapuoti	Z21638
<i>L. parva incertae sedis</i> **		W. A. Ellis (Veterinary Research Laboratories, Belfast, UK)	Z21636
<i>Leptonema illini</i> **		K. Hovind-Hougen (National Veterinary Laboratory, Copenhagen, Denmark)	Z21632
<i>L. biflexa sensu lato</i> *	<i>ancona</i> Ancona Porto	B. Cacciapuoti	Z21629
	<i>canela</i> Canela		Z21631
	<i>jequitia</i> Jequitia		Z21633

* Designation based on serological studies (Kmety & Dikken, 1988).

† Unless otherwise stated, the source of strains was L. H. Turner (Leptospira Reference Laboratory, FAO/WHO Collaborating Centre, County Hospital, Hereford HR1 2ER, UK).

‡ Accession numbers were obtained from the EMBL data library. The accession number for *canicola* Moulton was X17547 (Fukunaga *et al.*, 1990) and those for *S. hyodysenteriae* and *S. innocens* were M57741 and M57744, respectively (Stanton *et al.*, 1991).

§ Species considered to be pathogenic for man and animals (Yasuda *et al.*, 1987).

¶ Reference strains assigned to a species on the basis of DNA hybridization (hydroxyapatite method; Yasuda *et al.*, 1987).

¶ Reference strains assigned to a species on the basis of DNA hybridization (slot-blot method; Ramadass & Marshall, 1990; Ramadass *et al.*, 1992).

** Species considered to be saprophytic (Yasuda *et al.*, 1987; Johnson & Faine, 1984).

at 30 °C for 7–10 d and checked for the presence of contaminating aerobic bacteria by overnight culture on 8% (w/v) horse-blood agar at 30 and 37 °C.

Isolation of chromosomal DNA. Chromosomal DNA was prepared by the method of Hookey & Palmer (1991).

Preparation of PCR-amplified 16S rDNA. DNA was amplified by PCR using a primer pair specific for 16S rDNA [pU (34–53): Hookey, 1992; pH⁻: Edwards *et al.*, 1989]. Primer oligonucleotides were made using a DNA-synthesizer (model 381, Applied BioSystems) and the oligomers purified according to the manufacturer's recommendations. Reactions were performed on a Perkin-Elmer 480 Thermal Cycler in sterile thin-walled tubes that contained approximately 100 ng (1–2 µl) chromosomal DNA, the reaction mix comprising 10 µl of 10× PCR reaction buffer (500 mM-KCl, 100 mM-Tris/HCl, pH 8.3, 35 mM-MgCl₂, 0.01% gelatin; EIA grade, Bio-Rad), 200 µM each of dATP, dCTP, dGTP and dUTP (Pharmacia), 100 pM of each primer. Glycerol at 10% (w/v), 2.5 U AmpliTaq DNA polymerase (ILS Ltd) and sterile 'Millipure' water were added to a final volume of 100 µl. The reaction mix was overlaid with 150 µl mineral oil (ILS Ltd). A reaction mix containing no DNA served as a negative control.

Following denaturation of the DNA template at 95 °C for 5 min, the temperature was lowered to and held at 80 °C while the reaction mix was added to the DNA. Thermal cycling (×30) then proceeded at

95 °C for 40 s, 55 °C for 25 s and 72 °C for 3 min. A final step at 72 °C for 10 min was included.

The amplified DNA was extracted once with an equal volume of phenol/chloroform, precipitated in the presence of 0.1 vols 3.0 M-sodium acetate (pH 5.2) and 3 vols absolute ethanol (AnalaR; BDH), and redissolved in 25 µl Millipure water.

Purification of 16S rDNA product. The amplified 16S rDNA was purified prior to DNA sequencing according to standard protocols (Sambrook *et al.*, 1989). Briefly, unwanted primers, deoxyribonucleotides and DNA polymerase were separated from the amplification product by electroelution through a 1.0% (w/v) agarose gel (MB grade; Bio-Rad) at 100 mA onto activated DEAE-cellulose membranes (NA-45; Schleicher & Schuell).

Automated PCR-directed cycle sequencing. The purified double-stranded DNA template was sequenced using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied BioSystems) on an ABI model 373-A automated fluorescent DNA sequencer according to the manufacturer's instructions. The primers used were pC, pD, pD⁻, pH⁻ (Edwards *et al.*, 1989), pU (Hookey, 1992), p4 [5'...TAA GGG TTG CGC TCG TTG...3' (1132–1115)] and pE4 [5'...CGC AAC GAG CGC AAC CCT...3' (1113–1130)].

The thermal cycle sequencing programme used on the Pharmacia-LKB thermal cycler was 96 °C for 30 s, 50 °C for 40 s, and a 4 min

Fig. 1. Unrooted consensus Jukes & Cantor (1969) correction and Fitch & Margoliash (1967) distance tree showing the position of *Leptospiraceae* and related spirochaetes. Bootstrap percentages for the following were: *L. interrogans sensu stricto* and *L. canicola* Moulton, 78%; *L. kirschneri*, 98%; *L. noguchii* and *L. meyeri*, 34%; *L. santarosai*, 67%; *L. weilii* and *L. borgpetersenii*, 75%; *ancona* Ancona Porto, 100%; *jequitia* Jequitia, 39%; *canela* Canela, 27%; *L. biflexa sensu stricto* and *L. wolbachii*, 96%; *L. parva incertae sedis*, 56%; *Leptonema illini*, 100%; *E. coli*, 97%; *S. hyodysenteriae* and *S. innocens*, 100%. The bootstrap percentages quoted are the percentage times a taxa at that node occurred. The scale bar represents 0.02 substitutions per sequence position (K_{nuc}).

extension at 60 °C for 25 cycles. Following amplification, excess DyeDeoxy Terminators were removed from the sequencing reaction by spin-column centrifugation according to the manufacturer's instructions (Bio-Spin 30 chromatographic column; Bio-Rad), ethanol-precipitated, dried and redissolved in 4 µl loading buffer. DNA fragments were separated on conventional 0.4 mm, 6.0% (w/v) denaturing acrylamide gels at 100 W.

Analysis of sequence data. The primary sequence data have been deposited with the GenBank/EMBL data libraries and the accession numbers are given in Table 1. Sequences were aligned using the MULTALIN program (Corpet, 1988; Chervell Scientific Publishing Ltd, Oxford, UK). The 11 *Leptospira* spp., *Leptonema illini*, four reference serovars and two related spirochaetes were checked against the secondary structure model of Neefs *et al.* (1991). Those regions of hypervariability and base positions that could not be aligned unambiguously were omitted from the final alignment (about 140 nucleotides similar to those omitted by Paster *et al.*, 1991). A total of 1339 bases comprised the final alignment; this is available from the authors on request.

Distance matrices. Distance matrices were constructed from bootstrapped data by DNADIST contained in the Phylogenetic Inference Package, PHYLIP 3.4 (J. Felsenstein, University of Washington, USA) and user trees computed using neighbour-joining (NEIGHBOR; Saitou & Nei, 1987), and Fitch–Margoliash (FITCH; Fitch & Margoliash, 1967) using either the Jukes & Cantor (1969) correction for multiple substitutions or the maximum likelihood method (DNAML) employing the empirical frequencies of the bases as given in the input data. Majority-rule consensus trees were computed from the user trees using the CONSENSE program.

The bootstrap percentages quoted for a species or serovar in the legend to Fig. 1 are the percentage times that a taxa to the right of that node occurred and it provides some indication of the stability of the branching order.

Parsimony. Bootstrapped ($\times 100$) sequence data was analysed using the DNAPARS and CONSENSE programs.

Reliability of trees. Majority-rule consensus trees were produced from bootstrapped data sets via distance matrix and parsimony treeing. The aligned sequence data was resampled $\times 100$ using a general bootstrapping program, SEQBOOT. Parsimony and distance metric consensus trees were evaluated for statistical significance using the modified test of Templeton (Templeton, 1983; Felsenstein, 1985) by invoking the user tree option in DNAPARS. The topologies were not significantly different and only one is presented (Fig. 1). Specific topologies are available from the authors on request.

Results and Discussion

Sequencing generated between 1451 and 1374 bases (97–92%) of 16S rDNA sequence for the 15 strains studied. The matrix of evolutionary distance (K_{nuc}) values are given in Table 2.

Phylogenetic trees based on sequence data must be interpreted with caution (Felsenstein, 1988). In this study, bootstrapped data, out-grouping to *E. coli* and the statistical comparisons of topologies indicated that the

Table 2. Nucleotide substitution rates (K_{mc} values; Jukes & Cantor model, 1969) for 1339 bases of 16S rDNA from Leptospiraceae and related spirochaetes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. <i>L. interrogans sensu stricto</i>	0.0000																		
2. - <i>canicola</i> Moulton	0.0015	0.0000																	
3. <i>L. weilii</i>	0.0092	0.0108	0.0000																
4. <i>L. santarosai</i>	0.0146	0.0162	0.0123	0.0000															
5. <i>L. borgpetersenii</i>	0.0076	0.0092	0.0038	0.0100	0.0000														
6. <i>L. meyeri</i>	0.0092	0.0107	0.0131	0.0162	0.0100	0.0000													
7. <i>L. noguchii</i>	0.0131	0.0146	0.0154	0.0193	0.0154	0.0154	0.0000												
8. <i>L. kirschneri</i>	0.0015	0.0031	0.0092	0.0146	0.0076	0.0092	0.0131	0.0000											
9. <i>L. inadai</i>	0.0320	0.0320	0.0368	0.0375	0.0328	0.0352	0.0408	0.0320	0.0000										
10. <i>L. wolbachii</i>	0.1102	0.1121	0.1131	0.1120	0.1111	0.1157	0.1184	0.1111	0.1204	0.0000									
11. - <i>jequitaita</i> Jequitaita	0.1023	0.1042	0.1069	0.1014	0.1032	0.1077	0.1121	0.1032	0.1123	0.0139	0.0000								
12. - <i>ancona</i> Ancona Porto	0.1009	0.1027	0.1053	0.1026	0.1018	0.1063	0.1116	0.1018	0.1072	0.0162	0.0085	0.0000							
13. <i>L. biflexa sensu stricto</i>	0.1076	0.1094	0.1104	0.1094	0.1085	0.1130	0.1157	0.1085	0.1177	0.0084	0.0146	0.0123	0.0100	0.0147					
14. - <i>canela</i> Canela	0.1042	0.1061	0.1088	0.1060	0.1051	0.1096	0.1141	0.1051	0.1133	0.0170	0.02130	0.0217	0.02172	0.02155	0.0000				
15. <i>L. parva incertae sedis</i>	0.1800	0.1812	0.1773	0.1838	0.1760	0.1841	0.1871	0.1810	0.1755	0.2162	0.2130	0.2117	0.2172	0.2155	0.0000	0.1611			
16. <i>Leptonema illini</i>	0.1783	0.1794	0.1795	0.1791	0.1763	0.1745	0.1833	0.1783	0.1748	0.1960	0.2020	0.1977	0.2000	0.1976	0.1611	0.0000	0.0000		
17. <i>S. hyodysenteriae</i>	0.2838	0.2863	0.2866	0.2869	0.2849	0.2830	0.2852	0.2849	0.2812	0.2877	0.2787	0.2761	0.2877	0.2792	0.2546	0.2799	0.0046	0.0000	
18. <i>S. innocens</i>	0.2838	0.2841	0.2866	0.2869	0.2849	0.2830	0.2852	0.2849	0.2812	0.2912	0.2821	0.2795	0.2912	0.2815	0.2516	0.2788	0.0046	0.0000	
19. <i>E. coli</i>	0.2766	0.2768	0.2793	0.2807	0.2766	0.2801	0.2835	0.2777	0.2729	0.2998	0.2964	0.2950	0.2964	0.2992	0.2777	0.3052	0.3119	0.3096	0.0000

branching order was reliable and that the consensus tree (Fig. 1) may be a good reconstruction of the relationships between the *Leptospiraceae*. The overall topology between the *Serpulina* spp., *Leptonema illini*, *Leptospira interrogans* and *L. biflexa* was identical to that given by Paster *et al.* (1991).

The consensus trees summarize the evolutionary relationships between taxa examined and their relative positions (Fig. 1). The aetiological agent of swine dysentery, *Serpulina hyodysenteriae* and the saprophyte *S. innocens* were distinct species (Stanton *et al.*, 1991; Fig. 1; Table 2). Their exact phylogenetic position relative to the *Leptospiraceae* was not resolved by the current data and their position as an in-group in Fig. 1 was merely one of convenience.

L. parva incertae sedis was isolated as a contaminant in EMJH medium (Hovind-Hougen *et al.*, 1981). Morphologically it is similar to the leptospires, has phenotypic characteristics of both the pathogenic and the saprophytic forms, low DNA:DNA similarity values compared to other *Leptospira* sp. (Yasuda *et al.*, 1987) and comparatively large evolutionary distance values (Table 2). Collectively these data support the ad hoc proposal for a new genus, *Turneria* ('*L. parva* reference strain H; Anon., 1992).

Leptonema illini was unexpectedly more related to the *Leptospiraceae* than was *Leptospira parva incertae sedis* and the sequence data gave qualified support to it being a distinct genus (Hovind-Hougen, 1979).

The pathogenic leptospires (Table 1) have been divided broadly into two groups (*L. interrogans* and *L. kirschneri*, and *L. weilii*, *L. borgpetersenii* and *L. santarosai*) on the basis of such criteria as G+C content, sensitivity to 2,6-diaminopurine, lipase production (Haapala *et al.*, 1969), DNA homologies (Ramadass *et al.*, 1992) and the presence or absence of DNA fragments hybridizing on Southern blots to the *sphA* sphingomyelinase gene (Segers *et al.*, 1992). These results are in agreement with those presented here though a third group, *L. noguchii* and *L. meyeri*, may be delineated (Fig. 1). Although the relatively low percentage bootstrap value indicated branching instability (Fig. 1), the *L. noguchii* and *L. meyeri* group shared a 16S rDNA sequence value of approximately 96.6%, an evolutionary distance value of 0.0154 (Table 2) and a mol% G+C of about 35.0% (Yasuda *et al.*, 1987). Sequence values of between 95 and 97% are generally expected for different species within a genus (Paster & Dewhirst, 1988; Dewhirst *et al.*, 1989). The strain *L. meyeri ranarum* ICF was isolated from the kidneys of a leopard frog (*Rana pipiens*) and had the characteristics of being a saprophyte, growing at 13 °C and being unable to establish leptospirosis in laboratory animals (Diesch *et al.*, 1966). Yet it formed part of a pathogenic 'complex', being sensitive to 8-azaguanine,

2,6-diaminopurine and copper sulphate (Brendle *et al.*, 1974; Diesch *et al.*, 1966; Ellinghausen, 1968). Furthermore, it was positive in a PCR test for pathogenic leptospires (Hookey, 1992), gave multiple putative sphingomyelinase gene patterns similar to strains of a pathogenic nature (Segers *et al.*, 1992), an *Eco*R1 0.75–0.8 kb 16S rDNA fragment resembling the pathogens on 'ribotyping' (Hookey, 1993) and it occupied a central phylogenetic position within the pathogenic domain. In view of the weight of evidence, *L. meyeri* should be regarded as being related to the pathogens.

The type strain of the genus *L. interrogans icterhaemorrhagiae* RGA and *L. kirschneri* had about 99.84% sequence similarity and could be regarded as belonging to the same species. However, DNA:DNA hybridization values (approximately 31.1%) have clearly established both as separate entities (Yasuda *et al.*, 1987; Ramadass *et al.*, 1992). Similar results were also in evidence between the saprophytes, *L. weilii* and *L. biflexa sensu stricto* (Fig. 1; Table 2; Yasuda *et al.*, 1987). The lack of correlation between 16S rDNA sequence similarity and DNA hybridization data is not unknown (Ash *et al.*, 1991; Boddingtonhaus *et al.*, 1990; Collins *et al.*, 1989; Whiley *et al.*, 1990) and it has been suggested that such recently diverged strains should be treated as part of a single rRNA species or super-species complex (Fox *et al.*, 1992). In contrast, the serovar *canicola* may be regarded as a member of *L. interrogans sensu stricto* on both DNA relatedness (> 70%, Ramadass *et al.*, 1992) and 16S rDNA sequence identity (Table 2).

The group, *L. weilii*, *L. borgpetersenii* and *L. santarosai* have between 68 and 62% DNA:DNA relatedness to each other (Yasuda *et al.*, 1987), an average K_{nuc} value of 0.0087 (Table 2) and similar G+C content (Yasuda *et al.*, 1987). This level of 16S rDNA sequence divergence supports their division into three species.

The phylogenetic position of *L. inadai* was such that it bisected the pathogenic and saprophytic domains. Whether this represents an emerging subline or a transitional stage between the two domains is not known. The organism was isolated from a skin biopsy of a patient with Lyme disease (Schmid *et al.*, 1986), was pathogenic for laboratory animals, had the biological, genetic (Yasuda *et al.*, 1987) and fatty acid composition (Johnson *et al.*, 1970; Cacciapuoti *et al.*, 1991) of both the pathogens and the saprophytes, yet was akin to the free-living leptospires in not having putative sphingomyelinase genes (Segers *et al.*, 1992).

There was clear division between *L. biflexa sensu stricto* and the salt-water isolate *L. biflexa sensu lato ancona* Ancona Porto (Fig. 1). These strains were separated by a K_{nuc} value of 0.0085 (Table 2) and share only 3.8% DNA hybridization (Yasuda *et al.*, 1987). Also, *L. biflexa* was assigned to genetic group 5 and that

of *L. biflexa ancona* Ancona Porto to group 4 on DNA relatedness (Ramadass *et al.*, 1992). Consistent with this scenario would be a proposal to assign *L. biflexa sensu lato ancona* Ancona Porto to a new species.

In the absence of hybridization data and the relatively low consensus among the bootstrapped 16S rDNA sequence data sets (Fig. 1), little can be inferred of the genetic relationships between the freshwater isolates, *L. biflexa sensu lato canela* Canela and *L. biflexa sensu lato jequitaita* Jequitaita other than that they were distinct from each other and from the other strains examined.

In this study a comparative 16S rDNA sequencing analysis of all recognized species of *Leptospiraceae* revealed that the pathogenic species, though distinct from each other, comprised three groups and that the saprophytes, as currently circumscribed by *L. biflexa sensu stricto* and *L. wolbachii*, comprised a heterogeneous complex requiring further analyses. The lineages for *L. inadai* and *L. parva incertae sedis* were distinct. There was a correlation between groupings formed on total DNA hybridizations and those on 16S rDNA sequence data that may provide the framework for a natural classification of these spirochaetes.

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