Revised group classification of the genus *Spiroplasma*

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Significant changes have been made in the systematics of the genus *Spiroplasma* (class *Mollicutes*) since it was expanded by revision in 1987 to include 23 groups and eight sub-groups. Since that time, two additional spiroplasmas have been assigned group numbers and species names. More recently, specific epithets have been assigned to nine previously designated groups and three sub-groups. Also, taxonomic descriptions and species names have been published for six previously ungrouped spiroplasmas. These six new organisms are: *Spiroplasma alleghenense* (strain PLHS-1^T) (group XXVI), *Spiroplasma lineolae* (strain TALS-2^T) (group XXVII), *Spiroplasma platyhelix* (strain PALS-1^T) (group XXVIII), *Spiroplasma montanense* (strain HYOS-1^T) (group XXXI), *Spiroplasma helicoides* (strain TABS-2^T) (group XXXII) and *Spiroplasma tabanidicola* (strain TAUS-1^T) (group XXXIII). Also, group XVII, which became vacant when strain DF-1^T (*Spiroplasma chrysopicola*) was transferred to group VIII, has been filled with strain Tab 4c. The discovery of these strains reflects continuing primary search in insect reservoirs, particularly horse flies and deer flies (Diptera: Tabanidae). In the current revision, new group designations for 10 spiroplasma strains, including six recently named organisms, are proposed. Three unnamed but newly grouped spiroplasmas are strain TIUS-1 (group XXIX; ATCC 51751) from a typhiid wasp (Hymenoptera: Tiphiidae), strain BIUS-1 (group XXX; ATCC 51750) from floral surfaces of the tickseed sunflower (*Bidens* sp.) and strain BARC 1901 (group XXXIV; ATCC 700283). Strain BARC 2649 (ATCC 700284) from *Tabanus lineola* has been proposed as a new sub-group of group VIII. Strains TIUS-1 and BIUS-1 have unusual morphologies, appearing as helices at only certain stages in culture. In this revision, potentially important intergroup serological relationships observed between strain DW-1 (group II) from a neotropical *Drosophila* species and certain sub-group representatives of group I spiroplasmas are also reported.

Keywords: *Spiroplasma*, *Mollicutes*

INTRODUCTION

The group classification of spiroplasmas had its beginnings in 1976 when spiroplasma workers, under the auspices of the International Research Programme

on Comparative Mycoplasma and the International Subcommittee on the Taxonomy of *Mollicutes*, agreed to determine spiroplasma species concepts cooperatively (23). Five years later, the first group classification, which included five groups and

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Abbreviations: DF test, deformation test; MI test, metabolism inhibition test.

four sub-groups, was proposed by Junca *et al.* (25). This informal classification scheme was later refined by proposal of criteria for groups and sub-groups (48) and by proposal of standards for assignment of species names to sub-groups. Three revisions of the *Spiroplasma* group classification were published (42, 48, 51), resulting in an inventory of classified spiroplasmas of 23 groups and eight sub-groups by 1987.

Since then, the Chinese flower spiroplasma (strain CCH^T) has been designated to group XXV and named *Spiroplasma chinense* (16), and the CUAS-1^T mosquito spiroplasma was assigned to group XXV and named *Spiroplasma diminutum* (67). Also during this period, nine previously established *Spiroplasma* group representatives and three sub-group representatives were further characterized and, using standard criteria (24), assigned specific epithets. These included: group I, sub-group 6 (*Spiroplasma insolitum*, strain M55^T) (21); group VII (*Spiroplasma monobiae*, strain MQ-1^T) (61); group IX (*Spiroplasma clarkii*, strain CN-5^T) (63); group XI (*Spiroplasma velocicrescens*, strain MQ-4^T) (28); group XII (*Spiroplasma diabroticae*, strain DU-1^T) (9); group XIV (*Spiroplasma corruscae*, strain EC-1^T) (20); group XVI, sub-group 1 (*Spiroplasma cantharicola*, strain CC-1^T) (49); group XVIII (*Spiroplasma litorale*, strain TN-1^T) (27), group XX (*Spiroplasma leptinotarsae*, strain LD-1^T) (19); and group XXIII (*Spiroplasma gladiatoris*, strain TG-1^T) (53).

In the case of group VIII sub-groups (13), strain EA-1^T, the initial sole representative of the group, was redesignated as group VIII, sub-group 1 and given the species name *Spiroplasma syrphidicola* (56). Strain DF-1^T (previously assigned to group XVII) was redesignated as group VIII, sub-group 2, and designated *Spiroplasma chrysopicola* (53). A tabanid strain (TAAS-1) was designated VIII-3. These changes left group XVII vacant. A spiroplasma (strain Tab 4c) from tabanid flies in France has recently been characterized and found to be unrelated to other established groups or sub-groups. Strain Tab 4c has been assigned to group XVII (22).

More recently, eight additional spiroplasma strains were reported to be unassignable to any of the existing 25 groups (71). Six of these strains were considered to represent significant additions to the genus *Spiroplasma* and have been recently named. These include group XXVI (*Spiroplasma alleghenense*, strain PLHS-1^T) (2), group XXVII (*Spiroplasma lineolae*, strain TALS-1^T) (12), group XXVIII (*Spiroplasma platyhelix*, strain PALS-1^T) (65), group XXXI (*Spiroplasma montanense*, strain HYOS-1^T) (54), group XXXII (*Spiroplasma helicoides*, strain TABS-2^T) (53) and group XXXIII (*Spiroplasma tabanidicola*, strain TAUS-1^T) (53).

In this revision, we present a revised *Spiroplasma* group classification, and list species names (where available), type strains and numbers, major biochemical and genomic characteristics, and host origin of the

organisms. We also report experimental work fulfilling criteria (48) for designation of three new spiroplasma groups. These new groups represent strains from both plants and insects, including strain TIUS-1 (group XXIX, ATCC 51751) from a typhiid wasp (Hymenoptera: Tiphidae) and strain BIUS-1 (group XXX, ATCC 51750) from the floral surface of *Bidens* (tickseed sunflower) flowers. Strains TIUS-1 and BIUS-1 have unusual morphologies and/or growth cycles in culture. We also provide a group assignment (XXXIV) for a new strain, BARC 1901 (ATCC 700283) (52), from *Tabanus lineola* horse flies. Strain BARC 2649 (ATCC 700284), which appeared at first to represent a new group, is identified as a representative of a candidate new sub-group of group VIII.

We also report some recent and potentially important intergroup relationships between the group II representative strain DW-1 (the sex ratio spiroplasma from a neotropical *Drosophila* species) and various members of the eight sub-groups within group I spiroplasmas.

METHODS

Spiroplasma strains. The plant and insect spiroplasma strains whose properties are described here were isolated using standard techniques (11, 29). Strain TIUS-1 was isolated from an unknown species of the insect family Tiphidae (order Hymenoptera) collected by T. B. Clark on 27 May 1983, in Beltsville, MD, USA. After primary isolation, the strain was thought to be non-helical, but helical forms of the organism were noted after several passages in culture media. Strain BIUS-1 was isolated from a *Bidens* sp. flower surface by K. J. Hackett on 11 September 1983, in Beltsville, MD, USA. Strains BARC 1901 and BARC 2649 were isolated from gut contents of *Tabanus lineola* collected in Georgia, USA, in 1995 by F. E. French and colleagues (52).

Culture medium and cultivation techniques. Spiroplasmas were grown in M1D liquid broth (47) at 30 °C, a temperature at which most spiroplasma species grow well (26). The strains were cloned (38, 58) in M1D broth and on M1D solid medium. Other culture media used were SP-4 (47), conventional horse serum medium, and serum-fraction broth containing 1% bovine serum fraction (Difco) (39).

Morphological studies. Cells of strains cultivated in M1D broth were examined during exponential phase at a magnification of $\times 1250$ by dark-field microscopy (37). For electron microscopic examination of membrane structure, exponential-phase cultures of approximately 2 ml in M1D broth were pelleted by centrifugation at 4 °C. Most pellets were fixed for 2 h in 3% glutaraldehyde in phosphate buffer (pH 7.3) supplemented with 0.25 M sucrose. In some cases, higher molarities of sucrose were used. The pellets were then postfixed in Palade-buffered 1% osmium tetroxide for 1 h, dehydrated in acetone and embedded in Spurr low-viscosity embedding medium. The prepared blocks were sectioned at 60–90 nm, and sections were stained with 1% aqueous uranyl acetate and Reynold's lead citrate (64).

Serological tests. Antiserum to the four spiroplasmas was raised in rabbits as previously described (68). Hyperimmune antisera to all established groups, putative groups, and sub-

Table 1. Revised classification of spiroplasmas

Group	Species	Representative strain (ATCC)	G + C (± 1 mol %)	Glucose/arginine	Host/habitat
I-1	<i>S. citri</i>	Maroc-R8A2 ^T (27556)	26	+ / +	Phloem/leafhopper
I-2	<i>S. melliferum</i>	BC-3 ^T (33219)	26	+ / +	Honey bee
I-3	<i>S. kunkelii</i>	E275 ^T (29320)	26	+ / +	Phloem/leafhopper
I-4		277F (29761)	26	+ / +	Rabbit tick
I-5		LB-12 (33649)	26	+ / -	Plant bug
I-6	<i>S. insolitum</i>	M55 ^T (33502)	26	+ / -	Flower surface
I-7		N525 (33287)	26	+ / +	Plant surface
I-8	<i>S. phoeniceum</i>	P40 ^T (43115)	26	+ / +	Phloem/vector
II		DW-1 (43153)	26	ND	<i>Drosophila</i>
III	<i>S. floricola</i>	23-6 ^T (29989)	26	+ / -	Plant surface
IV	<i>S. apis</i>	B31 ^T (33834)	30	+ / -	Honey bee
V	<i>S. mirum</i>	SMCA ^T (29335)	30	+ / +	Rabbit tick
VI	<i>S. ixodetis</i>	Y32 ^T (33835)	25	+ / -	Ixodid tick
VII	<i>S. monobiae</i>	MQ-1 ^T (33825)	28	+ / -	<i>Monobia</i> wasp
VIII-1	<i>S. syrphidicola</i>	EA-1 ^T (33826)	30	+ / +	Syrphid fly
VIII-2	<i>S. chrysopicola</i>	DF-1 ^T (43209)	29	+ / -	Deer fly
VIII-3		TAAS-1 (51123)	31	+ / +	Horse fly
IX	<i>S. clarkii</i>	CN-5 ^T (33827)	29	+ / +	Green June beetle
X	<i>S. culicicola</i>	AES-1 ^T (35112)	26	+ / -	Mosquito
XI	<i>S. velovirescens</i>	MQ-4 ^T (35262)	26	+ / -	<i>Monobia</i> wasp
XII	<i>S. diabroticae</i>	DU-1 ^T (43210)	25	+ / -	Beetle
XIII	<i>S. sabaudiense</i>	Ar-1343 ^T (43303)	29	+ / -	Mosquito
XIV	<i>S. corruscae</i>	EC-1 ^T (43212)	26	+ / -	Horse fly/beetle
XV		I-25 (43262)	26	+ / -	Leafhopper
XVI-1	<i>S. cantharicola</i>	CC-1 ^T (43207)	26	+ / -	Cantharid beetle
XVI-2		CB-1 (43208)	26	+ / -	Cantharid beetle
XVI-3		Ar-1357 (51126)	26	+ / -	Mosquito
XVII		Tab 4c (700271)	25	+ / -	Horse fly
XVIII	<i>S. litorale</i>	TN-1 ^T (43211)	25	+ / -	Horse fly
XIX	<i>S. lampyridicola</i>	PUP-1 ^T (43206)	25	+ / -	Firefly
XX	<i>S. leptinotarsae</i>	LD-1 ^T (43213)	25	+ / +	Colorado potato beetle
XXI		W115 (43260)	24	+ / -	Flower surface
XXII	<i>S. taiwanense</i>	CT-1 ^T (43302)	26	+ / -	<i>Culex</i> mosquito
XXIII	<i>S. gladiatoris</i>	TG-1 ^T (43225)	26	+ / -	Horse fly
XXIV	<i>S. chinense</i>	CCH ^T (43960)	29	+ / -	Flower surface
XXV	<i>S. diminutum</i>	CUAS-1 ^T (49235)	26	+ / -	<i>Culex</i> mosquito
XXVI	<i>S. alleghenense</i>	PLHS-1 ^T (51752)	31	+ / +	Scorpionfly
XXVII	<i>S. lineolae</i>	TALS-2 ^T (51749)	25	+ / -	Horse fly
XXVIII	<i>S. platyhelix</i>	PALS-1 ^T (51748)	29	+ / +	Dragonfly
XXIX		TIUS-1 (51751)	28	+ / -	Tiphid wasp
XXX		BIUS-1 (51750)	28	+ / -	Flower surface
XXXI	<i>S. montanense</i>	HYOS-1 ^T (51745)	28	+ / +	Horse fly
XXXII	<i>S. helicoides</i>	TABS-2 ^T (51746)	27	+ / -	Horse fly
XXXIII	<i>S. tabanidicola</i>	TAUS-1 ^T (51747)	26	+ / -	Horse fly
XXXIV		BARC 1901 (700283)	25	+ / -	Horse fly
		BARC 2649 (700284)	28	+ / +	Horse fly

groups (16, 41, 42, 62, 67, 71) of the genus *Spiroplasma* (Table 1) were taken from the reference collections at the Beltsville Agricultural Research Center and the National Institute of Allergy and Infectious Diseases laboratory in Frederick, MD, USA. Antisera and spiroplasmas were tested reciprocally in metabolism inhibition (MI) (68, 69) and spiroplasma deformation (DF) tests (55, 57, 68, 69, 70).

In these tests, serological relationships between candidate strains and existing strains were indicated by reciprocal cross-reactions with the candidate strain represented by antigen or antiserum. The existence of reciprocal serological cross-reactions in both tests was deemed a reliable indicator of relationships. In rare cases, the propensity of some strains to exhibit one-way crosses made this determination difficult.

Table 2. Serological reactivities of newly proposed spiroplasma group representatives (in bold)

Antigen and antisera of the strains were tested in all heterologous combinations against all known and putative groups and sub-groups. All cross-reactions were negative (—) except where shown. Values in the 'Antigen' columns are reciprocals of end points in which the designated antigen was tested versus heterologous antiserum. Values in 'Antiserum' columns are reciprocals of end points in which the designated antiserum was tested versus heterologous antiserum.

Group/strain	Deformation		Metabolism inhibition	
	Antigen	Antiserum	Antigen	Antiserum
Strain TIUS-1 (group XXIX)				
I-3/E275 ^T	—	—	54	—
I-7/N525	20	—	—	—
II/DW-1	—	—	—	18
VIII-3/TAAS-1	—	—	18	—
XIII/Ar-1343	—	—	54	—
XIV/EC-1 ^T	—	—	54	—
XVI-2/CB-1	—	—	18	—
XVI-3/Ar-1357	—	—	18	—
XIX/PUP-1 ^T	—	—	—	162
XXI/W115	—	—	—	18
XXII/CT-1 ^T	—	20	—	—
XXVI/PLHS-1 ^T	—	—	—	162
XXVII/TALS-2 ^T	—	—	18	—
XXIX/TIUS-1	640*	640*	39000*	39000*
Strain BIUS-1 (group XXX)				
I-1/R8A2 ^T	20	—	—	—
I-4/277F	—	—	—	1458
I-5/LB-12	—	—	—	54
I-7/N525	—	—	—	54
II/DW-1	—	—	—	18
III/23-6 ^T	160	20	—	1458
IV/B31 ^T	—	—	18	—
VII/MQ-1 ^T	80	—	—	486
VIII-2/DF-1 ^T	—	—	18	—
VIII-3/TAAS-1	—	—	54	—
XI/MQ-4 ^T	20	—	—	—
XII/DU-1 ^T	1280	—	162	—
XIII/Ar-1343 ^T	—	—	18	—
XV/I-25	—	—	54	—
XVIII/TN-1 ^T	—	—	18	—
XIX/PUP-1 ^T	—	40	—	1458
XX/LD-1 ^T	—	—	—	54
XXIII/TG-1 ^T	—	—	18	—
XXVI/PLHS-1 ^T	—	40	—	1458
XXVIII/PALS-1 ^T	—	—	—	486
XXX/BIUS-1	10240*	10240*	39000*	39000*
XXXIII/TAUS-1 ^T	—	—	18	—
XXXIV/BARC 1901	160	—	—	—
BARC 2649	20	—	—	—
Strain BARC 1901 (group XXXIV)				
I-5/LB-12	—	—	18	—
I-6/M55 ^T	—	—	18	—
I-7/N525	—	—	18	—
I-8/P40 ^T	—	—	18	—

Table 2 (cont.)

Group/strain	Deformation		Metabolism inhibition	
	Antigen	Antiserum	Antigen	Antiserum
Strain BARC 1901 (cont.)				
II/DW-1	—	—	—	486
IV/B31 ^T	—	—	—	54
VII/MQ-1 ^T	—	20	—	54
VIII-1/EA-1	—	—	18	—
VIII-2/DF-1 ^T	—	—	—	18
VIII-3/TAAS-1	—	20	—	—
IX/CN-5 ^T	—	—	18	—
XIII/Ar-1343 ^T	—	—	18	—
XV/I-25	—	—	18	—
XVI-3/Ar-1357	—	—	18	—
XIX/PUP-1 ^T	—	20	—	4374
XXII/CT-1 ^T	—	—	—	18
XXVI/PLHS-1 ^T	—	—	—	54
XXVII/TALS-2 ^T	—	—	—	54
XXVIII/PALS-1 ^T	—	—	—	162
XXX/BIUS-1	—	160	—	—
XXXIV/BARC 1901	320*	320*	39000*	39000*
Strain BARC 2649				
II/DW-1	—	—	—	1458
VIII-1/EA-1 ^T	—	—	486	—
VIII-2/DF-1 ^T	—	—	—	54
VIII-3/TAAS-1	—	—	486	—
X/AES-1 ^T	—	—	18	—
XIII/Ar-1343 ^T	—	—	—	18
XVII/Tab 4c	—	—	24	—
XIX/PUP-1 ^T	—	20	—	4374
XX/LD-1 ^T	—	—	18	—
XXI/W115	—	—	18	—
XXV/CUAS-1 ^T	—	—	18	—
XXVI/PLHS-1 ^T	—	20	—	18
XXX/BIUS-1	—	20	—	—
XXXII/TABS-2 ^T	—	—	4374	—
BARC 2649	320*	320*	117000*	117000*

* Homologous titres of the strains in the respective test system. The reaction, obtained only once, is listed in each column for completeness.

On the other hand, some relationships that have been eventually established by genomic studies (5, 6) are reflected only by one-way crosses. For these reasons, it was necessary to consider the results of both MI and DF tests in assessing relationships.

Biochemical tests. Strains were examined by standard methods for their ability to utilize glucose (3), arginine (17) and urea (3). Prior to testing, each strain was adapted to conventional mycoplasma medium supplemented with 1% bovine serum fraction.

Genomic determinations. G+C content of the DNA of the strains was determined by buoyant density and melting temperature as previously described (7, 8). The genome size of some strains was measured by PFGE (5, 14).

RESULTS

Serological reactions

Four candidate strains were included in this study: TIUS-1, BIUS-1, BARC 1901 and BARC 2649. Reciprocal serological tests (Table 2) between these strains and representatives of all existing spiroplasma groups and sub-groups indicated that three of the candidate strains (TIUS-1, BIUS-1 and BARC 1901) are unrelated to known taxa or to previously defined spiroplasma groups or sub-groups, or to each other. Strains BARC 1901 and BARC 2649 showed variable crossing patterns with some group strains, especially in the MI test. Some serological analyses of strains BARC

Table 3. Serological reactivities of the group II spiroplasma strain DW-1

See the legend to Table 2.

Group/strain	Deformation		Metabolism inhibition	
	Antigen	Antiserum	Antigen	Antiserum
Strain DW-1 (group II)				
I-1/R8A2 ^T	20	20	162	—
I-2/BC-3 ^T	160	—	486	54
I-3/E275 ^T	160	20	486	—
I-4/277F	160	80	4374	—
I-5/LB-12	160	—	39000	—
I-6/M55 ^T	320	20	4374	—
I-7/N525	320	40	39000	—
I-8/P40 ^T	640	20	1458	54
II/DW-1	1280*	1280*	> 117000*	> 117000*
III/23-6 ^T	—	—	18	—
IV/B31 ^T	20	—	54	—
VI/Y32 ^T	—	—	18	—
VII/MQ-1 ^T	—	—	54	—
VIII-1/EA-1 ^T	—	—	54	—
VIII-2/DF-1 ^T	—	—	1458	—
IX/CN-5 ^T	—	—	162	—
X/AES-1 ^T	—	—	486	—
XII/DU-1 ^T	—	—	18	—
XIII/Ar-1343 ^T	—	—	54	—
XV/I-25	—	—	54	—
XVI-1/CC-1 ^T	20	—	—	—
XVI-2/CB-1	—	—	54	—
XVI-3/Ar-1357	—	—	54	—
XVIII/TN-1 ^T	40	—	18	—
XIX/PUP-1 ^T	—	40	18	—
XX/LD-1 ^T	—	—	54	18
XXI/W115	—	—	18	—
XXII/CT-1 ^T	—	—	18	—
XXIII/TG-1 ^T	—	—	54	—
XXIV/CCH ^T	—	—	18	—
XXV/CUAS-1 ^T	20	—	18	—
XXVI/PLHS-1 ^T	—	—	—	54
XXIX/TIUS-1	—	—	18	—
XXX/BIUS-1	—	—	18	—
XXXI/HYOS-1 ^T	—	—	54	—
XXXII/TABS-2 ^T	—	—	18	—
XXXIII/TAUS-1 ^T	—	—	18	—
XXXIV/BARC 1901	—	—	486	—
BARC 2649	—	—	1458	—

* See footnote to Table 2.

1901 and BARC 2649 are reported elsewhere (52) in a comparison of spiroplasmas from tabanid hosts.

In the serological analyses performed for this study, we observed relatively high levels of serological cross reactivity between group II strain DW-1 spiroplasmas and group I antisera (Table 3). In two reciprocal group I vs group II comparisons (strain DW-1 vs sub-group I-3 strain E275^T, and sub-group I-8 strain P40^T),

crosses were observed in both directions with both techniques.

Biochemical tests

All four candidate strains produced acid from glucose, but only BARC 2649 was able to hydrolyse arginine. None hydrolysed urea.

Fig. 1. Electron micrograph of cells of strains (a) TIUS-1, (b) BIUS-1, (c) BARC 1901 and (d) BARC 2649. Arrows indicate unit membrane. Bar, 200 nm.

Morphology

Strains BIUS-1 and TIUS-1 were unusual in that they often appeared to grow in a non-helical form when examined by conventional dark-field techniques. Each of these strains, however, exhibited motile helical forms in late-exponential or early-stationary phase, at a time when the medium appeared to approach its lowest pH (6.0) from acid production by glucose fermentation. Electron microscopic observations revealed (Fig. 1a, b) that the cells were bound by a single cytoplasmic membrane.

Strains BARC 1901 and BARC 2649, when grown in MID broth medium, exhibited typical spiroplasma morphology in dark-field microscopy. Cells of strain BARC 1901 possessed long helices with six or more turns, but cells of strain BARC 2649 exhibited short helices with less than six turns. Ultrastructural examination of cells of both strains showed that the cells of

both strains were bound only by a single cytoplasmic membrane (Fig. 1c, d).

G + C content of DNA

The G + C content (± 1 mol%) of the strains were: TIUS-1, 28; BIUS-1, 28; BARC 1901, 25; and BARC 2649, 28. The genome (in kbp) of strain TIUS-1 was 840, and of strain BARC 2649 was 1325.

DISCUSSION

Table 1 reflects recent corrections to earlier data (42) on the responses of sub-groups I-5 and I-6 to tests for glucose fermentation and arginine hydrolysis. We also hereby correct the identification of several spiroplasmas in electron micrographs in the 1987 publication by Tully *et al.* (42). Figs 1(H, I) of that report

actually show cell membranes of group XXI strain W115 and group XXIII strain TG-1^T. An electron micrograph of group XX strain LD-1^T was inadvertently omitted from Fig. 1, but has been recently published (19).

To complete the serological comparisons required to establish the serological classification of spiroplasmas, 3362 serological cross-reactions (1681 tests in reciprocal sets of both DF and MI test procedures) were done. Although laborious to complete, this data set gives a reasonable basis for assessing serological relationships among spiroplasmas. Valid relationships (i.e. those confirmed by other criteria) are usually reflected by complete reciprocity in DF and MI tests. This is not universally so; the relatedness among sub-groups of group VIII (13) and group XVI (1, 49) was obscured at first by a lack of reciprocity in DF or MI tests between some strains in the matrix of interrelated organisms. Growth inhibition tests (60), although applied earlier to some groups (42, 51, 59, 60) were abandoned as the matrices to be compared grew larger and larger. The meaning of the many one-way crosses among spiroplasma strains is unclear. Of course, when a pair of spiroplasma strains eventually proves to be part of an interrelated matrix (e.g. group VIII and XVI strains), the one-way cross-reactions are clearly meaningful. On the other hand, some spiroplasmas, including especially group XIX (*Spiroplasma lampyridicola*, strain PUP-1^T) (35), group XX (*S. leptinotarsae*, strain LD-1^T) (19), group XXVI (*S. alleghenense*, strain PLHS-1^T) (2), and to some extent, group XXVIII (*S. platyhelix*, strain PALS-1^T) (65), when used as test antigens, reacted promiscuously with a broad array of spiroplasma antisera. These one-way crosses involve a large number of spiroplasma pairs that show no other related feature. Such pairs often have very discrepant G+C contents, different glucose/arginine utilization patterns, different host or geographic origins and, when tested, share little or no DNA-DNA homology. Such one-way crosses can be clearly seen to be spurious and must be ignored. However, the meaning of one-way crosses observed with spiroplasmas that do not show a promiscuous one-way crossing pattern is much less clear, and probably needs to be examined in the context of a polyphasic (44) classification of spiroplasma species and groups. Analysis of 16S rDNA sequences of various mollicutes, where available (15, 36, 45, 46), would provide an excellent primary tool for such an analysis. It is not clear how useful whole genomic data (genome size and base composition) would be in intergroup classification. The variation in genome size among spiroplasmas (5, 6, 30–32) is great, suggesting that this character is evolutionarily labile. G+C contents are problematic, since values may vary substantially even within a group, as with *S. insolitum* (sub-group I-6), compared to other group I members (21). In general, with either genome size or G+C contents, widely different values between strains indicate that the strains are different species; however, similar values are not unequivocally indicative of

relationship, since both G+C and genome size data are composite overall values that may be derived from genomes that may be very different genetically. With spiroplasmas, as with other mollicutes, DNA-DNA hybridization data continue to be the criterion of last resort in deciding whether to lump strains into a single species. For interspecies classifications, including cladistic reconstructions, 16S rDNA sequence data are much more useful than any phenotypic characters. However, until sequence data are available for representatives of all spiroplasma groups and sub-groups, phenotypic characters, including serological data, will provide a basis for construction of polyphasic classifications.

The serological analyses provided in this paper revealed a hitherto unappreciated distant relationship between group I and the group II spiroplasma (strain DW-1) from *Drosophila* species. Because group I spiroplasmas (4, 11, 33, 34, 50) are associated with plant diseases, sap-sucking insects, ticks and flower surfaces, and because group II spiroplasmas are very fastidious host-associated organisms that induce sex-ratio abnormalities in their *Drosophila* hosts (66), this relationship had not been suspected. Furthermore, group II spiroplasmas are extremely difficult to cultivate in artificial medium (18), and the tests summarized in the 1987 revision (42) had, by necessity, been performed with haemolymph-derived antigens. It is interesting to note that group I and II spiroplasmas had been found by Weisburg *et al.* (46) to be closely clustered in the phylogenetic analysis of their 16S rDNA. In addition, the G+C content of their DNA is indistinguishable (42). Further clarification of this suggested relationship may eventually require an attempt to assess the DNA-DNA homology between group I and group II spiroplasmas.

Data from partial serological crossing may occasionally provide an inaccurate impression of intergroup relationships. Thus, in 1992, it was proposed (59) that the CUAS-1^T strain from mosquitoes and the group VII *S. monobiae*, MQ-1^T strain from a *Monobia* wasp were serologically related, and perhaps represented sub-groups of a single group. However, analysis of the polyphasic set of characters for the two groups, particularly genome size, established that strain CUAS-1^T was in fact distinct from strain MQ-1^T and all other existing groups. This strain was eventually described as a new species (*S. diminutum*) (group XXV) (67) in the current classification scheme.

Recent studies of an extensive collection of tabanid spiroplasmas (52) have suggested the possibility of a cluster of strains that includes groups XXIII, XXVII, XXXII, XXXIII and possibly other strains. All of the strains exhibit aggressive glucose fermentation, fail to hydrolyse arginine, and have a G+C content of 25–27 mol%. In addition, these strains, and some related strains, show a complex pattern of inter-relationships in one-way serological crosses (52). It may be necessary to perform 16S rDNA sequence

analysis to test the hypothesis that these spiroplasma may form a monophyletic spiroplasma clade.

Also, sequence analysis of 16S rDNA will probably be necessary to clarify several other interesting questions concerning some of the spiroplasmas designated herein. The morphologies of *S. platyhelix* (strain PALS-1^T), and strains TIUS-1, and BIUS-1 reported here are unusual for spiroplasmas, and it is possible that one or more of these strains could represent paraphyletic lineage(s). The group VI *Spiroplasma ixodetis* (43) strain Y32^T presented a similar dilemma, in that its morphology also differed substantially from that of conventional spiroplasmas. In this instance, 16S rDNA analysis (46) confirmed the monophyly of the Y32^T strain and other spiroplasmas.

Since the last revision, the concept of spiroplasma sub-groups has undergone subtle modification. In both group VIII (13) and XVI spiroplasmas (1, 49), there are now sub-groups that represent some of the known variability within the group. When we attempted to apply this classification to a large existing cluster of spiroplasma isolates from tabanids in Georgia, however, we were unable to place the most prevalent group VIII serovar in any of the existing sub-groups (52). Reciprocal serological analysis has subsequently shown that this serovar (strain BARC 1357) probably represents a fourth unrecognized sub-group of group VIII. The serological data presented here suggest that strain BARC 2649 represents a fifth unrecognized sub-group of group VIII. Other group VIII isolates from tabanid flies appear to differ considerably from any and all of these five representatives. The large number of possible sub-groups of this cluster, given the requirements for sub-group designation (48), appears to preclude their taxonomic designation.

Lastly, a phylogenetic analysis of mollicutes reported in 1989 (46) indicated that the spiroplasmas represent an important evolutionary divergence from the achleplasma/anaeroplasmata branch of mollicutes. From this lineage, one cluster of mycoplasmas evolved, which includes *Mycoplasma mycoides*, and a cluster of mollicutes (including those currently placed in *Entomoplasma* and *Mesoplasma*) from insects. These organisms and the major clades of mycoplasmas (e.g. *Mycoplasma hominis* and *Mycoplasma pneumoniae*) are polyphyletic. These major phylogenetic distinctions prompted a proposed revision in the classification of the class Mollicutes (40). In this revision, the order Entomoplasmatales was created to provide taxonomic status for the emerging collections of arthropod-associated mollicutes, including the spiroplasmas (family Spiroplasmataceae, genus Spiroplasma) and for many newly described non-helical mollicutes from insect and/or plant hosts that possessed distinct growth requirements (family Entomoplasmataceae, genus Mesoplasma and genus Entomoplasma).

The association of spiroplasmas with insects (10), coupled with the high global biodiversity of insects, should assure the continuing collection of new Spiro-

plasma strains. Further evaluation and reclassification of these remarkable prokaryotes will undoubtedly continue to be a taxonomic necessity.

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