Genetic structure of the genus *Leptospira* by multilocus enzyme electrophoresis

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Thirty strains from the 11 species of the genus *Leptospira* were studied by multilocus enzyme electrophoresis at 12 enzyme loci, all of which were polymorphic. The mean number of alleles per locus was 6.5. Twenty-five electrophoretic types were distinguished. Grouping of the strains by cluster analysis was in general agreement with species delineation as determined by DNA–DNA hybridization, except for the strains of *Leptospira meyeri* and *Leptospira inadai*, which were scattered throughout the genus, reflecting previously recognized taxonomic uncertainties. Analysis of the clonality within *Leptospira interrogans sensu stricto* indicated that this population was relatively heterogeneous and a lack of gene linkage disequilibrium could not be excluded. There was a genetic discrimination between the pathogenic species and the saprophytic ones. The phenotypically intermediate species (*L. inadai* and *Leptospira fainei*) were also genetically separated and were probably closer to the saprophytes than to the pathogens.

Keywords: *Leptospira*, multilocus enzyme electrophoresis, genetics, phylogeny, taxonomy

INTRODUCTION

Bacteria of the genus *Leptospira* are the causative agent of leptospirosis, a worldwide zoonosis (Johnson & Faine, 1984). Virulence for susceptible animals is a differential character which led to the primary subdivision of *Leptospira* into two nomenclatures: *Leptospira interrogans*, including the pathogens, and *Leptospira biflexa*, comprising the non-pathogens. For epidemiological purposes, pathogenic isolates were further classified on serological grounds into 24 serogroups and more than 200 serovars (Dikken & Kmety, 1978).

During the past decade, molecular taxonomy, especially DNA–DNA hybridizations, has greatly im-

proved knowledge of the genus. These studies have shown that the two nomenclatures, *L. interrogans* and *L. biflexa*, are in fact a mixture of several genomic species: seven pathogenic species (Ramadass *et al.*, 1992; Yasuda *et al.*, 1987), *Leptospira borgpetersenii*, *Leptospira inadai*, *L. interrogans sensu stricto*, *Leptospira kirschneri*, *Leptospira noguchii*, *Leptospira santarosai* and *Leptospira weilii*, and three saprophytic species, *L. biflexa sensu stricto*, *Leptospira meyeri* and *Leptospira wolbachii*. Recently, an eighth pathogenic species, *Leptospira fainei*, was described (Perolat *et al.*, 1998).

Other molecular approaches have been applied to taxonomic and epidemiological investigations of *Leptospira* genetics. These include restriction endonuclease analysis (Ellis *et al.*, 1991), pulsed-field gel electrophoresis (PFGE) (Herrmann *et al.*, 1991, 1992), ribotyping (Perolat *et al.*, 1993), mapped restriction site polymorphism (MRSP) and arbitrarily primed PCR (AP-PCR) (Perolat *et al.*, 1994; Ralph *et al.*, 1993). Such methods have allowed characterization of the leptospires at the species and subspecies level.

However, the taxonomy of *Leptospira* needs to be studied further as some questions remain. (i) The

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Abbreviations: ACO, aconitase; ADK, adenylate kinase; ALP, alkaline phosphatase; AP-PCR, arbitrarily primed PCR; CAT, catalase; ET(s), electrophoretic type(s); FUM, fumarase; GD2, glutamate dehydrogenase 2; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; MEE, multilocus enzyme electrophoresis; MPI, mannose-phosphate isomerase; MRSP, mapped restriction site polymorphism; PEP, peptidase; PFGE, pulsed-field gel electrophoresis; PGI, phosphoglucose isomerase.

Table 1. List of the *Leptospira* strains used in this study

Genospecies	ET	Serovar	Strain	Serogroup	Origin
<i>L. biflexa</i>	22	patoc	Patoc 1 ^T	Sema	Italy (water)
	23	andamana	CH11	Anda	Andaman islands (human)
<i>L. borgpetersenii</i>	6	javanica	Veldrat Batavia 46 ^T	Java	Indonesia (rat)
	8	ballum	Mus 127	Ball	Denmark (mouse)
	7	hardjo	Hardjobovis Sponselee	Sejr	Netherlands (cattle)
	9	sejroe	M 84	Sejr	Denmark (mouse)
<i>L. fainei</i>	24	hurstbridge	But6 ^T	Hurs	Australia (pig)
<i>L. inadai</i>	17	lyme	10 ^T	Lyme	USA (human)
	18	malaya	H6*	Cani	Malaysia (human)
<i>L. interrogans</i>	1	icterohaemorrhagiae	RGA ^T	Icte	France (human)
	2	autumnalis	Akiyami A	Autu	Japan (human)
	3	bataviae	Van Tienen	Bata	Indonesia (human)
	1	bratislava	Jez-Bratislava	Aust	Czechoslovakia (hedgehog)
	2	canicola	Hond Utrecht IV	Cani	Netherlands (dog)
	21	evansi	267-1348	Rana	Malaysia (water)
	1	hardjo	Hardjoprajitno	Sejr	Indonesia (human)
	4	hebdomadis	Hebdomadis	Hebd	Japan (human)
	1	lai	Lai	Icte	China (human)
	5	pomona	Pomona	Pomo	Australia (human)
<i>L. kirschneri</i>	3	pyrogenes	Salinem	Pyro	Indonesia (human)
	10	cynopteri	3522 C ^T	Cyno	Indonesia (human)
	11	grippotyphosa	Moskva V	Grip	Russia (human)
<i>L. meyeri</i>	19	ranarum	ICF ^{T*}	Rana	USA (frog)
	20	sofia	Sofia 874*	Java	Bulgaria (human)
<i>L. noguchii</i>	12	panama	CZ 214 ^T	Pana	Panama (opossum)
	13	cristobali	1996 K	Pana	Panama (opossum)
<i>L. santarosai</i>	14	shermani	1342 K ^T	Sher	Panama (rat)
	15	navet	TRVL 109873	Tara	Trinidad (human)
<i>L. weilii</i>	16	celledoni	Celledoni ^T	Cell	Australia (human)
<i>L. wolbachii</i>	25	codice	CDC ^T	Codi	USA (water)

* Identification of these strains is discussed in the text.

status of some strains, an even species, is uncertain, as in the case of *L. meyeri* reference strain (serovar ranarum strain ICF), for which controversial taxonomic results are provided in the literature: although it was reported to represent a new species by Yasuda *et al.* (1987), this strain was previously reported in numerous studies to be related to the pathogenic group (Brendle *et al.*, 1974; Gravekamp *et al.*, 1993; Hookey, 1992; Hookey *et al.*, 1993; Letocart *et al.*, 1997; Murgia *et al.*, 1997; Segers *et al.*, 1992). (ii) Though great diversity is evident within the genus, the degree of proximity of the strains within the species has been poorly investigated. (iii) Lastly, little information is available on the genetic distinction between the saprophytic and pathogenic species.

In order to investigate these questions, the genetic structure of the genus *Leptospira* was studied by multilocus enzyme electrophoresis (MEE), which has not previously been performed on these bacteria. This method allows the analysis of many loci, representing several different metabolic activities, at the same time

(Selander *et al.*, 1986). MEE has a large spectrum of applicability and it can distinguish nearly clonal strains (Beutin *et al.*, 1990; Boerlin, 1997; Patton *et al.*, 1991; Yakus *et al.*, 1992) as well as different species of a genus (Altwegg *et al.*, 1991; Boerlin *et al.*, 1991, 1992). MEE and DNA-DNA hybridization are strongly correlated (Altwegg *et al.*, 1991; Boerlin, 1997; Boerlin *et al.*, 1991, 1992), so this study could attempt to determine whether uncertainties at the species level could be due to the method used or to discrepancies between reference collections.

METHODS

Bacterial strains. Thirty reference strains of leptospires were analysed (Table 1), including two *L. biflexa*, four *L. borgpetersenii*, one *L. fainei*, two *L. inadai*, 11 *L. interrogans*, two *L. kirschneri*, two *L. meyeri*, two *L. noguchii*, two *L. santarosai*, one *L. weilii* and one *L. wolbachii*. These bacteria were supplied initially by the WHO Collaborating Centre for Leptospirosis, Institute Pasteur, Paris, France, and subsequently maintained at the laboratory. Species assign-

ments are according to Brenner & Kaufmann (1994), which is the last genomic classification available on the basis of DNA–DNA hybridization.

Culture conditions. Each strain was grown to stationary phase in 1.5 l EMJH medium (Ellinghausen & McCullough, 1965) in the dark at 30 °C with shaking.

Preparation of lysates and electrophoresis. Lysate preparation, electrophoresis in starch gels (Sigma) and selective staining were performed as described by Selander *et al.* (1986), except for catalase, which was stained by the method of Harris & Hopkinson (1976). After testing more than 50 specific enzyme staining procedures and five different electrophoresis buffer systems, the following 12 enzymes and four buffer systems were chosen: buffer A, aconitase (ACO), adenylate kinase (ADK), fumarase (FUM), isocitrate dehydrogenase (IDH) and phosphoglucose isomerase (PGI); buffer B, malate dehydrogenase (MDH) and mannose-phosphate isomerase (MPI); buffer F, catalase (CAT) and peptidases 4 and 5 (PEP4 and 5, the respective substrates of which are Leu–Leu–Leu and Phe–Pro); and buffer G, alkaline phosphatase (ALP) and glutamate dehydrogenase 2 (GD2).

Statistical analysis of the data. Alleles were numbered in order of decreasing mobility and allele '0' represented a lack of detectable activity for the corresponding enzyme. Migration coefficients were unweighted. Under pure clonality, strong linkage disequilibrium should develop among loci. The GENEPOP software (Raymond & Rousset, 1995) was used to test whether loci in *L. interrogans* were in linkage equilibrium. Genetic distances among electrophoretic types (ETs) were calculated as shared allele distances, which is considered a strong method for tree topology restitution (Takezaki & Nei, 1996), and two trees were constructed using the neighbour-joining method (Saitou & Nei, 1987) implemented in NEIGHBOR, part of the PHYLIP suite of programs (J. Felsenstein and University of Washington, 1991–1993), and the unweighted pair group method with averages (UPGMA).

RESULTS

Genetic diversity at enzyme loci

Table 2 summarizes the genetic diversities at the 12 loci, which were all polymorphic. The number of alleles at each locus varied from 3 (ACO, CAT and MDH) to 12 (PEP4), with a mean value of 6.5. The genetic diversity between alleles at a given locus was calculated according to the formula previously described by Selander *et al.* (1990). It varied from 0.35 (MDH) to 0.90 (PEP4) with a mean value of 0.63 (Table 2). *L. kirschneri*, *L. inadai* and *L. meyeri* had no monomorphic locus with a specific allele (Table 3). *L. noguchii* had specific monomorphic alleles for ALP and MPI, *L. biflexa* for GD2, PEP5 and PGI, *L. weilii* for ADK, ALP, MPI and PEP4, and *L. wolbachii* for FUM, MPI, PEP4 and PEP5 (these last two species were represented by one strain only). *L. interrogans* was monomorphic at the ALP locus and only shared this allele with *L. inadai* malaya H6 and *L. meyeri* ranarum ICF among the other species. It should be noted that these last strains did not cluster with their species on the dendrogram, but were instead included

Table 2. Genetic diversity at the 12 loci studied

Locus*	No. of different alleles	Genetic diversity
ACO	3	0.52
ADK	7	0.62
ALP	8	0.75
CAT	3	0.39
FUM	5	0.45
GD2	8	0.78
IDH	4	0.53
MDH	3	0.35
MPI	10	0.79
PEP4	12	0.90
PEP5	7	0.81
PGI	8	0.72
Mean	6.5	0.63

* See Methods for locus abbreviations.

in *L. interrogans* (Figs 1 and 2). The same situation was observed for *L. borgpetersenii* with *L. meyeri* sofia Sofia 874 at the IDH and PEP5 loci.

No specific allele was identified that allowed the pathogens and saprophytes to be distinguished. However, a lack of detectable activity for ALP was evident in *L. biflexa* and *L. wolbachii* (but not in *L. meyeri*). It was also noticeable that CAT activity was not detected in most of the saprophytic strains, whereas it was present in nearly all pathogenic strains analysed. This observation confirms a previous report by Corin *et al.* (1978).

Genetic relationships between ETs

The 30 strains included in this study were distributed among 25 ETs (Table 3), each representing a particular combination of alleles for the 12 loci considered. ETs comprising several isolates all belonged to the same species, *L. interrogans*. The 11 strains analysed in that species were shared among six ETs, ET1 comprising four strains (icterohaemorrhagiae RGA, bratislava Jez-Bratislava, lai Lai and hardjo Hardjoprajitno) and ETs 2 and 3 each comprising two strains (autumnalis Akiyami A and canicola Hond Utrecht IV, and bataviae Van Tienen and pyrogenes Salinem, respectively) (Figs 1 and 2, Table 1). Estimation of the genetic relationships between ETs is summarized on the dendrogram in Fig. 1. Each species was clustered individually, except *L. inadai* and *L. meyeri*. Maximum genetic distances at the intraspecific level varied from 0.18 (*L. santarosai* and *L. kirschneri*) to 0.42 (*L. borgpetersenii*), which is largely consistent with the distance of 0.60 to 0.70 commonly accepted as delimiting different species (Boerlin *et al.*, 1991). No cluster of pathogenic species was obvious from the data obtained by MEE in the present study, although

Table 3. Allele profiles of 25 ETs of *Leptospira* species

ET	No. of alleles at the enzyme loci											
	ACO	ADK	ALP	CAT	FUM	GD2	IDH	MDH	MPI	PEP4	PEP5	PGI
1	2	7	8	2	5	5	2	2	4	4	3	5
2	2	7	8	2	5	5	2	2	4	4	4	5
3	2	7	8	2	5	5	2	2	4	5	3	5
4	2	7	8	2	5	4	2	1	4	5	4	5
5	2	7	8	0	5	4	2	2	8	4	4	5
6	2	5	1	1	5	3	4	2	11	11	7	0
7	2	5	2	1	5	2	4	3	5	8	7	7
8	2	6	1	1	5	3	4	2	11	0	7	7
9	2	5	1	1	5	3	4	2	11	11	7	7
10	1	6	6	2	5	2	2	2	3	1	2	8
11	1	5	6	0	5	1	2	2	3	1	2	8
12	2	7	10	2	5	5	2	2	1	5	3	1
13	3	7	10	2	5	5	2	2	1	7	2	2
14	3	7	5	3	2	5	2	2	3	11	4	3
15	3	7	5	0	2	5	2	2	3	12	5	3
16	2	1	3	2	5	5	2	2	6	10	5	5
17	3	5	9	0	2	7	3	3	2	1	3	6
18	2	7	8	2	5	4	2	2	4	6	3	5
19	2	7	8	2	3	4	2	2	4	13	4	5
20	2	6	1	2	5	3	4	2	11	11	7	7
21	2	7	8	2	5	4	2	2	4	13	4	5
22	1	2	0	0	3	8	1	1	5	8	0	4
23	1	4	0	0	2	8	1	1	7	8	0	4
24	3	7	6	0	6	6	3	3	4	2	1	2
25	1	3	0	0	1	7	3	2	10	3	8	5

Fig. 1. Dendrogram representing the genetic relationships within the genus *Leptospira*. The tree was generated by the average-linking method of clustering from a matrix of pairwise coefficients of genetic distances between ETs. See Table 1 for correspondence between ETs and strains. Taxonomic uncertainties for ETs 18, 19 and 20 are discussed in the text. P, Pathogen; I, intermediate; S, saprophyte.

Fig. 2. Dendrogram representing the genetic relationships within the genus *Leptospira*. The tree was generated by the neighbour-joining method. ET numbers are given at the ends of the branches (see Table 1 for correspondence with strains). Taxonomic uncertainties for ETs 18, 19 and 20 are discussed in the text. P, Pathogen; I, intermediate; S, saprophyte.

the pathogenic species appeared genetically separated from intermediate (*L. inadai* and *L. fainei*) and saprophytic species.

Gene linkage disequilibrium

None of the pairs of loci examined showed a statistically significant departure from random recombination, as expected for a non-clonal population structure (data not shown).

DISCUSSION

The polymorphism of the genus *Leptospira* has been demonstrated by several studies (Ralph *et al.*, 1993; Ramadass *et al.*, 1992; Yasuda *et al.*, 1987) and is confirmed by the present MEE analysis. All loci considered in the present study were polymorphic with a mean number of alleles of 6.5, which is comparable to that found for organisms like *Aeromonas* (Altwegg *et al.*, 1991) or *Campylobacter* (Aeschbacher & Piffaretti, 1989), with respective mean values of 6.5 and 6.9. Others, like *Mycobacterium* (Wasem *et al.*, 1991) and *Borrelia* (Boerlin *et al.*, 1992) seem to have less allelic diversity, with respective calculated mean values of 2.8 and 5.9. In contrast, the genus *Listeria* appears to be

more polymorphic, with a mean number of alleles per locus of 9.5 (Boerlin *et al.*, 1991).

The mean genetic diversity within the genus *Leptospira* is 0.63, which is equal to that found for *Campylobacter* (Aeschbacher & Piffaretti, 1989) and comparable to the values of 0.64 for *Treponema* (Lymbery *et al.*, 1990) and *Aeromonas* (Altwegg *et al.*, 1991) or 0.67 for *Borrelia* (Boerlin *et al.*, 1992).

The *L. interrogans* cluster is relatively heterogeneous, the 11 strains studied being dispersed among six different ETs. On the other hand, some strains could not be separated under the conditions used in this investigation. This reflects the ambivalent nature of this species, which exhibits at the same time some heterogeneity and some homogeneity, as already suspected from studies of the organization of the *Leptospira* genome (Herrmann *et al.*, 1991). The fact that this species was the only one in which ETs with multiple strains were observed is very interesting, as it suggests that the species of this genus could have different population dynamics. This possibility should be investigated by the analysis of more strains in the species concerned. Determination of linkage disequilibrium within the *L. interrogans* population suggests a possible lack of clonality within the species (Brown *et al.*, 1980), as no pair of loci showed significant disequilibrium. If this is confirmed by the analysis of a larger number of strains and loci, it should be interpreted as the consequence of a non-clonal population structure and would involve sufficiently frequent chromosomal rearrangements that no important linkage disequilibrium appears in these bacteria (Krawiec & Riley, 1990; Wasem *et al.*, 1991). This hypothesis could be supported by the presence of intervening sequences in the genomes of these bacteria, which would testify for their potential plasticity (Boursaux-Eudes *et al.*, 1995; Zuerner, 1993), and will stimulate research into possible transfers of DNA between *Leptospira*, a domain which is still little explored (Saint Girons *et al.*, 1990). If recombinational events are relatively frequent in the genomes of these bacteria, ETs comprising multiple strains could be the consequence of a relatively recent divergence of the strains concerned (Maynard Smith *et al.*, 1993), which could be distinguished by PFGE (Herrmann *et al.*, 1991, 1992) and AP-PCR. Nevertheless, care must be taken in the interpretation of these results, since linkage disequilibrium was calculated on a limited sample and more strains should be studied to confirm definitively the population structure of the species *L. interrogans*.

Two strains unrelated to *L. interrogans* were strongly integrated in its cluster. One of these was ranarum ICF, the type strain of the species *L. meyeri*, for which such a situation has already been observed. This strain was designated as representing a new species by Yasuda *et al.* (1987), while it had been previously recognized as an *L. interrogans* strain by Brendle *et al.* (1974). Similarly, *rrs* gene sequencing (Hookey, 1992; Hookey *et al.*, 1993), as well as PCR studies (Murgia *et*

al., 1997), revealed that strain ICF clustered with the pathogenic species. Its inclusion in the *L. interrogans* cluster by MEE was expected, since analysis of this strain in this laboratory, by molecular techniques including ribotyping (Perolat *et al.*, 1993), AP-PCR (Ralph *et al.*, 1993), MRSP (Ralph *et al.*, 1993) and species-specific probing (Letocart *et al.*, 1997), was always consistent with its assignment to *L. interrogans*. Indeed, for strain ICF, with the exception of Yasuda *et al.* (1987), who defined it as the type strain of *L. meyeri*, all other molecular studies (Gravekamp *et al.*, 1993; Hookey, 1992; Hookey *et al.*, 1993; Letocart *et al.*, 1997; Murgia *et al.*, 1997; Segers *et al.*, 1992) have assigned it to the pathogenic group. In the case of malaya H6, the results of MEE were once more in agreement with previous typing in this laboratory: this strain was recognized by a specific probe directed against *L. interrogans* (Letocart *et al.*, 1997) and its ribotype was identical to those of other strains known to belong to the *L. interrogans* species according to DNA-DNA hybridization (Brenner & Kaufmann, 1994; Perolat *et al.*, 1993). Lastly, these two strains share the same allele at the ALP locus with the *L. interrogans* strains, an allele not encountered in any other species in the present study. Heterogeneity among culture collections of reference strains is a more credible explanation for these discrepancies.

Information given by the trees in Figs 1 and 2 on the phylogeny of the genus generally confirms the coherence of the species delineation as established by DNA-DNA hybridization. This was not true, however, for the two species *L. meyeri* and *L. inadai*, strains of which were scattered on the trees. The case of the reference strain of *L. meyeri* (ranarum strain ICF) has been discussed above; the second strain, sofia Sofia 874, was distant from the former. Instead of representing the species *L. meyeri* in a separate cluster, it definitely belongs to the *L. borgpetersenii* cluster. In addition, strain Sofia 874 had identical alleles to the *L. borgpetersenii* strains at the IDH and PEP5 loci, which were monomorphic and specific to this species. When hybridized to species-specific probes (Letocart *et al.*, 1997), sofia Sofia 874 was recognized by the probe directed against *L. borgpetersenii* strains and not by another probe directed against the two species *L. interrogans* and *L. meyeri* (Letocart *et al.*, 1997). These results are also in agreement with ribotyping (Perolat *et al.*, 1993), where sofia Sofia 874 had the same ribotype as nona Nona, identified as an *L. borgpetersenii* strain by DNA-DNA hybridization (Brenner & Kaufmann, 1994). Similarly, PCR studies were in agreement with classification of strain sofia Sofia 874 among the pathogenic leptospires (Murgia *et al.*, 1997).

L. inadai and *L. fainei* are characterized by an intermediate response to the phenotypic characters used to distinguish pathogens and saprophytes (growth at 30 °C with 8-azaguanine and growth at 13 °C), which suggests a separate genetic status in view of these results. The cluster analysis also seems to be in

agreement with the close relationship previously suggested between *L. inadai* and *L. fainei* (Perolat *et al.*, 1998).

Concerning the comparison between pathogenic and saprophytic species, the species of the two groups are distinguished genetically on the two trees and *L. inadai* and *L. fainei*, which are phenotypically intermediate, seem to be separated genetically from both pathogens and non-pathogens and closer to the saprophytes (Figs 1 and 2). The topologies of the trees seem to be in agreement with the hypothesis that the saprophytic leptospires are the most ancestral, from which the intermediate and pathogenic strains could have been derived by the acquisition of pathogenic characteristics.

In conclusion, the main contribution of this study is the demonstration of a close correlation between the results of MEE and those obtained by other molecular methods, in particular DNA-DNA hybridization. The data presented here provide a clear picture of the heterogeneity of the genus *Leptospira* at the species and subspecies levels. Genetic distinction between pathogenic and saprophytic species is evident on both trees, confirming previous reports related to *rrs* gene sequencing (Hookey *et al.*, 1993). An important taxonomic question exhibited by this work is the accuracy of the delineation and positioning of the two species *L. meyeri* and *L. inadai*. The case of *L. meyeri* has been discussed extensively here and elsewhere (Letocart *et al.*, 1997), with the same conclusion that there must be collection discrepancies that should be taken into account by the reference laboratories and resolved finally by the comparative typing of samples of these strains originating from reference laboratories. In the case of *L. inadai*, the lack of molecular characterization of serovars related to this species necessitates a comprehensive taxonomic investigation to clarify the status of these strains. The need for such studies is supported by *rrs* gene sequencing data confirming heterogeneity among the world collections of leptospires (D. Postic, personal communication), which seems responsible for the discrepancies concerning *L. meyeri* and *L. inadai* members.

This initial work with MEE that focused on a representative set of strains should be considered as the basis for a comprehensive and new approach of the study of the genetic structure of the genus *Leptospira*. Further investigations including additional reference strains from serovars of public health interest will contribute to knowledge of the taxonomy of these bacteria.

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