**Spiroplasmas: evolutionary relationships and biodiversity**

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## 1. ABSTRACT

Spiroplasmas are wall-less descendants of Gram-positive bacteria that maintain some of the smallest genomes known for self-replicating organisms. These helical, motile prokaryotes exploit numerous habitats, but are most often found in association with insects. Co-evolution with their insect hosts may account for the highly speciose nature of the genus *Spiroplasma*, with many spiroplasmas existing in obligate insect/plant transmission cycles. In addition to insect and plant hosts, spiroplasmas are found in association with ticks and crustaceans. Although most spiroplasma associations appear to be commensal, some cases of pathogenicity or mutualism have been described. Most notably, spiroplasmas have been identified as the causative agents of agricultural and aquacultural diseases and the sex ratio disorder in insects. Some spiroplasmas exhibit strict host and/or geographical ranges, but others are relative generalists. Species of the genus *Spiroplasma* have been traditionally classified into 34 groups based on cross-reactivity of surface antigens. Three of the serogroups contain closely related strain complexes that are further divided into subgroups. Phylogenetic reconstructions based on 16S rDNA sequence strongly support the closely related serogroups. To date, less than 40 *Spiroplasma* species have been fully characterized and given binomial names. Complete characterization of a new species involves numerous phenotypic and genotypic tests as outlined in the minimal standards document; this document is currently under revision to include phylogenetic data and a reevaluated set of required phenotypic and genotypic tests. The area of spiroplasma research is poised for major advances with new criteria for naming species in preparation, a dramatic increase in available molecular characters, the promise of full genome sequences, and advances in genetic tools for manipulation of these organisms.

## 2. INTRODUCTION

Spiroplasmas are among the smallest self-replicating prokaryotes known, with genomes ranging in size from approximately 780-2,220 kbp (1,2). They are helical, motile bacteria that apparently evolved via simplification of Gram-positive bacteria, although they lack a cell wall (3,4). As the organisms radiated to occupy multiple habitats, they became able to invade the arthropod gut lumen. Some species expanded their habitat range to include arthropod hemolymph, ovaries, fat bodies, hypodermis, and salivary glands. With much lower frequency spiroplasmas have also been found in association with ticks, plants and higher order invertebrates (5-11). Most spiroplasmas appear to be commensals, but there are a few cases of mutualism or pathogenicity associated with spiroplasma infections (12,13,14).

Although insects are the main reservoir for spiroplasmas (15), other early isolations were made from secondary hosts that were adversely affected by spiroplasma infections. Description of the genus *Spiroplasma* began with the isolation and characterization of the causative agent of citrus stubborn disease (16), *S. citri* (17). *S. citri* infects the plant phloem sieve tubes, with the pathogenic spiroplasma being delivered to the plants via sap-feeding insects. The infected sieve elements then serve as a reservoir for transmission to other sap-feeding insects (reviewed in 18). The second described member of the genus *Spiroplasma*, *S. kunkelii*, causes corn stunt disease and also resides in an obligate cyclical habitat that consists of both plant phloem and an insect vector (19-21). Another early member of the genus *Spiroplasma* was the previously described microorganism that causes a sex-ratio abnormality in *Drosophila*; it was originally thought to be a spirochaete but was later identified as a spiroplasma and named *S. poulsonii* (22-24). Spiroplasmas were also isolated from ticks (25-29). One of the tick isolates, *S. mirum*, is referred to as the suckling mouse cataract agent due to its ability to establish experimental infections and disease in suckling mice (27,28,30-32). As research on spiroplasmas proceeded and more isolates were characterized, it became apparent that insects served as the major reservoir for spiroplasma maintenance and dispersal (14,15), and that plant surfaces were more important in dispersal and transmission than the plant phloem (33-35).

This early work on spiroplasmas led to the development of methods for cultivation and characterization, resulting in a proliferation of spiroplasma isolates from varied hosts and diverse geographical locales. To date, 36 *Spiroplasma* species have been completely described and given binomial names. Hundreds of other isolates have been partially described, and some of these undoubtedly represent new *Spiroplasma* species. The wide range of hosts and geographic distribution offers some challenges for thoroughly describing this genus, but it also provides some interesting opportunities to explore questions of biodiversity, co-evolution and phylogenetic relationships. This article will explore the areas of spiroplasma biodiversity and their evolutionary relationships.

## 3. SPIROPLASMA SYSTEMATICS

### 3.1. Taxonomy

The taxonomic requirements for naming new spiroplasma species are governed by the 1995 guidelines published by the International Committee on Systematic Bacteriology (ICSB) Subcommittee on Taxonomy of *Mollicutes* (36); this committee has since been renamed the International Committee on Systematics of Prokaryotes (ICSP). The 1995 guidelines are revisions of earlier documents outlining the minimal standards for description of Mycoplasma species. The first document providing general guidelines for naming species of *Mycoplasmatales* was published in 1967 by the ICSB Subcommittee on the Taxonomy of *Mycoplasmatales* (37) In 1972, modifications to the original document resulted in publication of the minimal standards for description of mycoplasma species (38); this document was then revised in 1979 and 1995. The revisions reflect changes in *Mollicutes* systematics over time, and there is currently a movement within *Mollicutes* systematics to introduce another revision to the classification system. The ICSP Subcommittee on Taxonomy of *Mollicutes* reported that a newly revised minimal standards document for description of Mycoplasma species was being drafted (ICSP Subcommittee, 11 July 2004 meeting, Athens Georgia; R. F. Whitcomb, personal communication). This section will review the current requirements for *Spiroplasma* species designation and discuss some of the issues that have led to a call to revise the criteria.

### 3.1.1. *Spiroplasma* species concept

Although the species is the basal category for taxonomic classification, the definition of a bacterial species is relatively vague and artificial (39,40). Nature often represents a continuum rather than discrete points, such as clearly differentiated species (41). As more and more spiroplasmas are isolated and characterized, the continuous nature of spiroplasma biodiversity, at least in some serogroups, is becoming clear. For example, the group VIII strains once represented several clearly defined species but are now a huge collection of inter-related strains that cannot be clearly defined using the current criteria (42,43).

The basic species concept for Spiroplasma is rooted in the species concept for other prokaryotic taxa (44,45), which requires genomes exhibiting less than 70% similarity for separate species designations (46,47). In theory, this requirement was satisfied by completing DNA-DNA reassociation analyses; the limited reassociation data that was generated was found to be consistently complementary to the analysis of surface antigens (48-52). These comparisons validated the use of serological analyses as the basis for *Spiroplasma* species designations (36). A detailed historical review on the movement toward a serological classification system was recently provided by Gasparich (53).

### 3.1.2. Current requirements for classification of *Spiroplasma* species

The current minimal standards for spiroplasma species designation rely upon phenotypic and genotypic information. As described by the ICSB Subcommittee on

Taxonomy of *Mollicutes*, an isolate must be examined for morphology and serology as well as molecular and biological traits before classification as a member of the genus *Spiroplasma* (36). The requirements from the minimal standards document (36) are outlined below, but the reader should be aware that these standards are currently under revision (ICSP Subcommittee, 11 July 2004 meeting, Athens Georgia; R. F. Whitcomb, personal communication)

All isolates must be cultivable for species designation. Due to specialized habitats, some isolates require co-culture with insect cells for initial isolation or other modifications such as very rich media or anaerobic growth conditions (54,55). Extensive work has been done to examine different growth requirements and media (56-58). If an isolate cannot be cultivated, then it can only be considered for *Candidatus* status (59). To date, there have been no *Candidatus* spiroplasma described, but other *Candidatus* mollicutes have been reported (e.g. phytoplasmas). A non-cultivable isolate could be characterized by molecular methods (e.g. 16S rDNA sequence) that would tentatively place it within the *Spiroplasma* genus.

An isolate must be clonal before final species characterizations can be completed. Preliminary isolations often result in mixed cultures that may contain more than one spiroplasma, as well as other microorganisms. A pure culture is obtained by initial filtration through a 220 nm filter, which will block most microbes except mollicutes. The filtrate is then used in a dilution cloning technique to isolate a pure culture (60,61). This procedure is repeated three times. The emerging strain is then designated as the representative strain, which, if the taxon is named, will become the type strain.

Microscopic morphology is determined using dark field and electron microscopy. A dark field microscope can be used to observe the isolate for motility and helicity. The degree of helicity may vary, but all spiroplasmas exhibit helicity at some point in their life history. Electron microscopy is used to verify the lack of a cell wall; the cell should be enclosed within a single membrane. The size of *S. citri* cells is approximately 200 nm in length and 3-12 nm in width (62). Cells of other species differ in both size and type of helicity. For example, cells of *S. ixodetis* are tightly coiled and cells of group VIII spiroplasmas are generally very small. Spiroplasma cell morphology has recently been described in detail by Gasparich and colleagues (42).

Numerous biological characteristics must be determined; however, none of the traits tested are genus-specific. Some of the biological characters tested correlate with cell morphology, including filterability through a 220 nm membrane and resistance to penicillin (500U/ml). Other characters are based on cell biochemistry (63), such as glucose fermentation (64-66), arginine hydrolysis (67) and urea hydrolysis (68). The original requirement to determine cholesterol utilization (69) has been questioned, since some spiroplasmas do not have a sterol requirement

(e.g. *S. floricola* (70), *S. gladiatoris* (71)). The range of temperatures for growth, including the optimal temperature and the growth rate of the species (72) are characteristics that are probably rooted in the organism's habitat. A complete species characterization requires a detailed description of the spiroplasma habitat, including geographic and host/source information. If known, the microhabitat within the host should also be described. The ability to hydrolyze arginine and the optimal growth temperature vary for spiroplasmas, but all known spiroplasmas are filterable through a 220 nm membrane, resistant to penicillin, ferment glucose and are unable to hydrolyze urea.

Only one molecular characteristic was required by the 1995 minimal standards document for species designation, G + C base composition (mol%). The guanine and cytosine composition of the genome is determined using a melting temperature method, with reported compositions falling within the range of 24-31% (73). Although not required, determination of genome size and 16S rDNA sequence was recommended. Genome sizes for spiroplasmas range from 780-2,220 kbp (1,2) and are determined using pulsed-field gel electrophoresis (74). 16S rDNA sequence determination permits taxonomic placement of individual species within the genus *Spiroplasma* and provides valuable insights into evolutionary relationships with previously described species based on phylogenetic analyses (3,4,42,75,76). The ICSP Subcommittee on Taxonomy of *Mollicutes* recently recommended that 16S rDNA sequence information be published for all newly described species (ICSP Subcommittee, 11 July 2004 meeting, Athens Georgia; R. F. Whitcomb, personal communication).

As discussed above, serological relatedness was accepted as a surrogate for DNA-DNA reassociation analyses and formed the basis for the *Spiroplasma* species concept. Surface serology has been the definitive character for *Spiroplasma* species placement for several decades. The serological classification system for *Spiroplasma* has provided a pragmatic way to screen and tentatively classify hundreds of new isolates until binomial names could be established for the species type strain (47). The serological classification scheme places new isolates into serological groups or subgroups based on the degree of surface antigen cross reactivity. Originally, members of a single serogroup demonstrated strong serological relatedness and had genomes that exhibited more than 70% DNA-DNA homology (46,47). As strains were identified that appeared to be weakly to moderately related to previously identified groups, the subgroup designation was initiated to reflect this degree of relatedness within the serological group system. These strains exhibited 30-70% homology in DNA-DNA reassociation assays and weak serological cross-reactivity to previously identified group members (36). Each subgroup is considered a putative species as members of subgroups have been shown to have high degrees of DNA-DNA homology (>70%) within the subgroup. However, the ICSB Subcommittee urged that names be applied only to subgroups of some importance (77). There are currently 34 recognized groups; three of

**Table 1.** *Spiroplasma* species and/or representative strains by serogroup [1]

| Serogroup[2] | Binomial Name | Type Strain[3] | Host(s) | Disease | Reference |
|---|---|---|---|---|---|
| I-1 | *Spiroplasma citri* | Maroc-R8A2 (27556) | Dicots, leafhoppers | Citrus stubborn | 17 |
| I-2 | *S. melliferum* | BC-3 (33219) | Honey bees | Honeybee spiroplasmosis | 165 |
| I-3 | *S. kunkelii* | E275 (29320) | Maize, leafhoppers | Corn stunt | 20 |
| I-4 | *S.* sp. | 277F (29761) | Rabbit ticks | NK[4] | 6 |
| I-5 | *S.* sp. | LB-12 (33649) | Green leaf bugs | NK | 6 |
| I-6 | *S. insolitum* | M55 (33502) | *Eristalis* flies, flowers | NK | 166 |
| I-7 | *S.* sp. | N525 (33287) | Coconut palms | NK | 6 |
| I-8 | *S. phoeniceum* | P40 (43115) | *Catharanthus roseus* | Periwinkle disease | 137 |
| I-9 | *S. penaei* | SHRIMP (BAA-1082) | *Penaeus vannamei* shrimp | Shrimp disease | 78 |
| II | *S. poulsonii* | DW-1 (43153) | *Drosophila* | Sex ratio trait | 24 |
| III | *S. floricola* | 23-6 (29989) | Insects, flowers | Beetle "lethargy" | 167 |
| IV | *S. apis* | B 31 (33834) | Bees, flowers | May disease | 134 |
| V | *S. mirum* | SMCA (29335) | Rabbit ticks | Suckling mouse cataract disease | 28 |
| VI | *S. ixodetis* | Y32 (33835) | *Ixodes pacificus* ticks | NK | 29 |
| VII | *S. monobiae* | MQ-1 (33825) | *Monobia* wasps | NK | 168 |
| VIII-1 | *S. syrphidicola* | EA-1 (33826) | *Eristalis arbustorum* flies | NK | 169 |
| VIII-2 | *S. chrysopicola* | DF-1 (43209) | *Crysops* sp. Flies | NK | 71 |
| VIII-3 | *S.* sp. | TAAS-1 (51123) | Horse fly | NK | 52 |
| IX | *S. clarkii* | CN-5 (33827) | *Cotinus* beetles | NK | 170 |
| X | *S. culicicola* | AES-1 (35112) | *Aedes* mosquitoes | NK | 171 |
| XI | *S. velocicrescens* | MQ-4 (35262) | *Monobia* wasps | NK | 172 |
| XII | *S. diabroticae* | DU-1 (43210) | *Diabrotica undecimpunctata* beetles | NK | 173 |
| XIII | *S. sabaudiense* | Ar 1343 (43303) | *Aedes* mosquitoes | NK | 174 |
| XIV | *S. corruscae* | EC-1 (43212) | *Ellychnia corrusca* beetles, horse flies | NK | 105 |
| XV | *S.* sp. | I-25 (43262) | Leafhopper | NK | 6 |
| XVI-1 | *S. cantharicola* | CC-1 (43207) | Cantharid beetle | NK | 175 |
| XVI-2 | *S.* sp. | CB-1 (43208) | Cantharid beetle | NK | 51 |
| XVI-3 | *S.* sp. | Ar 1357 (51126) | Mosquito | NK | 51 |
| XVII | *S. turonicum* | Tab4c (700271) | Horse fly | NK | 176 |
| XVIII | *S. litorale* | TN-1 (43211) | *Tabanus nigrovittatus* | NK | 177 |
| XIX | *S. lampyridicola* | PUP-1 (43206) | *Photuris pennsylvanicus* beetles | NK | 178 |
| XX | *S. leptinotarsae* | LD-1 (43213) | *Leptinotarsa decemlineata* | NK | 156 |
| XXI | *S.* sp. | W115 (43260) | *Prunus sp.* flowers | NK | 7 |
| XXII | *S. taiwanense* | CT-1 (43302) | *Culex tritaeniorhynchus* | NK | 179 |
| XXIII | *S. gladiatoris* | TG-1 (43525) | *Tabanus gladiator* | NK | 71 |
| XXIV | *S. chinense* | CCH (43960) | *Calystegia hederaceae* | NK | 180 |
| XXV | *S. diminutum* | CUAS-1 (49235) | *Culex* mosquito | NK | 181 |
| XXVI | *S. alleghenense* | PHLS-1 (51752) | Scorpionfly | NK | 182 |
| XXVII | *S. lineolae* | TALS-2 (51749) | Horse fly | NK | 183 |
| XXVIII | *S. platyhelix* | PALS-1 (51748) | Dragonfly | NK | 2 |
| XXIX | *S.* sp. | TIUS-1 (51751) | Tiphiid wasp | NK | 7 |
| XXX | *S.* sp. | BIUS-1 (51750) | Flower surface | NK | 7 |
| XXXI | *S. montanense* | HYOS-1 (51745) | Horse fly | NK | 184 |
| XXXII | *S. helicoides* | TABS-2 (51746) | Horse fly | NK | 71 |
| XXXIII | *S. tabanidicola* | TAUS-1 (51747) | Horse fly | NK | 71 |
| XXXIV | *S.* sp. | BARC 1901 (700283) | Horse fly | NK | 7 |
| Ungrouped[5] | *S. atrichopogonis* | GNAT3597 (BAA-520) | Biting midge | NK | 185 |

[1] Table modified from Williamson *et al.* (7) and Gasparich (53), [2] Serogroups are designated by Roman numerals; subgroups are indicated by hyphenated numbers, [3] Common strain name (American Type Culture Collection (ATCC) strain reference number), [4] NK, none known, [5] No group number yet assigned to this serologically distinct species.

these groups encompass 15 subgroups of inter-related strains (7). Group I has nine subgroups and groups VIII and XVI have three subgroups each (7,50-52, 78). Most groups and subgroups have been given binomial names (Table 1).

Serological relatedness can be tested by several methods, but the deformation test (DF) is widely accepted for *Spiroplasma* characterization (7,79,80). The DF test screens new isolates against antisera to representatives of existing serogroup strains. During the initial screening, the new isolate is generally tested against pools of representative antisera and then examined for deformation. Tentative serological relatedness is determined by observation of 50% or greater cells deformed (e.g. clumping, blebs). This initial screening is followed by reciprocal DF tests against antisera to individual strains as indicated by the preliminary screen. Reciprocal, terminal titers of 320 or greater indicate strong serological relatedness, and would allow placement of a novel isolate in a pre-existing serogroup. Generally, homologous reactions are much stronger. Weak reciprocal reactions are indicative of partial relatedness, and may indicate the need for more reciprocal DF tests using other related strains. Subgroup strains often show weak reciprocal cross reactivity with one or more representative strains from a group (47). Strong one-way reactions are discounted in this assay as they are not reliable indicators of serological relatedness. The second serological confirmatory test generally chosen is the metabolism inhibition test (80,81). Other options for secondary tests include the growth inhibition and enzyme-linked immunosorbent assays (82).

The criteria described above allow assignment of a novel isolate to the species/group level (47). A triply cloned isolate that is insensitive to penicillin and is bound by only a cell membrane is placed in the Class *Mollicutes* (within the Phylum *Firmicutes*, Domain Bacteria). Demonstration of helicity and motility allows for placement in the Family *Spiroplasmataceae* (within the Order *Entomoplasmatales*). Members of the genus *Spiroplasma*, as far as known, utilize glucose but do not utilize urea. *Spiroplasma* species status is determined by serological testing that includes a DF test (79) and a second confirmatory serological test (i.e. metabolism inhibition (81) or growth inhibition (83)).

### 3.1.3. Polyphasic taxonomy

Polyphasic taxonomy calls for the inclusion of phenotypic, genotypic and phylogenetic information in classification (84). The goal is to use all available information about an organism to accurately portray its relationship to other organisms, which makes polyphasic taxonomy an especially powerful tool for classifying closely related organisms. The required minimal standards for spiroplasma species designation adopted in 1995 required only phenotypic and genotypic information (36), but recent work demonstrates that a more inclusive approach may be more informative (42,76). As the field of *Spiroplasma* (and *Mollicutes*) systematics has changed, the ICSP Subcommittee on the Taxonomy of *Mollicutes* has begun to develop a revised minimal standards document (ICSP Subcommittee, 11 July 2004 meeting, Athens Georgia; R. F. Whitcomb, personal communication).

One of the core issues involves inclusion of phylogenetic information in the required classification scheme. Historically, the 16S rDNA sequence has been the main genomic sequence used for this purpose based on the pioneering work of Carl Woese (reviewed in 85). Several studies have demonstrated the utility of the 16S rDNA sequence for examining evolutionary relationships of the genus *Spiroplasma* (3,4,42,75,76). 16S rDNA analysis provides excellent resolution for interspecific and intergroup comparisons in which high, moderate, or even fairly low degrees of sequence divergence has occurred; and trees generated by maximum parsimony and other analyses often divide the genus into clades that have clear biological significance. Sequences of 16S rDNA provide a fairly reliable characteristic for species designation in general; however, its utility may be limited in differentiating recently diverged or rapidly evolving species that often exhibit 16S rDNA sequences with >97% identity (86). For example, *Spiroplasma* subgroups represent closely related strain complexes that cannot always be easily differentiated based on 16S rDNA sequence analysis (42). Several alternative sequences have recently been examined that may enhance the 16S rDNA reconstructions of *Spiroplasma* phylogenetics, including the 16S-23S rDNA intergenic region, the *gyrB* gene and the translated sequence of the metabolic gene *pgk* (43;87; G.E. Gasparich, unpublished data). Ultimately, a multilocus approach that is representative of the whole genome may provide the best evidence for evolutionary relationships.

It has been suggested that 16S rDNA phylogenies could be used in concert with serology to serve as primary characters for taxonomic classification (76,88). Directed serological analyses could be used to confirm phylogenetic placement. This approach would greatly simplify the current requirement to serologically test novel isolates against all known groups/species (36). This requirement is already difficult for many laboratories to complete and as the number of species continues to increase it will only become more unwieldy. To date, all of the positive serological cross-reactions that have been observed are strongly supported by 16S rDNA phylogenetic reconstructions (42); however, this congruency covers only a small part of the total matrix of species. Most pair-wise phylogenetic comparisons reported appear to involve evolutionary distances that are beyond those that can be detected by serology (R. F. Whitcomb, personal communication).
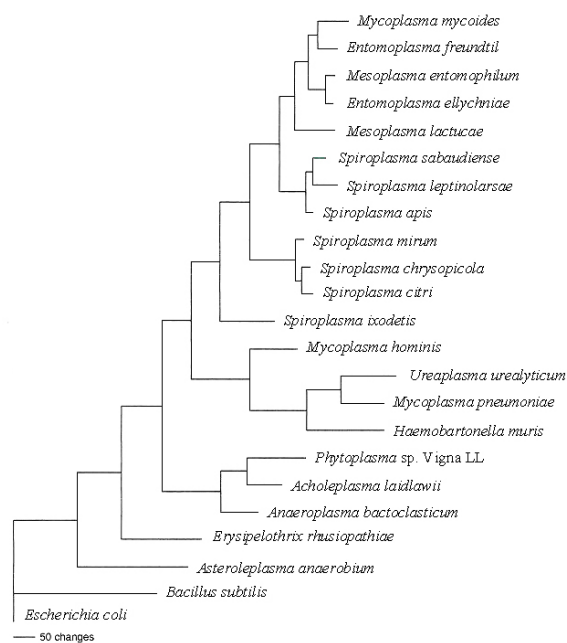
Molecular phylogeny and directed serology would need to be used in concert with other characteristics. The minimal standards document outlines numerous phenotypic and genotypic requirements for *Spiroplasma* species designation, as described above. There is a real need to thoroughly reevaluate the required tests, as some of the tests are mooted by newer techniques or no longer predictive (42,88). Until a revised minimal standards document is approved and published, new *Spiroplasma* species designations must continue to follow the criteria outlined in the 1995 document (36). This has hampered the description of new species, as many laboratories lack the resources to complete all required tests.
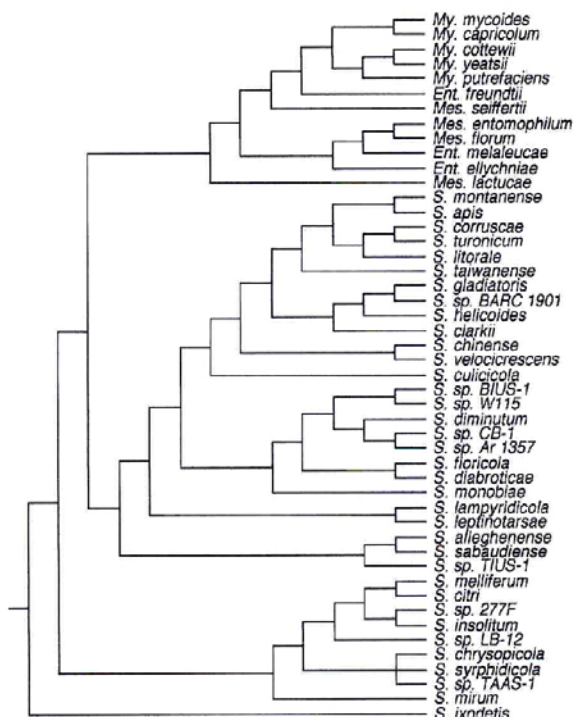
### 3.2. Molecular phylogeny

Initial phylogenetic analysis of the genus *Spiroplasma* began with the work of Woese and colleagues using the 16S rDNA sequence (3). Their early work provided a basic framework for examining the evolution of spiroplasmas, and most subsequent studies have continued to rely heavily on 16S rDNA analysis.

### 3.2.1. Evolutionary relationship of spiroplasmas to other Eubacteria

The general placement of the genus *Spiroplasma* in relation to other Eubacteria is well established (Figure 1). Several studies, using multiple phylogenetic algorithms, have produced a consistent picture of *Spiroplasma* evolution (3,4,42,75,76). Phylogenetic analyses indicate that the class *Mollicutes* is a terminus in the evolution of Gram-positive bacteria, arising from a gram-positive, low G + C content Clostridial lineage of the Eubacteria (3). Recent phylogenetic analyses using the amino acid sequence of phosphoglycerate kinase (Pgk) as a molecular marker indicate a similar derivation, but also suggest that modern mollicutes may be more closely related to the *Streptococcus/Lactobacillus* spp. than to *Clostridium* and *Bacillus* spp. (87). Within the class *Mollicutes*, in a deep split, the *Acholeplasma-Anaeroplasma* and *Mycoplasmatales-Entomoplasmatales* lineages diverged (3,4,42,76). This split is reflected in the codon usages in the two lineages. The *Mycoplasma-Ureaplasma* lineage and the *Spiroplasma* lineage then diverged. It is presumed

**Figure 1.** Phylogenetic analysis of *Mollicutes* based on 16S rDNA sequence. Phylogram generated using the Maximum Parsimony algorithm. Specific parameters and GenBank accession numbers previously described by Gasparich and co-workers (42).



**Figure 2.** Majority Rule Maximum Parsimony tree showing position of major clades of the Spiroplasma-Entomoplamataceae-Mycoides (SEM) clade. Specific parameters and GenBank accession numbers previously provided by Gasparich and co-workers (42).

that the *Mycoplasma-Ureaplasma* lineage derived from a spiroplasmal ancestor, since the complex cytoskeletal structure (89,90) and large genome size of *Spiroplasma* (1) appear to be primitive. Some cytoskeletal elements are present in the pneumoniae group of *Mycoplasma*, but in the terminal taxa *Mycoplasmatales*, cytoskeletal structure is largely absent. Taken as a whole, the phylogenetic data indicates that the *Spiroplasma* evolved from a eubacterial lineage following divergent and degenerative evolutionary events. This evolutionary pathway clearly shows that the *Spiroplasma* genus arose due to reductive evolution (85), rather than from a primitive organism that evolved prior to the development of the cell wall (91). Recent divergence within *Spiroplasma* can probably be explained by co-evolution with a range of hosts.

**3.2.2. Evolutionary relationships within the genus *Spiroplasma***

The bulk of the molecular phylogenetic data generated for spiroplasmas is based on 16S rDNA sequence. Although a more complete picture of genetic relatedness may eventually be learned from inclusion of other discrete sequences or from comparative genomics, the 16S rDNA sequence is the current standard for phylogenetic analysis of spiroplasma. It reliably indicates all levels of classification above the level of species (92-94). At the species level, 16S rDNA sequence can distinguish most species unless they are part of closely related strain clusters, such as serologically related subgroups (42,95). For these closely related species a different set of characters will be required to improve resolution. The 16S-23S rRNA spacer region and the *gyrB* gene are promising areas that may yield additional DNA sequence characters to enhance molecular phylogenies (43; G.E. Gasparich, unpublished data).

Recent phylogenetic reconstructions of the genus *Spiroplasma* examined the evolutionary relationship of 36 *Spiroplasma* spp. along with other members of the order *Entomoplasmatales* within the class *Mollicutes* (42). This recent work has confirmed and extended prior studies by the addition of 26 new *Spiroplasma* 16S rDNA sequences (4,75,76). This section will use the clade terminology proposed by Gasparich and co-workers (42). The order *Entomoplasmatales* contains four major clades: the Mycoides-Entomoplasmataceae clade, the Apis clade, the Citri-Chrysopicola-Mirum clade, and the Ixodetis clade (Figure 2). These four clades, which are described below, include *Spiroplasma, Mycoplasma, Entomoplasma* and *Mesoplasma* species. Based on the current phylogenetic analyses, the genus *Spiroplasma* is not monophyletic (Figure 2).

**3.2.2.1. The Ixodetis clade**

The Ixodetis clade represents a basal divergence in the spiroplasma lineage, and its evolutionary distance from other *Spiroplasma* spp. is significant. The clade contains a single member, *S. ixodetis* (serogroup VI), which was isolated from a tick (29). *S. ixodetis* represents the known upper limit for spiroplasma genome size at 2220 kbp and it exhibits a unique, tightly coiled helicity. It is not clear if this unusual morphology is a recently derived

characteristic or if it represents a primitive, ancestral character. Studies examining the mechanisms of motility and helicity allow for the possibility that helicity could be altered without substantial genomic change (90,96). Other insect-associated spiroplasmas have been identified as potential members of this clade based on 16S rDNA sequence similarity in BLAST searches; however, BLAST searches alone cannot provide phylogenetic placement (97) and none of the organisms in question have been cultured and visualized microscopically. The organisms with similar 16S rDNA sequences are associated with sex-ratio abnormalities in a butterfly (*Danaus chrysippus*) and coccinellid beetles (*Adalia bipunctata*) (98-100) or were isolated from the bamboo pseudococcid and green pea aphid (101,102).

### 3.2.2.2. The Citri-Chrysopicola-Mirum clade

Phylogenetic analyses provide strong support for a monophyletic Citri-Chrysopicola-Mirum clade. At the time that this clade diverged, the helicity and motility associated with most *Spiroplasma* species had been firmly established. The Citri-Chrysopicola-Mirum clade is composed of three component clades: (i) the Citri-Poulsonii clade, (ii) the Chrysopicola-Syrphidicola-TAAS-1 clade and (iii) the Mirum clade.

The Citri-Poulsonii clade contains nine serogroup I spiroplasmas and the serogroup II species *S. poulsonii* (42,78). Although all members of the clade clearly fall within this grouping based on multiple phylogenetic algorithms, resolution of some nodes within this group proved problematic due to 16S rDNA similarity coefficients of 0.986 to 0.991 (42). Members of this clade represent diverse host associations, including honeybees, ticks, leafhoppers, plant/flower surfaces, *Drosophila* and shrimp (6,23,78). This clade is also of special interest due to the pathogenicity of some members (see section 4.1). Diseases include citrus stubborn (*S. citri*), honey bee spiroplasmosis (*S. melliferum*), corn stunt (*S. kunkelii*), periwinkle disease (*S. phoeniceum*), the sex ratio trait in *Drosophila* (*S. poulsonii*), and the recently described shrimp aquaculture disease (*S. penaei*). Given the diverse habitats and important diseases associated with the Citri-Poulsonii clade, accurate resolution of all evolutionary relationships within this clade is critical. Interestingly, strain LB-12 from the green leaf bug (serogroup I-5, 103) is basal to the Citri-Poulsonii clade, suggesting that an ancestral habitat of the Citri-Poulsonii clade may have been the plant phloem/sucking insect habitat.

The Chrysopicola-Syrphidicola-TAAS-1 clade is a monophyletic grouping of serogroup VIII strains. Each of the three serogroup VIII strains in this clade represents a subgroup: *S. syrphidicola* (VIII-1), *S. chrysopicola* (VIII-2), and TAAS-1 (VIII-3). Two additional strains, BARC 2649 and BARC 1357, also belong to this clade. Strain BARC 2649 was described by Williamson and co-workers (7) as an undesignated subgroup of serogroup VIII. The strains of this cluster are very closely related (16S rDNA similarity coefficients 0.992-0.999; 42), limiting the utility of this sequence for detailed phylogenetic analyses. The high degree of sequence similarity among these strains may

be due to a common host source; nearly all serogroup VIII strains are isolated from tabanid flies. Not surprisingly, the serogroup VIII strains have other similarities including smaller genomes as compared to serogroup I, relatively high G + C content (28-31%), rapid growth, arginine utilization, and an unusually small size that allows them to pass quantitatively through a 220 nm filter. In contrast to members of the Citri-Poulsonii clade, which occupy specialized insect/plant habitats, members of this clade appear to be radiating rapidly in dipterous insects and are distributed worldwide.

To date, over 100 additional tabanid-associated members of serogroup VIII have been identified based on antigenic cross-reactivity (F.E. French, personal communication). The net result of these efforts has been to blur the distinctions that seemed clear when only a few strains, each representing separate subgroups, were available. A recent study by Regassa and colleagues (43) examined the utility of the 16S-23S rDNA intergenic sequence for phylogenetic analysis of these closely related isolates. The 16S-23S rDNA phylogenetic reconstructions consistently placed BARC 2649 in a separate clade along with a novel isolate (GSU5367), supporting the classification of BARC 2649 as a new serogroup VIII subgroup. While the 16S-23S rDNA intergenic sequences are promising, additional characters will be needed to generate trees with sufficiently stable topology to resolve all of the nodes in this rapidly expanding clade.

The Mirum clade contains only a single member, the tick spiroplasma *S. mirum*. In all phylogenetic reconstructions, *S. mirum* was always basal to the other Citri-Chrysopicola-Mirum component clades. It is the most basal species identified to date that consistently exhibits the characteristic spiroplasma helicity and motility. In a limited phylogenetic analysis using maximum parsimony, the causative agent of tremor disease in the Chinese mitten crab was identified as a member of the genus *Spiroplasma* that was closely related to *S. mirum* (9).

### 3.2.2.3. The Apis clade

The majority of the isolates examined by Gasparich and co-workers (42) fell into the Apis clade, which is comprised of 14 composite clades. The Apis clade resulted from a major split that occurred after the divergence that led to the Citri-Chrysopicola-Mirum clade. It contains two basal clades (the Lampyridicola-Leptinotarsae and Sabaudiense-Alleghenense-TIUS-1 clades) that are more closely related to the non-helical members of the Mycoides-Entomoplasmataceae clade than to the other members of the Apis clade. The two members of the Lampyridicola-Leptinotarsae clade, *S. lampyridicola* (serogroup XIX) and *S. leptinotarsae* (serogroup XX), were isolated from a firefly beetle and Colorado potato beetle, respectively (15,104). Both of these species exhibit irregular helicity with respect to other spiroplasmas. Interestingly, two members of the Sabaudiense-Alleghenense-TIUS-1 clade (*S. alleghenense* and TIUS-1) also exhibit poor helicity. The Sabaudiense-Alleghenense-TIUS-1 clade is difficult to interpret from an ecological perspective. The three strains in this clade span serogroups

XIII, XXVI and XXIX and were isolated from a diverse group of insects (mosquitoes, tiphiid wasps, and scorpionflies).

The remainder of the 12 composite Apis clades contains 21 closely related *Spiroplasma* strains. Resolution of tree nodes for these closely related strains proved problematic in some cases. Several of the clades contain a single isolate (monospecific clades: Taiwanense, Clarkii, Culicicola, Diminutum, and Monobiae) and will only be discussed below if their association with another composite clade is strongly supported. The Apis-Montanense clade contains *S. apis* (serogroup IV) and *S. montanense* (serogroup XXXI), both of which have been isolated from tabanid flies. *S. apis* has also been isolated from other insect hosts and is the causative agent of May Disease in honey bees. The Litorale-Turonicum-Corruscae clade contains members from serogroups XIV, XVII and XVIII that have all been isolated from tabanid flies. *S. corruscae* has also been isolated from an over-wintered lampyrid beetle, suggesting a possible over-wintering method for the spiroplasmas (105). If the beetles provide a winter reservoir for the spiroplasmas, then tabanid flies could acquire them in the spring at feeding sites shared with the beetles (106). The Chinense-Velocicrescens clade contains two flower-associated spiroplasmas from serogroups XI and XXIV. The Helicoides-Gladiatoris-BARC1901 clade includes three serogroup XXIII, XXXII and XXXIV strains that were isolated from tabanid flies. Clade BIUS-1-W115 contains strains W115 (serogroup XXI) and BIUS-1 (serogroup XXX). Both strains were isolated from flower surfaces and no insect host has been identified. Clade CB-1-Ar 1357 is comprised of two serogroup XVI subgroup strains (CB-1 (XVI-2) and Ar 1357 (XVI-3)). The serogroup XVI-1 strain (*S. cantharicola*) was not included in the analysis. Members of serogroup XVI have been isolated from flower surfaces, cantharid beetles and mosquitoes. *S. diminutum*, a mosquito-associated spiroplasma, was a sister lineage to the CB-1-Ar 1357 clade. Resolution of the Floricola-Diabroticae clade was problematic in some trees, but in general this clade contained serogroup III and XII strains that were either isolated from flower surfaces or from beetles that frequent flowers.

### 3.2.2.4. The Mycoides-Entomoplasmataceae clade

The Mycoides-Entomoplasmataceae clade is a sister lineage to the Apis clade, and it contains the composite Mycoides and Entomoplasmataceae clades. The Entomoplasmataceae clade has both *Entomoplasma* and *Mesoplasma* species, but the two genera do not form distinct clades. Instead, they are intermixed and exhibit very small evolutionary distances. In addition, they share a common host habitat, the insect/plant surface. Given the phylogenetic status of these two genera and a lack of compelling phenotypic characters to differentiate between the two, there have been calls to combine the two into a single genus (76). The Mycoides clade is a derivative of the Entomoplasmataceae clade and contains several *Mycoplasma* species from ruminant animals, including *M. mycoides.* The placement of the Mycoides clade as an evolutionary terminus that is derived from a spiroplasmal ancestor creates a taxonomic dilemma, since *M. mycoides* is the type strain for *Mycoplasma*. Based on the current classification, the *Mycoplasma* genus is split into two phylogenetically separate groups that do not share a common ancestor (Figure 1). A simple change of nomenclature to resolve this discrepancy is not feasible given the importance of the *Mycoplasma* species for veterinary medicine and mycoplasmology.

### 3.2.3. Comparative genomics

The *Mollicutes* were among the first organisms targeted for whole genome sequencing because they have relatively small genomes and many are pathogenic to plants, animals or humans. From an evolutionary perspective, the small genomes of mollicutes are of interest because they may provide information about the minimal gene complement required for independent cellular life (107-110). Since the initial *Mollicutes* genome project was completed for *Mycoplasma genitalium* (111), ten additional mollicute genomes have been completed (112) and others are underway including *S. citri* and *S. kunklii* (113). As the cost and ease of whole genome sequencing projects continues to improve, it is anticipated that the growing genomic dataset will lead to an increased reliance on comparative genomics.

Comparative genomics offers the possibility of assessing some fundamental questions about evolutionary relatedness and biodiversity. By comparing whole genomes of different strains or species, insights can be gained into the degree to which horizontal gene transfer, rearrangements, deletions or insertion events helped to shape the genomic organization of the organisms (114-116). This same information may help to explain the life history of distinct strains/species. For example, comparing a pathogenic isolate to a closely related non-pathogenic organism can help to identify genes that are likely to play a role in pathogenicity (117-120). Similar studies can answer questions about host specificity and habitats. Early work by Gasparich and colleagues (121) identified the widespread occurrence of extrachromosomal DNA in spiroplasmas, both of viral and plasmid origin, which when integrated into the chromosome can interfere with genomic analysis. Subsequent studies by Ye and co-workers (122) compared *S. melliferum* and *S. citri* using physical and genomic maps, revealing some chromosomal rearrangements between the two closely related species. More detailed examination of *S. citri and S. melliferum* genomes has also highlighted the important role that viral DNA may play in genome organization and function (122,123). On a more defined level, comparison of individual genes has yielded insights into strain/species variability. For example, a truncated version of the *recA* gene is highly conserved among six strains of *S. citri* but is absent in *S. melliferum, S. phoeniceum* and *S. apis* (124). In contrast, significant variability was observed among the spiralin gene sequences in six *S. citri* strains (125).

Although comparative genomics offers many intriguing possibilities, the full potential of this approach for examining spiroplasmas is currently limited (reviewed in 113). Multiple genomes for organisms that occupy

different habitats and that pursue different life styles must be completed. In addition, these genomes must be accurately and consistently annotated to allow meaningful comparisons. Proteomics is likely to play a pivotal role in annotation of genomic information. For example, the *M. pneumoniae* genome has been annotated twice (126,127) but recent proteomic data suggests that several coding sequences should be revised (128,129). A similar situation arose from proteomic work on *S. melliferum* that demonstrated production of RecA despite a *recA* gene interrupted by a termination codon (130), indicating that the strain was not completely RecA-deficient as indicated by the DNA sequence. As the dataset of mollicute genomes continues to increase in size, new ways to manage and search the information must be developed. It is also critical that gene expression and biochemical data be linked to the genomic databases in a meaningful way.

## 4. BIODIVERSITY

### 4.1. Host range and interactions

Spiroplasmas are most often found in association with insects and ticks (14,15), including Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, Odonata and Acari. They appear to be restricted to insects of the Holometabola family and the highest Heteropteran orders. Among the insect hosts, the tabanid fly/spiroplasma association is the most widely characterized (*Diptera:Tabanidae*) (7,131). Because of their close association with arthropods, spiroplasmas may prove to be one of the most speciose groups of microbes on earth (21,53,104). In the insect host, spiroplasmas most often multiply within the insect gut but some pathogenic species have expanded their habitat range to include the hemolymph, ovaries, fat bodies, hypodermis or salivary glands (see section 4.1.1). At a much lower frequency, spiroplasmas have also been isolated from plants and higher order invertebrates (see sections 4.1.2 and 4.1.3).

Spiroplasma/host interactions can be classified as commensal, pathogenic or mutualistic (21,35). Most interactions with the host appear to be commensal (21). In the arthropod host, spiroplasmas attach to the epithelial cells of the midgut without any apparent adverse effect on the host. Spiroplasma pathogenicity in an insect host is generally linked to the ability to invade host tissues beyond the midgut. The same general pattern holds true for plant infection sites. Dispersal and transmission of spiroplasmas on plant or flower surfaces is not known to adversely affect the plant, but infection of internal plant tissues is pathogenic. The pathogenic interactions are the most thoroughly studied to date, and are described in detail below. There is also some evidence for mutualistic interactions between spiroplasma and leafhopper hosts. *S. kunkelii* can improve the fitness of the leafhopper *Dalbulus maidis* by enhancing its ability to survive cold temperatures (132). In return, *S. kunkelii* benefits by the maintenance of an over-wintering host in the absence of maize.

### 4.1.1. Insect pathogens

Some insect-associated spiroplasmas are entomopathogens. A critical difference between the incidental commensals and the pathogens appears to be the ability of the pathogenic spiroplasma to move from the initial site of attachment at the gut epithelial cells into the hemolymph. For example, both *S. melliferum* and *S. apis* are honey bee pathogens (14,133,134). They cross the insect gut barrier and reach the hemolymph, where they multiply abundantly and kill the bee.

A different mode of infection and transmission are used for spiroplasmas that cause sex ratio disorders. These sex ratio organisms are transmitted transovarially and kill the male progeny of an infected female fly. The most thoroughly studied example to date is *S. poulsonii*, which was isolated from the neotropical species *Drosophila willistoni* (22-24). In its most extreme form, a *S. poulsonii* infection is able to eliminate all male progeny in infected females. Recently, a closely related *Spiroplasma* isolate was recovered from naturally infected *D. melanogaster* (135). Given the extensive genetic tools available for *D. melanogaster*, this recent isolation may allow for a more thorough understanding of a sex ratio organism in its natural host. Other sex ratio organisms have been reported in association with the coccinelid beetles *Adalia bipunctata* (98) and *Harmonia axyridis* (136), and *Danaus chrysippus* butterflies (100).

### 4.1.2. Plant pathogens

There are three characterized phytopathogenic spiroplasmas, *S. citri, S. kunkelii* and *S. phoeniceum*. Each of these spiroplasmas is maintained in the plant phloem/sucking insect (Heteroptera) habitat. As they cannot survive outside of their plant or insect hosts, they are completely dependent upon transmission to an appropriate host for survival and dispersal. These spiroplasmas are maintained in the insect host and then transmitted to the plant phloem in saliva during feeding; spiroplasma replication and maintenance are confined to the phloem tubes. Once infected, a plant can transmit the spiroplasma to another feeding host as it sucks phloem sap from the sieve tubes. After ingestion the spiroplasmas must cross the insect midgut barrier to the hemolymph, where they replicate before infecting other organs including the salivary glands. In contrast to the insect host pathogens described above, no pathological effects are associated with multiplication of these spiroplasmas in their usual insect host and an infected insect host can serve as a transmission vector throughout its life.

The phytophathogenic spiroplasmas can cause a range of severe symptoms in the infected plant host including stunting, leaf yellowing, sterility, fruit size reduction and deformations, flower malformations, and short internodes. Taken together, these symptoms can severely impact commercial crops. *S. citri* causes citrus stubborn and brittle root disease (16,17), *S. kunkelii* is the causative agent of corn stunt (19-21), and *S. phoeniceum* infections result in aster yellow (or Periwinkle) disease (137). Recent work has focused on elucidating the molecular mechanisms responsible for spiroplasma pathogenicity in the plant host.

*S. citri* is by far the most well-studied of the plant pathogenic spiroplasmas, and we direct the reader to

recent reviews for an in-depth analysis of *S. citri* pathogenicity and transmission (18, 138). A general picture of important determinants for *S. citri* pathogenicity is beginning to emerge. Although translational motility is a core feature of spiroplasmas, motility is not required for transmission to the plant nor is it essential for pathogenicity. A non-motile *S. citri* mutant (*scm1*::Tn*4001*) was transmitted from a leafhopper vector (*Circulifer haematoceps*) to a periwinkle plant resulting in symptoms indistinguishable from those caused by the non-mutated parent strain (139). Two genes have been identified that appear to be involved in insect transmissibility, the Sc76 and spiralin genes. Inactivation of a putative solute binding protein of an ABC transporter (*Sc76*::Tn*4001*) results in a 30-fold decrease in the number of spiroplasmas delivered by the insect host; a similar decrease in the total number of spiroplasma is seen in the salivary glands (140). These results suggest that the mutant spiroplasma is deficient in its ability to either access or replicate within the salivary glands. Spiralin is the major surface antigen of *S. citri* and its most abundant membrane protein. Spiralin is not essential for pathogenicity but is required for efficient transmission from the insect host, with transmission of a spiralin-deficient mutant being decreased about 100-fold (141). Another surface protein, SARP1 (spiroplasma adhesion related protein 1), appears to be involved in attachment of *S. citri* to the insect vector gut membrane (142). Recent studies have also examined the role of carbohydrate catabolism on pathogenicity. Inactivation of genes in the fructose operon reduced pathogenicity (143,144); however, mutations that prevented glucose utilization by *S. citri* had no effect (145). Interestingly, *S. citri* preferentially uses fructose over glucose when both sugars are present (145). Utilization of fructose by the spiroplasma may interfere with the plant's normal physiology by depleting carbon sources in sink tissues and allowing them to accumulate in source leaves (18). It should be noted that fructose is the main carbohydrate for spiroplasmas only in plants. When spiroplasmas inhabit arthropods, they catabolize trehalose as the main sugar source via a process that utilizes part of the glucose uptake system (146).

### 4.1.3. Higher order invertebrate pathogens

Historically, spiroplasmas were associated with insects, ticks and plants; however, recent isolations from crustaceans are beginning to change our understanding of the host range. With the growing economic importance of aquaculture, spiroplasmas are being identified as the causative agents of diseases that adversely affect harvests. The causative agent of tremor disease in the Chinese mitten crab (*Erocheir sinensis*) has been identified as a member of the genus *Spiroplasma* based on phylogenetic reconstructions using the 16S rDNA sequence and confirmed by electron microscopy; this spiroplasma is closely related to *S. mirum* (9). A disease affecting crayfish that were reared in the same aquaculture ponds as the infected Chinese mitten crabs has been attributed to a spiroplasma-like organism (10). Another member of the genus *Spiroplasma* was identified as the infectious agent in lethal outbreaks on shrimp farms causing 10-90% mortality (11). This organism has been serologically placed in the

new serogroup I-9 and named *S. penaei* (78). These recent isolations must affect how we define the spiroplasma host range and may influence future biodiversity studies to include aquatic environments. It will be interesting to learn if spiroplasmas play an ongoing role in the epizootics of aquatic species in natural environments or if the spiroplasma-related outbreaks described above are an anomaly due to the environmental stresses imposed by aquaculture.

### 4.1.4. Vertebrate pathogenicity

Although the bulk of spiroplasma isolations have been from insect and plant habitats, many of these microbes are able to grow at 37°C allowing for the possibility of replication within a vertebrate host. Only one characterized *Spiroplasma* sp., *S. mirum*, has been shown to experimentally infect immunocompromised vertebrates (chick embryos and suckling mice, rats, Syrian hamsters and rabbits) (28, 147,148). *In vitro* growth of *S. mirum* has also been demonstrated in an epithelial cell line derived from the rabbit eye lens (149). Recent reports have suggested that *Spiroplasma* sp. may be associated with transmissible spongiform encephalopathies of humans and animals (150,151); however, studies have failed to amplify spiroplasma or other bacterial 16S rDNA from scrapie infected hamsters (G.E. Gasparich, unpublished data). Additional work needs to be done to establish a causative link between a spiroplasma-like organism and these diseases. Spiroplasma have been implicated in another human disease in a single case report involving a premature baby with a rapidly progressive unilateral cataract associated with severe anterior uveitis; spiroplasma was detected within the lens tissue (152).

### 4.2. Host specificity

Although a vast array of insects and plants can serve as hosts for spiroplasmas, some spiroplasmas appear to exhibit stricter host specificity than others. It seems reasonable that members of the plant phloem/sucking insect habitat would have more tightly defined insect vector host ranges than those inhabiting the insect/plant (or flower) surface habitat due to more complex transmission cycles. The infection of salivary glands in sucking insects requires a more specialized interaction of spiroplasmas with the insect host. For similar reasons, the sex ratio spiroplasmas would be expected to have strict insect host specificity. Very little is known about the mechanisms that allow for adherence and penetration of the spiroplasmas within different insect tissues (e.g. salivary glands, ovaries), but recent studies have suggested that spiroplasma may have pili-like structures and an attachment tip (153,154). A spiroplasma adhesion related protein 1 (SARP1) has also been identified that is correlated with adherence of *S. citri* to leafhopper cells (142,155).

Unfortunately, there are no broad studies that have experimentally tested or environmentally surveyed host specificity for spiroplasmas; there are only a few discrete cases. Some examples are given below to demonstrate the range of host specificity. *S. leptinotarsae* apparently has very strict host specificity. It has only been isolated from the Colorado potato beetle, and attempts to

infect other insects, including beetles, were unsuccessful (21,156,157). In contrast, *S. diabroticae* was isolated from a corn rootworm beetle host but was able to experimentally infect the Colorado potato beetle (21). In general, tabanid-associated spiroplasmas are believed to have a less restrictive host range due to transmission on plant surfaces that are frequented by other spiroplasma-harboring insects (e.g. other *Tabanus* species, fireflies). For example, *S. gladiatoris* was isolated from 11 *Tabanus* species and often occurred in mixed infections with other spiroplasmas (158,159). Common carbohydrate feeding sites (e.g. nectarines and honeydew deposits) would allow for efficient transmission of spiroplasmas among hosts (21); tabanids can become infected with spiroplasmas by ingestion of 5% sucrose solutions that contain spiroplasmas (160). This mode of transmission would also help to explain hosts that harbor infections by multiple spiroplasmas or that obtain spiroplasmas by horizontal transmission from another infected tabanid. To a large extent, plant host specificity is dictated by the insect vector. A sucking insect with a very specific feeding requirement will limit the plant host range for the spiroplasma, whereas a generalist may visit many plant surfaces involved in spiroplasma transmission.

### 4.3. Biogeography

Based on the large number of tabanid-associated spiroplasma isolations in North America, geographical ranges for some species have begun to emerge (159,161). *S. chrysopicola* has a wide range of distribution, spanning isolation sites from Georgia to Wyoming. Both *S. gladiatoris* and *S. tabanidicola* are apparently restricted to a southeastern range encompassing Florida, Georgia and North Carolina. *S. helicoides* has been isolated from collection sites in Oklahoma and the Southeast. *S. litorale* has a very restricted range and has only been isolated at coastal sites in Georgia and North Carolina.

To expand on the previous work in North America, field isolates of Apis clade spiroplasmas were obtained from female tabanid flies at seven locations in Australia, five in Costa Rica, and one in Ecuador (Table 2; 162,163; F.E. French, unpublished data). The isolation sites included temperate and tropical locales with elevations of 2-2000 meters. The spiroplasmas were isolated using standard methods (106), and pure cultures were prepared using a dilution cloning technique (61). Forty-three Australian isolates from 19 tabanid species in Queensland and New South Wales were obtained. A total of 57 isolations from the highlands and lowlands of Costa Rica were made from 14 tabanid species. Eight isolates from Ecuador were obtained from three tabanid species. This study also included four serologically distinct groups from the U.S.A. that represent over 100 individual isolates.

All of the isolates were screened by spiroplasma deformation (DF) tests against 12 combinations of 34 antisera to tabanid-associated spiroplasma species or groups from the U.S.A. and France (164). Isolates that failed to cross-react with any of the antisera combinations were used for polyclonal antiserum production. The antisera produced were individually examined in reciprocal DF tests with tabanid-associated spiroplasma antigens or antibodies from characterized North American and French isolates (79,164), as well as from the novel serogroups identified in this study (162;163; F.E. French, unpublished data). Group placement was achieved by reciprocal deformation reactions at antisera dilutions ≥1:320.

The serological analyses resulted in the identification of 26 independent serogroups (Table 2). For the Australian isolates, one serogroup, represented by strain GSU5603, contained 21 isolates. The 22 other isolates were placed into three serogroups represented by GSU5510, GSU5478 and GSU5508. Serological analysis of the Costa Rican isolates revealed that three of the Costa Rican serogroups were closely related to species previously identified in the U.S.A.; *S. litorale* (7 isolates), *S. lineolae* (6 isolates), and *S. helicoides* (8 isolates). Three additional isolates were related to another U.S.A. tabanid-associated isolate, BARC 4689. The remaining 33 isolates were unique to Costa Rica and were classified into 11 new serogroups. From Ecuador, three isolates could be placed in a new serogroup represented by strain GSU5862. Five additional isolates represented four distinct serogroups. Four unique U.S.A. serogroups contained a total of 111 isolates. Although the U.S.A. and Costa Rica shared four common serogroups, serogroups from Australia or Ecuador were confined to those regions. Thus, geography appears to play a role in serogroup distribution. Phylogenetic reconstructions based on 16S rDNA sequences for these strains are currently underway to determine their evolutionary relationships (L.B. Regassa, unpublished data).

## 5. PERSPECTIVES

Spiroplasma research is poised for major advances with new criteria for naming species under revision, a dramatic increase in available molecular characters and the promise of full genome sequences, and the availability of genetic tools for manipulation of these organisms. The unique qualities and life histories of spiroplasma make them ideally suited to ask a number of fundamental questions about degenerative evolution, host/pathogen relationships and co-evolution, host specificity, and insect- and plant-vectored maintenance/dispersal cycles.

Revision of the minimal standards document for *Spiroplasma* species designation will permit *Spiroplasma* systematics to move forward. The field can only progress if information can be shared using a common set of published criteria that are in line with modern systematic tools. Toward that end, the new guidelines will increase the reliance on molecular phylogenies (R.F. Whitcomb, personal communication). The current minimal standards document adopted in 1995 (36) requires tests that are beyond the resources of many laboratories. Streamlining and updating the criteria will encourage more species descriptions. By facilitating incorporation of new species into the known spiroplasma strain matrix, we will enhance our understanding of spiroplasma biodiversity and evolution. Revised guidelines will also encourage new researchers to enter the field.

**Table 2.** Apis clade *Spiroplasma* from *Tabanidae:Diptera* of Australia, Costa Rica, Ecuador and U.S.A.

| Serogroup representatives by country of origin | Number of serologically similar isolates[1] | Host of serogroup representative | Locality of serogroup representative host[2] |
|---|---|---|---|
| **Australia** | | | |
| GSU5603 | 21 | *Scaptia lasiophthalma* | Batemans Bay, NSW |
| GSU5510 | 11 | *Tabanus townsvilli* | Karyina Station, QLD |
| GSU5478 | 6 | *Tabanus pallipenis* | Eungella, QLD |
| GSU5508 | 5 | *Tabanus particaecus* | Karyina Station, QLD |
| **Costa Rica** | | | |
| TABS-2 (*S. helicoides*) | 8 | *Tabanus abactor* | Oklahoma, U.S.A. |
| TN-1 (*S. litorale*) | 7 | *Tabanus nigrovittatus* | North Carolina, U.S.A. |
| TALS-2 (*S. lineolae*) | 6 | *Tabanus lineola* | Georgia, U.S.A. |
| BARC 4689 | 3 | *Tabanus lineola* | Georgia, U.S.A. |
| BARC 4906 | 4 | *Poeciloderas quadripunctatus* | Sto. Domingo, Heredia Prov |
| BARC 4900 | 2 | *Poeciloderas quadripunctatus* | Sto. Domingo, Heredia Prov |
| BARC 4886 | 1 | *Poeciloderas quadripunctatus* | San Gerardo de Dota, San José Prov. |
| GSU5450 | 2 | *Poeciloderas quadripunctatus* | Sto. Domingo, Heredia Prov. |
| GSU5382 | 8 | *Tabanus punctipleura* | Pto Vargas N.P., Lemon Prov. |
| GSU5360 | 4 | *Dichelacera princessa* | Pto Vargas N.P., Lemon Prov. |
| GSU5373 | 3 | *Tabanus occidentalis* | Pto Vargas N.P., Lemon Prov. |
| GSU5443 | 3 | *Tabanus oculus* | Curu, Puntarenas Prov. |
| GSU5446H | 3 | *Tabanus nebulosus* | Curu, Puntarenas Prov. |
| GSU5366 | 2 | *Tabanus secundus* | Pto Vargas N.P. Lemon Prov. |
| GSU5405 | 1 | *Tabanus occidentalis* | Hiltoy Cerere, Lemon Prov. |
| **Ecuador** | | | |
| GSU5862 | 3 | *Tabanus occidentalis* | Tiputini Bio. Sta., Napo Prov |
| GSU5867 | 2 | *Tabanus occidentalis* | Tiputini Bio. Sta., Napo Prov |
| GSU5865 | 1 | *Tabanus occidentalis* | Tiputini Bio. Sta., Napo Prov |
| GSU5858 | 1 | *Phaeotabanus nigriflavus* | Tiputini Bio. Sta., Napo Prov. |
| GSU5853 | 1 | *Phaeotabanus prasiniventris* | Tiputini Bio. Sta., Napo Prov |
| **U.S.A.** | | | |
| TAUS-1 (*S. tabanidicola*) | 93 | *Tabanus sp.* | Beltsville, Maryland |
| BARC 4689 | 4 | *Tabanus lineola* | Bulloch Co., Georgia |
| GSU4980 | 3 | *Tabanus lineola* | Evangeline Parrish, Louisiana |
| TALS-2 (*S. lineolae*) | 11 | *Tabanus lineola* | Bulloch Co., Georgia |

[1] Number of serologically similar isolates from country of origin (not including serologically related isolates from another country), [2] Specific locale information follows: Batemans Bay, NSW, Australia, 5 km N in Benandarah St. Forest, S35°40.25'E150°12.84'; Curu, Refruge Nac. de Vida Silvestré, Puntarenas Prov., Costa Rica N9°48.17W84°55.52 ele. 35m; Eungella, QLD, Australia, Frank Kerr Dairy, S21°10.04'E148°30.41'; Heredia Prov., Costa Rica, INBio, N9°58.5'W84°5.5', ele. 1,100m, Sto. Domingo; Hiltoy Cerere Bio.Sta. Lemon Prov., Costa Rica, N09°40.37W83°01.44, ele.100m; Jimna St.Forest QLD, Australia, Sunday Creek Environment Studies Center S26°42'E152°32.18' ; Karyina Station, QLD, Australia, S. adjacent to Isla George Natl.Park, S25°13.19'E149°57.84'; Narrabri, NSW, Australia, 11km S.Spring Creek & Hwy39, S30°24.42'E149°41.98'; Pto.Vargas Nat. Pk., Lemon Prov.,Costa Rica, N09°42.930W82°49.269, ele. 2m; San Gerardo de Dota on Rio Savegre, San José Prov., Costa Rica, N9°33.20'W83°48.38', ele. 2,000m; Tiputini Biodiversity Station, Napo Providence, Ecuador 0°38.3'S,76°8.9'W, ele. 220m.

Historically, the mollicutes have played a prominent role in whole genome sequencing projects. Their small genomes have made them attractive subjects, providing a relatively easy sequencing project and offering insights into the minimal gene complement required for independent cellular life. As the cost and ease of whole genome projects continues to improve, there is a hope that numerous spiroplasma genomes will be sequenced. Comparative genomics holds out the possibility of addressing questions about degenerative evolution, host/habitat specificity and pathogenicity. It may also highlight unique characters that could be used for multilocus phylogenetic analysis, thereby enhancing our overall understanding of the evolutionary relationships among the spiroplasmas.

Finally, the use of newly emerging genetic tools to answer discrete questions of pathogenicity and gene function will enhance our understanding of the host/spiroplasma interaction. To date, adaptation and use of genetic tools in spiroplasmas has been focused on plant pathogens that adversely affect agricultural crops. These studies are providing insights into spiroplasma attachment, host tissue invasion and spiroplasma biochemistry in the host (18). Some of the information learned will undoubtedly impact our understanding of non-pathogenic spiroplasmas as well. The genetic tools being developed and used may be functional across sufficiently broad host ranges to permit their use in other spiroplasmas to pursue questions of host/habitat specificity or pathogenicity in non-plant hosts (e.g. crustaceans).

**6. ACKNOWLEDGMENTS**

**7. REFERENCES**

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