Further determination of DNA relatedness between serogroups and serovars in the family *Leptospiraceae* with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies

Don J. Brenner, Arnold F. Kaufmann, Katherine R. Sulzer, Arnold G. Steigerwalt, Faye C. Rogers and Robbin S. Weyant

Author for correspondence: Don J. Brenner. Tel: +1 404 639 2841. Fax: +1 404 639 4421. e-mail: djb3@cdc.gov

Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, Bldg 1-2226 Mailstop D11, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

DNA relatedness was determined among 303 strains of *Leptospira* and *Leptonema.* Included in the analysis were reference strains from 228 well-characterized and recognized serovars. The study included 268 serovars from 29 named and one or more unnamed serogroups. The strains clustered into 17 DNA hybridization groups, representing 12 previously described species (292 strains) and five new genomospecies (11 strains). The largest groups included *Leptospira interrogans* (91 strains from 82 serovars), *Leptospira santarosai* (65 strains from 59 serovars), *Leptospira borgpetersenii* (49 strains from 43 serovars), *Leptospira kirschneri* (29 strains from 26 serovars) and *Leptospira noguchii* (20 strains from 20 serovars). The new genomospecies include *Leptospira* genomospecies 1 (two strains, serovars pinagchang and sichuan), *Leptospira* genomospecies 2 (six strains, serovars lushui, manhao 3, manzhuang, nanding, mengla and yunnan), *Leptospira* genomospecies 3 (one strain, serovar holland), *Leptospira* genomospecies 4 (one strain, serovar hualin) and *Leptospira* genomospecies 5 (one strain, serovar saopaulo). With the exception of Ballum, all serogroups with greater than one serovar studied were genetically heterogeneous. Phenotypic tests, including optimal growth temperature, lipase activity and growth inhibition by copper sulfate or 2,6-diaminopurine, were of little use in differentiating DNA relatedness groups. The name *Leptospira alexanderi* sp. nov. is proposed for *Leptospira* genomospecies 2 (type strain L 60^T = ATCC 700520^T, serovar manhao 3).

Keywords: *Leptospira*, *Leptonema*, *Leptospira alexanderi*, *Leptospira* genomospecies, DNA relatedness

INTRODUCTION

Before 1979, the genus *Leptospira* contained two valid species, *Leptospira interrogans* and *Leptospira biflexa*. *L. interrogans* contained 23 serogroups whose strains were either parasitic or pathogenic for humans or animals (Johnson & Faine, 1984; Kmety & Dikken, 1993). *L. biflexa* contained 28 serogroups whose strains were usually found in fresh surface waters or moist soil and were rarely isolated from man or animals (Johnson & Faine, 1984; Kmety & Dikken, 1988).

As early as 1969, DNA relatedness studies on leptospires indicated that the 15 pathogenic and non-pathogenic serovars tested were contained in six DNA relatedness groups (Haapala *et al*., 1969; Brendle *et al*., 1974). Despite this finding of heterogeneity within both *L. interrogans* and *L. biflexa*, additional species were not proposed. In 1979, Hovind-Hougen proposed the new family *Leptospiraceae*, which comprised the genus *Leptospira* and the new genus *Leptonema*, with its single species, *Leptonema illini* (Hovind-Hougen, 1979), and in 1981, Hovind-Hougen and colleagues described a third *Leptospira* species, *Leptospira parva* (Hovind-Hougen *et al*., 1981). In 1992, the Subcommittee on the taxonomy of *Leptospira* decided that *L.*

parva was sufficiently different from *Leptospira* and *Leptonema* to merit proposal as a new genus, '*Turneria*' (Marshall, 1992). Since this genus was never formally described, it currently has no standing in nomenclature.

Heterogeneity within *Leptospiraceae* was again studied in 1986, when Yasuda *et al*. (1987) used DNA relatedness to compare 44 serovars from 23 recognized and three unnamed serogroups of *L. interrogans* and single serovars from *L. parva* and *Leptonema illini*. Their DNA relatedness studies confirmed the validity of the then new species, *L. parva* and *Leptonema illini*, and demonstrated that, as then defined, both *L. interrogans* and *L. biflexa* were extremely heterogeneous. They described seven new species: *Leptospira borgpetersenii*, *Leptospira inadai*, *Leptospira noguchii*, *Leptospira santarosai*, *Leptospira weilii*, *Leptospira meyeri* and *Leptospira wolbachii*. They further demonstrated that serogrouping did not strictly equate with speciation, since some serovars in different subserogroups within the same serogroup belonged to different species. Ramadass *et al*. (1992) described the most recent species, *Leptospira kirschneri*, in a study that included strains of 66 serovars.

The purpose of the present study was to further survey strains of leptospire serovars in order to properly speciate them and to better determine the species-level specificity of serogroups and serovars.

METHODS

The nomenclature of serogroups and serovars generally follows that of Kmety & Dikken (1988, 1993). Serovars not included in the list of Kmety & Dikken are shown in bold type in Table 1. The methods used in this study for the cultivation of *Leptospiraceae*, for their biochemical characterization and for the determination of their DNA relatedness are essentially identical to those reported previously (Yasuda *et al*., 1987). These methods are summarized below.

Bacterial strains. Three-hundred-and-three strains were studied (Table 1). These organisms include representatives from 30 named and one or more unnamed serogroups in the genera *Leptospira* and *Leptonema* (Table 1). It has been the practice among leptospirologists to italicize serovars. We have not italicized taxa below the level of species. The term leptospires is used to refer to any members of the family *Leptospiraceae*.

DNA relatedness. Strains were grown at 30 °C in polysorbate albumin medium and harvested by centrifugation during late-logarithmic or stationary growth. DNA was isolated and purified as previously described (Brenner *et al*., 1982). DNA was labelled *in vitro* with [^{32}P]dCTP (Brenner *et al*., 1982). DNA relatedness and percentage divergence within related sequences were determined by the hydroxyapatite method, with 55 °C incubation used for optimal DNA reassociation and 70 °C incubation used for stringent DNA reassociation (Brenner *et al*., 1982). Percentage divergence within related DNA sequences was determined to the nearest 0·5 %.

G+C content of DNA. The G+C content (mol %) was determined for DNAs from type strains of each of the five new genomospecies by the thermal denaturation method (Mandel *et al*., 1970).

Phenotypic characteristics. The following tests, as previously described, were performed on strains incubated at 30 °C (unless stated otherwise) on polysorbate albumin medium: growth at 11, 30 and 37 °C; growth in the presence of 225 μg 8-azoguanine ml^{-1} or in the presence of 10 μg 2,6-diaminopurine ml^{-1}; growth at 30 °C in the presence of varying concentrations (25, 50, 100, 1000 and 10000 μg ml^{-1}) copper sulfate; and the presence of lipase (trioleinase) activity. Growth was measured by visual inspection for turbidity after 7, 14, 21 and 28 d incubation.

RESULTS AND DISCUSSION

The 303 leptospire strains in this study represent 268 serovars in 30 named serogroups (Table 1). Fourteen strains were in new, thus far undesignated serogroups. As defined in a previous study (Brenner *et al*., 1993), the term genomospecies is used to indicate a species determined on the basis of genetic methods. Genomospecies corresponds to a DNA relatedness group. It is convenient to use genomospecies for unnamed species until they have been formally described. DNA hybridization comparisons divided the 303 strains studied into 17 DNA relatedness groups, each of which corresponds to the genetic designation of a species as 'strains with approximately 70 % or greater DNA–DNA relatedness and with 5 °C or less ΔT_m. Both values must be considered.' (Wayne *et al*., 1987). In our laboratory, we have long used DNA relatedness at a stringent reassociation temperature (70 °C in the present study) as a third criterion for speciation, or for differentiating between species (Brenner, 1991). At the stringent criterion, strains of a single species are 60 % or more related, whereas strains from different species exhibit significantly less relatedness as shown in our previous study (Yasuda *et al*., 1987) and in Tables 2 and 3 of the present study. The DNA relatedness results shown in Tables 2 and 3 illustrate the use of 70 °C relatedness values as a rapid screening method to either include or exclude strains from any given species. For example, a large number of strains exhibiting 69–100 % relatedness in 70 °C reactions to labelled DNA from *L. interrogans* serovar icterohaemorrhagiae were included in this species without testing for relatedness at 60 °C or for divergence (Table 2). Similarly, relatedness values of less than 50 % obtained at 70 °C were considered sufficient to differentiate between species. DNA relatedness results for 44 of the serovars included in this study were previously reported by Yasuda *et al*. (1987). Some of these were retested and others were not. Data from the Yasuda *et al*. (1987) study are indicated as explained in Table 2.

The largest number of strains (91 strains representing 82 different serovars) were in *L. interrogans* (Tables 1 and 2). Other species containing large numbers of strains were *L. santarosai* (65 strains from 59 serovars), *L. borgpetersenii* (49 strains from 43 serovars), *L.*

Table 1. Strains of *Leptospiraceae* used in DNA relatedness studies

Blank spaces indicate that data are not available. Serovars given in bold type are not included in the list of Kmety & Dikken (1988, 1993).

Serovar	Serogroup	Strain	Species	Country of isolation	Source
abrahamson	Hebdomadis	Abrahamson	*L. santarosai*	Panama	Human
abramis	Pyrogenes	Abraham	*L. interrogans*	Malaysia	Human
agc	Undesignated	AGC	*L. interrogans*	Peru	Human
agogo	Djasiman	Agogo	*L. kirschneri*	Ghana	Human
aguaruna	Shermani	MW-4	*L. inadai*	Peru	Opossum
aguatia	Tarassovi	45-74	*L. santarosai*	Peru	Cattle
alexi	Pyrogenes	HS-616	*L. santarosai*	Puerto Rico	Human
alexi	Pyrogenes	Linaires	*L. santarosai*	Panama	Human
alice	Autumnalis	Alice	*L. santarosai*	Sri Lanka	Human
andamana	Andamana	CH 11	*L. biflexa*	Andaman Islands	Human
anhoa	Celledoni	LT 90-68	*L. borgpetersenii*	Vietnam	Human
arborea	Ballum	Arborea	*L. borgpetersenii*	Italy	Wood mouse
argentiniensis	Bataviae	Peludo	*L. noguchii*	Argentina	Armadillo
atchafalaya	Tarassovi	LSU 1013	*L. santarosai*	USA	Opossum
atlantae	Tarassovi	LT 81	*L. santarosai*	USA	Opossum
australis	Australis	Ballico	*L. interrogans*	Australia	Human
autumnalis	Autumnalis	Akiyami A	*L. interrogans*	Japan	Human
babudieri	Shermani	CI 40	*L. santarosai*	Peru	Pig
bac 1376	Tarassovi	Bac 1376	*L. noguchii*	Peru/Panama	Human
bafani	Canicola	Bafani	*L. kirschneri*	Zaire	Human
bagua	Pyrogenes	MW-12	*L. santarosai*	Peru	Opossum
bajan	Undesignated	Bajan	*L. noguchii*	Barbados	Toad
bakeri	Tarassovi	LT 79	*L. santarosai*	USA	Opossum
balboa	Bataviae	735 U	*L. santarosai*	Panama	Spiny rat
balcanica	Sejroe	1627 Burgas	*L. borgpetersenii*	Bulgaria	Human
balcanica	Sejroe	New Zealand	*L. borgpetersenii*	New Zealand	Opossum
ballum	Ballum	Mus 127	*L. borgpetersenii*	Denmark	Field mouse
ballum	Ballum	S102	*L. borgpetersenii*	Netherlands	
bananal	Undesignated	Aa 14	*L. santarosai*	Brazil	Field mouse
bangkinang	Autumnalis	Bangkinang I	*L. interrogans*	Indonesia	Human
bangkok	Australis	Bangkok D-92	*L. interrogans*	Thailand	Dog
barbudensis	Australis	Toad 67	*L. noguchii*	Barbados	Toad
bataviae	Bataviae	Van Tienen	*L. interrogans*	Indonesia	Human
bataviae	Bataviae	Schoolby	*L. santarosai*	Panama	Human
benjamini	Canicola	Benjamin	*L. interrogans*	Indonesia	Human
beye	Mini	1537 U	*L. santarosai*	Panama	Spiny rat
biflexa	Undesignated	965	*Leptonema illini*	USA	
biflexa	Undesignated	LT 430	*L. inadai*	USA	
biggis	Pyrogenes	Biggs	*L. interrogans*	Malaysia	Human
bim	Autumnalis	1051	*L. kirschneri*	Barbados	Dog
bindjei	Canicola	Bindjei	*L. interrogans*	Indonesia	Human
birkini	Icterohaemorrhagiae	Birkin	*L. interrogans*	Malaysia	Human
bogvere	Icterohaemorrhagiae	LT 60-69	*L. kirschneri*	Jamaica	Rat
borincana	Hebdomadis	HS 622	*L. santarosai*	Puerto Rico	Human
borincana	Hebdomadis	Norland	*L. santarosai*	Panama	Human
borincana	Hebdomadis	Samson	*L. santarosai*	Panama	Human
borincana	Hebdomadis	Woerner	*L. santarosai*	Panama	Human
brasiliensis	Bataviae	An 776	*L. santarosai*	Brazil	Opossum
bratislava	Australis	Jez Bratislava	*L. interrogans*	Czechoslovakia	Hedgehog
bravo	Tarassovi	Bravo	*L. santarosai*	Panama	Human
broomi	Canicola	Patane	*L. interrogans*	Australia	Human
budapest	Icterohaemorrhagiae	PV 1	*L. interrogans*	Hungary	Laboratory rat
bulgarica	Autumnalis	Mallika	*L. interrogans*	India	Human
bulgarica	Autumnalis	Nicolaevo	*L. kirschneri*	Bulgaria	Human
butembo	Autumnalis	Butembo	*L. kirschneri*	Zaire	Human
camlo	Pyrogenes	LT 64-67	*L. interrogans*	Vietnam	Human
canalzonae	Grippotyphosa	CZ 188	*L. santarosai*	Panama	Spiny rat
canicola	Canicola	Hond Utrech IV	*L. interrogans*	Netherlands	Dog
canicola	Canicola	Ruebush	*L. interrogans*		
caribe	Sejroe	TRVL 61866	*L. santarosai*	Trinidad	Rat
carimagua	Shermani	9160	*L. noguchii*	Argentina	
carlos	Autumnalis	C-3	*L. interrogans*	Philippines	Toad
castellonis	Ballum	Castellon 3	*L. borgpetersenii*	Spain	Wood mouse
celledoni	Celledoni	Celledoni	*L. weilii*	Australia	Human
cenepa	Pyrogenes	MW-2	*L. santarosai*	Peru	Opossum
ceylonica	Javanica	Piyasena	*L. borgpetersenii*	Sri Lanka	Human
chagres	Tarassovi	1913 K	*L. santarosai*	Panama	Spiny rat
claytoni	Bataviae	1348 U	*L. noguchii*	Panama	Spiny rat
codice	Codice	CDC	*L. wolbachii*	USA	
copenhageni	Icterohaemorrhagiae	M 20	*L. interrogans*	Denmark	Human
copenhageni	Icterohaemorrhagiae	‘Virulent’	*L. interrogans*		
copenhageni	Icterohaemorrhagiae	Wijinberg	*L. interrogans*	Holland	Human

Table 1 (*cont.*)

Serovar	Serogroup	Strain	Species	Country of isolation	Source
cornelli	Pomona	CB	*L. interrogans*	USA	Cow
coxi	Javanica	Cox	*L. weilii*	Malaysia	Human
cristobali	Panama	LT 940	*L. noguchii*	Panama	Opossum
cynopteri	Cynopteri	3522 C	*L. kirschneri*	Indonesia	Bat
dakota	Icterohaemorrhagiae	Grand River	*L. kirschneri*	USA	Water
dania	Pomona	K 1	*L. santarosai*	Denmark	Cow
darien	Tarassovi	637 K	*L. santarosai*	Panama	Opossum
dehong	Javanica	De 10	*L. borgpetersenii*	China	*Suncus murinus*
dikkeni	Sejroe	Mannuthi	*L. borgpetersenii*	India	Bandicoot
djasiman	Autumnalis	Djasiman	*L. interrogans*	Indonesia	Human
djatzi	Bataviae	HS 26	*L. kirschneri*	Puerto Rico	Human
dukou	Canicola	83194	*L. interrogans*	China	
erinaceiauriti	Autumnalis	Erineceus Auritus 670	*L. kirschneri*	Russia	Hedgehog
evansi	Ranarum	267-1348	*L. interrogans*	Malaysia	Water
figeiro	Hebdomadis	Figeiro	*L. santarosai*	Panama	Human
fluminense	Javanica	Aa 3	*L. santarosai*	Brazil	Field mouse
fortbragg	Autumnalis	Fort Bragg	*L. noguchii*	USA	Human
fugis	Australis	Fudge	*L. interrogans*	Malaysia	Human
galtoni	Canicola	LT 1014	*L. kirschneri*	Argentina	Cow
gatuni	Tarassovi	1473 K	*L. santarosai*	Panama	Opossum
gem	Icterohaemorrhagiae	Simon	*L. interrogans*	Sri Lanka	Human
gengma	Tarassovi	M 48	*L. borgpetersenii*	China	Pig
gent	Undesignated	Wa Gent	*L. wolbachii*	Belgium	Water
georgia	Mini	LT 117	*L. santarosai*	USA	Racoon
geyaweera	Sejroe	Geyaweera	*L. interrogans*	Sri Lanka	Human
goiano	Hebdomadis	Bovino 131	*L. santarosai*	Brazil	Cow
gorgas	Sejroe	1413 U	*L. santarosai*	Panama	Spiny rat
grippotyphosa	Grippotyphosa	Andaman	*L. interrogans*		
grippotyphosa	Grippotyphosa	Moskva V	*L. kirschneri*	Russia	Human
grippotyphosa	Grippotyphosa	DF	*L. kirschneri*	USA	Human
grippotyphosa	Grippotyphosa	GG	*L. kirschneri*	USA	Human
grippotyphosa	Grippotyphosa	STP	*L. kirschneri*	USA	Water
guangdong (Ballum 3)	Ballum	1853	*L. borgpetersenii*	China	*Rattus losea*
guaratuba	Pyrogenes	An 7705	*L. interrogans*	Brazil	Opossum
guaricura	Sejroe	Bov. G	*L. santarosai*	Brazil	Cow
guidae	Tarassovi	RP 29	*L. borgpetersenii*	Brazil	Pig
gurungi	Djasiman	Gurung	*L. interrogans*	Malaysia	Human
habaki	Bataviae	Habaki	*Leptonema illini*		
haemolytica	Sejroe	Marsh	*L. interrogans*	Malaysia	Human
hainan	Celledoni	6712	*L. weilii*	China	Human
hamptoni	Pyrogenes	Hampton	*L. borgpetersenii*	Malaysia	Human
harbola	Javanica	Harbola 20	*L. borgpetersenii*		
hardjo	Sejroe	K-125	*L. borgpetersenii*	USA	Cow
hardjo	Sejroe	T-20	*L. borgpetersenii*	USA	Cow
hardjo	Sejroe	Sponselee	*L. borgpetersenii*	Holland	Cow
hardjo	Sejroe	Hardjoprajitno	*L. interrogans*	Indonesia	Human
hardjo	Sejroe	Went 5	*L. meyeri*	Canada	
hawain	Australis	LT 62-68	*L. interrogans*	New Guinea	Bandicoot
hebdomadis	Hebdomadis	Hebdomadis	*L. interrogans*	Japan	Human via Guinea pig
hekou	Mini	H 27	*L. weilii*	China	Human
holland	Holland	WaZ Holland^T	Genomospecies 3	Netherlands	Water
honghe	Icterohaemorrhagiae	H 2^T	*L. interrogans*	China	Human
hualin	Icterohaemorrhagiae	LT 11-33	Genomospecies 4	China	
huallaga	Djasiman	M 7	*L. noguchii*	Peru	Opossum
icterohaemorrhagiae	Icterohaemorrhagiae	RGA	*L. interrogans*	Belgium	Human
icterohaemorrhagiae	Icterohaemorrhagiae	1 (Japan)	*L. interrogans*	Japan	Human
icterohaemorrhagiae	Icterohaemorrhagiae	1 (Kmety)	*L. inadai*	Japan	Human
illini	Leptonema	3055	*Leptonema illini*	USA	Cow
istrica	Hebdomadis	Bratislava	*L. borgpetersenii*	Czechoslovakia	Wood mouse
jalna	Australis	Jalna	*L. interrogans*	Czechoslovakia	Yellow throat mouse
javanica	Javanica	Veldrat Batavia 46	*L. borgpetersenii*	Indonesia	Field rat
jin	Sejroe	A81	*L. interrogans*	China	Human
jonsis	Canicola	Jones	*L. interrogans*	Malaysia	Human
jules	Hebdomadis	Jules	*L. borgpetersenii*	Zaire	Human
kabura	Hebdomadis	Kabura	*L. kirschneri*	Zaire	Human
kambale	Hebdomadis	Kambale	*L. kirschneri*	Zaire	Human
kamituga	Canicola	Kamituga	*L. kirschneri*	Zaire	Human
kanana	Tarassovi	Kanana	*L. borgpetersenii*	Kenya	Gerbil
kaup	Tarassovi	LT 64-68	*L. inadai*	New Guinea	Bandicoot
kennewicki	Pomona	LT 1026	*L. interrogans*	USA	Cow
kenya	Ballum	Nijenga	*L. borgpetersenii*	Kenya	Pouched rat
kisuba	Tarassovi	Kisuba	*L. borgpetersenii*	Zaire	Human
kobbe	Bataviae	CZ 320	*L. santarosai*	Panama	Spiny rat
kremastos	Hebdomadis	Kremastos	*L. interrogans*	Australia	Human
kremastos	Hebdomadis	2414 VAB	*L. santarosai*	Peru/Panama	Human
kunming	Pomona	K 5	*L. kirschneri*	China	*Apodemus chevrieri*
kuwait	Canicola	136/2/2	*L. interrogans*	Kuwait	Rat
kwale	Pyrogenes	Julu	*L. borgpetersenii*	Kenya	Human

Table 1 *(cont.)*

Serovar	Serogroup	Strain	Species	Country of isolation	Source
lai	Icterohaemorrhagiae	Lai	*L. interrogans*	China	Human
lambwe	Autumnalis	Lambwe	*L. kirschneri*	Kenya	Unstripped grass rat
langati	Tarassovi	M 39039	*L. weilii*	Malaysia	
lanka	Louisiana	R/740	*L. interrogans*	Sri Lanka	Human
liangguang	Grippotyphosa	1880	*L. interrogans*	China	Rat
lichuan (Manhao 4)	Manhao	Li 130	*L. inadai*	China	Human
lincang	Manhao	L 14	*L. inadai*	China	Human
longnan	Hebdomadis	L 573	*L. weilii*	China	Human
lora	Australis	Lora	*L. interrogans*	Italy	Human
losbanos	Bataviae	LT 101-69	*L. interrogans*	Philippines	Rat
louisiana	Louisiana	LSU 1945	*L. noguchii*	USA	Armadillo
luis	Shermani	M 6	*L. santarosai*	Peru	Opossum
lushui (Manhao 1)	Manhao	L 70	Genomospecies 2	China	
lyme	Lyme	10	*L. inadai*	USA	Human
machiguenga	Sarmin	MMD 3	*L. santarosai*	Peru	Opossum
malaya	Canicola	H 6	*L. inadai*	Malaysia	Human
mangus	Panama	TVRL/CAREC 137774	*L. inadai*	Trinidad	Mongoose
manhao 3	Manhao	L 60ᵀ	Genomospecies 2	China	
manilae	Pyrogenes	LT 398	*L. interrogans*	Philippines	Rat
mankarso	Icterohaemorrhagiae	Mankarso	*L. interrogans*	Indonesia	Human
manzhuang	Hebdomadis	A 23	Genomospecies 2	China	Human
maru	Hebdomadis	CZ 285	*L. santarosai*	Panama	Water via hamster
maru	Hebdomadis	Brinkman	*L. santarosai*	Panama	Human
maru	Hebdomadis	Clark	*L. santarosai*	Panama	Human
may	Javanica	May	*L. santarosai*	Panama	Human
medanensis	Sejroe	Hond HC	*L. interrogans*	Indonesia	Dog
mengdeng	Celledoni	M 6906	*L. weilii*	China	Human
mengla	Javanica	A 85	Genomospecies 2	China	Human
menglian	Pyrogenes	S 621	*L. weilii*	China	Human
mengma	Javanica	S 590	*L. weilii*	China	Human
menoni	Javanica	Kerala	*L. borgpetersenii*	Indonesia	Bandicoot
menrun	Javanica	A 102	*L. weilii*	China	Human
mini	Mini	Sari	*L. borgpetersenii*	Italy	Human
mogdeni	Tarassovi	Compton 746	*L. weilii*	United Kingdom	Sewage
moldaviae	Bataviae	114-2	*L. borgpetersenii*	Russia	
monjakov	Pomona	Monjakov	*L. interrogans*	Russia	Human
monymusk	Icterohaemorrhagiae	LT 75-68	*L. interrogans*	Jamaica	Rat
monymusk	Icterohaemorrhagiae	81552	*L. interrogans*	China	
mooris	Autumnalis	Moores	*L. interrogans*	Malaysia	Human
mozdok	Pomona	5621	*L. kirschneri*	Russia	Field vole
muenchen	Australis	München C 90	*L. interrogans*	Germany	Human
mwogolo	Icterohaemorrhagiae	Mwogolo	*L. kirschneri*	Zaire	Human
mwogolo	Icterohaemorrhagiae	Korea	*L. interrogans*	Korea	Human
myocastoris	Pyrogenes	LSU 1551	*L. noguchii*	USA	Nutria
naam	Icterohaemorrhagiae	Naam	*L. interrogans*	Indonesia	Human
nanding	Hebdomadis	M 6901	Genomospecies 2	China	Human
nanxi	Icterohaemorrhagiae	HK 6	*L. interrogans*	China	Human
naparuca	Cynopteri	NN-1	*L. santarosai*	Peru	*Galictis furax*
navet	Tarassovi	TRVL 109873	*L. santarosai*	Trinidad	Human
ndahambukuje	Icterohaemorrhagiae	Ndahambukuje	*L. kirschneri*	Zaire	Human
ndambari	Icterohaemorrhagiae	Ndambari	*L. kirschneri*	Zaire	Human
nero	Sejroe	Gamsulin	*L. borgpetersenii*	Russia	Human
nicaragua	Australis	1011	*L. noguchii*	Nicaragua	*Mustela nivalis*
nona	Hebdomadis	Nona	*L. borgpetersenii*	Zaire	Human
nyanza	Sejroe	Kibos	*L. borgpetersenii*	Kenya	Human
orleans	Louisiana	LSU 2580	*L. noguchii*	USA	Nutria
paidjan	Bataviae	Paidjan	*L. interrogans*	Indonesia	Human
panama	Panama	CZ 214 K	*L. noguchii*	Panama	Opossum
parva	Turneria	H	*L. parva*	England	Bacteriological media
patoc	Semaranga	Patoc I	*L. biflexa*	Italy	Water
perameles	Mini	Bandicoot 343	*L. meyeri*	Australia	Perameles
peru	Undesignated	MW 10	*L. santarosai*	Peru	Opossum
peruviana	Australis	V 42	*L. noguchii*	Peru	Cow
pina	Australis	LT 932	*L. borgpetersenii*	Panama	Opossum
pingchang	Ranarum	80-412	Genomospecies 1	China	Frog
poi	Javanica	Poi	*L. borgpetersenii*	Italy	Human
polonica	Sejroe	493 Poland	*L. borgpetersenii*	Poland	Hedgehog
pomona	Pomona	Pomona	*L. interrogans*	Australia	Human
pomona	Pomona	164	*L. interrogans*	USA	Cow
pomona	Pomona	S91	*L. interrogans*	USA	Pig
pomona	Pomona	Wickard	*L. interrogans*	USA	Cow
pomona	Pomona	Johnson	*L. interrogans*		
pomona	Pomona	24K	*L. noguchii*	Russia	
portlandvere	Canicola	My 1039	*L. interrogans*	Jamaica	Human
princestown	Pyrogenes	TRVL 112499	*L. santarosai*	Trinidad	Human
proechimys	Pomona	1161 U	*L. noguchii*	Panama	Spiny rat
pyrogenes	Pyrogenes	Salinem	*L. interrogans*	Indonesia	Human
pyrogenes	Pyrogenes	Northrup	*L. santarosai*	Panama	Human

Table 1 *(cont.)*

Serovar	Serogroup	Strain	Species	Country of isolation	Source
qingshui (Manhao 2)	Manhao	L 105	*L. weilii*	China	Human
qunjian	Canicola	7957	*L. interrogans*	China	Rat
rachmati	Autumnalis	Rachmat	*L. interrogans*	Indonesia	Human
rama	Tarassovi	316	*L. santarosai*	Panama	Opossum
ramisi	Australis	Musa	*L. kirschneri*	Kenya	Human
ranarum	Ranarum	ICF	*L. meyeri*	USA	Frog
ranarum shu	Undesignated	Ranarum shu	*L. interrogans*		
ratnapura	Grippotyphosa	Wumalasena	*L. kirschneri*	Sri Lanka	Human
recreo	Sejroe	380	*L. interrogans*	Nicaragua	Opossum
ricardi	Sejroe	Richardson	*L. interrogans*	Malaysia	Human
rio	Sarmin	Rr 5	*L. santarosai*	Brazil	Rat
rioja	Bataviae	MR 12	*L. santarosai*	Peru	Opossum
robinsoni	Pyrogenes	Robinson	*L. interrogans*	Australia	Human
roumanica	Sejroe	TM 294	*L. interrogans*	Romania	*Mus musculus*
ruparupae	Mini	M 3	*L. santarosai*	Peru	Opossum
rushan	Australis	507	*L. noguchii*	China	*Bombina orientalis*
sanmartini	Pyrogenes	CT 63	*L. santarosai*	Peru	Cow
saopaulo	Semaranga	Sao Paulo^T	Genomospecies 5	Brazil	Water
sarmin	Sarmin	Sarmin	*L. weilii*	Indonesia	Human
saxkoebing	Sejroe	Mus 24	*L. interrogans*	Denmark	Wood mouse
schueffneri	Canicola	Vleermuis	*L. interrogans*	Indonesia	Bat
sejroe	Sejroe	M 84	*L. borgpetersenii*	Denmark	Mouse
semaranga	Semaranga	Veldrat Samarang	*L. meyeri*	Indonesia	Rat
sentot	Djasiman	Sentot	*L. interrogans*	Indonesia	Human
shermani	Shermani	1342 K	*L. santarosai*	Panama	Spiny rat
sichuan	Undesignated	79601^T	*L.* genomospecies 1	China	Frog
smithi	Icterohaemorrhagiae	Smith	*L. interrogans*	Malaysia	Human
soccoestomes	Ballum	78-082387	*L. borgpetersenii*		
sofia	Javanica	Sofia 874	*L. meyeri*	Bulgaria	Human
sorexjalna	Javanica	Sorex Jalna	*L. borgpetersenii*	Czechoslovakia	Shrew
srebarna	Autumnalis	1409/69	*L. borgpetersenii*	Bulgaria	*Sorex auraneus*
sulzerae	Tarassovi	LT 82	*L. santarosai*	USA	
sumneri	Canicola	Sumner	*L. interrogans*	Malaysia	Human
szwajizak	Mini	Szwajizak	*L. interrogans*	Australia	Human
szwajizak	Mini	Oregon	*L. santarosai*	USA	Cow
tabaquite	Mini	TVRL 3405	*L. santarosai*	Trinidad	Human
tarassovi	Tarassovi	Perepelicin	*L. borgpetersenii*	Russia	Human
tingomariensis	Cynopteri	M 13	*L. santarosai*	Peru	Opossum
tonkini	Icterohaemorrhagiae	LT 96-68	*L. borgpetersenii*	Vietnam	Human
trinidad	Sejroe	TRVL 34056	*L. santarosai*	Trinidad	Human
tropica	Pomona	CZ 299	*L. santarosai*	Panama	Spiny rat
tsaratsovo	Pomona	B 81/7	*L. kirschneri*	Bulgaria	Harvest mouse
tunis	Tarassovi	P 2/65	*L. borgpetersenii*	Tunisia	Pig
Undesignated	Undesignated	965	*Leptonema illini*	USA	Unknown
Undesignated	Undesignated	LT 430	*L. inadai*	USA	Unknown
unipertama	Sejroe	K2-1	*L. weilii*	Indonesia	Cow
valbuzzi	Grippotyphosa	Valbuzzi	*L. interrogans*	Australia	Human
valbuzzi	Grippotyphosa	Dyster	*L. kirschneri*		
vanderhoedeni	Grippotyphosa	Kipod 179	*L. kirschneri*	Israel	Hedgehog
varela	Pyrogenes	1019	*L. santarosai*	Nicaragua	Opossum
vargonicas	Javanica	24	*L. santarosai*	Peru	Rodent
vughia	Tarassovi	LT 89-68	*L. weilii*	Vietnam	Human
waskurin	Sarmin	LT 63-68	*L. interrogans*	New Guinea	Bandicoot
wawain	Undesignated	MW 6	*L. santarosai*	Peru	Opossum
weaveri	Sarmin	CZ 390	*L. santarosai*	Panama	Human
weerasinghe	Autumnalis	Weerasinghe	*L. interrogans*	Sri Lanka	Human
wewak	Australis	LT 65-68	*L. interrogans*	New Guinea	Dog
whitcombi	Celledoni	Whitcomb	*L. borgpetersenii*	Malaysia	Human
wolffi	Sejroe	3705	*L. interrogans*	Indonesia	Human
worsfoldi	Hebdomadis	Worsfold	*L. borgpetersenii*	Malaysia	Human
yaan	Javanica	80-27	*L. borgpetersenii*	China	*Crocidura attenuata*
yunnan	Mini	A 10	Genomospecies 2	China	Human
yunxian	Tarassovi	L 100	*L. borgpetersenii*	China	Pig
zanoni	Pyrogenes	Zanoni	*L. interrogans*	Australia	Human
zhenkang	Javanica	L 82	*L. borgpetersenii*	China	Rat
K 128	Undesignated	K 128	*L. borgpetersenii*		
K 142	Undesignated	K 142	*L. borgpetersenii*		
X 47	Sejroe	X 47	*L. santarosai*	Indonesia	
21-74	Bataviae	21-74	*L. interrogans*	Brazil	
26-73	Bataviae	3859	*L. interrogans*	Indonesia	
27-75	Javanica	Azalia	*L. inadai*	Indonesia	Human
52-73	Javanica	457	*L. borgpetersenii*	Sri Lanka	Dog
82224	Icterohaemorrhagiae	82224	*L. interrogans*	China	
83-011457	Tarassovi	MO1K	*L. santarosai*	Panama	
83-015437	Undesignated	W16K	*L. noguchii*	Panama	
84-011370	Undesignated	2050	*L. noguchii*	Panama	Human
87-029496	Hebdomadis	KF001	*L. santarosai*	Panama	Human

Table 2. DNA relatedness of strains within *Leptospira* and *Leptonema* species

RBR, Relative binding ratio = [(percentage DNA bound to hydroxyapatite in heterologous reactions)/(percentage DNA bound in homologous reactions)] × 100. D, Percentage divergence (calculated to the nearest 0·5%); calculations of D assumed that a 1% decrease in the thermal stability of a heterologous DNA duplex compared with that of the homologous duplex was caused by 1% of the bases within the duplex that were unpaired. Values shown in bold type were taken from our previous study (Yasuda *et al.*, 1987). For example, in the reaction between serovar icterohaemorrhagiae RGA and serovar djasiman, the 55 °C value was obtained from the first study and the 70 °C value was obtained in the present study. For serovars where more than one strain was studied, the strain names are given in parentheses.

Unlabelled DNA from serovar	RBR at 55 °C	D	RBR at 70 °C
	Labelled DNA from *L. interrogans* serovar icterohaemorrhagiae strain RGA		
icterohaemorrhagiae (RGA)	100	0·0	100
agc	**100***		**100***
australis	**100***		**100***
autumnalis	**100***		**100***
djasiman	**100***		100
jalna	**100***		100
pyrogenes (Salinem)	**100***		**100***
schueffneri	**100***		100
lanka			100
mooris			100
pomona (Pomona)	**100**	**1·5**	100
saxkoebing	**100**	**1·0**	
bataviae (Van Tienen)	**100***		99
grippotyphosa (Andaman)	**100***	**0·0***	**99***
zanoni	**100**		**90**
82224	100	3·0	86
smithi	**100**		77
guaratuba	100†	2·5†	69†
bulgarica (Mallika)	99	1·0	97
broomi	99	1·0	93
wolffi	**98**	**0·0**	100
muenchen	98	1·5	85
honghe	97	0·5	87
robinsoni	95	2·0	73
mwogolo (Korea)			95
losbanos	94	1·0	89
copenhageni (Wijinberg)	94	0·5	89
21-74			94
fugis			94
canicola (Hond Utrech IV)	**93**	**1·0**	**93**
qunjian	93	1·5	78
huwain	93	4·5	77
roumanica			93
26-73			93
copenhageni (M20)	**92**	**0·0**	**100**
sumneri	92	0·5	93
abramis			92
sentot			92
copenhageni ('virulent')	91	1·0	100
liangguang	91	0·5	92
jin	91	1·5	87
nanxi	91	2·0	87
gurungi			91
monjakov			91
monymusk (LT 75-68)			91
manilae			91
ricardi			91

Table 2 *(cont.)*

Unlabelled DNA from serovar	RBR at 55 °C	D	RBR at 70 °C
	Labelled DNA from *L. interrogans* serovar icterohaemorrhagiae strain RGA		
biggis	90	0·5	96
birkini	90	0·5	96
kennewicki	90	1·0	89
mankarso	90	0·5	78
carlos	90	1·5	75
bratislava			90
icterohaemorrhagiae 1 (Japan)	89	0·5	91
hebdomadis	**89***	**3·0***	91
rachmati			89
cornelli	88	0·5	94
jonsis	88	2·5	83
dukou	88	1·0	79
bindjei			88
bangkok			87
pomona (164)			87
lai	86	1·5	91
benjamini			86
haemolytica	85	1·0	94
medanensis			85
valbuzzi (Valbuzzi)			85
weerasinghe			85
monymusk (81552)	84	2·0	96
ranarum shu	84	1·5	89
canicola (Ruebush)			84
hardjo (Hardjoprajitno)			84
geyaweera			83
waskurin	82	3·5	69
pomona (Johnson)			82
wewak	81	3·5	65
bangkinang			81
pomona (S 91)			81
camlo			80
evansi			80
portlandvere			80
gem			79
kremastos (Kremastos)			79
naam			79
pomona (Wickard)			79
lora			78
recreo			77
szwajizak (Szwajizak)			76
budapest			74
paidjan			72
kuwait			69
	Labelled DNA from *L. santarosai* serovar shermani strain 1342K		
shermani	100	0·0	100
sulzerae	100†		
alexi			100
gorgas			100
kobbe			100
princestown			100
ruparupae			100
sanmartini			100

Table 2 *(cont.)*

Unlabelled DNA from serovar	RBR at 55 °C	D	RBR at 70 °C
	Labelled DNA from *L. santarosai* serovar shermani strain 1342K		
tropica			100
varela			100
cenepa	97	1·5	95
bravo	97	3·5	
balboa	96	1·5	86
trinidad			96
figeiro	95	1·5	84
atlantae	**93**	**0·5**	**87**
bakeri	**92**	**2·0**	**88**
bagua			92
tingomaria			91
borincana	**90**	**0·5**	**91**
alice	90	1·5	79
beye			90
chagres			88
guaricura			88
machiguenga			88
peru	**86**	**0·5**	**91**
bananal	**86**	**0·5**	**90**
wawain	**86**	**0·0**	**86**
navet	**86**	**2·0**	**86**
X 47			86
maru			85
pyrogenes (Northrup)	85	2·0	77
naparuca			84
canalzonae			83
83-011457			83
atchafalaya	82	2·5	99
borincana (Woerner)	82	3·0	79
abrahamson	82	1·0	72
weaveri			82
babudieri			81
darien			81
rama			81
tabaquite			81
borincana (Sampson)	80	3·0	79
aquatia			80
gatuni			80
riojo	79	1·0	87
borincana (Norland)	79	3·0	76
goiano	78	1·0	
kremastos (2414 VAB)	78	3·0	68
may	78	2·0	66
maru (Clark)	78	2·5	66
luis			78
rio			78
bataviae (Schoolby)	76	1·5	69
alexi (Linaires)	74	1·0	
dania	74	1·5	69
caribe			74
vargonicus			74
szwajizak (Oregon)	71	1·0	53
fluminense			71

Table 2 (*cont.*)

Unlabelled DNA from serovar	RBR at 55 °C	D	RBR at 70 °C
	Labelled DNA from *L. santarosai* serovar shermani strain 1342K		
georgia			71
maru (Brinkman)	70	1·5	58
87-029496	69	1·0	72
brasiliensis			68
	Labelled DNA from *L. borgpetersenii* serovar javanica strain Veldrat Batavia 46		
javanica (Veldrat Batavia 46)	100	0·0	100
K 142	100	1·0	95
soccoestomes			100
yaan			100
zhenkang	98	1·0	89
dehong	98	0·5	88
sorexjalna			98
mini	**97**		**80**
kenya	96	0·5	**100**
kanana	96	0·5	92
castellonis			96
ceylonica			96
poi	95	2·0	94
K 148	95	0·5	92
hardjo (K125)	95	1·0	80
ballum (Mus 127)	**94**	**0·0**	**99**
menoni	94	1·5	
arborea			94
dikkeni	93	1·0	100
nyanza	93	1·0	96
nana	92	0·5	100
sejroe	**92**	**4·5**	**90**
pina	91	1·0	85
tarassovi	90	1·0	75
nero	89	1·5	93
guangdong	88	1·0	86
harbala	88‡	1·0‡	
ballum (S 102)	87	4·5	92
balcanica (New Zealand)	87	1·5	74
jules	86	1·5	98
hardjo (T 20)	86	2·0	85
guidae	86	2·5	76
balcanica (1627 Burgas)			86
polonica			86
yunxian	85	2·0	98
moldaviae	85	1·5	93
52-73	85	1·0	85
istrica			85
kwale			85
srebarna			84
hardjo (Sponselee)	83	2·0	82
anhoa	82	2·0	82
tonkini	80§	2·5§	
kisuba	78	1·5	74
gengma	77	2·0	89
tunis			77
worsfoldi			74
hamptoni	72‖	1·5‖	67‖
whitcombi			64

Table 2 *(cont.)*

Unlabelled DNA from serovar	RBR at 55 °C	D	RBR at 70 °C
	Labelled DNA from *L. kirschneri* serovar cynopteri strain 3522C		
cynopteri	100	0·0	**100**
grippotyphosa (DF)	100	2·0	97
ndahambukuje			100¶
tsaratsova	98	1·0	94
kunming	96	1·0	100
dakota	96	1·0	94
butembo			96¶
bafani	95	1·0	92
ratnapura	94	2·5	81
bogvere	93	1·0	84
grippotyphosa (GG)	92	2·0	
mozdok			92¶
ndambari			90
kabura	89	2·0	98
grippotyphosa (STP)	89	2·0	95
erinaceiauriti			89¶
djatzi			88¶
mwogolo (Mwogolo)			88¶
vanderhoedeni			88¶
galtoni			87¶
agogo	86	2·0	91
kambale	86	1·0	89
valbuzzi (Dyster)	86	1·5	83
bim			86¶
bulgarica (Nicolaevo)	85	2·0	80
lambwe	84	0·0	
grippotyphosa (Moskva V)			83¶
kamituga			82¶
ramisi			73¶
	Labelled DNA from *L. noguchii* serovar panama strain CZ 214 K		
panama	100	0·0	100
proechimys	100		89
pomona (24K)	100	0·5	84
rushon	99	1·0	78
carimagua	95	1·5	87
huallaga			92
barbudensis	90	2·5	87
nicaragua	90	3·5	66
orleans	90	3·5	62
83-015437	89	1·5	68
bac 1376	87	2·0	93
fortbragg	86	2·5	77
bajan	85	2·5	79
louisiana	84	2·0	77
claytoni			84
myocastoris	83	3·5	67
84-011370	81	3·5	71
argentiniensis			78
cristobali (LT 960)			76
peruviana			70

Table 2 (*cont.*)

Unlabelled DNA from serovar	RBR at 55 °C	D	RBR at 70 °C
	Labelled DNA from *L. weilii* serovar celledoni strain Celledoni		
celledoni	100	0·0	100
mengma	100	0·0	96
vughia	100	2·5	82
hainan			98
menrun			98
hekou	90	1·0	77
menglian	89	1·5	84
mogdeni			89
sarmin	**88**	**2·0**	80
coxi			87
mengdeng	86	1·0	93
longnan	86	1·0	83
unipertama	80	2·5	82
langati			77
qingshui			72
	Labelled DNA from *L. inadai* serovar lyme strain 10		
lyme	100	0·0	100
27-75	100	0·0	97
lichuan	100	1·5	94
lincang	100	2·0	90
mangus	99	0·5	100
malaya	99	0·0	88
kaup	98	0·0	96
aguarina	98	0·0	91
icterohaemorrhagiae 1 (Kmety)	89	0·0	98
undesignated (LT 430)	78	0·0	81
	Labelled DNA from *L. meyeri* serovar ranarum strain ICF		
ranarum	100	0·0	100
sofia	100	0·0	100
perameles	100		
hardjo (Went 5)	94	0·5	91
semaranga	83	2·5	86
	Labelled DNA from *Leptonema illini* serovar illini strain 3055		
illini	100	0·0	100
habaki	100	2·0	75
undesignated (965)	83	3·0	61
	Labelled DNA from *L. wolbachii* serovar codice strain CDC		
codice	100	0·0	100
gent	100	2·0	93
	Labelled DNA from *L. biflexa* serovar andaman strain CH 11		
andaman	100	0·0	100
patoc	98	1·0	74
	Labelled DNA from *L. parva* serovar parva strain H		
parva	100	0·0	100
	Labelled DNA from *Leptospira* genomospecies 1 serovar pingchang strain 80-412		
pingchang	100	0·0	100
sichuan	98†		

Table 2 (*cont.*)

Unlabelled DNA from serovar	RBR at 55 °C	D	RBR at 70 °C
	Labelled DNA from *Leptospira* genomospecies 2 serovar manhao strain L 60^T		
manhao 3	100	0·0	100
lushui	86	1·0	90
mengla	85	0·5	95
manzhuang	85	0·5	85
nanding	69	1·0	81
yunnan	66	1·5	60
	Labelled DNA from *Leptospira* genomospecies 3 serovar holland strain WaZ Holland^T		
holland	100	0·0	100
	Labelled DNA from *Leptospira* genomospecies 4 serovar hualin strain LT 11-33		
hualin	100	0·0	100
	Labelled DNA from *Leptospira* genomospecies 5 serovar saopaulo strain Sao Paulo^T		
saopaulo	100	0·0	100

* Values were obtained using labelled *L. interrogans* serovar copenhageni M 20 DNA.

† Values obtained were with labelled DNA from the strain listed as unlabelled. For example, the values for serovar guaratuba were obtained using labelled DNA from serovar guaratuba and unlabelled DNA from serovar icterohaemorrhagiae strain RGA.

‡ Results obtained using labelled DNA from serovar Menoni.

§ Results obtained using labelled DNA from serovar Anhoa.

‖ Results obtained using labelled DNA from serovar Tonkini.

¶ Results obtained using labelled DNA from serovar Ndambari.

kirschneri (29 strains from 26 serovars) and *L. noguchii* (20 strains from 20 serovars). The remaining 49 strains each represented a different serovar. Those that belonged to named species were: *L. weilii*, 15 strains; *L. inadai*, 10 strains, *L. meyeri*, five strains; *Leptonema illini*, three strains; *L. biflexa*, two strains, *L. wolbachii*, two strains; and *L. parva*, one strain. Eleven strains belonged to previously unidentified species, which we designated *Leptospira* genomospecies 1–5. Two strains were in *Leptospira* genomospecies 1, six strains were in *Leptospira* genomospecies 2 and one strain was in each of *Leptospira* genomospecies 3, 4 and 5.

L. parva and *Leptospira* genomospecies 3, 4 and 5 contained a single strain that showed less than species-level relatedness to the type strain of all other species. The remaining named species, as well as *Leptospira* genomospecies 1 and 2, contained two or more strains (see exceptions below) that fulfil the molecular definition of a species: 70 % relatedness at optimal DNA renaturation temperature (55 °C in these studies) and whose related sequences exhibit 5 % or less divergence [on the assumption that each 1 °C of decreased thermal stability of a heterologous DNA duplex compared with that of the homologous DNA duplex is caused by approximately 1 % unpaired bases (Wayne *et al.*, 1987)]. In our laboratory, we included the additional parameter that strains of the same species exhibit 60 % or greater relatedness at a stringent DNA renaturation temperature (70 °C in this study). DNA relatedness determined at the 70 °C temperature was used as the sole criterion for including 112 of the 303 strains in a given species. Partial or complete data from our previous study (Yasuda *et al.*, 1987) was used for 31 strains, as indicated by the bold numbers in Table 1. Of the 299 strains included in species containing two or more strains, all but four strains showed relatedness and divergence values within the limits of the species definition. The exceptions were: strain 87-029496, which was 69 % related to the type strain of *L. santarosai* at 55 °C with 1·0 % divergence and was 72 % related to it at 70 °C; strain Oregon of serovar szwajizak, which was 71 % related to the type strain of *L. santarosai* at 55 °C with 1·0 % divergence and was 53 % related to it at 70 °C; strain 6901 of serovar nanding, which was 69 % related to the type strain of *Leptospira* genomospecies 2 at 55 °C with 1·0 % divergence and was 81 % related to it at 70 °C; and strain A-10 of serovar yunnan, which was 66 % related to the type strain of *Leptospira* genomospecies 2 at 55 °C with 1·5 % divergence and was 60 % related to it at 70 °C.

By definition, the type strain of every species must exhibit less than species-level relatedness to the type strain (and any other strain tested) of every other species. DNA relatedness ranges between species are shown in Table 3. In these comparisons, labelled DNAs from type and reference strains of each named species and genomospecies were reacted with unlabelled DNAs from the same and different species (see Table 3). Relatedness between a number of species pairs is close, but in all cases, the levels of relatedness observed within strains of a species are substantially

Table 3. DNA relatedness between leptospire species

Strains used as sources of labelled DNA are as follows: *L. interrogans*, RGA; *L. santarosai*, 1342K; *L. borgpetersenii*, Veldrat Batavia 46; *L. kirschneri*, 3522C; *L. noguchii*, CZ 214 K; *L. weilii*, Celledoni; *L. inadai*, 10; *L. meyeri*, ICF; *Leptonema illini*, 3055; *L. wolbachii*, CDC; *L. biflexa*, CH 11; *L. parva*, H; *Leptospira* genomospecies 1, 80-412; *Leptospira* genomospecies 2, L 60ᵀ; *Leptospira* genomospecies 3, WaZ Hollandᵀ; *Leptospira* genomospecies 4, LT 11-33; *Leptospira* genomospecies 5, Sao Pauloᵀ. *n*, No. of strains studied.

Source of labelled DNA	Relatedness (%) to unlabelled DNAs from:																	
	55 °C	(*n*)	D	(*n*)	70 °C	(*n*)	55 °C	(*n*)	D	(*n*)	70 °C	(*n*)	55 °C	(*n*)	D	(*n*)	70 °C	(*n*)
	L. interrogans						***L. santarosai***						***L. borgpetersenii***					
L. interrogans	93	(48)	1·0	(38)	88	(90)	46	(10)	10·0	(5)	7	(66)	51	(4)	11·0	(1)	6	(43)
L. santarosai	34	(3)	12·5	(1)	6	(4)	84	(30)	1·5	(29)	82	(60)	62	(2)	8·0	(2)	18	(10)
L. borgpetersenii	33	(10)	13·5	(5)	13	(7)	53	(24)	10·0	(23)	30	(63)	89	(33)	1·5	(33)	88	(45)
L. kirschneri	39	(1)	12·0	(2)	30	(4)	50	(1)			9	(6)	50	(1)			9	(6)
L. noguchii	66	(8)	9·5	(4)	41	(9)	48	(8)	14·0	(4)	9	(24)	44	(3)	10·5	(1)	9	(6)
L. weilii	43	(2)	14·0	(1)	8	(6)	57	(9)	9·5	(9)	24	(26)	71	(2)	6·5	(2)	28	(3)
L. inadai	3	(2)			2	(2)	5	(1)			1	(2)	10	(2)			5	(1)
L. meyeri	2	(1)			3	(2)	1	(1)			1	(2)	5	(2)			1	(5)
Leptonema illini					0	(2)					0	(2)	5	(1)			0	(6)
L. wolbachii	3	(7)			0	(2)	6	(3)			1	(2)	3	(3)			0	(5)
L. biflexa	3	(2)			1	(2)	5	(1)			1	(2)	6	(1)			1	(5)
L. parva	0	(1)			0	(2)	0	(2)			0	(2)	3	(2)			0	(6)
Leptospira genomospecies 1	35	(1)			6	(2)	42	(2)			12	(3)	44	(4)			13	(4)
Leptospira genomospecies 2					2	(2)					14	(2)	61	(5)	4·0	(5)	28	(5)
Leptospira genomospecies 3	5	(1)			1	(1)	2	(1)			0	(1)	5	(2)			1	(2)
Leptospira genomospecies 4	4	(1)			5	(1)	5	(3)			2	(1)	4	(3)			2	(1)
Leptospira genomospecies 5	3	(1)					3	(2)					2	(3)				
	L. kirschneri						***L. noguchii***						***L. weilii***					
L. interrogans	67	(21)	7·5	(20)	41	(27)	70	(6)	8·0	(6)	34	(18)	38	(1)			7	(15)
L. santarosai	23	(2)	15·5	(1)	6	(24)	41	(1)	13·0	(1)	8	(10)	54	(2)	10·5	(2)	25	(9)
L. borgpetersenii	26	(1)	13·5	(1)	7	(28)	34	(5)	11·5	(5)	11	(18)	68	(8)	7·5	(7)	42	(15)
L. kirschneri	92	(15)	1·5	(15)	89	(26)					23	(4)					5	(2)
L. noguchii	66	(14)	9·0	(14)	31	(24)	90	(14)	2·0	(14)	78	(19)	50	(2)	13·5	(1)	8	(7)
L. weilii	47	(1)	11·5	(1)	7	(14)	50	(1)	13·5	(1)	5	(8)	90	(8)	1·5	(8)	86	(14)
L. inadai	5	(1)			0	(3)	5	(1)			0	(3)	7	(1)			0	(1)
L. meyeri	1	(1)			1	(3)					1	(3)	4	(1)			1	(1)
Leptonema illini					0	(4)					0	(3)					0	(1)
L. wolbachii					0	(3)	2	(3)			0	(3)	4	(1)			0	(1)
L. biflexa	3	(1)			1	(3)	11	(1)			1	(3)	5	(1)			3	(1)
L. parva	0	(1)			0	(4)	0	(1)			0	(3)					0	(1)
Leptospira genomospecies 1	33	(1)			2	(2)	21	(1)			4	(3)	43	(1)			3	(1)
Leptospira genomospecies 2					2	(3)					1	(4)	41	(1)	6·0	(1)	16	(1)
Leptospira genomospecies 3	5	(1)			1	(1)	4	(1)			1	(3)	5	(1)			0	(1)
Leptospira genomospecies 4	4	(1)			1	(1)	4	(1)			1	(3)	4	(1)				
Leptospira genomospecies 5	2	(1)					2	(1)					1	(1)				
	L. inadai						***L. meyeri***						***Leptonema illini***					
L. interrogans	5	(1)			2	(10)	4	(3)			2	(2)	7	(1)			8	(3)
L. santarosai	5	(1)			2	(10)	3	(1)			3	(5)					5	(3)
L. borgpetersenii	6	(1)			3	(10)	4	(2)			6	(4)	4	(1)			4	(2)
L. kirschneri					3	(10)					1	(2)					4	(3)
L. noguchii	7	(1)			3	(10)	3	(2)			3	(4)	6	(1)			2	(2)
L. weilii					3	(7)	5	(3)			5	(4)	14	(1)			5	(1)
L. inadai	96	(9)	0·5	(9)	93	(9)	3	(3)			4	(2)	6	(1)			0	(3)
L. meyeri	1	(1)			1	(10)	94	(4)	1·0	(3)	92	(3)	3	(1)			1	(3)
Leptonema illini					0	(1)					0	(2)	92	(2)	1·5	(2)	68	(2)
L. wolbachii					1	(1)	41	(3)	11·5	(1)	14	(2)	2	(1)			1	(2)
L. biflexa	3	(1)			1	(9)	30	(2)			3	(3)	0	(1)			1	(3)
L. parva	0	(1)			0	(1)	1	(3)			2	(1)	2	(1)			0	(3)
Leptospira genomospecies 1	6	(1)			0	(1)	5	(3)			0	(1)	1	(1)			0	(1)
Leptospira genomospecies 2					0	(1)					1	(2)					10	(1)
Leptospira genomospecies 3	5	(1)			1	(1)	57	(2)	11·0	(2)	28	(2)	4	(1)			0	(1)
Leptospira genomospecies 4	4	(1)			0	(1)	54	(2)			14	(1)	2	(1)			1	(1)
Leptospira genomospecies 5	1	(1)					29	(2)					2	(1)				

higher than those observed between species. In no case do strains not included in a given species fulfil more than one of the three criteria used here for the molecular definition of a species (70 % or greater relatedness at 55 °C, 5 % or less divergence within related sequences and 60 % or greater relatedness at 70 °C). Examples of closely related species are *L. interrogans* and *L. noguchii*, *L. borgpetersenii* and

Table 3 (*cont.*)

Source of labelled DNA	Relatedness (%) to unlabelled DNAs from:																	
	55 °C	(*n*)	D	(*n*)	70 °C	(*n*)	55 °C	(*n*)	D	(*n*)	70 °C	(*n*)	55 °C	(*n*)	D	(*n*)	70 °C	(*n*)
	L. wolbachii						***L. biflexa***						***L. parva***					
L. interrogans	7	(1)			2	(2)	27	(1)			9	(2)	0	(1)			2	(1)
L. santarosai					2	(2)					3	(2)	1	(1)				
L. borgpetersenii					6	(2)	9	(2)			0	(1)	2	(1)			1	(1)
L. kirschneri					2	(1)					1	(2)					1	(1)
L. noguchii	5	(1)			5	(2)	21	(2)			3	(2)	1	(1)			3	(1)
L. weilii					7	(2)	13	(2)			6	(2)	3	(1)			7	(1)
L. inadai	11	(1)			1	(2)	4	(1)			4	(1)	7	(1)			0	(1)
L. meyeri	25	(1)			16	(2)					4	(1)					1	(1)
Leptonema illini					0	(1)					0	(1)					0	(1)
L. wolbachii	100	(1)	2·0	(1)	92	(1)	15	(2)			6	(1)					1	(1)
L. biflexa	21	(1)			13	(1)	98	(1)	1·0	(1)	74	(1)	0	(1)			2	(1)
L. parva	1	(1)			0	(1)	0	(1)			0	(1)						
Leptospira genomospecies 1	2	(1)			0	(1)					0	(1)	2	(1)			0	(1)
Leptospira genomospecies 2					1	(1)					0	(1)					0	(1)
Leptospira genomospecies 3	74	(1)	6·0	(1)	45	(1)	41	(1)			6	(1)	8	(1)			0	(1)
Leptospira genomospecies 4	58	(1)	10·5	(1)	20	(1)	39	(1)			5	(1)	2	(1)			1	(1)
Leptospira genomospecies 5	26	(1)			41	(1)							1	(1)				
	***Leptospira* genomospecies 1**						***Leptospira* genomospecies 2**						***Leptospira* genomospecies 3**					
L. interrogans					7	(1)					5	(5)					4	(1)
L. santarosai					6	(1)	66	(1)			18	(6)					1	(1)
L. borgpetersenii					25	(1)	70	(5)	6·5	(5)	45	(6)					1	(1)
L. kirschneri					7	(1)					7	(5)					1	(1)
L. noguchii					5	(1)					5	(5)					2	(1)
L. weilii					18	(1)					42	(5)					2	(1)
L. inadai					1	(1)					1	(4)					1	(1)
L. meyeri					0	(1)					1	(5)					12	(1)
Leptonema illini					0	(1)					1	(5)					0	(1)
L. wolbachii					1	(1)					0	(5)	60	(1)	6·5	(1)	48	(1)
L. biflexa					0	(1)					1	(5)					6	(1)
L. parva					1	(1)					1	(6)					0	(1)
Leptospira genomospecies 1	98	(1)					40	(1)			16	(1)	3	(1)				
Leptospira genomospecies 2					11	(1)	79	(5)	1·0	(5)	82	(5)					2	(1)
Leptospira genomospecies 3	9	(1)			0	(1)	4	(1)										
Leptospira genomospecies 4	2	(1)					4	(1)					55	(1)			25	(1)
Leptospira genomospecies 5	2	(2)					1	(1)					26	(1)				
	***Leptospira* genomospecies 4**						***Leptospira* genomospecies 5**											
L. interrogans					7	(1)					3	(1)						
L. santarosai					1	(1)					1	(1)						
L. borgpetersenii					1	(1)					2	(1)						
L. kirschneri					1	(1)												
L. noguchii					2	(1)					2	(1)						
L. weilii					6	(1)					4	(1)						
L. inadai					1	(1)					3	(1)						
L. meyeri					14	(1)					7	(1)						
Leptonema illini					0	(1)					0	(1)						
L. wolbachii	10	(1)	10·5	(1)	21	(1)					8	(1)						
L. biflexa					5	(1)												
L. parva					0	(1)					0	(1)						
Leptospira genomospecies 1	4	(1)			0	(1)												
Leptospira genomospecies 2					1	(1)					1	(1)						
Leptospira genomospecies 3	63	(1)	13·5	(1)	20	(1)												
Leptospira genomospecies 4																		
Leptospira genomospecies 5	28	(1)																

Leptospira genomospecies 2, *L. weilii* and *L. borgpetersenii*, and *L. wolbachii* and *Leptospira* genomospecies 3.

The results of this study confirmed and extended our previous finding (Yasuda *et al*., 1987) and that of Ramadass *et al*. (1992) of species heterogeneity among serovars of a given serogroup. Of a total of 24 serogroups, in which two or more serovars were tested, only the seven strains in serogroup Ballum were confined to a single species (Table 4). All other serogroups in which more than one serovar were tested were identified in from two to six species. Similarly, all species containing more than one serovar contained serovars belonging to two to seventeen serogroups (Table 4). Species heterogeneity was also found when multiple strains of a single serovar were tested (Table 5). Two to six strains from each of 20 serovars were tested. In only eight of these did all strains belong to the same species. It is therefore not possible to determine the species of any serogroup or serovar without specifically identifying the strain.

Table 4. Distribution of serogroups among leptospire species

Species: 1, *L. interrogans*; 2, *L. santarosai*; 3, *L. borgpetersenii*; 4, *L. kirschneri*; 5, *L. noguchii*; 6, *L. weilii*; 7, *L. inadai*; 8, *L. meyeri*; 9, *Leptonema illini*; 10, *L. wolbachii*; 11, *L. biflexa*; 12, *L. parva*; 13, Genomospecies 1; 14, Genomospecies 2; 15, Genomospecies 3; 16, Genomospecies 4; 17, Genomospecies 5.

Serogroup	No. of strains	No. of strains in species																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Andamana	1											1						
Australis	15	9		1	1	4												
Autumnalis	16	8	1	1	5	1												
Ballum	7			7														
Bataviae	15	5	5	1	1	2				1								
Canicola	16	12			3			1										
Celledoni	5			2			3											
Codice	1										1							
Cynopteri	3		2		1													
Djasiman	4	2			1	1												
Grippotyphosa	11	3	1		7													
Hebdomadis	23	2	12	4	2		1								2			
Icterohaemorrhagiae	26	18		1		5		1									1	
Javanica	19		3	10			3	1	1						1			
Leptonema	1									1								
Louisiana	3	1				2												
Lyme	1							1										
Manhao	5						1	2							2			
Mini	10	1	5	1			1		1						1			
Panama	3					2		1										
Pomona	15	8	2		3	2												
Pyrogenes	20	8	8	2		1	1											
Ranarum	3	1							1					1				
Sarmin	5	1	3				1											
Sejroe	27	10	5	10			1		1									
Semaranga	3								1		1							1
Shermani	5		3			1		1										
Tarassovi	24		12	7		1	3	1										
Turneria	1												1					
Undesignated	15	2	3	2		3		1		1	1			1		1		

Our previous study indicated that the biochemical tests used to differentiate *L. interrogans sensu lato* from *L. biflexa sensu lato* were of no value in differentiating the then 11 species of *Leptospiraceae* (Yasuda *et al.*, 1987). This remains true for *L. kirschneri*, described by Ramadass *et al.* (1992), and for the five new *Leptospira* genomospecies identified in the present study. Results of phenotypic characterization of 119 strains are presented in Table 6. Only one of six *L. inadai* strains grew at 11 °C and only four of the 119 strains grew well at 37 °C. Although all leptospires grow slowly, all strains grew well at 30 °C. Strains of *L. inadai*, *L. biflexa* and *L. wolbachii* were variable for growth in the presence of the inhibitory compound 8-azaguanine. *L. meyeri* (two strains tested) and single strains of *L. parva*, *Leptonema illini* and *Leptospira* genomospecies 4 and 5 grew in the presence of 8-azaguanine; strains of all other species did not grow. Only *L. meyeri* (two strains) grew in the presence of the inhibitor 2,6-diaminopurine. Strains of *L. interrogans*, *L. santarosai*, *L. borgpetersenii*, *L. noguchii*, *L. weilii*, *L. inadai* and *L. biflexa* gave variable results for growth in 2,6-diaminopurine; strains of the remaining species did not grow. Growth in the presence of copper sulfate was positive in two *L. meyeri* strains and in single strains of *L. parva*, *Leptospira* genomospecies 4 and *Leptospira* genomospecies 5; growth was negative in strains of *L. interrogans*, *L. santarosai*, *L. borgpetersenii*, *L. noguchii*, *Leptonema illini*, *Leptospira* genomospecies 1 and *Leptospira* genomospecies 3 and variable in strains of the other species. The presence of lipase activity was variable among species; strains of *L. interrogans*, *L. noguchii*, *L. inadai*, *L. kirschneri* and *Leptospira* genomospecies 2 were variable, although largely positive. The small number of strains tested in *L. biflexa*, *L. meyeri*, *L. wolbachii*, *L. parva* and *Leptonema illini* all possessed lipase activity, whereas strains of *L. borgpetersenii*, *L. santarosai*, *L. weilii* and *Leptospira*

Table 5. Species distribution of serovars in which multiple strains were tested

Figures in parentheses indicate the number of strains in each species.

Serovar	No. strains tested	Species to which serovars belong
alexi	2	*L. santarosai* (2)
balcanica	2	*L. borgpetersenii* (2)
ballum	2	*L. borgpetersenii* (2)
bataviae	2	*L. interrogans* (1), *L. santarosai* (1)
borincana	4	*L. santarosai* (4)
bulgarica	2	*L. interrogans* (1), *L. kirschneri* (1)
canicola	2	*L. interrogans* (2)
copenhageni	3	*L. interrogans* (3)
grippotyphosa	5	*L. kirschneri* (4), *L. interrogans* (1)
hardjo	5	*L. borgpetersenii* (3), *L. interrogans* (1), *L. meyeri* (1)
icterohaemorrhagiae	3	*L. interrogans* (2), *L. inadai* (1)
kremastos	2	*L. interrogans* (1), *L. santarosa*i (1)
maru	3	*L. santarosai* (3)
monymusk	2	*L. interrogans* (2)
mwogolo	2	*L. kirschneri* (1), *L. interrogans* (1)
pomona	6	*L. interrogans* (5), *L. noguchii* (1)
pyrogenes	2	*L. interrogans* (1), *L. santarosai* (1)
szwajizak	2	*L. interrogans* (1), *L. santarosai* (1)
valbuzzi	2	*L. interrogans* (1), *L. kirschneri* (1)

Table 6. Phenotypic characteristics of leptospires

8-AG, 8-Azaguanine; 2,6-DAP, 2,6-diaminopurine; −, 10 % or fewer strains positive; +, 90 % or more strains positive; v, 10–89 % strains positive.

Species	No. of strains	Growth at temp. (°C) of:			Growth in the presence of:			Lipase activity
		11	30	37	8-AG	2,6-DAP	Copper sulfate	
L. interrogans	28	−	+	−	−	v	−	v
L. santarosai	28	−	+	−	−	v	−	−
L. borgpetersenii	16	−	+	−	−	v	−	−
L. noguchii	8	−	+	−	−	v	−	v
L. weilii	6	−	+	−	−	v	v	−
L. inadai	6	v	+	v	v	v	v	v
L. kirschneri	6	−	+	−	−	−	v	v
L. biflexa	3	−	+	v	v	v	v	+
L. meyeri	2	−	+	+	+	+	+	+
L. wolbachii	2	−	+	−	v	−	v	+
Leptonema illini	2	−	+	v	+	−	−	+
L. parva	1	−	+	+	+	−	+	+
Genomospecies 1	2	−	+	−	−	−	−	−
Genomospecies 2	5	−	+	−	−	−	v	v
Genomospecies 3	1	−	+	−	−	−	−	−
Genomospecies 4	1	−	+	−	+	−	+	−
Genomospecies 5	1	−	+	−	+	−	+	−

genomospecies 1, 3, 4 and 5 lacked lipase activity. These reactions are presently of little or no value in differentiating species of *Leptospiraceae*.

Our findings, although quite preliminary, suggest that the geographic distribution of species is not totally random. There are eight species in which we have identified six or more strains (Table 1). The six strains of *Leptospira* genomospecies 2 were all isolated in China. All but three of the 65 strains of *L. santarosai* were from North and South America, as were all but two of the 20 strains of *L. noguchii*. Nearly half of the 91 *L. interrogans* strains were isolated from countries in Oceania, representing more than 70% of the total isolates from this area. Additional study is needed to determine whether other factors are responsible for this restricted distribution. Thus far, all 37 African *Leptospira* strains characterized by DNA hybridization in this study or in the studies of Feresu belonged to either *L. kirschneri* or *L. santarosai* (Table 1; Feresu *et al.*, 1993, 1994, 1995, 1996, 1998, 1999). While these observations are intriguing, additional studies with substantially more strains are necessary to determine their accuracy.

The study of Yasuda *et al.* (1987) on speciation of leptospires was extended by Ramadass *et al.* (1992) who used slot-blot hybridization. They described the new species, *L. kirschneri*, with serovar cynopteri strain 3522C as its type strain. In the present study, all serovars used by Ramadass *et al.* (1992) were examined, except for serovar sumatrana, which they identified as *L. interrogans* and serovar vietnam, which they identified as *L. borgpetersenii*. Ramadass *et al.* (1992) did not speciate strains from nine serovars. One of these, serovar nicaragua strain 1011, was 100% related to *L. noguchii* according to their data, and we identified it as *L. noguchii*. Six strains, serovar atchafalaya strain LSU 1013, serovar borincana strain HS 622, serovar bravo strain Bravo, serovar gatuni strain 1473K, serovar luis strain M 6 and serovar rama strain 316, were identified by us as *L. santarosai*. Four of these gave high, but less than species-level relatedness to *L. santarosai* in the Ramadass *et al.* (1992) study. Serovar ballum strain Mus 127, which was most highly related to *L. borgpetersenii* in their study, was identified as *L. borgpetersenii* in our study and that of Yasuda *et al.* (1987). Serovar szwajizak strain Szwajizak, which was not highly related to any species in their study, was identified as *L. interrogans* in our study.

Of the 55 other strains identified in the study of Ramadass *et al.* (1992), their identification of seven strains differed from that obtained in either the present study and/or the study of Yasuda *et al.* (1987). They identified serovar atlantae strain LT 81 as *L. interrogans*, whereas it was identified as *L. santarosai* by Yasuda *et al.* (1987) and in our study. We have no explanation for this discrepancy. Ramadass *et al.* (1992) identified serovar grippotyphosa strain Moskva V as *L. kirschneri*. This strain was reported as *L. interrogans* by Yasuda *et al.* (1987). We confirm its identification as *L. kirschneri*. This discrepancy resulted from a strain designation error by Yasuda *et al.* (1987). They used serovar grippotyphosa strain Andaman, which is *L. interrogans*, but mistakenly reported it as strain grippotyphosa, which is *L. kirschneri*. Another inconsistency, due to an apparent strain designation error, is responsible for a discrepancy in the identification of serovar saxkoebing. Ramadass *et al.* (1992) reported its reference strain as M 84 and identified it as *L. borgpetersenii*, whereas Yasuda *et al.* (1987) and in our study, serovar saxkoebing was identified as *L. interrogans*. M 84 is the reference strain for serovar sejroe, not serovar saxkoebing, and serovar sejroe is *L. borgpetersenii*.

We have not resolved the four remaining inconsistencies. Ramadass *et al.* (1992) identified serovar dania strain K1 as *L. kirschneri*; we identified it as *L. santarosai*. They identified serovar muenchen strain München C 90 as *L. noguchii*; we identified it as *L. santarosai*. They identified serovar tunis strain P2/65 as *L. santarosai*; we identified it as *L. borgpetersenii*. They identified serovar worsfoldi strain Worsfold as *L. weilii*; we identified it as *L. borgpetersenii*.

Of the pairs of species involved in these discrepancies, only *L. weilii* and *L. borgpetersenii* pose any difficulty in differentiation if divergence of related sequences and relatedness at a stringent DNA reassociation (70 °C) are not done. Despite the substantial methodological differences in our study and that of Ramadass *et al.* (1992) (they used slot-blot hybridization only at 60 °C), it is doubtful that differences in hybridization methodology are responsible for the discrepancies.

The taxonomic DNA hybridization methods used in this study require the growth of leptospires in substantial quantity and the use of a radioactive isotope. These requirements limit the utility of these methods to very few laboratories and even then are not suited for routine use in speciation. In recent years, several molecular approaches have been developed for the identification of leptospires at the species and serovar level. These include whole chromosome restriction endonuclease patterns, PCR amplification of 23S rDNA and artibrarily primed DNA fingerprinting (Marshall *et al.*, 1981; Feresu *et al.*, 1994, 1995; Corney *et al.*, 1997; Woo *et al.*, 1997). Where compared, the results obtained using these methods are comparable to those obtained using DNA hybridization. These methods are significantly less resource-intensive and, therefore, more widely applicable. The findings presented in the present study should provide a molecular taxonomic framework for the continued development and application of these new identification approaches.

The five new *Leptospira* genomospecies identified in this study are described below. Only one of these, *Leptospira* genomospecies 2, is formally named, because the other genomospecies presently contain only one or two strains.

Description of *Leptospira alexanderi* sp. nov. (*Leptospira* genomospecies 2)

Leptospira alexanderi (a.lex.an′der.i. N.L. gen. n. *alexanderi* to honour Aaron D. Alexander, an

American microbiologist who has devoted more than 40 years to the study of leptospires). The first leptospiral DNA hybridization studies that were conducted in Professor Alexander's laboratory in 1969 and 1974 (Haapala *et al.*, 1969; Brendle *et al.*, 1974) inspired the study of Yasuda *et al.* (1987) and the present study.

Cells are Gram-negative, flexible and helical. Motile by means of two flagella (axial fibriles). Obligately aerobic and oxidase-positive. NaCl is not required for growth. No growth at 11 or 37 °C. Growth is inhibited by 8-azaguanine (225 μg ml^{-1}) and 2,6-diaminopurine (10 μg ml^{-1}). Growth in the presence of copper sulfate (100 p.p.m.) and production of lipase are variable. It contains serovars from serogroup Manhao (serovar lushui and manhao 3), Hebdomadis (serovar manzhuang and nanding), Javanica (serovar mengla) and Mini (serovar yunnan). DNA G+C content is 38·0 mol%. All strains isolated to date are from China. The type strain, L 60^T (= ATCC 700520^T; serovar manhao 3) was isolated in China from an unknown source. DNA relatedness among the strains in the species and their relatedness to other leptospires are shown in Tables 2 and 3.

Description of *Leptospira* genomospecies 1

Cells are Gram-negative, flexible and helical. Motile by means of two flagella (axial fibriles). Obligately aerobic and oxidase-positive. NaCl is not required for growth. No growth at 11 or 37 °C. Growth is inhibited by 8-azaguanine (225 μg ml^{-1}), 2,6-diaminopurine (10 μg ml^{-1}) and copper sulfate. Lipase is not produced. It contains serovars from serogroups Ranarum and an as yet undesignated serogroup. DNA G+C content is 39·8 mol%. The type strain is 79601^T (= ATCC 700521^T; serovar sichuan), isolated in China from a frog. The DNA relatedness relationships of the strains in the species and their relatedness to other leptospires are shown in Tables 2 and 3.

Description of *Leptospira* genomospecies 3

Cells are Gram-negative, flexible and helical. Motile by means of two flagella (axial fibriles). Obligately aerobic and oxidase-positive. NaCl is not required for growth. No growth at 11 or 37 °C. Growth is inhibited by 8-azaguanine (225 μg ml^{-1}), 2,6-diaminopurine (10 μg ml^{-1}) and copper sulfate. Lipase is not produced. It contains serovars from an as yet undesignated serogroup. The type and only strain isolated to date, is WaZ $Holland^T$ (= ATCC 700522^T; serovar holland), isolated from water in the Netherlands. DNA G+C content is 43·4 mol%. The DNA relatedness relationships to other leptospires are shown in Tables 2 and 3.

Description of *Leptospira* genomospecies 4

Cells are Gram-negative, flexible and helical. Motile by means of two flagella (axial fibriles). Obligately aerobic and oxidase-positive. NaCl is not required for growth. No growth at 11 or 37 °C. Growth is inhibited in the presence of 2,6-diaminopurine (10 μg ml^{-1}), but not by 8-azaguanine (225 μg ml^{-1}) or copper sulfate. Lipase is not produced. It contains serovars from serogroup Icterohaemorrhagiae. The type and only strain isolated to date is H 2^T (= ATCC 700639^T; serovar hualin), isolated from an unknown source in China. DNA G+C content is 38·9 mol%. The DNA relatedness relationships to other leptospires are shown in Tables 2 and 3.

Description of *Leptospira* genomospecies 5

Cells are Gram-negative, flexible and helical. Motile by means of two flagella (axial fibriles). Obligately aerobic and oxidase-positive. NaCl is not required for growth. No growth at 11 or 37 °C. Growth is inhibited in the presence of 2,6-diaminopurine (10 μg ml^{-1}), but not by 8-azaguanine (225 g ml^{-1}) or copper sulfate. Lipase is not produced. It contains serovars from serogroup Semaranga. The type strain is Sao $Paulo^T$ (= ATCC 700523^T; serovar saopaulo), isolated in Brazil from water. DNA G+C content is 37·9 mol%. The DNA relatedness relationships to other leptospires are shown in Tables 2 and 3.

REFERENCES

Brendle, J. J., Rogul, M. & Alexander, A. D. (1974). Deoxyribonucleic acid hybridization among selected leptospiral serotypes. *Int J Syst Bacteriol* **24**, 205–214.

Brenner, D. J. (1991). Taxonomy, classification, and nomenclature of bacteria. In *Manual of Clinical Microbiology*, 5th edn, pp. 209–215. Edited by A. Balows, W. J. Hausler, Jr, K. L. Herrman, H. D. Isenberg & H. J. Shadomy. Washington, DC: American Society for Microbiology.

Brenner, D. J., McWhorter, A. C., Leete-Knutson, J. K. & Steigerwalt, A. G. (1982). *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. *J Clin Microbiol* **15**, 1133–1140.

Brenner, D. J., Grimont, P. A. D., Steigerwalt, A. G., Fanning, G. R., Ageron, E. & Riddle, C. F. (1993). Classification of citrobacteria by DNA hybridization: designation of *Citrobacter farmeri* sp. nov., *Citrobacter youngae* sp. nov., *Citrobacter braakii* sp. nov., *Citrobacter werkmanii* sp. nov., *Citrobacter sedlakii* sp. nov., and three unnamed *Citrobacter* genomospecies. *Int J Syst Bacteriol* **43**, 645–658.

Corney, B. G., Colley, J. & Graham, G. C. (1997). Simplified analysis of pathogenic leptospiral serovars by random amplified polymorphic DNA fingerprinting. *J Med Microbiol* **46**, 927–932.

Feresu, S. B., Bolin, C. A. & Korver, H. (1993). A new leptospiral serovar in the Icterohaemorrhagiae serogroup isolated from an ox in Zimbabwe. *Int J Syst Bacteriol* **43**, 179–182.

Feresu, S. B., Bolin, C. A., Korver, H. & Terpstra, W. J. (1994). Classification of leptospires of the Pyrogenes serogroup isolated from cattle in Zimbabwe by cross-agglutinin absorption and restriction fragment length polymorphism analysis. *Int J Syst Bacteriol* **44**, 541–546.

Feresu, S. B., Bolin, C. A., Korver, H. & Van De Kemp, H. (1995). Identification of leptospires of the Pomona and Grippotyphosa serogroups isolated from cattle in Zimbabwe. *Res Vet Sci* **59**, 92–94.

Feresu, S. B., Korver, H., Riquelme, N., Baranton, G. & Bolin, C. A. (1996). Two new leptospiral serovars in the Hebdomadis serogroup isolated from Zimbabwe cattle. *Int J Syst Bacteriol* **46**, 694–698.

Feresu, S. B., Bolin, C. A. & Korver, H. (1998). A new leptospiral serovar, ngavi, in the Tarassovi serogroup isolated from Zimbabwe oxen. *Int J Syst Bacteriol* **48**, 207–213.

Feresu, S. B., Steigerwalt, A. G. & Brenner, D. J. (1999). DNA relatedness of *Leptospira* strains isolated from beef cattle in Zimbabwe. *Int J Syst Bacteriol* (in press).

Haapala, D. K., Rogul, M., Evans, L. B. & Alexander, A. D. (1969). Deoxyribonucleic acid base composition and homology studies of *Leptospires*. *J Bacteriol* **98**, 421–428.

Hovind-Hougen, K. (1979). *Leptospiraceae*, a new family to include *Leptospira* Noguchi 1917 and *Leptonema* gen. nov. *Int J Syst Bacteriol* **29**, 245–251.

Hovind-Hougen, K., Ellis, W. A. & Birch-Andersen, A. (1981). *Leptospira parva* sp. nov.: some morphological and biological characters. *Zentbl Bakteriol Parasitenkd Infektionskr Hyg Abt* 1 *Orig Reihe A* **250**, 343–354.

Johnson, R. C. & Faine, S. (1984). Family II. *Leptospiraceae*. In *Bergey's Manual of Systematic Bacteriology*, pp. 62–67. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Kmety, E. & Dikken, H. (1988). Revised list of *Leptospira* serovars. I. Alphabetical order. 14 pp. Groningen: University Press.

Kmety, E. & Dikken, H. (1993). Classification of the species *Leptospira interrogans* and history of its serovars. 104 pp. Groningen: University Press.

Mandel, M., Igambi, L., Bergendahl, J., Dodson, M. L., Jr & Scheltgen, E. (1970). Correlation of melting temperature and cesium chloride buoyant density of bacterial deoxyribonucleic acid. *J Bacteriol* **101**, 333–338.

Marshall, R. (1992). International Committee on Systematic Bacteriology Subcommittee on the Taxonomy of *Leptospira*. Minutes of the Meetings, 13 and 15 September 1990, Osaka, Japan. *Int J Syst Bacteriol* **42**, 330–334.

Marshall, R. B., Wilton, B. E. & Robinson, A. J. (1981). Identification of leptospiral serovars by restriction endonuclease analysis. *J Med Microbiol* **14**, 163–166.

Ramadass, P., Jarvis, B. D. W., Corner, R. J., Penny, D. & Marshall, R. B. (1992). Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. *Int J Syst Bacteriol* **42**, 215–219.

Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Woo, T. H., Smythe, L. D., Symonds, M. L., Norris, M. A., Dohnt, M. F. & Patel, B. K. (1997). Rapid distinction between *Leptospira interrogans* and *Leptospira biflexa* by PCR amplification of 23S ribosomal DNA. *FEMS Microbiol Lett* **150**, 9–18.

Yasuda, P. H., Steigerwalt, A. G., Sulzer, K. R., Kaufmann, A. F., Rogers, F. & Brenner, D. J. (1987). Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. *Int J Syst Bacteriol* **37**, 407–415.