Leptospirosis is a zoonosis of worldwide distribution (World Health Organization, 1999), caused by infection with pathogenic spirochaetes of the genus *Leptospira*. The disease is maintained in nature by chronic renal infection of carrier mammals, which excrete the organism in their urine (Faine et al., 1999). Humans become infected through direct exposure to infected animals or their urine or through indirect contact via contaminated water or soil (Levett, 2001). Leptospirosis has been recognized as an emerging infectious disease, in part because of recent large-scale outbreaks associated with recreational activities (Morgan et al., 2002; Sejvar et al., 2003).

The genus *Leptospira* presently consists of 12 named species and four unnamed genomospecies (Brenner et al., 1999; Levett et al., 2005; Pérolat et al., 1998). Phylogenetic analysis shows that leptospires cluster in three clades, representing species that contain pathogenic serovars, non-pathogenic serovars and an intermediate group (Pérolat et al., 1998). Within the latter clade are two species, *Leptospira inadai* and *Leptospira fainei* (Pérolat et al., 1998; Yasuda et al., 1987). *L. fainei* was first isolated from the uteri and kidneys of pigs in south-eastern Australia (Pérolat et al., 1998). Serological reactivity to *L. fainei* has been found in pigs and cattle in Australia (Pérolat et al., 1998) and in humans in Australia (Chappel et al., 1998) and in the Seychelles (Yersin et al., 1998). Three cases of human infection were recently reported in European countries (Arzouni et al., 2002; Petersen et al., 2001), from which leptospires identified as *L. fainei* were isolated. In this paper, we report the characterization of these isolates using DNA–DNA relatedness, G + C content, 16S rRNA gene sequence data and pulsed-field gel electrophoresis (PFGE) and the definition of a novel species to accommodate them.

Strains 5399^T^ and L065 were isolated in polysorbate medium (Arzouni et al., 2002; Petersen et al., 2001) from the blood of patients with acute leptospirosis. These and other strains were maintained at room temperature in semi-solid PLM-5 medium (Serologicals Corp.) containing 1·5 % agar (Difco). Subcultures in liquid PLM-5 medium were incubated at 30°C for 7 days. DNA was extracted and purified from strains 5399^T^ and L065, *L. fainei* BUT 6^T^ and *L. inadai* 10^T^ as described previously (Brenner et al., 1982). DNA from all strains except L065 was labelled with [³²P]dCTP (Brenner et al., 1982) and DNA relatedness and percentage divergence between the strains were determined by the hydroxyapatite method, with 55°C used for optimal reassociation (Table 1). The G + C content (mol%) was determined for strains 5399^T^ and L065 by the thermal denaturation method (Mandel et al., 1970). All samples were run at least three times, using DNA from *Escherichia coli* K-12 as a control.

DNA for 16S rRNA gene sequence determination was extracted and purified with a QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions and amplified by using the Expand high-fidelity PCR system (Roche) with primers fL1 and rL1 (Segonds et al., 1999). Amplification conditions were 94°C for 5 min, 35 cycles of 94°C for 15 s, 50°C for 15 s and 72°C for 90 s and finally a single extension of 72°C for 5 min followed by a 4°C hold. Products were confirmed by running 10 μl samples on a 1·0 % (w/v) agarose gel for 30 min at 75 V. Excess dNTPs and primers were removed with magnetic carboxylate beads (Agencourt Bioscience). Cycle sequencing was performed with Big Dye version 3.1 dye terminator chemistry (Applied Biosystems) by standard protocols with a 3-2 pM primer...
concentration. Excess dyes were removed with magnetic carboxylate beads and reaction products were sequenced on an ABI 3100 sequencer (Applied Biosystems). Sequences were assembled with Seqmerge (Genetics Computer Group) and trimmed to at least two confirming reads. Sequences were aligned with CLUSTAL X (Thompson et al., 1994) and a distance matrix was created. In TREECON, distances of aligned sequences were estimated by the Kimura two-parameter model, bootstrapped 1000 times and the tree topology was determined by the neighbour-joining method. The final phylogenetic tree was rooted with an outgroup and bootstrap values were displayed as percentages.

For PFGE analysis, late-exponential-phase cultures in liquid PLM-5 medium at 30°C were centrifuged to concentrate the bacteria used to prepare the agarose plugs. Slices cut from each agarose plug were digested with 30 U NotI restriction enzyme at 37°C for 2 h. *Salmonella enterica* serovar Braenderup strain H9812 was digested with 50 U XbaI for use as a DNA size standard. Plug slices containing digested DNA were placed in the wells of a 1% agarose gel and electrophoresed in a Bio-Rad CHEF Mapper XA or CHEF-DRIII for 18 h at 14°C with recirculating TBE buffer under the following conditions: 2.16 s initial switch time, 35-07 s final switch time, 120° angle, 6-0 V cm⁻¹ gradient, linear ramping factor. The gel was stained with ethidium bromide following PFGE and photographed under UV transillumination. Gel analysis was performed and Pearson correlation coefficients were calculated using BioNumerics version 3.0 software.

Strains 5399T and L065 showed morphology and motility typical of *Leptospira* strains under dark-field microscopy. DNA relatedness data are shown in Table 1. Strains 5399T and L065 showed no significant relatedness to *L. fainei* strain BUT 6T or *L. inadai* 10T. However, there was strong relatedness between the two strains 5399T and L065. These two strains meet the criteria for the molecular definition of a species (Brenner et al., 1999). The G+C contents of strains 5399T and L065 were 42 mol%, within the range of other *Leptospira* species (Yasuda et al., 1987). Phylogenetic analysis of 16S rRNA gene sequences of strains 5399T and L065, showed that these two strains formed a cluster distinct from *L. fainei* and were more closely related to, but still distinct from, *L. inadai* (Fig. 1). The DNA relatedness and phylogenetic analyses support the classification of strains 5399T and L065 in a novel species, part of the intermediate clade that also contains *L. inadai* and *L. fainei*.

PFGE analysis of strains 5399T and L065 (Fig. 2) showed that these strains were distinct from each other and from strains of *L. fainei* serovar Hurstbridge. There was also no match with any of 147 other serovars of *Leptospira* in a database constructed in Bionumerics (Galloway & Levett, 2004). Because these strains each produced unique PFGE profiles, they may represent previously unrecognized serovars. However, further serological and molecular study of these and future isolates will be necessary for this hypothesis to be confirmed.

**Table 1.** DNA relatedness of strains 5399T and L065 with related *Leptospira* strains

<table>
<thead>
<tr>
<th>Source of unlabelled DNA</th>
<th>BUT 6T</th>
<th>5399T</th>
<th>L. inadai 10T</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBR</td>
<td>D</td>
<td>RBR</td>
<td>D</td>
</tr>
<tr>
<td><em>L. fainei</em> BUT 6T</td>
<td>100</td>
<td>0-0</td>
<td>64</td>
</tr>
<tr>
<td>Strain 5399T</td>
<td>68</td>
<td>10-5</td>
<td>100</td>
</tr>
<tr>
<td>Strain L065</td>
<td>65</td>
<td>11-0</td>
<td>93</td>
</tr>
<tr>
<td><em>L. inadai</em> 10T</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Fig. 1.** Phylogenetic position of *Leptospira broomii* sp. nov. based on 16S rRNA gene sequences. *L. broomii* falls within the clade of ‘intermediate’ leptospires, including *L. inadai* and *L. fainei*. The tree was rooted with *L. interrogans* strain RGAᵀ. Bar, 2% sequence difference. GenBank accession numbers are shown in parentheses.
Leptospira broomii sp. nov.

Leptospira broomii (broo’mi.i. N.L. gen. masc. n. broomii after Dr J. C. Broom, a Scottish bacteriologist, who made substantial contributions to the study of leptospirosis).

Isolated from the blood, cerebrospinal fluid and urine of human patients with leptospirosis in Denmark and France. Morphology is as described previously for the genus (Brenner et al., 1999; Johnson & Faine, 1984). Cells are 10–15 μm in length and 0·1 μm in diameter, with wavelength of 0·5 μm and amplitude of approximately 0·3 μm (Petersen et al., 2001). The DNA G + C content is 42 mol%. The type strain is 5399T (= ATCC BAA-1107T = KIT 5399T).

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References


