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GENETIC ANALYSIS OF *LIPL32* AS THE TARGET GENE IN THE DEVELOPMENT OF DIAGNOSTIC ASSAYS FOR LEPTOSPIROSIS

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SUMMARY

Definitive identification of pathogenic leptospires is crucial to instigate treatment and control of leptospirosis. Polymerase chain reaction (PCR) which is considered as a sensitive and specific assay has been useful in the rapid identification of pathogenic leptospires. The *lipL32* gene encoding major outer membrane protein is an ortholog gene in all pathogenic leptospires. Construction of a precise evolutionary tree based on the complete *lipL32* gene sequence presents useful information in understanding the genetic evolution of pathogenic strains which facilitate the design of a definitive diagnostic assay. This present study showed the significance of *lipL32* gene and its application as the most suitable target in diagnostic assays for pathogenic leptospires. A comparative study was conducted via PCR using primers covering the *lipL32* gene. Amplicons of the correct size were cloned and sequenced followed by bioinformatics analysis. Comparison of *lipL32* DNA sequence revealed a high degree of sequence conservation with an average DNA sequence identity of 97.8%. Three field isolates obtained were shown to have their origin from a common ancestor. This study is believed to be the first investigation involving the complete *lipL32* gene among pathogenic species in the genus *Leptospira*.

Keywords: Genetic analysis, *Leptospira*, *lipL32* gene

INTRODUCTION

Leptospirosis is one of the most widespread zoonotic diseases and is of considerable importance to international public health largely due to its high morbidity and mortality of humans and global livestock industries (Levett, 2001). Since 1987, spirochetes have been known as one of the few major bacterial groups whose natural phylogenetic relationships are evident at gross phenotypic appearances (Yasuda *et al.*, 1987). The physical map obtained from *Leptospira* which falls under the order Spirochaetales showed unusual patterns of genetic organisation and unique mechanisms of genetic regulation (Saint Girons *et al.*, 1992). More recently, molecular classification that divides the genus *Leptospira* into several species on the basis of DNA relatedness (Ramadass *et al.*, 1992; Faine, 1994; Brenner *et al.*, 1999; Feresu *et al.*, 1999; Zuerner *et al.*, 2000) has been described. Phylogenetic analysis based on comparison of the 16S rRNA sequences has revealed divergence of saprophytic and pathogenic *Leptospira* and has provided further evidence of the genetic diversity among this species (Paster *et al.*, 1991). Classification based on serological characterisation has been complicated by the extreme diversity of the genus which currently comprises 18 genomospecies organised into 31 serogroups and over 250 serovars based on their antigenic relatedness (Pérolat *et al.*, 1998; Faine *et al.*, 1999; Bharti *et al.*, 2003; Levett et al., 2005a; Levett *et al.*, 2006; Picardeau *et al.*, 2008). Nonetheless, the genomospecies of *Leptospira* do not correspond to the previous classification into two species (*L. interrogans* and *L. biflexa*) and here in this new classification, both pathogenic and saprophytic serovars can occur within the same species (Levett, 2001). Leptospiral serovar diversity is a result of structural heterogeneity in the carbohydrate component of lipopolysaccharides (de la Pena *et al.*, 1999), with its composition generally specific for each serovar (Faine *et al.*, 1999). Hence, neither serogroup nor serovar can reliably disclose the species of *Leptospira*.

In spirochetes, the most abundant proteins are lipoproteins (Hayashi and Wu, 1990; Haake *et al.*, 2000) and *Leptospira* species are no exceptions. Various surface-exposed lipoproteins have been found to be protective immunogens as shown in animal models of bacterial diseases (Fikrig *et al.*, 1990; Danve *et al.*, 1993; Von Specht *et al.*, 1995). The amino acid sequences of leptospiral proteins, such as the major outer membrane protein (MOMP) LipL32, appear to be highly conserved among pathogenic *Leptospira* but absent in the non-pathogenic species (Guerreiro *et al.*, 2001; Haake *et al.*, 2000; Zhang *et al.*, 2005). Besides *LipL32*, other identified lipoproteins that have no orthologs in *L. biflexa* include

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LipL41, *LipL36* and several *LipL45* related proteins (Cullen *et al*., 2004). Gene encoding *LipL21*, however, which was reported as a pathogen-specific lipoprotein (Cullen *et al*., 2003) based on antibody reactivity, has an ortholog in *L. biflexa* with 50% similarity (Picardeau *et al*., 2008). This level of similarity is consistent with a different function for the *lipL21* orthologs in two *Leptospira* species. Expression of *LipL32* has been proven not only to be present during cultivation but also during mammalian infection (Haake *et al*., 2000). Previous reports showed that *LipL32* is a prominent immunogen in human leptospirosis in which anti- *LipL32* reactivity display the greatest sensitivity and specificity in both acute and convalescent-phase sera of human leptospirosis (Flannery *et al*., 2001). Therefore, sequence analysis of *lipL32* was selected to provide advanced understanding in molecular phylogeny of pathogenic species.

For epidemiological and public health surveillance in Malaysia, precise identification and evolutionary investigation of common pathogenic strains is critical since different serovars exhibit different host specificities and are responsible for different clinical forms of the disease (Faine, 1982; Pèrolat *et al*., 1990). In this study, we present insights into the evolution of pathogenic leptospires isolated in Malaysia based on the *lipL32* (MOMP) gene sequencing and investigate the reliability of primers derived from this gene in identifying heterogeneous pathogenic strains. It is believed that this is the first investigation involving the complete *lipL32* gene analysis among pathogenic species of the genus *Leptospira*.

MATERIALS AND METHODS

Leptospiral strains and DNA isolation

Leptospiral reference strains listed in Table 1 were kindly provided by WHO/FAO/OIE for Reference and Research on Leptospirosis, Queensland Health Scientific Services, Australia. Seven field isolates used in this study were obtained from the Faculty of Veterinary Medicine, Universiti Putra Malaysia. The leptospires were grown in EMJH liquid medium enriched with bovine serum albumin at 30°C. DNA was extracted from the leptospiral cultures using the Wizard genomic DNA purification kit in accordance with the manufacturer's instructions (Promega, USA).

Table 1: Leptospira reference strains and local isolates used in this study

Species	Serovar	Strain
L. interrogans	Australis	Ballico
	Autumnalis	Akiyami A
	Bataviae	Swart
	Canicola	Hond Utrecht IV
	Grippotyphosa	Moskva V
	Hebdomadis	Hebdomadis
	Pomona	Pomona
	Djasiman	Djasiman
	Icterohaemorrhagiae	RGA
L. borgpetersenii	Javanica	Veldrat Batavia 46
	Hardjobovis	Sponselee
	Ballum	Mus 127
	Mini	Sari
L. inadai	Malaya	H6
	Lyme	10
L. kirschneri	Cynopteri	3522C
L. weilii	Celledoni	Celledoni
L. meyeri	Semaranga	Veldrat Sem 173
L. fainei	Hurstbridge	BUT6
L. biflexa	Patoc	Patoc 1
(Local Isolates)	(GenBank accession no.)	
UPM-R37	EU871720	
UPM-R44	EU871721	
UPM-R48	EU871722	
UPM-ISO9	EU871717	
UPM-ND	EU871718	
UPM-KKB-15.2	EU882034	
UPM-P5	EU871719	

Nucleotide sequence determination

Gene encoding *LipL32* was amplified with a set of primers, SV1 (5'- ATG AAA AAA CTT TCG ATT TTG GC-3') and SV2 (5'- TTA CTT AGT CGC GTC AGA AGC-3'). Fragment amplification was conducted in the first cycle at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 1 minute with a final single extension of 72°C for 7 minutes.

Cloning and sequencing of lipL32 gene

The *lipL32* gene from twenty reference strains and seven local isolates were examined. Amplified PCR product was excised from agarose gel after electrophoresis and purified using Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Purified amplicons were ligated into pGEMT®-T Easy vector (Promega) and subsequently transformed into JM109 *E. coli* (Promega) competent cells via chemical transformation protocol (Sambrook *et al.*, 1989). Similarly, plasmid extractions were performed essentially as described by Sambrook *et al.*(1989). For confirmation, plasmids were digested with *Eco*RI endonuclease, electrophoresed in a 1.0 % agarose gel and stained with ethidium bromide before being visualised under a UV transilluminator.

Nucleotide sequence analysis

Nucleotide sequencing reactions were conducted at Macrogen Inc., Korea. Homological analysis was performed by BLAST against the nucleotide sequence database on the GenBank/NCBI website (*http://www.ncbi.nlm.nih.gov*). Sequences obtained were aligned with CLUSTALW v1.83 (Thompson *et al.*, 1997) with default parameters. Missing (gaps) and ambiguous characters were excluded from the analysis. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4 (Tamura *et al.*, 2007) on complete *Leptospira lipL32* gene sequences obtained from GenBank as listed in Table 2. One thousand bootstrap replications were used for the correct positioning of the strains into the branches. The assembled tree was constructed by the Neighbor-Joining method and the results are given with regard to *p*-distance.

Nucleotide sequence accession number

The DNA sequences of the *lipL32* gene from seven pathogenic leptospiral isolates and previously unpublished gene sequences have been deposited in the GenBank database under accession numbers EU871717, EU871718, EU871719, EU871720, EU871721, EU871722, and EU882034 respectively.

RESULTS

To assess the specificity of the primers, DNA samples from leptospiral reference strains were subjected to PCR amplification as controls, followed by local isolates (Table 1). All amplicons from PCR using the SV1/SV2 set of primers yielded single amplification of 819 bp indicating that the *lipL32* MOMP was present in pathogenic leptospires belonging to *L. borgpetersenii*, *L. interrogans*, *L. kirschneri*, *L. weilii*, *L. fainei* and *L. inadai* but was absent in the saprophytic species, *L. biflexa* and *L. meyeri*. However, no DNA amplification was seen in *L. inadai* strain 10.

The *lipL32* structural gene, consisting of 816 bases encoding a protein of 272 amino acids (exclude stop codon), was determined in each of the leptospiral strains listed in Table 2. Several sequences from various strains under the same species were found to be identical. Figures 1 and 2 show the dendrogram derived from the similarity matrix of both nucleotides and amino acids using the Neighbor-Joining (NJ) method.

The analysis of phylogenetic relatedness involving nucleotide sequences and amino acid sequences appear to be dissimilar between isolates examined in this study.

Table 2: Leptospiral *lipL32* gene sequences used in present study

Species	GenBank accession no.*
L. interrogans	AE010300, AF245281, AY442332, AY568679, AJ580493, AY609321, AY609323, AY609324, AY609325, AY609327, AY609328, AY609329, AY609332, EU871716, and EU871723.
L. borgpetersenii	CP000348, CP000350, AY609333, AY609330, AY609322, and AY568680.
L. kirschneri	AF121192
L. noguchii	AY609326
L. weilii	AY609331

* Only complete sequences of *lipL32* gene were analysed.

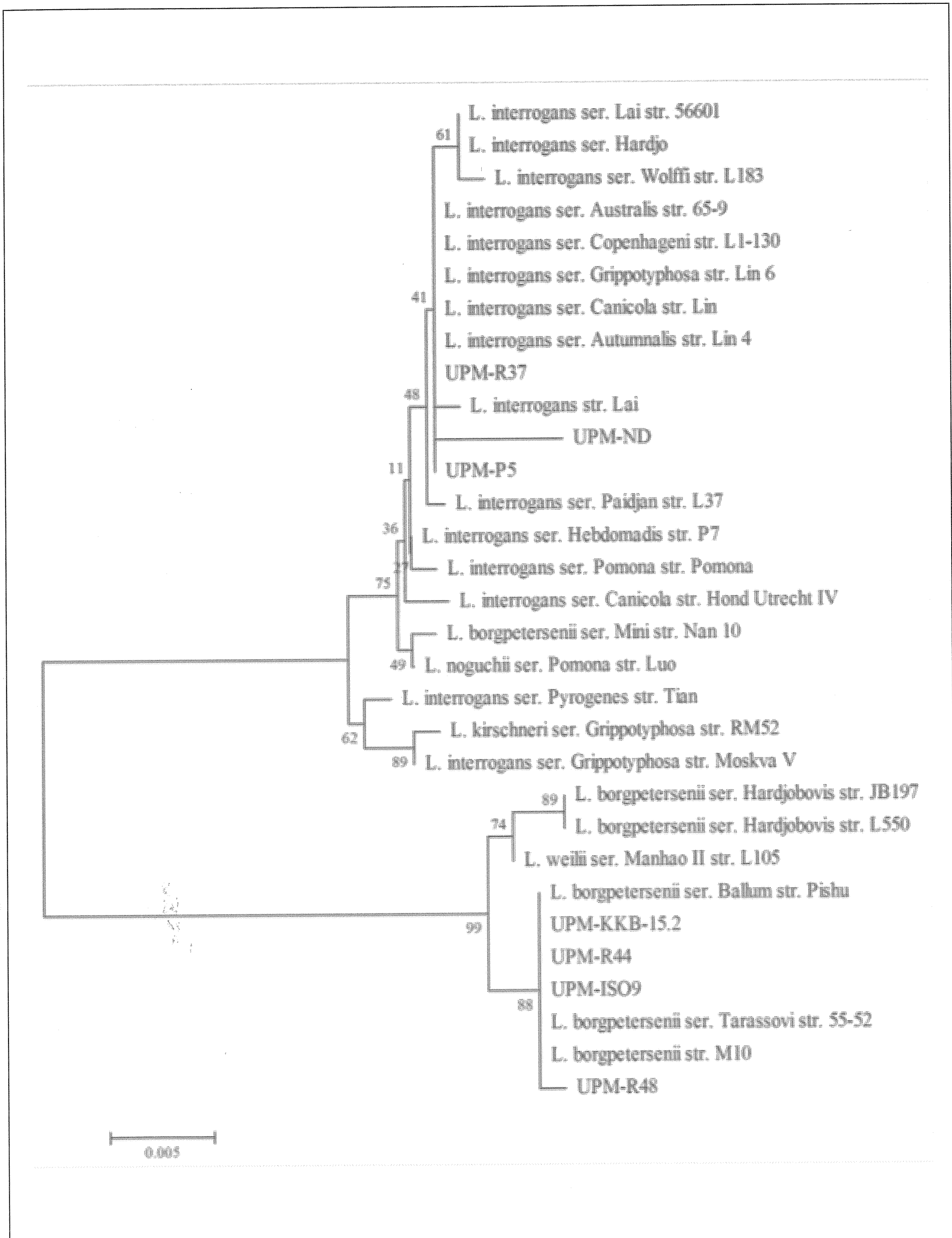

Figure 1: Dendrogram revealing the evolutionary relationships of Leptospira *lipL32* gene based on nucleotides. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

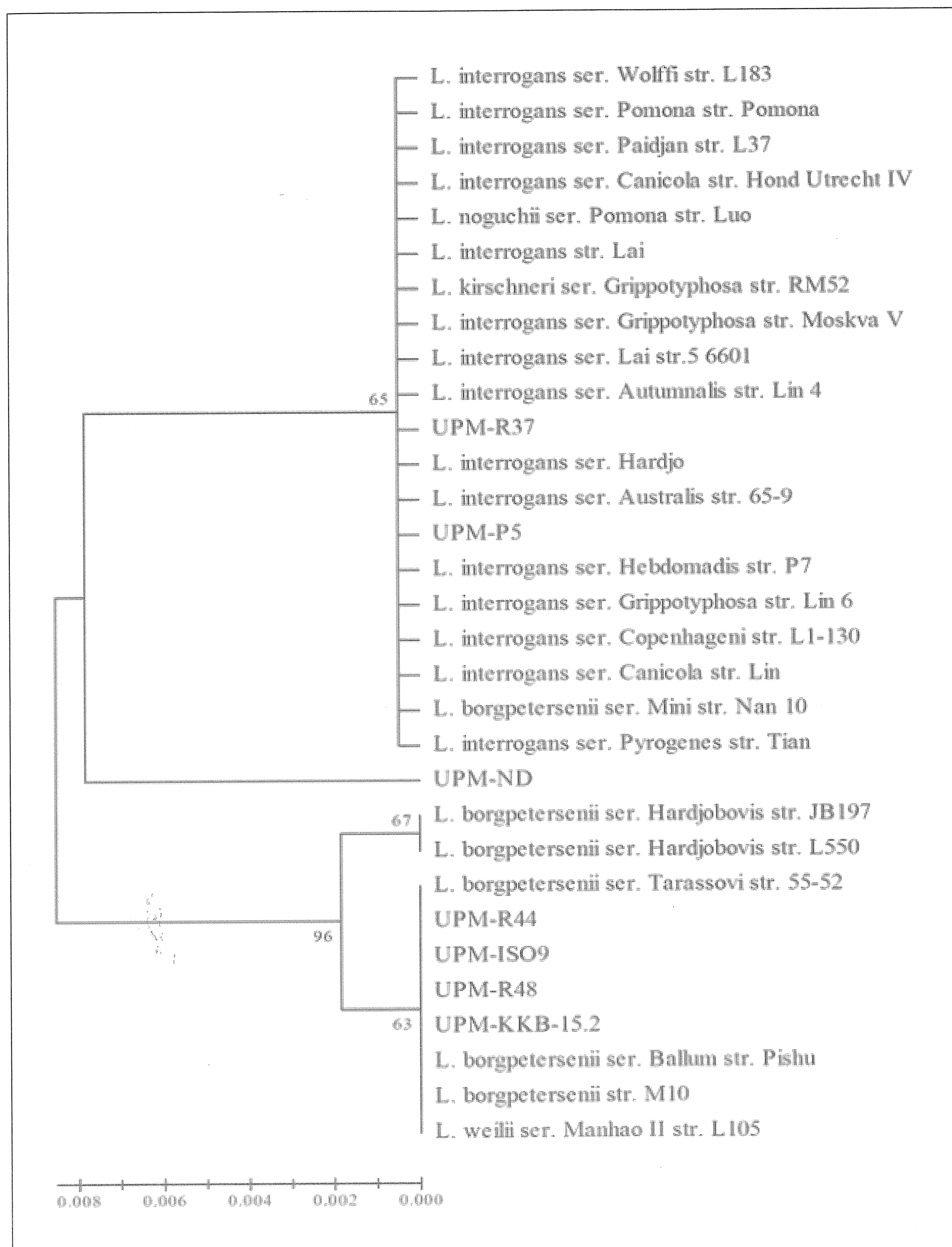

Figure 2: Dendrogram (linearise) revealing the evolutionary relationships of Leptospira *lipL32* gene based on amino acids. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The phylogenetic tree was linearised assuming equal evolutionary rates in all lineages (Takezaki *et al.*, 2004). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

The evolution of pathogenic isolates can be clearly seen in Table 3 based on sequence identity matrix and sequence difference count matrix. Sequence analyses were conducted in MEGA4 (Tamura *et al.*, 2007) and BioEdit (Hall, 1999). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Four of the seven local isolates were found to be *borgpetersenii* species. Amino acid alignment of *lipL32* gene provides further understanding of the molecular phylogeny of pathogenic isolates obtained in Malaysia (Figure 3). Significant deletions of one amino acid as well as several occasions of point mutation were observed in isolate UPM-ND.

DISCUSSION

Leptospirosis is an infectious disease caused by pathogenic species of the genus *Leptospira*. This zoonotic disease is common in tropical and subtropical regions, but has the utmost impact on public health in developing countries where it is usually under-diagnosed. The International Leptospirosis Society (ILS) has reported that there were over half a million people infected with leptospirosis annually (Hartskeerl, 2006). Clearly, a significant contribution to the diagnosis of leptospirosis is the precedence to improved patient management followed by immediate disease control. Being highly conserved in all pathogenic leptospires, *lipL32* gene is suggested to be the most suitable candidate for the development of diagnostic assay in identifying heterogeneous pathogenic strains.

The major outer membrane lipoprotein *lipL32* which is confined to pathogenic strains of all *Leptospira* species appears to be an important virulence factor in leptospirosis (Zhang *et al.*, 2005). Primers, SV1/SV2, were specially designed from the *lipL32* sequence to detect pathogenic leptospires. The current study showed that *lipL32* gene is found in all pathogenic strains except *L. inadai* strain 10. As expected, *lipL32* was not detected in the saprophytic species *L. biflexa* and *L. meyeri*. An earlier study reported that although *L. inadai* serovar Lyme strain 10 is pathogenic for laboratory animals, this organism is not considered to have a significant role in the patient's illness (George *et al.*, 1986). In a study conducted by Haake *et al.* (2000), *lipL32* expression was highly conserved among the pathogenic *Leptospira* species including *L. inadai* strain 10. Based on the immunoblot performed with *lipL32* antiserum, *L. inadai* strain 10 showed a positive result although the band appeared to be the weakest among the pathogenic species. This anomalous result may be due to diverse parameters being generated during PCR amplification. In contrast to a previous study, *lipL32* gene could also be amplified in leptospires from two intermediate species namely *L. inadai* serovar Malaya and *L. fainei* serovar Hurstbridge (Levett *et al.*, 2005b; Matthias *et al.*, 2008). On the other hand, Yasuda *et al.* (1987) have reported that *L. meyeri* strain Veldrat Semarang 173 belongs to the saprophytic species but this was found to be contradicting with an earlier experiment conducted using primers G1/G2 (Gravekamp *et al.*, 1993) (data not shown). The current study supports the findings of Yasuda *et al.* based on the absence of *lipL32* gene in the *L. myeri* strain. The results obtained are in accordance with the classification set by the International Committee on Systemic Bacteriology, Subcommittee on Taxonomy of *Leptospira*. The presence of *lipL32* gene has been considered characteristic of pathogenic leptospires (Haake *et al.*, 2000).

The *lipL32* gene, consisting of 816 bases encoding a protein of 272 amino acids, was sequenced from the seven local isolates. The segregation patterns established via *lipL32* sequences were remarkably similar to that of 16S RNA sequences (Hookey *et al.*, 1993). The similarity of the *lipL32* DNA and 16S rDNA phylogenetic trees indicate that the observed sequence differences are the result of genetic drift (Haake *et al.*, 2000). Comparison of the *lipL32* DNA sequence of thirty-one leptospiral strains, representing five of the seven pathogenic species, revealed a high degree of sequence conservation, with an average DNA sequence identity of 97.8% (range, 94.6 to 100%). Amazingly, *lipL32* gene from three isolates, UPM-R44, UPM-ISO9, and UPM-KKB-15.2, was identical to *L. borgpetersenii* strain Pishu, strain 55-52, and strain M10. Similarly, isolate UPM-R37 and isolate UPM-P5 were found to be identical to *L. interrogans* strain 65-9, strain Lin, strain Lin 4, and strain Lin 6. Results achieved support two earlier studies conducted based on *ompL1* (Reitstetter, 2006; Lee *et al.*, 2008). This explains that the isolates must have been derived from a common ancestor. The increasing number of pathogenic leptospires isolated from rats and the environment observed in this study warrant further investigation on the epidemiology of leptospirosis in Malaysia.

On the other hand, the different pattern in codon usage for bacterial genomes is important to detect and date lateral gene transfer events. The molecular phylogeny analysis of this *lipL32* gene sequence has disclosed the presence of some nucleotide substitutions. An amino acid was found to be deleted in a relatively complete *lipL32* gene sequence of UPM-ND isolated from a rat. The deletion of the three bases 'CGA' has resulted in the replacement of Asparagine with Lysine which carries different properties of amino acids. The significance of this deletion has not been determined. Although *lipL32* is the most highly expressed protein in pathogenic *Leptospira* species, codon bias was not the explanation for the greater degree of sequence conservation in this gene because codon adaptation index measurements for both *ompL1* and *lipL41* were found to be higher than *lipL32* (Haake *et al.*, 2004).

Apart from advancing the understanding of the reliability of *lipL32* as the target gene for development of diagnostic assay, capability of the primers SV1/SV2 in

Table 3: Estimation of the evolutionary relationships between sequences

Sequence	str. JB197	str. L550	str. Nan 10	str. 55-52	str. Pishu	str. M10	str. 56601	str. L183	str. L37	str. P7	str. Lin 6	str. 65-9	str. Lin 4	str. Tian	str. Lin	str. Hond Utrecht IV	str. Lai	str. -	str. L1-130	str. Pomona	str. Moskva V	str. RM52	str. Luo	str. L105	UPM-ND	UPM-P5	UPM-ISO9	UPM-R48	UPM-R44	UPM-R37	UPM-KKB-15.2
L. borg (str.JB197)	-	100	95.7	99.3	99.3	99.3	95.4	95.3	95.7	95.7	95.6	95.6	95.6	95.7	95.6	95.7	95.4	95.4	95.6	95.6	95.8	95.7	95.6	99.7	94.6	95.6	99.3	99.2	99.3	95.6	99.
L. borg (str.L550)	0	-	95.7	99.3	99.3	99.3	95.4	95.3	95.7	95.7	95.6	95.6	95.6	95.7	95.6	95.7	95.4	95.4	95.6	95.6	95.8	95.7	95.6	99.7	94.6	95.6	99.3	99.2	99.3	95.6	99.
L. borg (str.Nan 10)	35	35	-	95.8	95.8	95.8	99.5	99.3	99.5	99.7	99.6	99.6	99.6	99.5	99.6	99.5	99.5	99.5	95.6	95.6	99.3	99.2	99.8	95.9	98.6	99.6	95.8	95.7	95.8	95.6	95.
L. borg (str.55-52)	5	5	34	-	100	100	95.6	95.4	95.8	95.8	95.7	95.7	95.7	95.8	95.7	95.8	95.6	95.6	95.7	95.7	95.9	95.8	95.7	99.6	94.7	95.7	100	99.8	100	95.7	10
L. borg (str.Pishu)	5	5	34	0	-	100	95.6	95.4	95.8	95.8	95.7	95.7	95.7	95.8	95.7	95.8	95.6	95.6	95.7	95.7	95.9	95.8	95.7	99.6	94.7	95.7	100	99.8	100	95.7	10
L. borg (str.M10)	5	5	34	0	0	-	95.6	95.4	95.8	95.8	95.7	95.7	95.7	95.8	95.7	95.8	95.6	95.6	95.7	95.7	95.9	95.8	95.7	99.6	94.7	95.7	100	99.8	100	95.7	10
L. int (str.56601)	37	37	4	36	36	36	-	99.8	99.7	99.7	99.8	99.8	99.8	99.2	99.8	99.5	99.7	100	99.8	99.6	99.1	99.0	99.6	95.7	98.9	99.8	95.6	95.4	95.6	99.8	95.
L. int (str.L183)	38	38	5	37	37	37	1	-	99.6	99.6	99.7	99.7	99.7	99.1	99.7	99.3	99.6	99.8	99.7	99.5	99.0	98.9	99.5	95.6	98.7	99.7	95.4	95.3	95.4	99.7	95.
L. int (str.L37)	35	35	4	34	34	34	2	3	-	99.7	99.8	99.8	99.8	99.2	99.8	99.7	99.7	99.7	99.8	99.6	99.1	99.0	99.6	95.9	98.9	99.8	95.8	95.7	95.8	99.8	95.
L. int (str.P7)	35	35	2	34	34	34	2	3	2	-	99.8	99.8	99.8	99.5	99.8	99.7	99.7	99.7	99.8	99.8	99.3	99.2	99.8	95.9	98.9	99.8	95.8	95.7	95.8	99.8	95.
L. int (str.Lin 6)	36	36	3	35	35	35	1	2	1	1	-	100	100	99.3	100	99.6	99.8	99.8	100	99.7	99.2	99.1	99.7	95.8	99.0	100	95.7	95.6	95.7	100	95.
L. int (str.65-9)	36	36	3	35	35	35	1	2	1	1	0	-	100	99.3	100	99.6	99.8	99.8	100	99.7	99.2	99.1	99.7	95.8	99.0	100	95.7	95.6	95.7	100	95.
L. int (str.Lin 4)	36	36	3	35	35	35	1	2	1	1	0	0	-	99.3	100	99.6	99.8	99.8	100	99.7	99.2	99.1	99.7	95.8	99.0	100	95.7	95.6	95.7	100	95.
L. int (str.Tian)	35	35	4	34	34	34	6	7	6	4	5	5	5	-	99.3	99.2	99.2	99.2	99.3	99.3	99.6	99.5	99.3	95.9	98.4	99.3	95.8	95.7	95.8	99.3	95.
L. int (str.Lin)	36	36	3	35	35	35	1	2	1	1	0	0	0	5	-	99.6	99.8	99.8	100	99.7	99.2	99.1	99.7	95.8	99.0	100	95.7	95.6	95.7	100	95.
L. int (str.Hond Utrecht IV)	35	35	4	34	34	34	4	5	2	2	3	3	3	6	3	-	99.5	99.5	99.6	99.6	99.1	99.0	99.6	95.9	98.6	99.6	95.8	95.7	95.8	99.6	95.
L. int (str.Lai)	37	37	4	36	36	36	2	3	2	2	1	1	1	6	1	4	-	99.7	99.8	99.6	99.1	99.0	99.6	95.7	98.9	99.8	95.6	95.4	95.6	99.8	95.
L. int (str. -)	37	37	4	36	36	36	0	1	2	2	1	1	1	6	1	4	2	-	99.8	99.6	99.1	99.0	99.6	95.7	98.9	99.8	95.6	95.4	95.6	99.8	95.
L. int (str.L1-130)	36	36	3	35	35	35	1	2	1	1	0	0	0	5	0	3	1	1	-	99.7	99.2	99.1	99.7	95.8	99.0	100	95.7	95.6	95.7	100	95.
L. int (str.Pomona)	36	36	3	35	35	35	3	4	3	1	2	2	2	5	2	3	3	3	2	-	99.2	99.1	99.7	95.8	98.7	99.7	95.7	95.6	95.7	99.7	95.
L. int (str.Moskva V)	34	34	5	33	33	33	7	8	7	5	6	6	6	3	6	7	7	7	6	6	-	99.8	99.2	96.0	98.2	99.2	95.9	95.8	95.9	99.2	95.
L. kirschneri (str.RM52)	35	35	6	34	34	34	8	9	8	6	7	7	7	4	7	8	8	8	7	7	1	-	99.1	95.9	98.1	99.1	95.8	95.7	95.8	99.1	95.
L. noguchii (str.Luo)	36	36	1	35	35	35	3	4	3	1	2	2	2	5	2	3	3	3	2	2	6	7	-	95.8	98.7	99.7	95.7	95.6	95.7	99.7	95.
L. weilii (str.L105)	2	2	33	3	3	3	35	36	33	33	34	34	34	33	34	33	35	35	34	34	32	33	34	-	94.8	95.8	99.6	99.5	99.6	95.8	99.
UPM-ND	44	44	11	43	43	43	9	10	9	9	8	8	8	13	8	11	9	9	8	10	14	15	10	42	-	99.0	94.7	94.6	94.7	99.0	94.
UPM-P5	36	36	3	35	35	35	1	2	1	1	0	0	0	5	0	3	1	1	0	2	6	7	2	34	8	-	95.7	95.6	95.7	100	95.
UPM-ISO9	5	5	34	0	0	0	36	37	34	34	35	35	35	34	35	34	36	36	35	35	33	34	35	3	43	35	-	99.8	100	95.7	10
UPM-R48	6	6	35	1	1	1	37	38	35	35	36	36	36	35	36	35	37	37	36	36	34	35	36	4	44	36	1	-	99.8	95.6	99.
UPM-R44	5	5	34	0	0	0	36	37	34	34	35	35	35	34	35	34	36	36	35	35	33	34	35	3	43	35	0	1	-	95.7	10
UPM-R37	36	36	3	35	35	35	1	2	1	1	0	0	0	5	0	3	1	1	0	2	6	7	2	34	8	0	35	36	35	-	95.
UPM-KKB-15.2	5	5	34	0	0	0	36	37	34	34	35	35	35	34	35	34	36	36	35	35	33	34	35	3	43	35	0	1	0	35	-

(Upper right) Percentage sequence identity matrix (Lower left) Sequence difference count matrix. The number of base differences per site from analysis between sequences is shown. Analyses were conducted in MEGA 4 and BioEdit. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). (*L. borg*: *L. borgpetersenii*; *L. int*: *L. interrogans*; str.: strain).

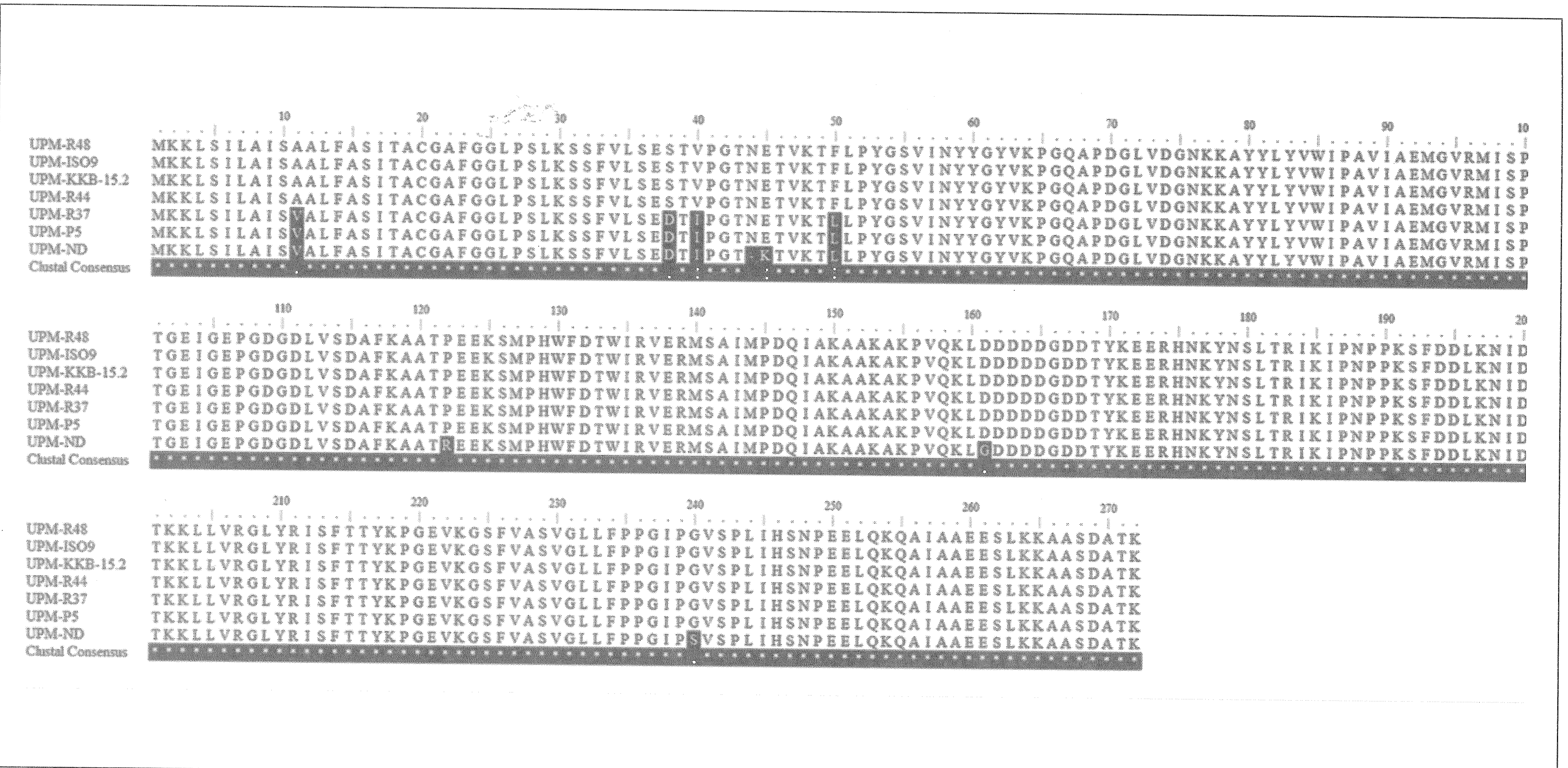

Figure 3: Alignment of *lipL32* amino acids variable regions. Locations of variable amino acids are indicated by dark boxes. In clustal consensus, columns marked with asterisk "*" represent positions which have a single, fully conserved amino acid. Positions where one of the strong amino acid groups is fully conserved are marked with a colon ":" and columns marked with a dot "." indicate that one of the 'weaker' amino acid groups is fully conserved. Locations without mark signify no similarity among amino acids in the same column. These are all the positively scoring groups that occur in the Gonnet Pam250 matrix. The strong and weak groups are defined as strong score >0.5 and weak score <0.5 respectively (Thompson *et al.*, 1997).

providing accurate identification of pathogenic *Leptospira* species consistently will definitely facilitate the diagnosis of leptospirosis in a timely manner. Since Malaysia is situated in the high risk region, a precise diagnostic assay may greatly assist improvement of patient management followed by immediate disease control.

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