Bioinformatics Describes Novel Loci for High Resolution Discrimination of *Leptospira* Isolates

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**Abstract**

**Background:** Leptospirosis is one of the most widespread zoonoses in the world and with over 260 pathogenic serovars there is an urgent need for a molecular system of classification. The development of multilocus sequence typing (MLST) schemes for *Leptospira* spp. is addressing this issue. The aim of this study was to identify loci with potential to enhance *Leptospira* strain discrimination by sequencing-based methods.

**Methodology and Principal Findings:** We used bioinformatics to evaluate pre-existing loci with the potential to increase the discrimination of outbreak strains. Previously deposited sequence data were evaluated by phylogenetic analyses using either single or concatenated sequences. We identified and evaluated the applicability of the *ligB*, *secY*, *rpoB* and *lipL41* loci, individually and in combination, to discriminate between 38 pathogenic *Leptospira* strains and to cluster them according to the species they belonged to. Pairwise identity among the loci ranged from 82.0–92.0%, while interspecies identity was 97.7–98.5%. Using the *ligB*-secY-rpoB-lipL41 superlocus it was possible to discriminate 34/38 strains, which belong to six *pathogenic* *Leptospira* species. In addition, the sequences were concatenated with the superloci from 16 sequence types from a previous MLST scheme employed to study the association of a leptospiral clone with an outbreak of human leptospirosis in Thailand. Their use enhanced the discriminative power of the existing scheme. The *lipL41* and *rpoB* loci raised the resolution from 81.0–100%, but the enhanced scheme still remains limited to the *L. interrogans* and *L. kirschneri* species.

**Conclusions:** As the first aim of our study, the *ligB*-secY-rpoB-lipL41 superlocus demonstrated a satisfactory level of discrimination among the strains evaluated. Second, the inclusion of the *rpoB* and *lipL41* loci to a MLST scheme provided high resolution for discrimination of strains within *L. interrogans* and *L. kirschneri* and might be useful in future epidemiological studies.

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**Introduction**

Leptospirosis is a zoonotic disease caused by pathogenic *Leptospira* spp. and is considered an emerging global public health problem [¹,²]. Furthermore, the impact of leptospirosis has increased, particularly in poverty stricken regions of the world, due to the high mortality (>50%) associated with the recent increase of severe pulmonary haemorrhage syndrome (SPHS) in patients with severe leptospirosis [³,⁴]. Based on serology, *Leptospira* spp. are traditionally classified into 29 serogroups and over 300 serovars [⁵–⁷]. More recently, genetic methods have attempted to replace the traditional classification methods and DNA-DNA hybridization studies have identified 20 *Leptospira* spp. to date [³,⁸–¹¹]. Several typing methods have been employed to classify isolates with differing degrees of success [¹²]. However, a major limitation is the lack of correlation between the serologic and genotypic classification methods [⁵,¹²,¹³].

Multilocus sequence typing (MLST) was originally developed for bacteria using *Neisseria meningitidis* isolates [¹⁴] and, so far, it has been successfully applied to over 30 bacteria [¹⁵,¹⁶]. In the field of leptospirosis, efforts to develop a typing method have focused on MLST [¹⁷,¹⁸]. MLST allows the adoption of a universal format...
for a particular bacterial species and permits the sequence data generated to be easily exchanged over the Internet. Traditionally, the loci chosen for MLST analyses are based on 6–10 housekeeping genes that are under selection for metabolic functionality [15]. Since this group is comprised of slowly evolving genes they are likely to be more conserved and stable within a particular species [19]. Unfortunately it has not been possible to identify a set of housekeeping genes with universal applicability to all bacterial pathogens. Rather, MLST loci are chosen empirically and evaluated for each individual pathogen [15]. Ahmed and colleagues presented the first MLST scheme based on loci from four housekeeping genes and two genes encoding outer-membrane proteins for typing L. alexandri, L. borgpetersenii, L. interrogans, L. kirschneri, L. noguchii and L. santarosai isolates [18]. An alternative MLST scheme using loci from seven housekeeping genes was used to type L. interrogans and L. kirschneri isolates and is available on the Internet (http://leptospira.mlst.net/). The database contains 109 sequence types (ST) and sequences from 263 isolates at time of writing [17]. Although this evidently moved the field forward, a limitation of this database is that it only applies to isolates from two Leptospira species, L. interrogans and L. kirschneri. The ideal MLST scheme should be valid for all Leptospira spp. or at least the pathogenic species [20], and provide discrimination beyond the species level [21].

High-resolution typing, such as that required during outbreak investigations, usually requires the inclusion of genes with greater diversity, e.g. antigen genes, rather than housekeeping genes [15]. The objective of this study was to carry out a bioinformatics-based analysis of Leptospira genes available in GenBank to identify potential targets for improved Leptospira discrimination. The genes ligB, secY, lipL41 and rpoB were identified as potential genes for use in an improved typing scheme.

Methods

DNA sequences

The DNA sequences for the ligB, secY, rpoB and lipL41 loci used in this study were obtained from GenBank and LepBank [22] (Table 1) or from the authors of the original Leptospira MLST scheme [18]. Most of these sequences were generated by the authors during previous studies and they belong to different reference strains and clinical isolates. The sizes of the loci analyzed were 214 bp (ligB), 245 bp (secY), 541 bp (rpoB) and 884 bp (lipL41) and correspond to nucleotide positions 2236–2449 (ligB), 771–1015 (secY), 1922–2462 (rpoB) and 73–956 (lipL41). Note that nucleotide positions are based on the L. interrogans Copenhagheni Fiocruz L1-130 genome (AE016823). These genes can be amplified by using the primers ligB (PSB1: 5’-ACWRVHVVHR-GYWDDCCTGTTCTTCC-3’; PSB2: 5’-TARRHDGCYBT-TAATAYCCGRWYTYTCTTAA-3’), [23]; secY (SeqYI: 5’-GAA-TTTCTCTTTGATCTTCG-3’; SeqYIV: 5’-GAGTTAGAG-CTGAAATCTAAG-3’), [24]; rpoB (Lept 1906F: 5’-CCCTCTGG-GTTCCCAACATGCA-3’; Lept 2500R: 5’-CGCATCCCTCTCRAAGTGTAWCCCTT-3’), [25] and lipL41 (lipL41F: 5’-TAG-GAATTGCCGAGCTACA-3’; lipL41R: 5’-GCAATCGAGAG-GAATTAACATCA-3’), [18]. The DNA sequences corresponding to the gltU (18 alleles), pncA (24 alleles), sucA (20 alleles), fadD (20 alleles), fkpA (30 alleles), ppbB (35 alleles) and mrcA (23 alleles) loci were downloaded from the Leptospira MLST database at http://leptospira.mlst.net [26].

Phylogenetic analysis

DNA sequences were aligned using ClustalW at the default settings (http://www.ebi.ac.uk/clustalw). The phylogenetic analyses were performed with Mega 4.1 [27] or Geneious Pro ver 4.7 [28], and the neighbour-joining method with no outgroup. The Tamura-Nei genetic distance model was selected and all trees were resampled using the bootstrap method and 1000 replicates. The phylogenetic trees constructed using sequences of 38 reference strains (Table 1) was based a 1884 bp superlocus composed of the concatenated sequences of the loci for each strain in the following order: ligB-secY-rpoB-lipL41.

Results

Phylogenetic analysis of the ligB, secY, rpoB and lipL41 loci

The main criteria to select genes for the presented MLST scheme were their ability to separate one or more species in different clusters and to discriminate the strains and clinical isolates within them. To identify candidate loci with these properties we searched those previously characterized by the authors and mined public databases to obtain additional representative sequences. The genes used to constitute this study (ligB, secY, rpoB and lipL41) showed an optimal discriminative power. Following alignment, the percentage of identical pairwise amino acids residues (PI) for the lipL41 locus was 92.0% and the percentage of identical sites (IS) among the DNA sequences was 77.9%, the rpoB locus was 91.8 and 75.2%, the secY locus was 87.8 and 71.4%, and the ligB locus was 82.0 and 48.6%, respectively. When all four loci for each strain were concatenated and when the resulting superloci were aligned the overall PI was 90.3% and the IS was 72.9%. Of note, interspecies identity was considerably higher, 97.7–98.5±0.2% (Figure 1). The phylogenetic tree formed two major clusters representing L. interrogans and the other species: L. interrogans, L. noguchii, L. borgpetersenii, L. santarosai and L. weilii (Figure 2).

The 15 L. interrogans strains, two L. noguchii strains, four L. weilii strains and three L. santarosai strains, listed in Table 1, could all be discriminated using the superlocus and of the seven L. kirschneri strains, only two proved to be identical at the sequence level (strains 5621 and 3522C). Among the seven L. borgpetersenii strains, the superlocus sequence was capable of discriminating all but two strains (JB197 and L550). Morphospecies, where one sampled sequence exhibits a unique base relative to the common nucleotide of the others were observed in 13 serovars and all species except L. weilii. The L. interrogans species included the largest number of polymorphic-containing serovars, six, followed by Pomona with two, then Autumnalis, Lai, Manilae, Muench and Pyrogenes with one. The L. noguchii serovars contained the largest number of unique polymorphic sites per serovar, Orleans and Panama had five each). Inclusion of the low polymorphic genes rs2 and lipL32 as in the scheme of Ahmed and colleagues [18] was assessed but showed no advantage to the presented scheme (results not shown).

Increased discrimination using the ligB, secY, rpoB and lipL41 loci in an existing MLST scheme

Based on analysis of the loci sequences from 38 Leptospira reference strains, ligB contained 15 alleles, secY 16 alleles, rpoB 19 alleles and lipL41 28 alleles, Figure 3 and Figure S1 B–D, respectively. Using the online Leptospira MLST scheme (http://leptospira.mlst.net, [17]) we identified 16 sequence types (ST), of 109 ST, where the corresponding ligB, secY, rpoB and lipL41 sequences for each strain were readily available. Using the concatenated sequences that corresponded to the gltU, pncA, sucA, fadD, fkpA, ppbB and mrcA loci a phylogenetic tree was constructed that identified 13 unique ST among the 16 different strains.
included in the analysis (Figure 4). The ligB, secY, rpoB and lipL41 loci were added to the MLST superloci from each strain to determine their impact on the level of discrimination. While inclusion of the ligB locus did not improve upon the original scheme (Figure S2 B), the secY and lipL41 loci resolved 14 ST (Figures S2 C & D, respectively) and the rpoB locus discriminated between 15 ST (Figure S2 E). All possible combinations of the candidate loci were used to create additional superloci to determine the assembly with the greatest discriminatory power (data not shown). Complete resolution of the 16 ST was achieved by inclusion of both lipL41 and rpoB loci in the original concatenated sequence of each strain, Figure S2 F.

**Discussion**

The MLST scheme proposed by Thaipadungpanit and colleagues is based on loci from seven housekeeping genes and identified 109 unique ST among 263 isolates from either *L.*

Table 1. *Leptospira* serovars and the candidate alleles for MLST.

| Species      | Serogroup | Serovar | Strain          | Accession numbers |
|--------------|-----------|---------|-----------------|-------------------|
|              |           |         |                 | ligB  | secY  | rpoB  | lipL41 |
| L. borgpetersenii | Javanica  | Ceylonica | Piyasena        | EU938500^a | EU358041^a | DQ296134 | AY461936m |
|              | Javanica  | Javanica | Veldrat Batavia 46 | EU938501^a | EU358040^a | DQ296134 | AY461938m |
|              | Javanica  | Poi      | Poi             | EU938502^a | EU358007^a | DQ296134 | n/a n |
|              | Mini      | Mini     | Sari            | n/a       | EU358032^a | DQ296134 | n/a n |
|              | Sejroe    | Hardjo-bovis | JB197          | CP000350^b | CP000350^b | CP000350 | CP000350^b |
|              | Sejroe    | Hardjo-bovis | L550           | CP000348^b | CP000348^b | CP000348 | CP000348^b |
|              | Tarassovi | Tarassovi | Perealetskin    | n/a       | EU358057^a | EU747307 | AY461937m |
|              |           |          |                 |           |           |         |       |
| L. interrogans | Australis | Australis | Ballico        | EU938484^a | DQ082850^b | DQ296144 | n/a n |
|              | Australis | Bratislava | Jez Bratislava  | EU938487^a | EU357933^a | EU747300 | AY461939m |
|              | Australis | Muencen   | Muencen C90    | EU938497^a | EU357938^a | DQ296133 | n/a n |
|              | Automulli | Autumnalis | Akiyami A      | EU938485^a | EU357943^a | DQ296145 | AY461940 |
|              | Bataviae  | Bataviae  | Van Tienen     | EU938486^a | EU357956^a | DQ296146 | AY461941m |
|              | Canicola  | Canicola  | Hond Utrecht IV | EU938488^a | EU357961^a | EU747299 | AY461942m |
|              | Icterohaemorrhagiae | Copenhageni | Fiocruz L1-130 | AE016823^e | AE016823^e | AE016823 | AE016823^e |
|              | Icterohaemorrhagiae | Icterohaemorrhagiae | RGA   | EU938493^e | EU365950^a | DQ296133 | AY461947m |
|              | Icterohaemorrhagiae | Lai      |               | S6601    | AE010300^d | AE010300 | AE01300^d |
|              | Pyrogenes | Manilae   | LT398          | EU938496^a | EU358049 | DQ296133 | n/a n |
|              | Pyrogenes | Pyrogenes | Salinem        | n/a       | DQ982863^h | DQ296147 | n/a n |
|              | Sejroe    | Hardjo-prajitno | Hardjo-prajitno | EU938491^a | EU357983 | EU747303 | AY461943m |
|              | Sejroe    | Wolffi   | 3705           | EU938499^a | EU357985 | EU747306 | AY461943m |
|              | Hebdomadis | Hebdomadis | Hebdomadis     | EU938492^a | EU357974 | EU747304 | n/a n |
|              | Pomona    | Pomona   | Pomona         | EU938498^a | EU358013 | EU747306 | AY461948m |
| L. kirschneri | Australis | Ramisi   | Musa           | EU938507^a | EU358020 | DQ296139 | AY461949m |
|              | Automulli | Erinaeciaurit | Erinaeus Auritus 670 | EU938504^a | EU358021 | DQ296139 | AY461950m |
|              | Bataviae  | Djazti   | HS 26          | EU938503^a | EU358027 | DQ296139 | AY461951m |
|              | Cynopteri | Cynopteri | 3522 C         | EU938508^a | EU358027 | DQ296139 | n/a n |
|              | Grippotyphosa | Grippotyphosa | RM52          | AY190126^p | EU358027 | EU747304 | AY461953m |
|              | Hebdomadis | Kamadebi | Kamadebi       | EU938505^a | EU358030 | DQ296139 | AY461954m |
|              | Pomona    | Mozrak   | 5621           | EU938506^a | EU358015 | DQ296139 | AY461955m |
| L. noguchii | Louisiana | Orleans  | LSU 2580       | EU938509^a | EU365958 | EU349500 | AY461957m |
|              | Panama    | Panama   | CZ 214 K       | EU938510^a | EU365958 | DQ296141 | n/a n |
|              | Pyrogenes | Alexi    | HS 616         | EU938512^a | EU358047 | DQ296131 | AY461964m |
|              | Sejroe    | Trinidad | TRVL 34056     | n/a       | EU358035 | DQ296131 | n/a n |
|              | Shermani  | Shermani | LT 821         | EU938511^a | DQ082866 | DQ296131 | AY461965m |
| L. weilii   | Celledoni | Celledoni | Celledoni      | EU938514^a | EU356960 | DQ296132 | n/a n |
|              | Hebdomadis | n/a     | EcoChallenge    | EU700274^y | AY034036 | DQ296132 | n/a n |
|              | Javanica  | Coxi     | Cox            | n/a       | EU358009 | DQ296132 | AY461967m |
|              | Tarassovi | Vugia    | LT 89–68       | EU938515^a | EU356960 | DQ296132 | AY461968m |

n/a - Not applicable. Source of sequence data: [26]^o; [32]^p; [33]^q; [34]^r; [35]^s; [30]^t; [28]u; Riediger, unpublished data; [36]v; Bomfimk, unpublished data; [37]^w; [27]^x; [18]^y.

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interrogans or *L. kirschneri* [17]. This represented a major advance in the molecular epidemiology of *Leptospira* isolates. Unfortunately, as noted by the authors themselves, a major limitation of this scheme is that it does not allow for the inclusion of the other common pathogens associated with human leptospirosis [20]. Previously, it was shown that the antigen encoding genes *ligB*, *secY*, *lipL41* and the *rpoB* gene are potentially useful for the molecular discrimination of *Leptospira* strains and that they can be readily amplified from

**Figure 1. Alignment analysis of the concatenated superlocus.** Comparison of the variability of the DNA sequences from the *ligB*-secY-*rpoB*-lipL41 super locus among the *Leptospira* species included in this study, where B – *L. borgpetersenii*, I – *L. interrogans*, K – *L. kirschneri*, N – *L. noguchii*, S – *L. santarosai* and W – *L. weilii*. Results are shown as means ± SD. The nucleotide positions used during the alignment analysis were: nt 2236–2449 (*ligB*), 771–1015 (*secY*), 1922–2462 (*rpoB*) and 73–956 (*lipL41*) and refer to the *L. interrogans* serovar Copenhageni L1-130 strain.

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**Figure 2. Phylogenetic analysis of 38 Leptospira serovars.** The tree was constructed based on the *ligB*-secY-*rpoB*-lipL41 super locus sequences. The loci were analyzed using the Neighbor-Joining method as implemented in Geneious Pro 4.7.5 [25]. The samples are represented by the serovar followed by the strain designations. Confidence in the topology of this tree was gauged by bootstrap resampling (1,000 times).

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all known pathogenic leptospires [23–25,29,30]. Furthermore, the authors of the original bacterial MLST scheme recommend the inclusion of loci from antigen coding genes to improve discrimination, especially during outbreak investigations [31].

To determine the potential benefits of using ligB, secY, rpoB and lipL41 loci in an MLST typing scheme focused on strain differentiation we identified the corresponding sequences in a reference collection containing 38 Leptospira strains (Table 1). The overall level of pairwise identity ranged from 82–92% among the individual loci while the intraspecies identity was even higher (Figure 1). Furthermore, when the sequences were concatenated to create a superlocus for each strain and analysed, the overall pairwise identity was >90%. Following the modelling of phylogenetic trees and in agreement with previous studies, two distinct evolutionary branches were observed, the first contained L. kirschneri, L. interrogans and L. noguchii strains and the second the L. borgpetersenii, L. santarosai and L. weilii strains (Figure 4 and Figure S2) [23,29]. There was some evidence that serovars from the same serogroup clustered together, serogroups: Icterohaemorrhagiae: L. interrogans Icterohaemorrhagiae RGA and Copenhageni Fiocruz L1-130; Australis: L. interrogans Australis Ballico and Muenchen Muenchen C90; and Javanica: L. borgpetersenii Javanica Veldrat Batavia 46, Poi Poi and Ceylonica Piyasena (Figure 2). This may indicate homoplasy (similarity due to convergent evolution) of the genes from these serovars. This analysis showed a separation of serovar Bratislava from the other L. interrogans serovars (Figure 2). Alignment analyses demonstrated this is probably due to the high similarity of the L. interrogans Jez Bratislava strain rpoB gene sequence with those from the L. borgpetersenii strains (data not shown). Despite this, serovar Bratislava was correctly located within the L. interrogans clade. We did not determine however, whether this was due to sequence mosaicism or horizontal gene transfer. The phylogenetic organisation of the Leptospira genus based on the superlocus supports the theory [29] that L. interrogans is more recently evolved from L. kirschneri, more recent evolutionary subdivisions resulted in the separation of L. borgpetersenii followed by L. santarosai and L. weilii clades.

Analysis of the discriminatory power of the ligB-secY-rpoB-lipL41 superlocus found that the L. interrogans, L. noguchii, L. santarosai and L. weilii strains could be separated into individual ST. However, among the L. kirschneri and L. borgpetersenii strains, two could not be resolved by the superlocus. Serological analysis of the two L. borgpetersenii strains indicated that both belonged to serogroup Sejroe serovar Hardjo-bovis and their genomes were found to be highly conserved. Yet they are distinct clonal subtypes, both strains established chronic infections in cattle yet differed in their ability to cause lethal infections in hamsters [32]. Despite this no specific polymorphisms were observed in either of the L. borgpetersenii L550 or JB197 strains. These polymorphic regions are normally useful for surveillance purposes, to monitor outbreaks or for epidemiological studies. Thirteen serovars, out of
In this study, exhibited unique polymorphic sites. These findings highlight the efficiency of the proposed ligB-secY-rpoB-lipL41 superlocus to discriminate Leptospira strains. The study previously performed by Ahmed and colleagues [18] was the pioneer in the use of a concatenated superlocus to discriminate among the Leptospira species. However, this work was intended as a step towards the study of pathogen evolution rather than strain discrimination. Inclusion of low polymorphic genes such as rrs2 and lipL32 used by Ahmed et al., [18] did not contribute to enhance the discriminative power of the MLST scheme presented here. In the present work, we included some of those sequences in combination with recently sequenced polymorphic genes to increase the resolution, and observed the occurrence of discrimination to the subspecies level. Although a reduced number of strains and isolates were included in our study we believe the proposed superlocus presents a solid basis for discriminating within large panels of Leptospira strains and isolates.

An analysis of the ligB, secY, rpoB and lipL41 loci found that the ligB locus was the most conserved with 15 alleles, followed by secY with 16 alleles, rpoB with 19 alleles and lipL41 with 28 alleles out of a potential 38 (Figure 3 and Figure S1). Following analysis of the 263 isolates (109 ST) contained in the Leptospira MLST database (leptospira.mlst.net), 16 strains (corresponding to 13 ST) were identified as having the corresponding ligB, secY, rpoB and lipL41 loci sequences available (Figure 4). To determine the utility of the loci proposed in this study the relevant sequence for each individual locus was concatenated to the glmU, pntA, sucA, fadD, tspA, tpfB and mreA superlocus of each strain (Figures S2 B–E). All possible variables were evaluated in order to identify the most useful additional loci. The combination of the original superlocus together with the tspB and the lipL41 loci was found to be the simplest superlocus that could discriminate between all 16 of the strains (Figure S2 F). This is in agreement with the ability of the superlocus determined by Ahmed and colleagues [18], which includes the lipL41 locus, to discriminate the Leptospira spp. in study.

The phylogenetic analysis of our sequences showed a great diversity of ST and no clustering, due to the use of epidemiologically unrelated strains. Thus, when the two new loci sequences were concatenated to the original ST sequences we observed the complete discrimination of the strains, although our adapted scheme remains limited to L. interrogans and L. kirschneri. We recommend that the rpoB and lipL41 loci be evaluated in existing or future MLST schemes to enhance their typing power during outbreak investigations.

Supporting Information

Figure S1 Continued from figure 3.
Figure S2 Continued from figure 4.

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Author Contributions

Conceived and designed the experiments: GMC AJAM. Performed the experiments: GMC AJAM MRE. Analyzed the data: GMC AJAM. Wrote the manuscript: GMC AJAM RAH NA OAD MRE ALTON.
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