Distribution of the leptospiral immunoglobulin-like (*lig*) genes in pathogenic *Leptospira* species and application of *ligB* to typing leptospiral isolates

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The family of leptospiral immunoglobulin-like (*lig*) genes comprises *ligA*, *ligB* and *ligC*. This study used PCR to demonstrate the presence of *lig* genes among serovars from a collection of leptospiral strains and clinical isolates. Whilst *ligA* and *ligC* appeared to be present in a limited number of pathogenic serovars, the *ligB* gene was distributed ubiquitously among all pathogenic strains. None of the *lig* genes were detected among intermediate or saprophytic *Leptospira* species. It was also shown that, similar to the previously characterized *secY* gene, a short specific PCR fragment of *ligB* could be used to correctly identify pathogenic *Leptospira* species. These findings demonstrate that *ligB* is widely present among pathogenic strains and may be useful for their reliable identification and classification.

INTRODUCTION

Leptospirosis is a re-emerging zoonotic disease caused by *Leptospira* species, which are transmitted to humans through direct or indirect contact with contaminated urine from a reservoir host, usually rats or other rodents (Faine *et al.*, 1999). DNA–DNA hybridization studies have identified 19 *Leptospira* species to date (Yasuda *et al.*, 1987; Brenner *et al.*, 1999; Levett, 2001; Levett *et al.*, 2006; Matthias *et al.*, 2008; Slack *et al.*, 2008). Among these, *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira santarosai*, *Leptospira noguchii*, *Leptospira weilii*, *Leptospira kirschneri* and *Leptospira alexanderi* are considered to be the main agents of leptospirosis (Levett *et al.*, 2006). Serological methods have identified >300 serovars of which more than 200 are considered pathogenic (Faine *et al.*, 1999; Levett, 2001; Bharti *et al.*, 2003).

The *lig* genes, *ligA*, *ligB* and *ligC*, encode virulence determinants in pathogenic strains (Palaniappan *et al.*, 2002; Matsunaga *et al.*, 2003; Choy *et al.*, 2007; Lin &

Chang, 2007). The Lig proteins were identified as markers for the early diagnosis of leptospirosis (Croda *et al.*, 2007; Srimanote *et al.*, 2008) and as potential vaccine candidates (Koizumi & Watanabe, 2004; Palaniappan *et al.*, 2006; Silva *et al.*, 2007; Faisal *et al.*, 2008; Yan *et al.*, 2009). Previously, we determined that the *lig* genes are highly conserved (70–99 % identity) in virulent pathogenic *Leptospira* isolates (McBride *et al.*, 2009). The *ligB* gene was present in all isolates, whilst *ligA* was limited to *L. interrogans* and *L. kirschneri* strains and *ligC* was a pseudogene in several isolates.

Molecular tools employed for the classification of *Leptospira* species include PFGE (Herrmann *et al.*, 1992; Galloway & Levett, 2008), RFLP (Brown & Levett, 1997; Barocchi *et al.*, 2001), arbitrarily primed PCR (Perolat *et al.*, 1994), fluorescent amplified fragment length polymorphism (Vijayachari *et al.*, 2004) and variable number tandem repeats (Majed *et al.*, 2005; Slack *et al.*, 2005; Salaün *et al.*, 2006). However, these techniques lack reproducibility or have low sensitivity or specificity (Levett *et al.*, 2006). 16S rRNA gene sequencing has been used in phylogenetic analyses (Hookey *et al.*, 1993) but these genes exhibit a low degree of polymorphism, limiting their usefulness in typing. A limitation of a previous investigation of *lig* genes was the small number of isolates

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studied (McBride *et al.*, 2009). To this end, we proposed to determine the presence of *lig* genes in an expanded collection of strains using a PCR-based assay. In addition, we found that it was possible to type the pathogenic leptospires to the species level using the *ligB* sequence. We therefore investigated the possibility of using the *ligB* sequences from the PCR assay for the molecular characterization of pathogenic *Leptospira* isolates.

METHODS

Bacterial strains and culture conditions. Reference and clinical strains belonging to 10 species and including 40 serovars were obtained from the collections maintained at the Gonçalo Moniz Research Centre, Salvador, Brazil, and the National Reference Centre for Leptospirosis at the Institut Pasteur, Paris, France. Clinical strains were isolated from both humans and animals and from diverse geographical regions, including Brazil, Russia, Croatia and Guadeloupe (Majed *et al.*, 2005; Silva *et al.*, 2008). All strains were cultured at 30 °C in liquid Ellinghausen–McCullough–Johnson–Harris modified Tween 80/bovine albumin medium (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967). A microscopic agglutination test was carried out using a standard method for putative serogroup determination (Levett *et al.*, 2003).

Oligonucleotide design. Primers were designed using Vector NTI 10 software (Invitrogen). The *lig* gene sequences deposited in GenBank were aligned, conserved regions were identified and degenerate primers were designed. Fragments from each of the *lig* genes were amplified and sequenced using primers specific for *ligA* (PSAF: 5′-CKGAWCTTGTRACYTGGARKTCYTC-3′; PSAR: 5′-TT-GTTAATGTTTTCATRTTAYGGC-3′), *ligB* (PSBF: 5′-ACWRVHV-HRGYWDCCTGGTCYTCTTC-3′; PSBR: 5′-TARRHDGCYBTAAT-ATYCGRWYYTCCTAA-3′) and *ligC* (PSCF: 5′-GAGAAATAYA-ATCTCCTTCTTCCGG-3′; PSCR: 5′-CCTRTTCGTGTTGGARGAA-TTCC-3′).

DNA manipulation. Genomic DNA was extracted using a GFX Genomic Blood DNA Purification kit following the protocol for Gram-negative bacteria recommended by the manufacturer (GE Healthcare). PCR amplification was performed using *Taq* DNA polymerase (Invitrogen) and the following cycling conditions: one denaturing cycle at 94 °C for 2 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 54 °C for 30 s and elongation at 72 °C for 45 s; and a final elongation at 72 °C for 10 min. The amplified products were analysed by 1 % agarose gel electrophoresis.

Sequencing. PCR products were purified using a GFX PCR DNA and Gel Band Purification kit according to the manufacturer's instructions (GE Healthcare). Sequencing was performed using a MegaBACE 500 DNA sequencer (GE Healthcare) and Dynamic ET Terminator technology. The assembled sequences were analysed by BLAST alignment (http://www.ncbi.nlm.nih.gov/BLAST) against the available *lig* gene sequences in GenBank. The *lig* sequences were aligned using AlignX software (Invitrogen).

Phylogenetic analysis. The *ligB* gene sequences from 48 pathogenic strains (Table 1) were used to assemble a phylogenetic tree with the MEGA 4 software (Tamura *et al.*, 2007). 16S rRNA gene sequences were obtained from GenBank (Table 1) and aligned as described. One thousand bootstrap replications were used to provide confidence in the nodes. The trees were constructed by the neighbour-joining method using the Jukes–Cantor model (Tamura *et al.*, 2007). Synonymous/non-synonymous data were calculated using MEGA

4.1*β* software. *rpoB* sequences used for comparison were obtained from GenBank (accession nos DQ296129–DQ296147; La Scola *et al.*, 2006).

Southern blotting. A total of 3 μg genomic DNA was digested with 20 U *Bam*HI (Invitrogen) and separated by agarose gel electrophoresis. DNA was transferred from the gel to a positively charged Hybond-N nylon membrane (GE Healthcare) with a vacuum blotter (Bio-Rad). Probes for each of the *lig* genes were based on pooled PCR products amplified using the primers described and labelled using an ECL Direct Nucleic Acid Labelling and Detection System (GE Healthcare). Pre-hybridization was carried out at 42 °C for 1 h in hybridization buffer supplemented with 0.5 M NaCl and 5 % blocking agent. Hybridization was carried out overnight at 42 °C in roller bottles. Following hybridization, the membrane was washed twice for 10 min at 55 °C in wash solution (0.4 % SDS, 0.5 × SSC). Finally, the membrane was washed twice in 2 × SSC for 5 min per wash at room temperature. After incubation with ECL detection reagents, hybridization products were detected by exposure of the membrane to Hyperfilm ECL X-ray film (GE Healthcare).

RESULTS AND DISCUSSION

Distribution of the *lig* genes in *Leptospira* species

In our previous study, pairwise alignment of the *lig* genes allowed the identification of highly conserved regions within the *lig* genes (interspecies identity ranged from 68 to 99 %; McBride *et al.*, 2009). Based on these regions, primers were designed to successfully amplify *lig* gene fragments from the *Leptospira* strains described in this study (Table 1). For *ligA*, the primers spanned nt 3482–3692 at the C-terminus, the *ligB* primers spanned nt 2125–2504 within the non-identical region and for *ligC* the primers spanned nt 1487–1734. The expected sizes of the amplicons were 211 bp (*ligA*), 380 bp (*ligB*) and 248 bp (*ligC*). The PCR results indicated that *ligB* was conserved in the genome of 100 % (52/52) of the pathogenic strains tested (Table 1) (Ren *et al.*, 2003; Nascimenta *et al.*, 2004; Bulach *et al.*, 2006). Notably, *ligA* was limited to *L. interrogans* and *L. kirschneri* strains and was found in only 26/44 isolates. As well as being present in certain *L. interrogans* and *L. kirschneri* strains, *ligC* was also detected in *L. noguchii* and *L. weilii* strains (31/44 strains in total).

To confirm the negative PCR results as true negatives, Southern blot analysis was carried out (Table 1). The hybridization results corroborated the PCR assay findings. These results support previous studies suggesting that the *lig* genes are only found in pathogenic strains and that of the three *lig* genes, only *ligB* is conserved in all pathogenic *Leptospira* strains (Matsunaga *et al.*, 2003; McBride *et al.*, 2009). The findings presented here add to the growing body of evidence suggesting that Lig proteins are essential virulence determinants in *Leptospira* species (Matsunaga *et al.*, 2005; Choy *et al.*, 2007; McBride *et al.*, 2009). To ensure that the PCR products were not artefacts, a selection of amplicons (see Table 1) were sequenced and analysed with the *lig* gene sequences available in GenBank.

Table 1. Distribution of *lig* genes

GenBank accession numbers for the 16S rRNA gene are given in parentheses. ND, Not determined.

Species	Serovar	Strain	16S rRNA gene*	PCR		
				ligA	*ligB*	*ligC*
Pathogens						
L. interrogans	Australis	Ballico	+ (FJ154556)	+	+	+†
	Autumnalis	Akiyami A	+ (FJ154543)	+	+	+
	Bataviae	Van Tienen	+ (FJ154566)	+	+	+
	Bratislava	Jez Bratislava	+ (FJ154547)	+	+	+
	Canicola	Hond Utrech IV	+ (FJ154561)	+	+	+†
	Canicola	Kito	+	+‡	+‡	+‡
	Canicola	Mex 1	+	+‡	+‡	+†‡
	Copenhageni	Fiocruz L1-130	+ (AY461869)	+‡	+‡	+‡
	Copenhageni	M 20	+ (FJ154542)	+	+	+
	Hardjo-prajitno	Hardjoprajitno	+ (FJ154553)	+	+	+
	Hebdomadis	Hebdomadis	+ (FJ154551)	+	+	+
	Icterohaemorrhagiae	RGA§	+ (FJ154549)	+	+	+
	Kennewicki	LT 1026	+ (FJ154571)	+	+	+
	Lai	56601	(AY461870)	−	+	+
	Lai	Lai	+	+	+	+
	Manilae	LT 398	+ (FJ154545)	+	+	+
	Muenchen	Munchen C90	+ (FJ154565)	+	+	+
	Pomona	PO-06-047	+	+‡	+‡	+‡
	Pomona	Pomona	+ (FJ154544)	+	+	+
	Wolffi	3705	+ (FJ154558)	+	+	+
L. kirschneri	Cynopteri	3522 C§	+ (FJ154546)	+†	+	+†
	Djatzi	HS 26	+	+†	+	+
	Erinaceiauriti	Erinaceus auritus 670	+ (FJ154560)	+†	+	+
	Grippotyphosa	2.002.297‖	+	ND	+†	ND
	Grippotyphosa	2.002.306‖	+	ND	+†	ND
	Grippotyphosa	2000.11.449‖	+	ND	+†	ND
	Grippotyphosa	RM52	+ (AY461877)	+‡	+‡	+‡
	Kambale	Kambale	+ (FJ154562)	+	+	+
	Mozdok	5621	+ (FJ154559)	+	+	+
	Ramisi	Musa	+ (FJ154573)	+†	+	+†
L. borgpetersenii	ND	2E02	+	−†‡	+†‡	−
	Ceylonica	Piyasena	+ (FJ154596)	−	+	−
	Istrica	M 18‖	+	ND	+†	ND
	Javanica	Veldrat Batavia 46§	+ (FJ154600)	−	+	−
	ND	2002.10.110‖	+	ND	+†	ND
	Mini	Sari	+ (FJ154592)	−	+†	−
	Poi	Poi	+ (FJ154597)	−	+	−
	Hardjo-bovis	L550	(NC_008508)	−	+	−
	Hardjo-bovis	JB197	(NC_008510)	−	+	−
	Tarassovi	Perepelitsin	+ (FJ154595)	−	+†	−
L. noguchii	Bataviae¶	Cascata	+ (EU349495)	−‡	+‡	−‡
	Orleans	LSU 2580	+ (FJ154588)	−	+	−
	Panama	CZ 214 K§	+ (FJ154582)	−	+	+
L. weilii	Hebdomadis¶	Ecochallenge	+ (AY034037)	−‡	+‡	+‡
	Celledoni	Celledoni§	+ (FJ154580)	−	+	−
	Coxi	Cox	+	−	+†	+†
	Vughia	LT 89-68	+ (FJ154590)	−	+	+
	ND	2007.025.92‖	+	ND	+	ND
L. santarosai	Alexi	HS 616	+ (FJ154585)	−	+	−
	Shermani	LT 821§	+(AY631883)	ND	+	ND
	Trinidad	TRVL 34056	+ (FJ154598)	−	+†	−
	ND	2008.010.55‖	+	ND	+	ND

Table 1. cont.

Species	Serovar	Strain	16S rRNA gene*	PCR		
				ligA	*ligB*	*ligC*
Intermediates						
L. fainei	Hurstbridge	But 6§	+ (FJ154578)	−	−	−
L. inadai	Lyme	10§	+	ND	−	ND
Saprophytes						
L. meyeri	Semaranga	Veldrat Semarang 173§	+	−	−	−
L. biflexa	Semaranga	Patoc 1§	+	−‡	−‡	−‡

*Internal PCR control (Postic *et al.*, 2000). For entries without a ' + ', the sequence from GenBank was used but they were not amplified as a control in the experiment.
†PCR products not sequenced.
‡Confirmed by Southern blot analysis.
§Type strain.
||Clinical isolate.
¶Serogroup.

Sequence variability of the *lig* gene fragments

The *ligB* amplicons exhibited considerable DNA sequence polymorphism, due to indels, particularly at the 5′ and 3′ ends of the 380 bp fragment. Therefore, the *ligB* sequences were trimmed to remove these hypervariable regions as they did not appear to differ among strains of the same species, and a 214 bp region (nt 2236–2449, *L. interrogans* Fiocruz L1-130 strain) was identified that exhibited a high level of conservation. The hypervariable region of the 380 bp fragment was identified by comparison with the *L. interrogans* Fiocruz L1-130 *ligB* gene. The probability of recombination among the *ligB* hypervariable regions was confirmed (overall $P < 0.05$) and the ratio between the non-synonymous and synonymous substitutions (d_N/d_S) was 2.41. This provided evidence for positive selection within this region. Of note, although we did not see any evidence of horizontal transfer within the 214 bp region of *ligB*, we cannot exclude the possibility that this may occur occasionally. The overall level of pairwise DNA sequence variability was determined to be 21.2 ± 3.9 % (20.6 ± 3.8 % at the amino acid level) for the *ligB* amplicon (Fig. 1). This DNA fragment demonstrated some interspecies polymorphism, but it was not significant (Fig. 1). The *ligB* gene is more variable than the previously evaluated *ompL1* (15 %), *lipL41* (9 %) and *lipL32* (3 %) genes (Haake *et al.*, 2004). The mean pairwise DNA sequence variability was 0.8 ± 0.4, 3.7 ± 1.5, 0, 1.2 ± 0.9, 0.9 ± 0.9 and 0 % among the *L. interrogans*, *L. kirschneri*, *L. noguchii*, *L. borgpetersenii*, *L. santarosai* and *L. weilii* strains, respectively (0.8 ± 0.4, 3.7 ± 1.5, 0, 1.7 ± 1.2, 0.9 ± 0.9 and 0 % at the amino acid level, respectively). Furthermore, 17 different *ligB* orthologues were identified among the 48 *Leptospira* strains that contained one or more base substitutions within the amplified region.

The *ligA* amplicons demonstrated a mean pairwise variability of 21.5 ± 2.4 % among *L. interrogans* strains and 0.8 ± 0.8 % among *L. kirschneri* strains (25 ± 3.9 and 0 % at the amino acid level, respectively). The overall mean pairwise DNA sequence variability of the *ligA* amplicons was 22.2 ± 2.7 % (26.8 ± 4.4 % at the amino acid level) (Fig. 1). The alignment of the *ligA* sequences revealed the presence of indels in some of the *L. interrogans* sequences corresponding to the loss of an amino acid codon. The *ligC* gene exhibited a mean pairwise variability of 1.9 ± 1.7 and 0 % (1.9 ± 1.8 and 0 % at the amino acid level, respectively) among the *L. interrogans* and *L. kirschneri* strains, respectively. The overall mean pairwise variability was 4.4 ± 2.8 % (4.4 ± 2.8 % at the amino acid level) (Fig. 1).

The *lig* genes encode an important family of outer-membrane proteins that are characterized by the presence of immunoglobulin-like domains (Palaniappan *et al.*, 2002; Matsunaga *et al.*, 2003) and are potential virulence determinants of *Leptospira* species (Choy *et al.*, 2007; Lin & Chang, 2007). These proteins are surface-exposed and are upregulated within mammalian hosts (Matsunaga *et al.*, 2005; Choy *et al.*, 2007). Previous studies have demonstrated their usefulness as markers for diagnosis of leptospirosis (Palaniappan *et al.*, 2004, 2005; Croda *et al.*, 2007; Srimanote *et al.*, 2008) and as potential vaccine candidates (Koizumi & Watanabe, 2004; Palaniappan *et al.*, 2006; Silva *et al.*, 2007; Faisal *et al.*, 2008, 2009). More recently, their presence and conservation among virulent pathogenic strains of *Leptospira* species was confirmed (McBride *et al.*, 2009). Of note, inactivation of *ligB* does not result in attenuation of virulence in animal models (Croda *et al.*, 2008). This is probably due to functional redundancy of the Lig proteins, as LigA was expressed in the LigB-knockout strain. Both LigB and LigA can bind the same extracellular matrix and plasma proteins, suggesting that they both play a role during the colonization and dissemination stages of leptospirosis (Choy *et al.*, 2007). In addition, we demonstrated that LigA was created from
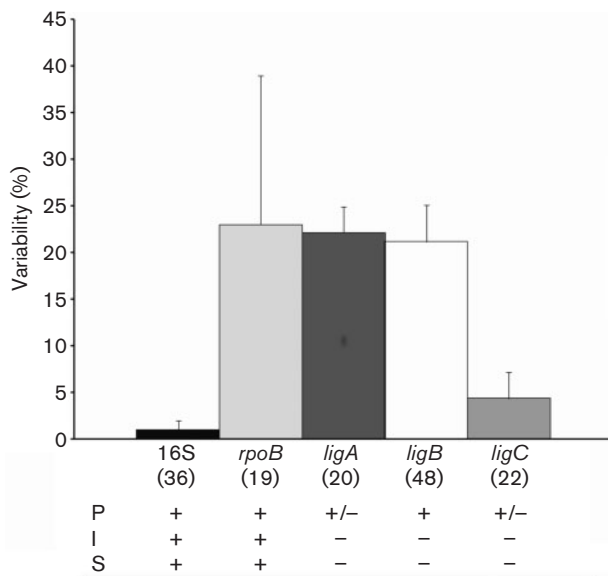
Fig. 1. Comparison of the variability of the DNA sequences from the 16S rRNA, *rpoB*, *ligA*, *ligB* and *ligC* genes from *Leptospira* species. Results are shown as means ± SD. The number of individual sequences used for the determination of sequence variability is indicated in parentheses. The presence (+) and absence (−) of each gene in pathogenic (P), intermediate (I) and saprophytic (S) strains is shown. The nucleotide positions used during the alignment analysis were: nt 75–1255 (16S rRNA gene), 1891–2462 (*rpoB*), 3482–3693 (*ligA*), 2236–2449 (*ligB*) and 1487–1734 (*ligC*).

LigB in a gene duplication event. The N-terminal region of LigB and the LigA paralogue are essentially identical, further supporting the idea that LigA could replace LigB during pathogenesis (McBride *et al.*, 2009). To clarify the role of the Lig proteins in virulence, a *ligB*/*ligA* double-knockout strain would be required. The findings of this study confirm the ubiquitous nature of LigB in pathogenic *Leptospira* species and that LigA and LigC are not present in all strains.

Phylogenetic analysis of *ligB*

The relatedness of the 48 *ligB* 214 bp DNA sequences is presented in Fig. 2(a). The *Leptospira* strains were resolved into two distinct clusters. The sequences from *L. interrogans*, *L. kirschneri* and *L. noguchii* grouped in one cluster, whilst those from *L. borgpetersenii*, *L. santarosai* and *L. weilii* formed the second cluster. The clustering pattern was similar to the phylogenetic tree based on the full-length *ligB* sequences (McBride *et al.*, 2009). The individual *Leptospira* species were thus easily determined based on the *ligB* internal sequence.

The *ligB* amplicon is situated within a region of the *ligB* gene that was found to be phylogenetically clonal based on a multiple change-point model in the majority of strains

(McBride *et al.*, 2009). Of the two strains that showed evidence of rearrangements (*L. interrogans* and *L. kirschneri*), the amplicon was located outside these recombination hotspots. The results demonstrate that the internal *ligB* sequence can be used to discriminate *Leptospira* to the species level. Within each major cluster, there was evidence of further subclustering. For example, three out of five of the *L. interrogans* serogroup Icterohaemorrhagiae strains clustered together, including serovars Copenhageni and Icterohaemorrhagiae. Within the *L. kirschneri* and *L. borgpetersenii* cluster, various subclusters were identified but they did not correspond to the serogroups (Fig. 2a). However, there was insufficient discriminatory power to type the serovars beyond the species level. This is a similar situation to that reported for the 16S rRNA gene in *Leptospira* species (Morey *et al.*, 2006). In addition, previous work by Victoria *et al.* (2008) demonstrated that the *S10-spc-α* locus is conserved within pathogenic *Leptospira* species, but less so among saprophytic and intermediate *Leptospira* species, indicating that it is a useful region for phylogenetic analysis. Sequence analysis of a short region (245 bp) of the *secY* gene that is normally used for PCR diagnosis found that it can be used to correctly identify *Leptospira* strains.

The number of synonymous substitutions within the *ligB* amplicons was equal or higher than the number of non-synonymous substitutions per site. The probability of the existence of recombination among the several *ligB* nucleotide sequences was not confirmed (overall $P=1.00$) and the d_N/d_S ratio was 0.34. This supports the hypothesis of sequence stability due to the absence of positive selection over this *ligB* locus. Rejection of the neutrality hypothesis (positive selection suggestive of recombination) in *ligB* was seen only in the *L. borgpetersenii* Poi and Veldrat Batavia 46 strains where d_N/d_S was 1.72 ($P=0.04$). However, this does not preclude the use of *ligB* for species typing, as both belong to the same species. The G+C content of the several *ligB* loci ranged from 37.8 mol% in *L. kirschneri* to 50 mol% in *L. borgpetersenii* (data not shown).

Phylogenetic analysis of the 16S rRNA gene sequences

The phylogenetic tree based on the available 16S rRNA gene sequences (Table 1) is presented in Fig. 2(b). The tree describes the relatedness for 36 sequences and the clustering pattern is similar to that described in previous studies (Haake *et al.*, 2004; Levett *et al.*, 2006). The strains clustered according to species: sequences from *L. interrogans*, *L. kirschneri*, *L. noguchii* and *L. santarosai* formed one cluster, whilst those from *L. borgpetersenii* and *L. weilii* formed a second cluster. The major difference between the predicted relatedness patterns is the clustering of the *L. santarosai* strains.
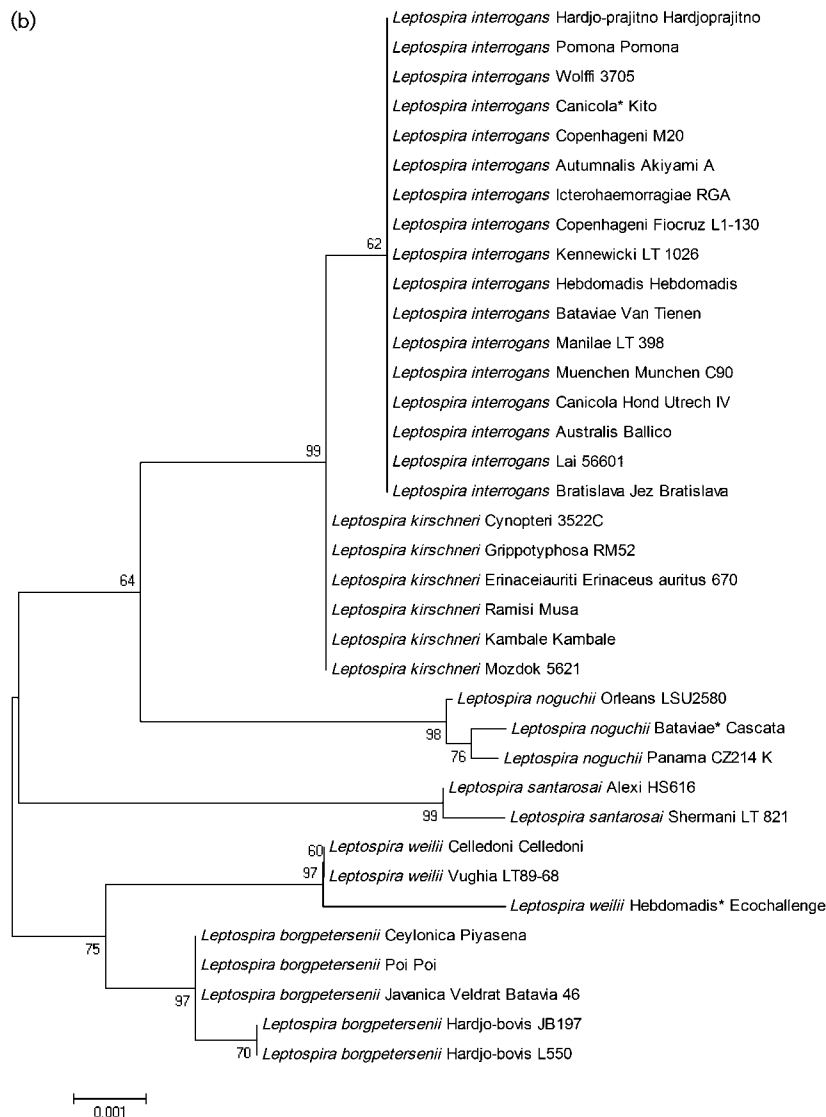
Traditionally, 16S rRNA gene sequences have been used for *Leptospira* species classification (Postic *et al.*, 2000; Morey

(a)
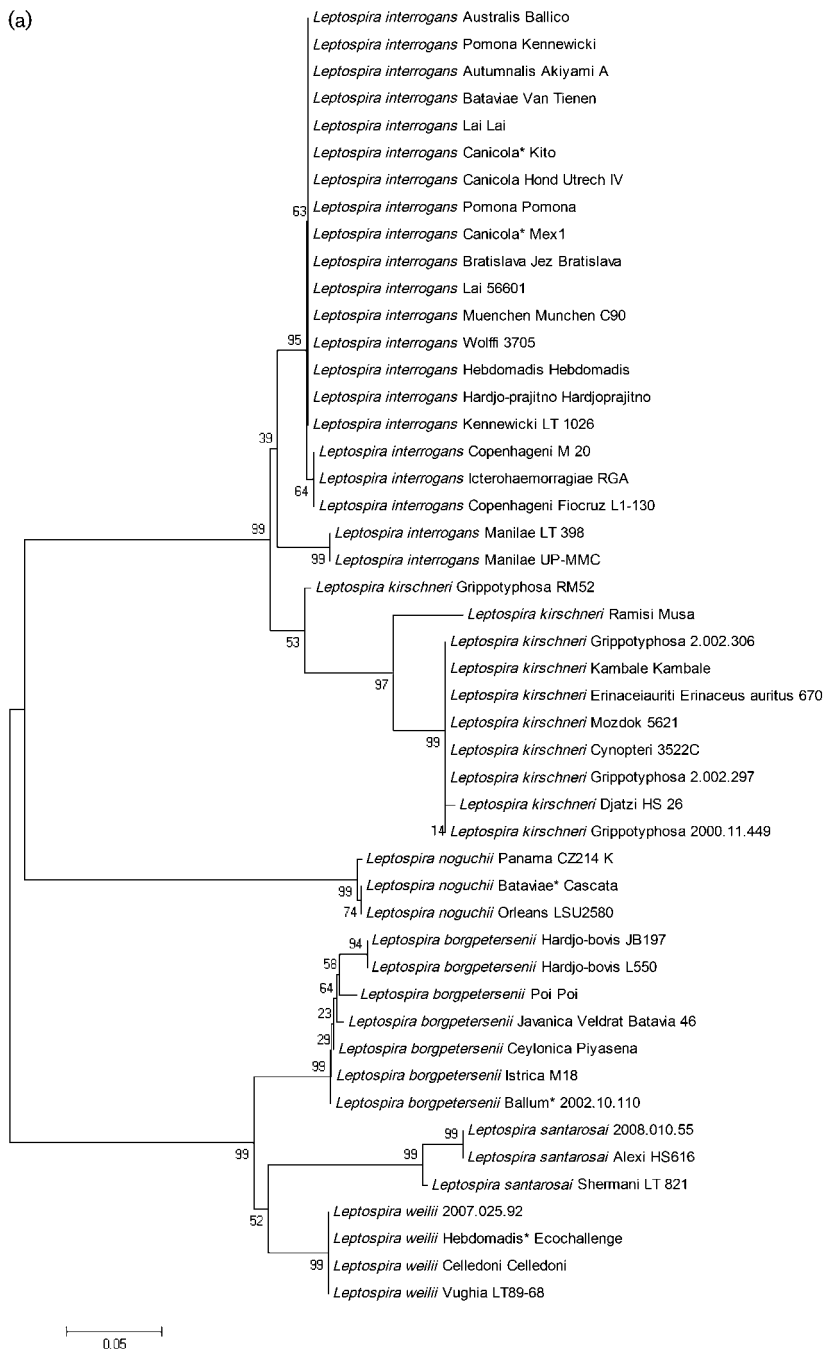
Fig. 2. Unrooted phylogenetic trees were constructed from the *ligB* gene (214 bp) (a, this page) and the 16S gene (1181 bp) (b, opposite page). Bootstrap consensus values are indicated. Asterisks indicate serogroups rather than serovars.

et al., 2006). However, this gene has few polymorphisms throughout its 1500 bp in *Leptospira* species (Janda & Abbott, 2007). Efforts to identify new markers for species differentiation have focused on the evaluation of partial *rpoB* (La Scola *et al.*, 2006) and *wzy* (Wangroongsarb *et al.*, 2007) polymerases, the gyrase subunit B (*gyrB*; Slack *et al.*, 2006), the pre-protein translocase *secY* (Victoria *et al.*, 2008) and the genes encoding the surface proteins LipL32, LipL41 and OmpL1 (Haake *et al.*, 2004; Ahmed *et al.*, 2006). The main advantage of selecting housekeeping genes for classification is the constant selection pressure over

these genes in the genome. Indeed, *secY* PCR data alone were able to distinguish leptospires in three distinct lines of evolution, based on their pathogenic potential, and when associated with sequence-based data, conclusions regarding strain classification were possible: *Leptospira meyeri* strain ICF clustered with the pathogenic strains and *Leptospira inadai* strain H6 was in fact an *L. interrogans* strain (Victoria *et al.*, 2008). However, as is the case for the 16S rRNA genes, this is associated with a low accumulation of polymorphisms and hence a lower resolution power in terms of strain differentiation. Genes such as *rpoB* and *gyrB*

(b)

Leptospira interrogans Hardjo-prajitno Hardjoprajitno
Leptospira interrogans Pomona Pomona
Leptospira interrogans Wolffi 3705
Leptospira interrogans Canicola* Kito
Leptospira interrogans Copenhageni M20
Leptospira interrogans Autumnalis Akiyami A
Leptospira interrogans Icterohaemorragiae RGA
Leptospira interrogans Copenhageni Fiocruz L1-130
Leptospira interrogans Kennewicki LT 1026
Leptospira interrogans Hebdomadis Hebdomadis
Leptospira interrogans Bataviae Van Tienen
Leptospira interrogans Manilae LT 398
Leptospira interrogans Muenchen Munchen C90
Leptospira interrogans Canicola Hond Utrech IV
Leptospira interrogans Australis Ballico
Leptospira interrogans Lai 56601
Leptospira interrogans Bratislava Jez Bratislava
Leptospira kirschneri Cynopteri 3522C
Leptospira kirschneri Grippotyphosa RM52
Leptospira kirschneri Erinaceiauriti Erinaceus auritus 670
Leptospira kirschneri Ramisi Musa
Leptospira kirschneri Kambale Kambale
Leptospira kirschneri Mozdok 5621
Leptospira noguchii Orleans LSU2580
Leptospira noguchii Bataviae* Cascata
Leptospira noguchii Panama CZ214 K
Leptospira santarosai Alexi HS616
Leptospira santarosai Shermani LT 821
Leptospira weilii Celledoni Celledoni
Leptospira weilii Vughia LT89-68
Leptospira weilii Hebdomadis* Ecochallenge
Leptospira borgpetersenii Ceylonica Piyasena
Leptospira borgpetersenii Poi Poi
Leptospira borgpetersenii Javanica Veldrat Batavia 46
Leptospira borgpetersenii Hardjo-bovis JB197
Leptospira borgpetersenii Hardjo-bovis L550

0.001

offer the advantage of being shorter and more polymorphic. Recently, La Scola *et al.* (2006) described three nucleotides that accounted for the differences between the *L. kirschneri* serovar Cynopteri and *L. interrogans* serovar Canicola 16S rRNA genes. In addition, Morey *et al.* (2006) reported that the difference between *L. interrogans* and *L. kirschneri* type strains was due to only two nucleotides. This is consistent with descriptions of the high degree of conservation of the 16S rRNA gene among other bacterial species (Janda & Abbott, 2007). *rpoB* was found to contain 51 polymorphisms over 600 bp when the Cynopteri and Canicola serovars were compared. In this study, the 214 bp *ligB* sequence contained 23 and 24 polymorphisms between the Cynopteri and Canicola serovars and the *L. interrogans* and *L. kirschneri* type strains, respectively.

The taxonomic analysis performed in this study demonstrated the discriminatory power of the *ligB* gene. We showed that *ligB* is a molecular marker that is able to differentiate serovars into their respective species (Fig. 2). Recently, we showed that some *ligB* genes contain mosaic sites, but they were located at the C-terminal end of the gene (McBride *et al.*, 2009). Furthermore, some of the *ligB* domains were involved in the duplication events that led to the creation of *ligA*. In this study, we specifically chose a region outside the potential mosaic region that did not include the domains involved in the gene duplication events. In conclusion, the *ligB* molecular typing scheme demonstrates several major advantages: (i) the ability to differentiate strains to the species level, (ii) discrimination between pathogenic and non-pathogenic strains and (iii) the potential to be employed in multilocus sequence typing or multi-virulence-locus sequence typing analysis for identification of clonal derivation events during the seasonal epidemics and outbreaks associated with urban leptospirosis.

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