Differentiation of group VIII Spiroplasma strains with sequences of the 16S–23S rDNA intergenic spacer region

Laura B. Regassa, Kimberly M. Stewart, April C. Murphy, Frank E. French, Tao Lin, and Robert F. Whitcomb

Abstract: Spiroplasma species (Mollicutes: Spiroplasmataceae) are associated with a wide variety of insects, and serology has classified this genus into 34 groups, 3 with subgroups. The 16S rRNA gene has been used for phylogenetic analysis of spiroplasmas, but this approach is uninformative for group VIII because the serologically distinct subgroups generally have similarity coefficients >0.990. Therefore, we investigated the utility of the 16S–23S rRNA spacer region as a means to differentiate closely related subgroups or strains. We generated intergenic sequences and detailed serological profiles for 8 group VIII Spiroplasma strains. Sequence analyses using Maximum Parsimony, Neighbor Joining, and Maximum Likelihood placed the strains into 2 clades. One clade consisted of strains BARC 2649 and GSU5367. The other clade was divided into clusters containing representatives of the 3 designated group VIII subgroups (EA-1, DF-1, and TAAS-1) and 3 previously unclassified strains. The stability of the positions of the strains in various analytical models and the ability to provide robust support for groupings tentatively supported by serology indicates that the 16S–23S intergenic rDNA sequence will prove useful in intragroup analysis of group VIII spiroplasmas.

Key words: Mollicutes, Spiroplasma, phylogeny, Tabanidae.

Introduction

Spiroplasmas (Procaryotae: Mollicutes: Entomoplasmatales: Spiroplasmataceae) are among the smallest self-replicating procaryotes known, with genomes ranging in size from approximately 780 to 2220 kbp (Carle et al. 1995; Williamson et al. 1997). They apparently evolved via simplification of Gram-positive bacteria (Woese 1980; Weisburg et al. 1989). As the organisms radiated to occupy multiple habitats, they became able to invade the arthropod gut lumen and, either as part of their life cycle or incidentally, to invade other habitats in the hemolymph, ovaries, fat bodies, hypodermis, and salivary glands. Because of their diverse hosts, spiroplasmas may be one of the most abundant groups of microbes on earth (Hackett and Clark 1989; Hackett et al. 1992). They are found in association with a wide variety of arthropods, such as, horse flies and deer flies (Diptera: Tabanidae) (Bové 1997; Williamson et al. 1998). To date,
tabanids have been recorded as hosts for 11 of the 34 known groups of *Spiroplasma*, including almost all group VIII strains (Clark et al. 1984; French et al. 1990; Whitcomb et al. 1997; Williamson et al. 1998).

Classification of spiroplasmas into provisional taxa based on serological and genomic properties began with the proposal of 5 groups (Junca et al. 1980). In time, 29 other groups were added (Williamson et al. 1998). Since serology has approximately the same resolving power as DNA–DNA reassociation, it has been used exclusively to define groups and the species into which they morph after characterization by a mandated set of taxonomic tests (International Committee on Systematic Bacteriology (ICSB) 1995). The serological criteria, upon which the group classification is based, have been “calibrated” against the standard technique of DNA–DNA reassociation (Junca et al. 1980; Mouches et al. 1982), which is the basis for the bacterial species concept (Johnson 1993; Wayne et al. 1987). The accepted standard of 70% or greater DNA–DNA homology for a species worked well for the initial group designations. However, several distinct groups were identified with 30%–70% homology and various degrees of reciprocal cross-reactive serology, suggesting the need to introduce the subgroup concept to describe these related isolates (ICSB 1995). Currently, groups I (Bové et al. 1983), VIII (Gasparich et al. 1993), and XV (Abalain-Colloc et al. 1993) are divided into 8, 3, and 3 subgroups, respectively. The group VIII subgroups were recognized as such only after the isolation of a “bridge” strain that serologically cross-reacted with both EA-1 (group VIII) and DF-1 (*Spiroplasma chrysopica*, then “group XVII”), resulting in the current designation of the group VIII subgroups (Gasparich et al. 1993). Note that after the removal of the group XVII designation from strain DF-1, the group XVII designation was subsumed by *Spiroplasma turonicum* (Helias et al. 1998).

Recently, phylogenetic reconstructions of spiroplasma evolution based on 16S rDNA sequences have been completed, indicating a strong correlation between the serological groups and subgroups and molecular phylogeny (Gasparich et al. 2004). 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; trees generated by maximum parsimony and other analyses divide the genus into clades that have clear biological significance (Gasparich et al. 2004). However, the lack of sequence divergence in the 16S rRNA genes of group VIII spiroplasmas (similarities >0.990) has prevented detailed phylogenetic analysis of this group; there is insufficient variability to clearly differentiate even the existing subgroups (Gasparich et al. 2004), without consideration for the many new isolates from tabanids (F.E. French, unpublished data). Serology, which reliably identifies groups and subgroups, also appears unable to make fine distinctions among group VIII strains. Therefore, in the absence of a means for strain classification, nomenclatural taxonomy of group VIII spiroplasmas has come to a standstill.

Sequences of the 16S–23S rDNA intergenic region may be better suited for intragroup analyses rather than gene sequences because they are generally less highly conserved than the adjacent 16S and 23S rRNA genes. This approach has been used to successfully determine the evolutionary relationships among closely related species of several bacterial genera, including non-group VIII *Spiroplasma*, *Ureaplasma*, *Mycoplasma*, *Listeria*, and *Mycobacterium* species (Graham et al. 1997; Harasawa 1999; Harasawa and Kanamoto 1999; Van der Giessen et al. 1994; Von der Schellenburg et al. 2000). In this study, we used the 16S–23S rDNA intergenic sequences from 8 group VIII spiroplasmas to assess strain relatedness by the method of phylogenetic reconstruction. We then compared this reconstruction with the serological interrelatedness of the 8 strains.

Materials and methods

Collection and isolation of spiroplasmas

The 8 *Spiroplasma* strains used in this study were isolated from female tabanids in temperate to tropical locations within the continental United States, Costa Rica, and Australia (Table 1). Five of the strains have been characterized previously as group VIII spiroplasmas and are as follows: *S. chrysopica* DF-1 (ATCC 43209), *Spiroplasma syphricola* EA-1 (ATCC 33826), TAAS-1 (ATCC 51123), BARC 2649 (ATCC 700284), and BARC 1357 (ATCC BAA-961). The remaining 3 strains were produced by cloning novel isolates designated GSU5367 and GSU5431 (ATCC BAA-963 and ATCC BAA-964, respectively; Stewart 2001) and GSU5485 (ATCC BAA-962; Murphy 2003). The novel isolates were obtained from tabanids using standard methods (Wedincamp et al. 1996). The host flies were captured using Gressitt/Malaise traps or hand nets. Flies were chilled, surface-sterilized with 0.5% NaOCl for at least 45 s, then rinsed in distilled water for 45 s, and the terminal abdominal segment was cut off. The viscera were removed, minced in 1.5 mL of medium, passed through a 0.45-μm filter, incubated in M1D broth (Whitcomb 1983) at 30 °C, and observed daily for growth. *Spiroplasma* cultures were monitored by dark-field microscopy at 1200× magnification, and suitable cultures were stored at −70 °C.

To obtain pure cultures of strains GSU5367, GSU5431, and GSU5485, a dilution cloning technique was used (Whitcomb and Hackett 1987). Actively growing *Spiroplasma* cultures were serially diluted in M1D broth containing phenol red indicator. Culture dilutions of 10−7 to 10−11 were distributed on 96-well microtiter plates with 200 μL per well. As cultures grew and acidified, the media changed from red to yellow. The plates were observed daily for color change. Organisms growing within a single, isolated yellow well at the highest possible dilution were subcultured after microscopic confirmation of their identity as spiroplasmas. This cloning process was completed a total of 3× to obtain triply cloned strains, which were stored at −70 °C.

Antiserum production

Triply cloned cultures of strains GSU5367, GSU5431, and GSU5485 were used for antiserum production. Each strain was grown in 500 mL of M1D broth at 37 °C. The cells were collected by centrifugation, washed in 20 mL of phosphate buffered saline (PBS (pH 7.3); 137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na2HPO4·H2O, 1.4 mmol/L KH2PO4), and then resuspended in 10 mL of PBS. A 2.4-mL aliquot of the cells was added to lyophilized RIBI adjuvant...
**Table 1. Spiroplasma isolates used in this study and their respective site of isolation and host.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fly/host</th>
<th>Locality</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BARC 2649</td>
<td>Tabanus lineola</td>
<td>Georgia, USA</td>
<td>Whitcomb et al. 1997</td>
</tr>
<tr>
<td>(ATCC 700284)</td>
<td></td>
<td>(N32°31.1′, W81°47.6′)</td>
<td></td>
</tr>
<tr>
<td>BARC 1357</td>
<td>Tabanus lineola</td>
<td>Georgia, USA</td>
<td>Whitcomb et al. 1997</td>
</tr>
<tr>
<td>(ATCC BAA-961)</td>
<td></td>
<td>(N32°31.1′, W81°47.6′)</td>
<td></td>
</tr>
<tr>
<td>GSUS5367</td>
<td>Diachlorus curvipes</td>
<td>Province Limón, Costa Rica</td>
<td>This study</td>
</tr>
<tr>
<td>(ATCC BAA-963)</td>
<td></td>
<td>(N9°42.9′, W82°49.3′)</td>
<td></td>
</tr>
<tr>
<td>GSUS5431</td>
<td>Tabanus occidentalis</td>
<td>Province Puntarenas, Costa Rica</td>
<td>This study</td>
</tr>
<tr>
<td>(ATCC BAA-964)</td>
<td></td>
<td>(N9°48.2′, W84°55.5′)</td>
<td></td>
</tr>
<tr>
<td>GSUS5485</td>
<td>Cydistomyia sp.</td>
<td>Queensland, Australia</td>
<td>This study</td>
</tr>
<tr>
<td>(ATCC BAA-968)</td>
<td></td>
<td>(S21°10.0′, E148°30.4′)</td>
<td></td>
</tr>
<tr>
<td>Spiroplasma chrystopica DF-1</td>
<td>Chrysops sp.</td>
<td>Beltsville, Maryland, USA</td>
<td>Gasparich et al. 1993</td>
</tr>
<tr>
<td>(ATCC 43209)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spiroplasma syrphidica EA-1</td>
<td>Eristalis arbustum</td>
<td>Beltsville, Maryland, USA</td>
<td>Gasparich et al. 1993</td>
</tr>
<tr>
<td>(ATCC 33826)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAAS-1</td>
<td>Tabanus atratus</td>
<td>Big Bend National Park, Texas, USA</td>
<td>Gasparich et al. 1993</td>
</tr>
<tr>
<td>(ATCC 51123)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*S* diptera: Tabanidae;  
*S* diptera: Syrphidae.

(Sigma Chemical Company, St. Louis, Missouri) and two 1.0-mL doses were administered to a rabbit at a three-week interval according to the manufacturer’s instructions. The animals were cared for in accordance with approved guidelines set forth in the *Guide for the Care and Use of Laboratory Animals*, and their use was reviewed and approved by the Institutional Animal Care and Use Committee of Georgia Southern University.

**Serological analysis**

The group affiliation of strains GSUS5367, GSUS5431, and GSUS5485 were first determined by screening primary isolates against 12 combinations of 34 antisera to spiroplasmas associated with tabanids (Whitcomb et al. 1999). Antisera were obtained from the reference collections at the Beltsville Agricultural Research Center (Beltsville, Maryland), the National Institute of Allergy and Infectious Diseases Laboratory (Frederick, Maryland), and our laboratory. Actively growing *Spiroplasma* cultures were mixed with combinations of antisera, with each antiserum at a 1:20 reaction dilution. Each isolate was allowed to react with the screening antisera for 30 min at room temperature. Group placement was established by observation of at least 50% cell deformation in a minimum of 3 independent screening assays.

Detailed serological analyses on triply cloned strains derived from the isolates were completed using the spiroplasma deformation (DF) test with individual antisera as described by Williamson et al. (1978). The antisera were diluted in M1D broth to initial concentrations of 1:10 to 1:1280 and then reacted with an equal volume of an actively growing *Spiroplasma* culture for 30 min at room temperature. Positive reactions were identified microscopically by deformation or clumping of at least 50% of the cells. Strain resolution, or identification, was achieved by reciprocal reactions at dilutions ≥1:320. Each test was performed at least 3×, with the highest dilution that consistently reacted positively being reported.

**DNA isolation, amplification, and sequencing**

Genomic DNA was isolated using the method described by Duret et al. (1999). Briefly, cultures were grown in M1D broth at 30 °C until a cell density of approx. 10⁶ cells/mL was achieved. Cells were then harvested by centrifugation, resuspended in STE buffer (10 mmol/L Tris–HCl (pH 7.5), 1 mmol/L EDTA (pH 8.0), 10 mmol/L NaCl), and lysed with 10% SDS at 67 °C. The lysate was treated with RNase A (Promega Corporation, Madison, Wisconsin) and then phenol-extracted. DNA was recovered by ethanol precipitation and resuspended in TE (10 mmol/L Tris–HCl (pH 7.5) 1 mmol/L EDTA (pH 8.0)). The DNA concentration for each sample was determined on a 1% agarose gel after ethidium bromide staining.

To isolate 16S–23S intergenic DNA, genomic DNA was used as a template for PCR amplification. A PCR product of approximately 450 bp was amplified using primers that we designed with homology to the 16S and 23S rRNA genes (5'-CGGTGAAATACGTTCGTCG-3' and 5'-CAAGGCGATCCACCATAC-3', respectively). Amplification was carried out in a 50-µL reaction mixture containing 100 ng of genomic DNA, 100 pmol of each primer, 1× *Taq* polymerase Buffer A, 0.2 mmol/L deoxynucleotide triphosphate (dNTPs), 2.5 U *Taq* polymerase, and 1.5 mmol/L MgCl₂. All PCR reagents were purchased from Promega Corporation (Madison, Wisconsin). Amplification cycles were completed as follows: denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 45 s, 46 °C for 45 s, 72 °C for 60 s; and 1 cycle at 72 °C for 2 min. PCR products were separated on a 2% agarose gel and purified using a Qiagen Gel Extraction Kit (Qiagen Inc., Valencia, California). Amplified products were sequenced at the Davis Sequencing Facility (Davis, California) using the PCR primers described above. All regions were double-strand sequenced, with any discrepancies between the 2 strands being resolved by additional sequencing reactions. Sequences were aligned using CLUSTAL W (Thompson et al. 1994), and a 293-bp consensus sequence was chosen for phylogenetic analysis. The nucleotide sequences determined in this study have been deposited in the GenBank database with accession numbers AY549209 (*S. syrphidica* EA-1), AY549210 (GSUS5485), AY549211 (BARC 1357), AY549212 (GSUS5431), AY549213 (GSUS5467), AY549214 (BARC 2649), AY549215 (TAAS-1), and AY549216 (*S. chrysopica* DF-1).

**Phylogenetic analysis**

All phylogenetic trees were constructed with 293 characters in the dataset; no gaps were present. Sequences were first aligned using CLUSTAL W software (Higgins and Sharp 1989) and then adjusted manually. Neighbor Joining, Maximum Parsimony, and Maximum Likelihood trees were constructed with PAUP (phylogenetic analysis using parsi-
**Table 2. Serological cross-reactivity of group VIII *Spiroplasma.***

<table>
<thead>
<tr>
<th>Antigen</th>
<th>BARC 2649</th>
<th>GSU 5367</th>
<th>BARC 1357</th>
<th>GSU 5431</th>
<th>TAAS-1</th>
<th>EA-1</th>
<th>GSU 5485</th>
<th>DF-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BARC 2649</td>
<td>320(^a)</td>
<td>640</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GSU5367</td>
<td>640</td>
<td>≥2560(^c)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BARC 1357</td>
<td>— (^b)</td>
<td>1280</td>
<td>20</td>
<td>1280</td>
<td>640</td>
<td>20</td>
<td>—</td>
<td>80</td>
</tr>
<tr>
<td>GSU5431</td>
<td>—</td>
<td>320</td>
<td>80</td>
<td>160</td>
<td>≥2560</td>
<td>40</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TAAS-1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>40</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EA-1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1280</td>
<td>160</td>
<td>160</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GSU5485</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>20</td>
<td>640</td>
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<tr>
<td>DF-1</td>
<td>—</td>
<td>20</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>≥2560</td>
</tr>
</tbody>
</table>

\(^a\)The reciprocal of the greatest dilution exhibiting at least 50% cell deformation is shown.

\(^b\)No reaction observed at a dilution ≥1:20.

\(^c\)The highest dilution of antibody in our assay was 1:2560.

**Results**

**Isolation and characterization of novel group VIII strains**

Three new *Spiroplasma* isolates (GSU5367, GSU5431, and GSU5485) were obtained from tabanids in Costa Rica and Australia (Table 1). Preliminary screening with a bank of sera directed against tabanid spiroplasmas showed that the isolates were reactive with a mixture of antisera to group VIII strains BARC 1357, BARC 2649, DF-1, EA-1, and TAAS-1. No positive screening reactions were observed with 29 other antisera, in various combinations, to known tabanid-associated spiroplasmas (data not shown). After identification to group VIII, the novel isolates were triply cloned to obtain pure cultures and designated strains GSU5367, GSU5431, and GSU5485.

**Serological analysis of group VIII strains**

Cross-reactivity among the 8 group VIII strains was determined using the DF test (Table 2). The titres of the individual antisera varied, with homologous reactions exhibiting positive results at dilutions of 1:320 to 1:2560. For 68% of the heterologous reactions, no deformation was observed. If weak or relatively weak serological reactions (dilutions ≤1:80) are added to this grouping, then the number increases to 88%. This is typical of group VIII strains, which rarely show strong serological cross-reactivity with available heterologous group VIII antisera (Gasparich et al. 1993; Whitcomb et al. 1997; F. E. French, unpublished data). The identity of strain GSU5367 was resolved by this method; it serologically matched BARC 2649 with reciprocal reactions of 1:640 (Table 2). The reactivity of BARC 2649 antisera with GSU5367 antigen (1:640) was actually greater than that seen for the BARC 2649 homologous reaction (1:320); this type of anomaly has been occasionally observed by others (Whitcomb et al. 1997). There were 3 one-way reactions with scores of 1:640 or 1:320, but none were reciprocal (Table 2). A meaningful serological relationship between GSU5431 and TAAS-1 may exist with reciprocal titres of 1:160 and 1:640 (Table 2).

**Molecular and phylogenetic analysis of group VIII strains**

PCR was used to amplify the 16S–23S rRNA spacer region from each of the group VIII strains, and approx. 450 bp of confirmed sequence was obtained. Between 125–150 bp corresponded to the 16S rRNA gene and at least 293 bp were 16S–23S rDNA intergenic sequence. The short segment of 16S rDNA sequence was identical for all strains as expected (data not shown); group VIII strains generally exhibit ≥99% identity within the 16S rDNA gene (Gasparich et al. 2004). The 16S–23S intergenic DNA sequence was less highly conserved, with 11 substitution sites over the 293-bp sequence. Each nucleotide variation at the 11 substitution sites was observed in at least 2 of the 8 strains (data not shown).

A phylogenetic tree for the 8 *Spiroplasma* isolates was derived from the 293-bp 16S–23S rDNA intergenic sequences (Fig. 1). The Maximum Parsimony method generated 43 equally parsimonious trees, with the strict consensus Maximum Parsimony, Maximum Likelihood, and Neighbor Joining trees all showing similar topologies. All 3 analyses separated the strains into 2 main groupings, with respective Maximum Parsimony and Neighbor Joining bootstrap values of 0.81–0.99 and 0.99–1.00. The Maximum Parsimony and Maximum Likelihood analyses accord these major groupings the status of clades. Strains BARC 2649 and GSU5367 formed 1 clade that was separated from the other group VIII strains by an appreciable evolutionary distance. In the other clade, distinct clusters of strains were observed. In one cluster, strain BARC 1357 was shown to be a sister to strain EA-1 with Maximum Parsimony and Neighbor Joining bootstrap values of 0.66 and 0.65, respectively. All 3 phylogenetic reconstructions tentatively placed strains DF-1 and TAAS-1 in one cluster and strain GSU5485 in a separate cluster (bootstrap values <0.50). Strain GSU5431 was tentatively classified into a distinct cluster in the Maximum Parsimony analysis, but it was more closely associated with the EA-1/BARC 1357 cluster in the Neighbor Joining and Maximum Likelihood analyses (bootstrap values <0.50).
Discussion

Serological and 16S rDNA gene analysis provide the current basis for Spiroplasma classification. Serology has clearly delimited 34 groups (species or putative species), with 3 of these groups being divided into a total of 14 subgroups. However, serology becomes progressively less useful as attempts are made to achieve finer and finer distinctions. 16S rDNA sequence analysis permits excellent differentiation of spiroplasmas at the intergroup level if the sequence similarities are <0.970. It also provides some information when the similarities are 0.970–0.990, as is the case among group I spiroplasmas (Gasparich et al. 2004). Phylogenetic analyses correlate well with the currently characterized serological groups and subgroups; every case of reciprocal serological cross-reactivity has been supported by phylogenetic reconstructions based on 16S rDNA sequence analyses (Gasparich et al. 2004). However, the converse is not always true, as phylogenetic reconstructions sometimes imply strong relationships among serologically dissimilar strains; these apparent discrepancies may be resolved as more samples are analyzed. For example, group VIII strains form a tight phylogenetic cluster. However, 2 of the group VIII subgroups were originally placed in separate serogroups until a bridge strain (BARC 1357) was identified that exhibited a complex set of serological reactions with the other 2 groups, resulting in the current subgroup designations (Gasparich et al. 1993). If the group VIII bridge strain had not been discovered, then the tight group VIII cluster identified by phylogeny would not have been supported by the serological evidence.

As useful as 16S rDNA sequence data is for group and subgroup taxonomy of the vast majority of spiroplasmas, the sequence analysis (like the serological analysis) reaches its limits when attempts are made to make fine distinctions among strains. This is particularly true for group VIII strains that often exhibit 16S rDNA sequence similarities of approximately 0.990 (Gasparich et al. 2004) and that rarely show strong serological cross-reactivity (Gasparich et al. 1993; Whitcomb et al. 1997; F.E. French, unpublished data). Thus, group VIII spiroplasmas present an insurmountable barrier to the use of either technique to define intraspecific taxa. As stated above, this group of tabanid-associated spiroplasmas was originally divided into 3 subgroups on the basis of serological and DNA–DNA reassociation data (Gasparich et al. 1993). To date, more than 100 group VIII isolates have been characterized serologically, and the net result of all these efforts has been to blur the distinctions that seemed clear when only a few isolates were available. The status of strain clusters within group VIII is now sufficiently complicated that an effective moratorium exists on defining additional group VIII subgroups. And, since group VIII subgroups have been accorded putative species status, the moratorium places an effective halt on all nomenclatural systematics of group VIII spiroplasmas.

Perhaps the best strategy for defining the relationships among group VIII strains will be to rely on phylogenetic reconstructions based on a chromosomal region that is not as highly conserved as the 16S rRNA genomic region. In this study, we investigated the utility of the 16S–23S rRNA spacer region as a tool for differentiating these closely related strains because it is usually less conserved than the flanking 16S or 23S RNA genes. The consensus sequences used for our phylogenetic tree spanned 293 bp within the 16S–23S rRNA spacer region and had 11 non-conserved sites (3.75% variability). This level of sequence divergence fell within the range (1.21%–18.88%) previously reported for the spacer region of 5 non-group VIII spiroplasmas (Von der Schulenburg et al. 2000). The group VIII 16S–23S rDNA intergenic sequences provided sufficient variability to differentiate the majority of strains.

Sequence analyses using Maximum Parsimony, Neighbor Joining, and Maximum Likelihood divided the group VIII strains into 2 clades. One of the clades, consisting of strains BARC 2649 from Georgia and GSUS367 from Costa Rica, was firmly supported by bootstrapped Maximum Parsimony (0.81, Fig. 1) and Neighbor Joining (1.00) trees. The 2nd clade, which contained representatives of the 3 designated group VIII subgroups, included strains from the United States (Maryland, Georgia, Texas), Costa Rica, and Australia. Detailed phylogenetic analysis of this clade identified distinct strain clusters, with the relative positions of the strains being very similar in trees generated by Maximum Parsimony, Neighbor Joining, and Maximum Likelihood. In one cluster, strain EA-1 (subgroup VIII-1: Spiroplasma syrphidace) from Maryland was shown to be a sister to strain BARC 1357 from Georgia in all 3 analyses (bootstrap values 0.65–0.66). Another cluster that was tentatively maintained in all analyses contained strains DF-1 (subgroup VIII-2; S. chrysopicola) from Maryland and TAAS-1 (subgroup VIII-3) from Texas (bootstrap values <0.50). Strains GSUS541 from Costa Rica and GSUS5485 from eastern Australia were classified into separate clusters in the analyses, although GSUS5431 showed a tentative relationship with the EA-

Fig. 1. Phylogenetic tree derived from the 293-bp 16S–23S rDNA intergenic sequence for the 8 group VIII Spiroplasma isolates by Maximum Parsimony analysis; Spiroplasma citri (AF005327) was designated as the outgroup. The analysis was performed as a heuristic search procedure using the tree bisection reconnection algorithm for branch swapping. Branch length is proportional to percent divergence (scale bar shown at bottom). Bootstrap confidence levels above 50% are indicated to the left of each relevant cluster. Each entry on the tree is represented in the following order: strain designation / isolation site / host.

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I/BARC 1357 cluster in the Neighbor Joining and Maximum Likelihood analyses (bootstrap values <0.50).

Comparison of the serological results and the phylogenetic relationships showed congruency between strong reciprocal serology and phylogeny; however, detailed phylogenetic relationships and low-level serological cross-reactions often lacked congruency. In our study, strains BARC 2649 and GSU5367 exhibited strong reciprocal serology, but showed no significant cross-reactivity with any of the other group VIII strains (Table 2). These results agree with those of Williamson et al. (1998), who identified strain BARC 2649 as an undesigned subgroup of group VIII. Thus, the serological and phylogenetic results for the BARC 2649 clade were congruent. The second clade was not supported by strong (≥1:320) reciprocal serological reactions, but this is to be expected, given the number of strains within the clade and the known strong proclivity for rapid serological divergence of group VIII strains. The diffuse pattern of low-level serological reactions for many of the group VIII strains suggests that these isolates may represent a pattern of continuous antigenic variation rather than discrete antigenic variants, thereby blurring serological strain distinctions. Overall, the clade was tentatively supported by the complex pattern of low-level reciprocal cross-reactions. The low-level cross reactions suggested that these strains were linked directly or via “bridge strains”, with the exception of DF-1, which did not exhibit reciprocal cross-reactivity with any of the strains in this study. The specific low-level serological linkages in the large clade were not reflected in the detailed phylogenetic relationships of the strains as determined by 16S–23S intergenic sequence analysis. Thus, the low-level serological cross-reactions seemed to reflect the phylogenetic positions imperfectly, providing some support at the clade level but not for the detailed phylogeny.

In summary, there is, at present, no effective method for determining relationships among group VIII Spiroplasma strains. In this report, we demonstrate that the 16S–23S rDNA intergenic sequence provided stable differentiation of 8 group VIII spiroplasma strains. Further, it permitted robust differentiation of a clade of group VIII spiroplasmas from Georgia and Costa Rica that had been tentatively identified as group VIII spiroplasma strains. Further, it permitted robust differentiation of closely related Spiroplasma strains.

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