**Leptospira idonii** sp. nov., isolated from environmental water

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Strain Eri-1T was isolated from a water sample on the campus of Kyushu University, Fukuoka, Japan. The motility and morphology of the isolate were similar to those of members of the genus *Leptospira*, but the spiral structure of the isolate was sharper under dark-field microscopy. Cells were 10.6±1.3 µm long and 0.2 µm in diameter, with a wavelength of 0.9 µm and an amplitude of 0.4 µm. Strain Eri-1T grew in Korthof’s medium at both 13 and 30°C, and also in the presence of 8-azaguanine. 16S rRNA gene-based phylogenetic analysis placed strain Eri-1T within the radiation of the genus *Leptospira* where it formed a unique lineage within the clad of the known saprophytic species of the genus *Leptospira*. The strain was not pathogenic to hamsters. Strain Eri-1T exhibited low levels (11.2–12.6 %) of similarity by DNA–DNA hybridization to the three most closely related species of the genus *Leptospira*. The DNA G+C content of the genome of strain Eri-1T was 42.5±0.1 mol%. These results suggest that strain Eri-1T represents a novel species of the genus *Leptospira*, for which the name *Leptospira idonii* sp. nov. is proposed. The type strain is Eri-1T (=DSM 26084T = JCM 18486T).

Members of the genus *Leptospira* are aerobic, Gram-negative bacteria whose cells are long, thin, flexible rods with a regular right-handed helical coiling pattern (Carleton *et al.*, 1979). In 1915, Ryokichi Inada and Yutaka Ido, the first and second professors of The First Medical Clinic of The Imperial University in Kyushu, which is presently known as Faculty of Medicine, Kyushu University, discovered spirochaetes and specific antibodies in the blood of Japanese coal miners with infectious jaundice (Weil’s disease) in Fukuoka, Japan (Inada *et al.*, 1916). This organism is now known as *Leptospira interrogans* strain Ictero No.1. Since this discovery, many serovars of this organism have been reported. Some strains are pathogenic to humans and animals, while others are saprophytic and found in freshwater and marine environments. The taxonomy of the genus *Leptospira* has undergone substantial revision with the use of 16S rRNA gene sequence comparison and DNA–DNA reassociation studies (Zuerner, 2011). Some researchers have classified 303 strains representing the genus *Leptospira* to a refined definition of 12 different species, and five new genomospecies have been identified (Brendle & Alexander, 1974; Yasuda *et al.*, 1987; Ramadass *et al.*, 1992; Brenner *et al.*, 1999). One species, *Turneriella parva*, was placed in a new genus of the family *Leptospiraceae* (Levett *et al.*, 2005). Recently, four novel species, *Leptospira broomii* (Levett *et al.*, 2006), *Leptospira licerasiae* (Matthias *et al.*, 2008), *Leptospira wolffii* (Slack *et al.*, 2008) and *Leptospira...
*Leptospira* strain Eri-1<sup>T</sup> was isolated from environmental water beside a swimming pool at Kyushu University, Fukuoka, Japan. We collected 10 ml of environmental water in a sterile 15 ml screw-capped tube. The tube was kept in a vertical position for 1 h to allow the sediment to settle. After that, 2.0 ml supernatant from the sample was added to 2.5 ml 2× concentrated Korthof’s medium (WHO & ILS, 2003) supplemented with 500 μl 10× concentrated STAFF (Chakraborty *et al.*, 2011). This tube was incubated at 30 °C and checked daily by dark-field microscopy (BX41, Olympus, Tokyo, Japan) for the presence of *Leptospira*, which was confirmed by observing their characteristic thin helical structures with prominent hooked ends and motility. For single colony isolation, solid medium was prepared by the incorporation of 1% (w/v) agar in liquid Korthof’s medium. The bacteria in the liquid culture medium were counted and diluted to approximately 10<sup>4</sup> cells ml<sup>−1</sup>. The solid medium was inoculated by spreading 0.1 ml diluted bacterial culture evenly over the surface of the medium with a glass spreader. The inoculated plate was sealed with tape and incubated at 30 °C. After seven days, subsurface colonies appeared. A single colony was picked up with a Pasteur pipette, transferred into fresh liquid Korthof’s medium, and incubated at 30 °C.

The isolated strain, Eri-1<sup>T</sup>, showed motility and morphology that were similar to those of members of the genus *Leptospira* under dark-field microscopy. However, the spiral structure of the isolate was sharper (Fig. 1). Fig. 2 shows an electron micrograph of strain Eri-1<sup>T</sup>. The cells were helical, 10.6 ± 1.3 μm long and 0.2 μm in diameter, with a wavelength of 0.9 μm and an amplitude of 0.4 μm. The diameter and wavelength of the strain were slightly larger and longer, respectively, than those of previously recognized species of the genus *Leptospira*. The larger diameter and longer wavelength of strain Eri-1<sup>T</sup> may be the reason why it is seen more sharply under dark-field microscopy.

**Fig. 1.** Dark-field micrograph of strain Eri-1<sup>T</sup> (a) and *Leptospira biflexa* strain Patoc I (b). Bars, 10 μm.
Phenotypic characterization of strain Eri-1T was performed by assessing its growth at varying temperatures and in the presence of 8-azaguanine (Johnson & Rogers, 1964). Duplicate Korthof’s medium or Ellinghausen-McCullough-Johnson-Harris (EMJH) medium were inoculated with strain Eri-1T (2.0 × 10^6 cells ml^-1) and incubated at 13 or 30 °C for 30 days (WHO & ILS, 2003). *L. interrogans* serovar Icterohaemorrhagiae strain Ictero No. 1 and *Leptospira biflexa* serovar Patoc strain Patoc I were used as controls to represent pathogenic and saprophytic species, respectively. Strain Eri-1T as well as *L. biflexa* grew in Korthof’s medium, and also in the presence of 8-azaguanine at 30 °C, and entered stationary phase after 5 days (1.5 × 10^8 cells ml^-1). At 13 °C in Korthof’s medium, the novel strain grew slowly and entered stationary phase after 13 days (6.3 × 10^7 cells ml^-1). Based on these results, strain Eri-1T was considered to be a non-pathogenic *Leptospira*. In EMJH medium the isolate required 30 days to reach stationary phase, and the bacterial count in the stationary phase was 5.6 × 10^7. This result suggested that EMJH medium was inadequate for the growth of strain Eri-1T compared with Korthof’s medium.

Serological identification of strain Eri-1T was performed by using the microscopic agglutination test (MAT) (WHO & ILS, 2003). Positive agglutination of strain Eri-1T was observed with three antisera against reference *Leptospira* serovars, anti-Hebdomadis, anti-Sejroe and anti-Mini. No agglutination was observed with the antisera against other serovars tested, namely Icterohaemorrhagiae, Australis, *Djasiman, Louisiana, Grippo yorkosha, Celledoni, Javanica, Pomona, Shermani, Canicola, Sarmin, Manhao, Patoc, Tarassovi, Autumnalis, Panama, Cynopteri, Manilae and Losbanos*. Hyperimmune antiserum against strain Eri-1T (with titres of 12,800) was produced in rabbits by using standard methods (Graves & Faine, 1970). The hyperimmune sera against Eri-1T was reactive to serovar Hebdomadis (strain Hebdomadis, 1:25,600), serovar Andamana (strain CH11, 1:50) and serovar Sao Paulo (strain Sao Paulo, 1:50).

Because of the serological similarity of strain Eri-1T and strain Hebdomadis, a cross-agglutinin absorption test (CAAT) was performed between strain Eri-1T and serovar Hebdomadis (strain Hebdomadis) according to Dikken and Kmety (1978). The results of the CAAT showed that strain Eri-1T does not belong to serovar Hebdomadis (Table 1). Whether strain Eri-1T belongs to serovar Jules or serovar Kremastos (other serovars in serogroup Hebdomadis) remains to be determined.

PCR amplification of the 16S rRNA gene and sequencing of the purified PCR products were carried out (Kawamura et al., 1999). After confirming amplicons of the 16S rRNA gene on 1% agarose gels, the sequence was determined using a 3130 Genetic Analyzer (Applied Biosystems). The sequences of the other species of the genus *Leptospira* used for alignment and for calculating levels of similarity were obtained from GenBank. Multiple sequence alignments of

### Table 1. CAAT between strain Eri-1T and *L. interrogans* serovar Hebdomadis strain Hebdomadis

<table>
<thead>
<tr>
<th>Antiserum against serovar/strain</th>
<th>Homologous titre before absorption</th>
<th>Absorbing strain</th>
<th>Homologous titre after absorption</th>
<th>Remaining homologous titre (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eri-1T</td>
<td>1:12,800</td>
<td>Hebdomadis</td>
<td>1:1600</td>
<td>12.5</td>
</tr>
<tr>
<td>Hebdomadis</td>
<td>1:3200</td>
<td>Eri-1T</td>
<td>1:3200</td>
<td>100</td>
</tr>
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</table>
DNA sequences were performed using CLUSTAL W software (Thompson et al., 1997). Phylogenetic distances were calculated using the neighbour-joining method (Saitou & Nei, 1987). The phylogenetic tree was reconstructed using TreeView software (Page, 1996). 16S rRNA gene sequence similarity between strain Eri-1T and the 20 previously described species of the genus Leptospira was found to be less than 97%. The type strains of L. biflexa (96.9%), Leptospira meyeri (96.8%), L. yanagawae (96.6%), L. terpstrae (96.9%) and L. vanthieli (96.9%) showed the highest 16S rRNA gene sequence similarity to strain Eri-1T. Phylogenetic analysis showed that strain Eri-1T was placed within the radiation of the genus Leptospira and formed a unique lineage within the clade of the saprophytic species of the genus Leptospira (Fig. 3).

To confirm that strain Eri-1T was not pathogenic to humans or animals, PCR assays and animal experiments were performed. Sets of PCR primers specific for both pathogenic (flaB) and saprophytic (ssl) Leptospira have been described (Murgia et al., 1997; Kawabata et al., 2001). It was possible to identify pathogenic strains and saprophytic strains by using these primer sets. Bacterial cultures of strain Eri-1T, L. interrogans strain K37 (Villanueva et al., 2010) and L. biflexa strain Patoc I were prepared for DNA isolation by centrifugation, followed by genomic DNA extraction using Illustra Bacteria genomicPrep Mini Spin kit (GE Healthcare). PCR was performed as described by Murgia et al. (1997) and Kawabata et al. (2001). The ssl-PCR assay yielded products of the expected sizes from DNA extracted from strain Eri-1T and L. biflexa strain Patoc I. The flaB-PCR assay produced a positive PCR product only for the pathogenic L. interrogans strain K37. The results from these two PCR assays strongly suggested that strain Eri-1T should be classified as a saprophytic Leptospira. For the pathogenicity experiment, four-week-old male golden Syrian hamsters were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). Five hamsters were inoculated subcutaneously with 10^8 cells of strain Eri-1T in 1 ml PBS (in-house) NaCl 8 g/L, KCl 0.2 g/L, Na2HPO4 1.13 g/L, KH2PO4 0.2 g/L, and observed for 28 days. The experimental protocol was reviewed by the Committee of Ethics on Animal Experiments of the Faculty of Medical Sciences, Kyushu University, and carried out in accordance

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**Fig. 3.** Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence analysis showing the phylogenetic position of strain Eri-1T. Bar, 1 inferred nucleotide substitution per 100 nt. Numbers at nodes are bootstrap values (% based on 1000 samplings). GenBank accession numbers are shown in parentheses.
with the Animal Experiment Guidelines of the Faculty, Law No. 105 and Notification No. 6 of the Japanese Government. No hamster died from infection even at such a high bacterial dose. Taken together, these results confirmed that strain Eri-1<sup>T</sup> represents a non-pathogenic or saprophytic species of the genus *Leptospira*.

For DNA–DNA hybridization and DNA G + C content analysis, DNA was isolated from strain Eri-1<sup>T</sup>, *L. biflexa* strain Patoc I, *L. yanagawae* (genomospecies 5) strain Sao Paulo and *L. meyeri* strain Verdrat Semarang 173 by using standard methods (Wilson, 1987). DNA–DNA hybridization was performed, as described by Tomida *et al.* (2011). Briefly, purified DNA (100 µg ml<sup>−1</sup>) of each strain was heat denatured and then diluted to 10 µg ml<sup>−1</sup> with PBS containing 0.1M MgCl<sub>2</sub>. The diluted DNA solution was distributed onto a microplate (Nunc-Immunoplate) at 100 µl per well, and the plate was incubated at 30 °C for 12 h. The solution was discarded and the plate was dried. DNA from the four strains was labelled with photobiotin (Vector Laboratories). The plate was pre-hybridized for 30 min and then hybridized for 2 h at 29 °C (optimal conditions) or 39 °C (stringent conditions) using 2 × SSC containing 50 % formamide. The plate was washed three times with 1 × SSC, and 100 µl of streptavidin-β-d-galactosidase (diluted 1 : 1000 with 0.5 % BSA in PBS; Calbiochem) was added to each well. The plate was incubated at 37 °C for 30 min and washed three times with 1 × SSC. Following this, 100 µl of the substrate (4-methylumbelliferyl-β-d-galactopyranoside, 100 µg ml<sup>−1</sup>) was added to each well and the fluorescence intensity was measured using a DTX 800 Multimode Detector (Beckman Coulter). DNA relatedness was expressed as a mean percentage of the homologous DNA binding value. Strain Eri-1<sup>T</sup> showed no significant relatedness to *L. biflexa* strain Patoc I (12.4 % under optimal conditions), *L. yanagawae* strain Sao Paulo (11.2 %) or *L. meyeri* strain Verdrat Semarang 173 (12.6 %).

The DNA G + C content (mol%) was determined by HPLC (Mesbah *et al.*, 1989; Tomida *et al.*, 2011). A total of 5 µg denatured DNA was hydrolysed with P1 nuclease (Yamasu Syoyu, Chiba, Japan) for 1 h at 50 °C. Alkaline phosphatase (Sigma) was then added, and the mixture was incubated at 37 °C for 30 min for nucleotide dephosphorylation. The nucleosides were quantified with a GC analysis standard (Yamasu Syoyu) using a model L-2400 HPLC system (Hitachi) and an Inertsil ODS-3 HPLC Column (GL Sciences). The nucleosides were eluted with a solvent containing 0.2 M NH₄H₂PO₄ and acetonitrile (20 : 1, v/v). DNA G + C content (mol%) was determined using the mean values of three experiments. The DNA G + C content of strain Eri-1<sup>T</sup> was 42.5 ± 0.1 mol%, which is within the range 33–43 mol% reported for members of the genus *Leptospira* (Zuernier, 2011).

These results provide sufficient evidence through molecular taxonomic characterization to justify the inclusion of strain Eri-1<sup>T</sup> within a novel species of the genus *Leptospira*, for which the name *Leptospira idonii* sp. nov. is proposed.

### Description of *Leptospira idonii* sp. nov.

*Leptospira idonii* (i.do’ni.i. N.L. gen. masc. n. *idonii* of Ido, named after Yutaka Ido, a Japanese physician and microbiologist who is one of the discoverers of leptospires in the blood of patients with Weil’s disease).

Motility and morphology of the isolate are similar to those of other members of the genus *Leptospira* but spiral structure of the isolates is sharper under dark-field microscopy. Grows well in Korthof’s media at 13 °C and 30 °C, and in EMJH media at 13 °C, but not in EMJH media at 30 °C. Grows in the presence of 8-azaguanine. Cells are 10.6 ± 1.3 µm long and 0.2 µm in diameter, with a wavelength of 0.9 µm and an amplitude of 0.4 µm. Shares less than 97 % 16S rRNA gene sequence similarity with all other recognized species of the genus *Leptospira* and exhibits low levels of DNA–DNA relatedness to the three most closely related species.

The type strain, Eri-1<sup>T</sup> (=DSM 26084<sup>T</sup> =JCM 18486<sup>T</sup>), was isolated from an environmental water sample on the campus of Kyushu University, Fukuoka, Japan. The DNA G + C content of the genome of strain Eri-1<sup>T</sup> is 42.5 ± 0.1 mol%.

### Acknowledgements

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### References


