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COMPARATIVE SEROLOGICAL AND MOLECULAR
ANALYSIS OF GROUP VIII SPIROPLASMA FROM
AUSTRALIA AND NORTH AMERICA

April Carmita Murphy



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COMPARATIVE SEROLOGICAL AND MOLECULAR
ANALYSIS OF GROUP VIII SPIROPLASMA FROM
AUSTRALIA AND NORTH AMERICA

A Thesis

Presented to

the College of Graduate Studies of

Georgia Southern University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

In the Department of Biology

by

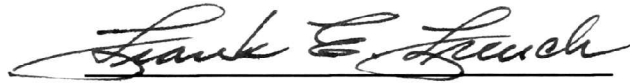
April Carmita Murphy

August 2003

June 26, 2003

To the Graduate School:

This thesis, entitled "Comparative Serological and Molecular Analysis of Group VIII *Spiroplasma* from Australian and North America," and written by April C. Murphy, is presented to the College of Graduate Studies of Georgia Southern University. I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Biology in the Department of Biology.

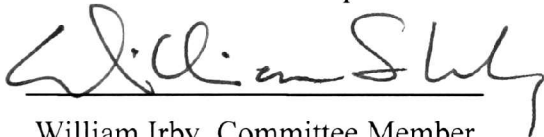


Frank E. French, Committee Co-chair

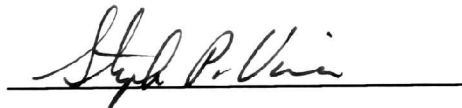


Laura B. Regassa, Committee Co-chair

We have reviewed this thesis
and recommend its acceptance:



William Irby, Committee Member



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Accepted for the College of Graduate Studies



Charles J. Hardy
Acting Dean, College of Graduate Studies

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Abstract

COMPARATIVE SEROLOGICAL AND MOLECULAR ANALYSIS OF GROUP VIII

SPIROPLASMA FROM AUSTRALIA AND NORTH AMERICA

August 2003

APRIL CARMITA MURPHY

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Spiroplasma bacteria (Mollicutes: Spiroplasmataceae) are characterized by motility, helical morphology and are most frequently found in insect guts and phloem tubes of plants. Traditionally, *Spiroplasma* have been classified by serology. Recent work has generated 16S rDNA sequences that generally correlate with the serological findings. Although the serology and the 16S rDNA sequence analysis clearly classifies strains to the group level, they do not distinguish between strains within the same group. The goal of this project was to investigate the utility of the 16S-23S rDNA intergenic spacer region sequence as a means to distinguish these closely related strains.

We chose Group VIII strains for this analysis because they were not separated by 16S rDNA analysis. We generated 16S-23S rDNA intergenic sequence and detailed serological profiles for eight Group VIII spiroplasmas isolated from North American and Australian horse flies. Within 293 nucleotides of 16S-23S rDNA intergenic spacer region, there was 96% identity among the eight strains. The sequence analysis grouped the strains into 3 main clusters, with the type strains *Spiroplasma chrysopicola*, *S. syrphidicola*, and TAAS-1 and BARC2649 each falling in to a different cluster. The phylogenetic analysis did not correlate precisely with the detailed serology. In addition, the phylogenetic groups did not correspond with geographic or host diversity.

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Chapter I

Introduction

Mollicutes are the smallest self-replicating Prokaryota known (Harasawa and Kanamoto, 1999) and readily pass through 200nm pores. The genus *Spiroplasma* was placed in the Family Spiroplasmataceae, Order Entomoplasmatales, Class Mollicutes, of the Division Tenericutes (Tully et al., 1993). *Spiroplasma* are characterized by helical morphology and motility. They lack a peptidoglycan cell wall and are descendents of a clostridial branch of gram-positive bacteria (Woese, 1987).

Mollicutes that are pathogenic to plants multiply in and are transmitted by insect vectors which are phloem-feeding species, namely leafhoppers (Anonymous, 1995; Bové, 1997). Spiroplasmas were first discovered in 1973 through the etiology of two plant pathogens: corn stunt and citrus stubborn diseases. Both diseases resulted in stunted growth of the infected plant as well as small ill-formed fruit. These plant pathogens are restricted to the sieve tube elements of the plant. The causative agent of Citrus Stubborn disease, *Spiroplasma citri*, was the first mollicute to be characterized and named (Saglio et al., 1973). *Spiroplasma kunkelii*, the causative agent of Corn Stunt disease, was fully characterized and named by 1986 (Whitcomb et al., 1996).

Morphological changes occur in spiroplasmas throughout their life cycle, but at some point they all possess a helical structure (Whitcomb et al., 1999) that is primarily observed during the exponential growth phase of the organism (Vazeille-Falcoz et al., 1997). Nutritional quality and various environmental parameters of culture media also

influence morphology (Chang, 1989). *Spiroplasma* are devoid of periplasmic fibrils, flagella, and axial filaments, but they are capable of achieving motility. The types of motility displayed by the spiroplasmas are rotational and flexional movement (Razin, 1989; Whitcomb et al., 1999). Rotary movement is accomplished through slow undulation, flexing, or twitching of the cell (Razin, 1978).

The *Spiroplasma* branch may have evolved as a lineage adapted to exploit various habitats in the hemolymph, ovaries, fat bodies, hypodermis, salivary glands, and gut lumen of insects. Because of this relationship, the genus *Spiroplasma* may be one of the most abundant groups of microbes on the earth (Hackett and Clark, 1989). *Spiroplasma* species are found in association with a wide variety of arthropods, including several tick species, Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera, Lepidoptera, and Odonata (Bové, 1997). Although most complete life cycles are not known, spiroplasmas are probably deposited by insect defecation or regurgitation of fluids during feeding (Hackett and Clark, 1989; Wedincamp et al., 1996). Some members of the genus *Spiroplasma* are maintained in cycles in the phloem of plants and the bodies of plant-sucking insects that act as vectors (Whitcomb, 1981). *Spiroplasma* may sometimes be transmitted from infected insect to uninfected insects via plant phloem (Fukatsu et al., 2001). Alternatively, horizontal transmission via parasitoids is also possible (Huigens et al., 2000).

The vast majority of mollicutes are host-restricted (Hackett and Clark, 1989). The issue of host specificity has important implications for pest management programs based on these microbes. For example, the Colorado potato beetle is a major pest of potatoes, tomatoes, and eggplant in North America (Hackett et al., 1996). These beetles are often

infected with host-specific, gut-inhabiting spiroplasmas, which could be used in biocontrol upon addition and expression of an insect-lethal gene (Konai et al., 1996). However, a better understanding of the nature of host specific associations will be needed before utilizing spiroplasmas as biological control agents (Hackett et al., 1992).

Most members of the genus *Spiroplasma* are known or suspected to be parasitic [sic], although the degree of effects are extremely diverse between different species and under different conditions, ranging from clearly detrimental to almost neutral or sometimes slightly beneficial (Whitcomb, 1981). Some insect spiroplasmas are entomopathogens. *Spiroplasma melliferum* and *S. apis* are honey bee pathogens that cross the gut barrier into the hemolymph where they multiply and kill the bee (Clark et al., 1985; Mouches et al., 1983). *Spiroplasma poulsonii* infects neotropical *Drosophila* (fruit flies) and kills the male progeny (Williamson et al., 1999). However, many insect spiroplasmas are not pathogenic, often restricted to the gut, and may be regarded as mutualists or incidental commensals (Bové, 1997). Most of the isolates multiply at 37°C which raises the question of whether or not they could be pathogenic to vertebrates (Konai et al., 1996). *Spiroplasma mirum* from ticks was an experimental pathogen of vertebrates when injected into the eye of fetal mice (Tully et al., 1995).

Clark et al. (1984) reported that tabanid flies (Diptera: Tabanidae) contain many spiroplasmas. To date, Tabanidae are recorded hosts for 11 of the 34 known Groups of *Spiroplasma* (Williamson et al., 1998) including one that contains three subgroups (Group VIII) (Clark et al., 1984; French et al., 1990; Whitcomb et al., 1997). Tabanid flies (horse flies) exhibit a high incidence of spiroplasmal microbes (French et al., 1992) in the abdominal viscera. It is common to isolate three or four strains from a single fly

and for the faster growing strains to out compete the slower strains in cultures (Whitcomb and Hackett, 1996). To date, none have been shown to be pathogenic to their hosts.

Certain tabanid associated strains appear to be geographically limited (Whitcomb et al., 1999). Recent analysis of isolates from tabanids of Costa Rica, Ecuador, U.S.A., Australia and France revealed putative new species (French, unpublished data). Some serologically identical strains were found in tabanids from both U.S.A. and France, while others were isolated only in France (Le Goff et al., 1991; Vazeille-Falcoz et al., 1997).

The spiroplasmas from tabanids have one of two fundamentally different morphologies. The Group VIII spiroplasmas exhibit short cells (four turns or less) during some point in their growth phase and they catabolize arginine. The other cluster of tabanid spiroplasmas is characterized by long cells that usually do not catabolize arginine, but ferment glucose. In addition, the Group VIII spiroplasmas have a significantly higher G+C ratio of the chromosomal DNA than do longer spiroplasmas (Whitcomb et al., 1990; Carle et al., 1990).

Before assignment of binomial names, putative species are currently classified in a group system (Gasparich et al., 1993). In 1976, the International Committee of Systematic Bacteriology Subcommittee assembled a group of *Spiroplasma* workers whose task was to evaluate various techniques for classification of *Spiroplasma*. By 1980, the assembly published criteria for *Spiroplasma* classification as well as a set of type strains (Whitcomb et al., 1999). The revised criterion for the classification system of spiroplasmas has resulted in 34 Groups and 14 subgroups (Anonymous, 1995; Williamson et al., 1998).

Traditionally, spiroplasmas are classified by serology based on surface antigens and utilization of other phenotypic characteristics. The first step is to place unknown strains into a known *Spiroplasma* Group based on surface reactions to combinations of specific *Spiroplasma* antibodies (Anonymous, 1995). After screening, the deformation (DF) test is the most widely used serological test for further evaluation of spiroplasmas (Williamson et al, 1978). The spiroplasmas tested are mixed with individual antiseras from the positive screening group and reacted at dilutions from 1:20 to 1:2560. The endpoint titer is the point at which half of the spiroplasma cells react and half do not react; the identity of the unknown spiroplasma is indicated at this point (Williamson et al., 1978). If the identity of the strain is not resolved by the serological DF test, dilution cloning is done to obtain a culture that originates from a single cell so that antibody/antigen reactivity will be homogeneous. Cloning is essential in many cases because mixed cultures can not be correctly identified. Dilution cloning is usually completed three times and reciprocal DF tests are done (Whitcomb and Hackett, 1987).

Detailed serology shows considerable differences among spiroplasmas (Whitcomb et al., 1999). Serology of spiroplasmas has been studied in great detail and careful examination of the totality of spiroplasma data indicates a strong correlation between serology and molecular phylogeny (Williamson et al., 1998). Group classification of spiroplasmas is based to a considerable degree on serological data. It is recognized that reciprocal cross reactivity is required to establish groups, since one-way cross-reactions, which in some cases can be of considerable magnitude, are not unusual in serology (Whitcomb et al., 1987). Every combination of *Spiroplasma* antigen and antibody reaction has been tested by DF serology in the course of defining the 34 groups

and 14 subgroups (Williamson et al., 1998). Everyone of the cross reactions observed in these tests has been consistent with the topologies of phylogenetic reconstructions (Gasparich et al., 2003).

Phenotypic characters are not able to serve as a sole basis for phylogenetic classification (Razin, 1989). The early genetic analysis of spiroplasmas indicated G+C values from 26 to 31 mol% and a genome that ranges from 1,100 kbp to 2,200 kbp (Dodge et al., 1998; Gasparich et al., 2003). Evolutionary relationships based on 16S rDNA sequence indicate that Mollicutes arose by simplification of Gram-positive bacteria (Woese, 1987). However, the lack of sequence divergence in the 16S rDNA region of the spiroplasmas prevents detailed phylogenetic analysis (Dodge et al., 1998). When examining Group VIII *Spiroplasma*, the G+C content values range from 28-31%, evolutionary distances among the type strains are very small (similarity coefficients of 0.992-0.999), and the 16S rDNA sequences failed to resolve the relationships among them (Stackebrandt and Goebel, 1994). The 16S-23S spacer region may be better suited to this type of phylogenetic analysis because it includes both conserved and variable regions in a short stretch and is less conserved than the adjacent 16S and 23S rRNA genes (Harasawa et al., 1996).

In my study, the 16S-23S spacer region from eight serologically distinct Group VIII isolates was analyzed. Genomic DNA was isolated, PCR amplified, and sequenced. The 16S-23S spacer region sequence was aligned and employed for phylogenetic analyses to separate the Group VIII *Spiroplasma* strains and show relatedness among the isolates from two continents, North America and Australia. The sequence analysis grouped the strains into 3 main clusters, with the type strains *Spiroplasma chrysopicola*,

S. syrphidicola, and TAAS-1 and BARC2649 each falling into a different cluster. The phylogenetic analysis did not correlate precisely with the detailed serology. In addition, the phylogenetic groups did not correspond with geographic or host diversity.

Chapter II

Materials and Methods

Collection and isolation of bacteria. *Spiroplasma* were isolated from female horse flies (Diptera: Tabanidae) in temperate and tropical locations on two continents. The fly hosts were captured using various methods including Gressitt/Malaise traps and hand nets. The host and isolation locale of putative species and the American type culture collection strains examined in detail in this report were: (1) GSU5485 from *Cydistomyia* sp., in temperate Engella, Queensland, Australia (S 21° 10.0' E 48° 30.4'; 725m above sea level; 57km inland), 6 February 1999 by Frank E. French; (2) GSU5603 from *Scaptia lasiophthalma* in temperate Batemans Bay, South Wales, Australia (S 35° 40.2' E 150° 12.8'; 40m above sea level; 5km inland), 24 February 1999 by Frank E. French; (3) GSU5367 from *Diachlorous curvipes* in tropical Puenta Vargas National Park, Province Limón, Costa Rica (N 9° 42.9' W 82° 49.3', 2m above sea level), 6 August 1998 by Frank E. French; (4) GSU5431 from *Tabanus occidentalis* in tropical Refuge Vida Silvestre, near Curu, Province Puntarenas, Costa Rica (N 9° 48.17 W 84° 55.52'; 35m above sea level), 14 August 1998 by Frank E. French; (5) BARC1357 from *Tabanus lineola* in temperate Bulloch Co., Georgia, U.S.A., 12 July 1989 by Frank E. French; (6) BARC2649 from *Tabanus lineola* in temperate Bulloch Co., Georgia, U.S.A., 25 April 1991 by Brenda A. Hester, ATCC 700284^T (^T= type culture); (7) *Spiroplasma syrphidicola* (EA-1) from *Eristalis arbustorum* (Diptera: Syrphidae) in temperate Prince Georges Co., Maryland, U.S.A., July 1980, T.B. Clark, ATCC 33826^T;

(8) *Spiroplasma chrysopicola* (DF-1) from *Chrysops* sp. in temperate Prince Georges Co., Maryland, U.S.A., August 1983, T.B. Clark, ATCC 43209^T; and (9) TAAS-1 from *Tabanus atratus* in Big Bend National Park, Texas, U.S.A., September 1987, by Robert F. Whitcomb, ATCC 51123^T (Clark, 1982; Clark et al., 1984; Gasparich et al., 1993).

Spiroplasma isolates were obtained as described by Wedincamp et al. (1996). The tabanids were chilled, surface sterilized with 0.5% NaOCl for at least 45 seconds, rinsed in distilled water for 45 seconds, and the terminal abdominal segment was removed. The viscera were removed, minced in 1.5 ml of M1D broth, passed through a 0.45 μ m filter, incubated at 30°C, and observed daily for growth. The recipe for 500 ml of M1D broth is 332 mg Glucose, 332 mg Fructose, 3,320 mg Sucrose, 25,150 mg Sorbital, 7,000 mg BBL Mycoplasma Broth Base, 2,320 mg Tryptone, 2,660 mg Peptone, and 8 ml 0.1% Phenol red, 534 ml Schneider's Drosophila Medium, 166 ml Fetal Bovine Serum, and 322 mg Penicillin G with a pH of 7.8 (Whitcomb, 1983). *Spiroplasma* cultures were examined by dark field microscopy at 1200 X magnification to ensure that helical cells were present. Isolates were stored at -70°C in M1D broth until tests were administered.

Serological Analysis. *Spiroplasma* cultures were evaluated serologically to determine group placement. Group placement was accomplished by screening isolates against 12 combinations of 32 antisera to spiroplasmas from tabanid hosts (Whitcomb et al., 1999); positive identification was determined based on 50% or greater cell deformation. For those placed in Group VIII by the screening assay, serological deformation (DF) tests were performed against the five specific antisera making up the screening cocktail combination for Group VIII (EA-1, DF-1, TAAS-1, BARC1357, and BARC2649). The individual antisera were diluted 10-fold in M1D broth and reacted

with an equal volume of *Spiroplasma* cultures for 30 minutes at room temperature.

Positive reactions were determined microscopically by observing deformation or clumping of at least 50% of the cells (Williamson et al., 1978). Identity was resolved by testing against individual antisera and a positive DF test score at a dilution of 1:320 or greater resolved identity.

Cloning was completed for isolates whose identity was not resolved. Cultures were serially diluted in M1D broth from 10^{-1} through 10^{-11} . For each dilution of 10^{-7} through 10^{-11} , 18 ml of broth was distributed on 96-well microtiter plates, with 200 μ l of broth in each well, and observed daily for color change of the M1D broth. The M1D contained phenol red, an acid indicator. If spiroplasmas grew, the color changed from red to yellow upon acidification indicating growth. To isolate a clone, broth from a single yellow well was chosen. All strains used in this study were cloned by dilution three times. DF tests were repeated after each round of cloning.

Triply-cloned cultures of selected strains were used for antisera production. Each strain was grown in 500 ml of M1D broth at 37°C, the cells were collected by centrifugation, resuspended in 10 ml of phosphate buffered saline (1.16% Na_2HPO_4 , 0.24% NaH_2PO_4 , and 0.584% NaCl , pH 7.5). A 2.4 ml aliquot of the cells was added to lyophilized RIBI[®] adjuvant (Sigma Chemical Co., St. Louis, MO), and a 1.0 ml dose was administered as 300 μ l intradermally, 400 μ l intramuscularly, 100 μ l subcutaneously, and 200 μ l intraperitoneally to a rabbit. The immunization schedule was distributed over three week intervals until antibody levels crested at a 1:640 or greater dilution, with 1.0 ml of resuspended cells in adjuvant injected every three weeks. Serum was recovered from the blood and aliquots were diluted 1:10 in M1D broth for subsequent serology.

Two way deformation tests were completed by doing reciprocal DF tests with all other cloned Group VIII strains and their respective antisera. I completed the serology for GSU5485 and GSU5603 and utilized the data from Stewart (2001) for the others.

DNA isolation, amplification, and sequencing. Genomic DNA was isolated using the method described by Duret et al. (1999). Twenty milliliters of culture was grown in M1D broth at 37°C and examined by dark field microscopy for concentration and morphology. When cells reached a density of 90-100 cells per field of view, they were harvested by centrifugation. The pellet was resuspended in 600 µl of STE buffer (10 mM Tris-HCl, 1 mM EDTA, 10 mM NaCl, and 5 M NaCl) and cells lysed with 67µl of 10% SDS at 67°C for 15 minutes. The lysate was treated with 2.3 µg/ml of RNase for 30 minutes at 37°C and then extracted with phenol: chloroform: isoamyl alcohol (24:24:1). The pellet was EtOH precipitated from the aqueous phase and resuspended in 50 µl TE buffer.

A 16S-23S rDNA spacer region of approximately 330 bp was PCR amplified using primers with homology to the 16S and 23S rDNA genes (5'-CGGTGAATACGTTCTCG-3' and 5'-CAAGGCATTCACCATAC-3', respectively). Amplification was carried out in a 50 µl reaction mixture containing 100 ng of genomic DNA, 1X Buffer A (Promega Corp., Madison, WI), 0.2 mM dNTPs, 2.5 units *Taq* polymerase (Promega Corp., Madison, WI), and 1.5 mM MgCl₂. Amplification cycles were completed as follows: denaturation at 94°C for 5 minutes, 35 cycles at 94°C for 45 seconds, 46°C for 45 seconds, 72° for 60 seconds, and one cycle at 72°C for 2 minutes. PCR products were separated on a 2% agarose gel and purified using a Qiagen Gel Extraction Kit (Qiagen, Inc., Valencia, CA). Amplified products were sequenced at the

Davis Sequencing Facility (Davis, CA) with the same primers used for PCR amplification. All DNA sequences were confirmed by at least two independent sequencing reactions. The DNA sequences were aligned using CLUSTALW (www.ebi.ac.uk/clustalw/) and a 293 bp region was chosen for subsequent analysis.

Phylogenetic Analysis. Phylogenetic trees were constructed by Dr. Tao Lin from the nucleotide sequence of the 16S-23S intergenic spacer regions. The neighbor-joining tree was constructed with PAUP (phylogenetic analysis using parsimony) software and was based on a comparison of the 293 bp of nucleotide sequence in the spacer region. Bootstrap confidence levels above 50% were obtained for each branch point.

Chapter III

Results

Serological analysis. Spiroplasma were isolated on two continents with climates ranging from tropical to temperate. Spiroplasma hosts included five genera and seven species of tabanid fly and one syrphid fly, *E. arbustorum*. Detailed serological analysis of Group VIII strains *Spiroplasma syrphidicola* (EA-1), *Spiroplasma chrysopicola* (DF-1), TAAS-1, BARC1357, BARC2649, GSU5431, and GSU5367 had been previously completed (Stewart, 2001). To add to this body of work, I completed serological analysis of two new strains from Australia, GSU5485 and GSU5603. I triply cloned these two strains and confirmed that the isolates serologically reacted with Group VIII screening antisera combinations. Interestingly, GSU5603 also reacted with other group antisera combinations inferring that GSU5603 is not exclusively related to Group VIII *Spiroplasma*.

Antisera was then produced for GSU5485 and GSU5603 and reciprocal DF tests were completed for all nine strains (Table I). No two-way reactions were observed between the Australian isolates GSU5485 and GSU5603 and the other 7 Group VIII strains, but deformation was observed with *S. syrphidicola* (EA-1) cells against GSU5485 antisera. Stewart (2001) reported a strong two-way reaction between BARC2649 and GSU5367. She also suggested that GSU5431 may be a bridge strain because of its reactivity with TAAS-1, BARC1357, and *S. chrysopicola* (EA-1). In general, the Group

Table I. Degree of spiroplasmal cell deformation against specific antisera.¹

Antigen	Antibody								
	BARC 2649 ⁴	GSU 5367 ⁴	BARC 1357 ⁴	GSU 5431 ⁴	TAAS-1 ⁴	EA-1 ²	GSU 5485	DF-1 ³	GSU 5603
BARC2649 ⁴	320 ⁴	640 ⁴	---- ^{4,5}	---- ⁴	---- ⁴	---- ⁴	----	---- ⁴	----
GSU5367 ⁴	640 ⁴	2560 ⁴	---- ⁴	---- ⁴	---- ⁴	---- ⁴	----	---- ⁴	----
BARC1357 ⁴	---- ⁴	---- ⁴	1280 ⁴	---- ⁴	---- ⁴	---- ⁴	----	---- ⁴	----
GSU5431 ⁴	---- ⁴	20 ⁴	320 ⁴	1280 ⁴	640 ⁴	20 ⁴	----	80 ⁴	----
TAAS-1 ⁴	---- ⁴	---- ⁴	40 ⁴	160 ⁴	2560 ⁴	40 ⁴	----	80 ⁴	----
EA-1 ²	---- ⁴	20 ⁴	80 ⁴	320 ⁴	---- ⁴	1280 ⁴	160	---- ⁴	40
GSU5485	----	----	----	----	----	20	640	----	----
DF-1 ³	---- ⁴	20 ⁴	---- ⁴	---- ⁴	---- ⁴	---- ⁴	----	2560 ⁴	----
GSU5603	40	----	----	----	----	----	----	80	2650

¹ Inverse of the greatest dilution exhibiting at least 50% cell deformation is shown; ² *Spiroplasma syrphidicola*; ³ *Spiroplasma chrysopicola*; ⁴ From K.M. Stewart, 2001; ⁵ ----Indicates no reaction at a dilution of 1:20 or greater.

VIII spiroplasmas have little to no cross reactivity with each other; 90% of the heterologous DF reactions had values of ≤ 80 .

Initial serology indicated that GSU5603 was not a typical Group VIII strain. GSU5603 screened positive for the Group VIII cocktail and seven other screening cocktails. GSU5603 did not have positive DF reactions against Group VIII antisera at a level above 1:80 (Table I). DF scores for GSU5603 versus antisera of Tab4C (Group XVII) and *Spiroplasma helictum* (Group XXXIII) were both 1:160. Antisera GSU5603 had strong one-way DF scores with 20 additional isolates from seven species representing three genera of tabanids collected in temperate eastern Australia (Table II). Reactions at 1:160 to 1:2560 were seen in the 20 strains. Two of these isolates also reacted with antisera GSU5485. Isolates 5573 (*Dasybasis* sp. from Canaberra, Australia) and 5542

(*Tabanus particaecus* from Narrabri, Australia) had strong one-way DF test scores of 1:320 and 1:640, respectively (Table II). Interestingly, isolate 5542 (*Tabanus particaecus*, from Narrabri, Australia) had strong one-way DF reactions with antisera from both GSU5603 and GSU5485 (Table II).

Table II. Deformation test scores for additional Australian isolates. ¹

Isolate	Coordinates	Host	Antisera	
			GSU5485	GSU5603
GSU5485	S 21° 10.0', E 48° 30.4'	<i>Cydistomyia</i> sp.	640 ¹	0
5573	S 35° 19.1', E 148° 50.4'	<i>Dasybasis</i> sp.	320	0
5542 ⁵	S 30° 24.4', E 149° 42.0'	<i>Tabanus particaecus</i>	640	320
5486 ²	S 21° 10.0', E 148° 30.4'	<i>Cydistomyia bancroftiae</i>	0	640
5492 ²	S 21° 10.0', E 148° 30.4'	<i>Scaptia aurifiva</i>	0	1280
5493 ⁴	S 21° 10.0', E 148° 30.4'	<i>Scaptia aurifiva</i>	0	320
5529 ^{5,6}	S 26° 42.0', E 152° 32.2'	<i>Cydistomyia</i> sp.	0	2560
5591	S 35° 40.3', E 150° 12.8'	<i>Scaptia clavata</i>	0	640
5593 ³	S 35° 40.3', E 150° 12.8'	<i>Scaptia clavata</i>	0	1280
5594	S 35° 40.3', E 150° 12.8'	<i>Scaptia clavata</i>	0	640
5595	S 35° 40.3', E 150° 12.8'	<i>Scaptia clavata</i>	0	2560
5597	S 35° 40.3', E 150° 12.8'	<i>Scaptia clavata</i>	0	640
5598 ²	S 35° 40.3', E 150° 12.8'	<i>Scaptia clavata</i>	0	160
5600	S 35° 40.3', E 150° 12.8'	<i>Scaptia clavata</i>	0	640
5601	S 35° 40.3', E 150° 12.8'	<i>Scaptia lasiophthalma</i>	0	640
5602	S 35° 40.3', E 150° 12.8'	<i>Scaptia lasiophthalma</i>	0	320
GSU5603	S 35° 40.3', E 150° 12.8'	<i>Scaptia lasiophthalma</i>	0	2560
5604 ²	S 35° 40.3', E 150° 12.8'	<i>Scaptia lasiophthalma</i>	0	2560
5605 ⁵	S 35° 40.3', E 150° 12.8'	<i>Scaptia lasiophthalma</i>	0	1280
5606 ³	S 35° 40.3', E 150° 12.8'	<i>Scaptia lasiophthalma</i>	0	320
5611	S 35° 40.3', E 150° 12.8'	<i>Scaptia testacae</i>	0	2560
5612 ⁵	S 35° 40.3', E 150° 12.8'	<i>Scaptia testacae</i>	0	1280
5613 ⁵	S 35° 40.3', E 150° 12.8'	<i>Scaptia testacae</i>	0	1280

¹ Inverse of the greatest dilution exhibiting at least 50% cell deformation; ² Completed by Alex Zarzuela; ³

Completed by Amanda Slider; ⁴ Completed by Amy Gray; ⁵ Completed by Michael Gutierrez; ⁶ Completed

by Frank French.

Molecular and phylogenetic analysis of Group VIII strains. Genomic DNA was isolated from the nine strains that were examined serologically (Table I). All nine were double strand sequenced, yielding approximately 450 base pairs of confirmed sequence data. Approximately 150 bp corresponded to the 16S rDNA gene and 293 bp was 16S-23S spacer region. Confirmed sequences were aligned using CLUSTALW with the expectation that the 16S rDNA sequence would show at least 99% identity for all Group VIII strains (Dodge et al., 1998). The 16S rDNA gene was 100% identical for all strains except GSU5603. GSU5603 was only 91% identical over the 126 bp of 16S rDNA gene sequence, and therefore was excluded from subsequent analysis.

When the 293 bp 16S-23S intergenic spacer sequence was aligned using CLUSTALW, there was 97% sequence identity among the eight Group VIII strains, with substitutions occurring at a total of 11 sites (Figure 1). GSU5367 and BARC2649 had identical spacer sequences but differ from the other six sequences at sites 28, 31, 43, 62, 84, 91, 110, and 287 with the following substitutions: T/A, T/C, C/A, G/A, T/C, A/G, A/G, and A/T, respectively. GSU5367, BARC2649, GSU5485, and TAAS-1 differ from *S. chrysopicola*, GSU5431, *S. syrphidicola*, and BARC1357 at site 85 with the substitution of an A for a G. At site 124, TAAS-1 and *S. chrysopicola* differ from the other six strains with the substitution of G for an A. At site 288, BARC1357 and *S. syrphidicola* differ from the other strains with the substitution of a T for an A.

A phylogenetic tree was produced from the 16S-23S spacer region of the eight *Spiroplasma* isolates by analyzing the number of changes between each pair in the group of sequences (Figure 2; Mount, 2001). We chose the Neighbor-joining method because it

Figure 1. CLUSTALW multiple sequence alignment of 293 bp from the 16S-23S intergenic spacer region of eight Group VIII *Spiroplasma*.

<i>S. syrphidicola</i>	TTTCTATGGAGTTATACTTTATAGTAAATACGGCTATAATGAAGTTATGTTTAGTTTTCA	60
BARC1357	TTTCTATGGAGTTATACTTTATAGTAAATACGGCTATAATGAAGTTATGTTTAGTTTTCA	60
GSU5431	TTTCTATGGAGTTATACTTTATAGTAAATACGGCTATAATGAAGTTATGTTTAGTTTTCA	60
<i>S. chrysopicola</i>	TTTCTATGGAGTTATACTTTATAGTAAATACGGCTATAATGAAGTTATGTTTAGTTTTCA	60
TAAS-1	TTTCTATGGAGTTATACTTTATAGTAAATACGGCTATAATGAAGTTATGTTTAGTTTTCA	60
GSU5485	TTTCTATGGAGTTATACTTTATAGTAAATACGGCTATAATGAAGTTATGTTTAGTTTTCA	60
GSU5367	TTTCTATGGAGTTATACTTTATAGTAAATACGGCTATAATGACGTTATGTTTAGTTTTCA	60
BARC2649	TTTCTATGGAGTTATACTTTATAGTAAATACGGCTATAATGACGTTATGTTTAGTTTTCA	60
	***** ** *****	
<i>S. syrphidicola</i>	GAGATTAGTTTCTCTGAATTAATCGAAATTGAACAAGTAGTTAAATTTTGATTAATAAAT	120
BARC1357	GAGATTAGTTTCTCTGAATTAATCGAAATTGAACAAGTAGTTAAATTTTGATTAATAAAT	120
GSU5431	GAGATTAGTTTCTCTGAATTAATCGAAATTGAACAAGTAGTTAAATTTTGATTAATAAAT	120
<i>S. chrysopicola</i>	GAGATTAGTTTCTCTGAATTAATCGAAATTGAACAAGTAGTTAAATTTTGATTAATAAAT	120
TAAS-1	GAGATTAGTTTCTCTGAATTAATCGAAATTGAACAAGTAGTTAAATTTTGATTAATAAAT	120
GSU5485	GAGATTAGTTTCTCTGAATTAATCGAAATTGAACAAGTAGTTAAATTTTGATTAATAAAT	120
GSU5367	GGGATTAGTTTCTCTGAATTAATTAATAAATAAACAAGTAGTTAAATTTTAATTAATAAAT	120
BARC2649	GGGATTAGTTTCTCTGAATTAATTAATAAATAAACAAGTAGTTAAATTTTAATTAATAAAT	120
	* ***** *****	
<i>S. syrphidicola</i>	GTTCTTTGAAAACCTGGATAATAGACATCTAGTTATTTTCATTTTAAATGAAATAACAAAAT	180
BARC1357	GTTCTTTGAAAACCTGGATAATAGACATCTAGTTATTTTCATTTTAAATGAAATAACAAAAT	180
GSU5431	GTTCTTTGAAAACCTGGATAATAGACATCTAGTTATTTTCATTTTAAATGAAATAACAAAAT	180
<i>S. chrysopicola</i>	GTTCTTTGAAAACCTGGATAATAGACATCTAGTTATTTTCATTTTAAATGAAATAACAAAAT	180
TAAS-1	GTTCTTTGAAAACCTGGATAATAGACATCTAGTTATTTTCATTTTAAATGAAATAACAAAAT	180
GSU5485	GTTCTTTGAAAACCTGGATAATAGACATCTAGTTATTTTCATTTTAAATGAAATAACAAAAT	180
GSU5367	GTTCTTTGAAAACCTGGATAATAGACATCTAGTTATTTTCATTTTAAATGAAATAACAAAAT	180
BARC2649	GTTCTTTGAAAACCTGGATAATAGACATCTAGTTATTTTCATTTTAAATGAAATAACAAAAT	180

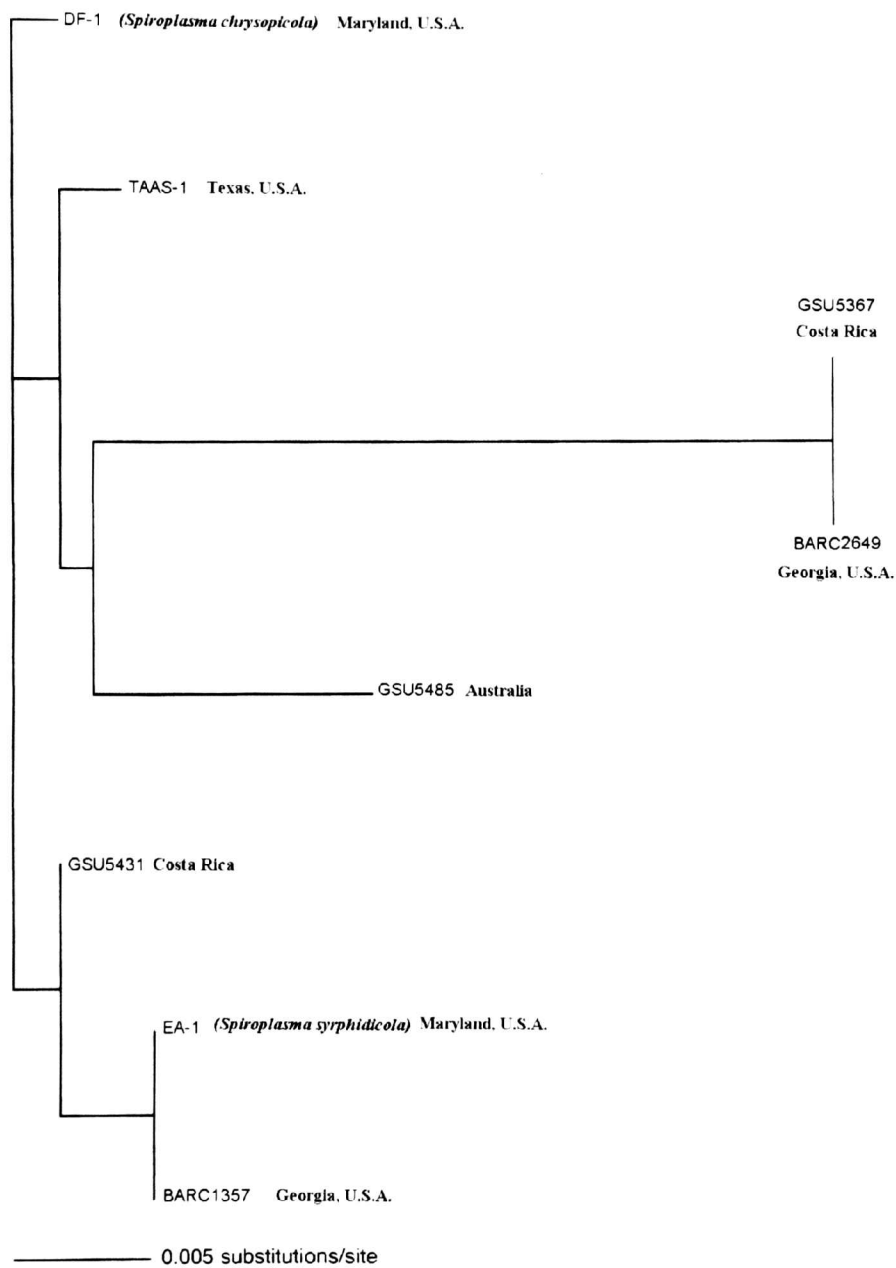
<i>S. syrphidicola</i>	AATTCAAATTTTCTGTTATTAAGTAATTTTAAATTAATAACTAAAAATTCACAGTTAT	240
BARC1357	AATTCAAATTTTCTGTTATTAAGTAATTTTAAATTAATAACTAAAAATTCACAGTTAT	240
GSU5431	AATTCAAATTTTCTGTTATTAAGTAATTTTAAATTAATAACTAAAAATTCACAGTTAT	240
<i>S. chrysopicola</i>	AATTCAAATTTTCTGTTATTAAGTAATTTTAAATTAATAACTAAAAATTCACAGTTAT	240
TAAS-1	AATTCAAATTTTCTGTTATTAAGTAATTTTAAATTAATAACTAAAAATTCACAGTTAT	240
GSU5485	AATTCAAATTTTCTGTTATTAAGTAATTTTAAATTAATAACTAAAAATTCACAGTTAT	240
GSU5367	AATTCAAATTTTCTGTTATTAAGTAATTTTAAATTAATAACTAAAAATTCACAGTTAT	240
BARC2649	AATTCAAATTTTCTGTTATTAAGTAATTTTAAATTAATAACTAAAAATTCACAGTTAT	240

<i>S. syrphidicola</i>	ATTTTGTAATGATTCTCAAAAAAATTATAAAAACCTTGATTAATTTAATTA	293
BARC1357	ATTTTGTAATGATTCTCAAAAAAATTATAAAAACCTTGATTAATTTAATTA	293
GSU5431	ATTTTGTAATGATTCTCAAAAAAATTATAAAAACCTTGATTAATTTAATTA	293
<i>S. chrysopicola</i>	ATTTTGTAATGATTCTCAAAAAAATTATAAAAACCTTGATTAATTTAATTA	293
TAAS-1	ATTTTGTAATGATTCTCAAAAAAATTATAAAAACCTTGATTAATTTAATTA	293
GSU5485	ATTTTGTAATGATTCTCAAAAAAATTATAAAAACCTTGATTAATTTAATTA	293
GSU5367	ATTTTGTAATGATTCTCAAAAAAATTATAAAAACCTTGATTAATTTAATTA	293
BARC2649	ATTTTGTAATGATTCTCAAAAAAATTATAAAAACCTTGATTAATTTAATTA	293

* indicates conserved nucleotides.

best described the situation between the serology and phylogeny of Group VIII *Spiroplasma*. This method does not assume a molecular clock and produces an unrooted tree. As with our samples, the levels of evolutionary change were unknown, therefore, neighbor-joining was the most reliable in predicting the correct tree (Saitou and Nei, 1987). No outgroup was chosen because eight of the nine strains were included in the analysis. GSU5603 was the most distant of the strains sequenced for 16S-23S spacer region, and its sequence divergence (51% identity) was great enough that an incorrect phylogenetic prediction would have been produced by its inclusion. A neighbor-joining relationship indicated two pairs of indistinguishable strains, GSU5367 and BARC2649, and BARC1357 and *Spiroplasma syrphidicola* (EA-1). The type strains, *S. syrphidicola*, *S. chrysopicola*, TAAS-1, and BARC2649, separated into three main branchings in the topology of the tree. *Spiroplasma chrysopicola* (DF-1) branches alone. TAAS-1, GSU5485, GSU5367 and BARC2649 constitute a second branch. The third branch includes *Spiroplasma syrphidicola* (EA-1), BARC1357, and GSU5431.

Figure 2. Neighbor-joining phylogenetic tree derived from 293 base pairs of 16S-23S rDNA spacer region sequence.



Chapter IV

Discussions

Serology has been the basis for *Spiroplasma* categorization for the past 20 years (Anonymous, 1995). Serology, particularly the DF test, has been a very effective means of differentiating strains and showing homology, especially in cases of strong reciprocal reactions, but giving little information concerning phylogeny (Whitcomb et al., 1999; Gasparich et al., 2003).

Although vital, serology has had problems. Initially, serological methods worked well with spiroplasmas. In particular, Group VIII placement is verified by positive screening reactions, one-way DF test scores, two-way DF test scores, and unique phenotypic characteristics, such as short cell morphology and catabolism of arginine. A rare case of failed identification by serology occurred when the strain DF-1 (*S. chrysopicola*) was initially placed in Group XVII. Eventually, this strain proved to be distantly related to other Group VIII strains. Another incidence of failed serology was when BARC2649 failed to cross-react with known Group VIII antisera (Whitcomb et al., 1999). Because of this particular flaw with serology, the 16S rDNA gene sequence was explored as a plausible means for properly grouping bacteria (Woese, 1987).

Analysis of the 16S gene sequence indicates a general agreement with serology for strains and Groups of *Spiroplasma* (Dodge et al., 1998; Gasparich et al., 2003). However, some clades of serologically distinct strains have identical 16S rDNA sequences. For example, Group VIII strains show $\geq 99\%$ identity among 16S rDNA

sequences (Gasparich et al., 2003) making it impossible to differentiate these strains based on 16S gene analysis.

Recent work has utilized the 16S-23S intergenic spacer region as a means to distinguish among closely related bacteria. Harasawa and Kanamoto (1999) recently used the 16S-23S spacer region to differentiate two biovars of *Ureaplasma urealyticum*. It was hoped that sequence divergence of the 16S-23S spacer region would be species specific within the Group VIII spiroplasmas, distinguishing them one from another. Phylogenetic analysis of eight Group VIII *Spiroplasma* using the 16S-23S spacer region resulted in clear divisions among the strains, placing them in 3 main groups.

We compared the phylogenetic analysis to detailed serological tests for the 8 strains, but did not find a convincing correlation. Two cases showed a strong correlation between serology and the phylogenetic analysis. BARC2649 and GSU5367 exhibited strong two-way reactions during serological testing and were indistinguishable by phylogenetic analysis. In addition, *Spiroplasma chrysopicola* (DF-1) had no strong serological reactions and separated alone on the phylogenetic tree. For most strains, the serology did not correlate with our phylogenetic tree. TAAS-1 showed moderate cross-reactivity with GSU5431, but groups with GSU5367 and BARC2649, and GSU5485. GSU5485 had no serological reactivity but groups with GSU5367 and BARC2649, and TAAS-1. *Spiroplasma syrphidicola* (EA-1) had a moderate reaction with GSU5431 but is phylogenetically indistinguishable from BARC1357, which showed no serological reactivity. GSU5431 has serological reactivity with BARC1357 and TAAS-1, but groups with BARC1357 and *S. syrphidicola* (EA-1).

Examination of the geographical isolation sites or the spiroplasma hosts in relation to the phylogenetic groups did not show a correlation. *Spiroplasma chrysopicola* (DF-1) and *S. syrphidicola* (EA-1) were isolated from Maryland, U.S.A., but separate onto different branches. GSU5431 and GSU5367 were isolated from Costa Rica and likewise separated onto different branches. As well, BARC1357 and BARC2649 were isolated from Georgia, U.S.A., and separated onto separate branches. BARC1357 isolated from Georgia, U.S.A. and *S. syrphidicola* (EA-1) isolated from Maryland, U.S.A., and GSU5431 isolated from Province Puntarenas, Costa Rica branch together. This branch represents both temperate and tropical regions as well as two host fly families, two genera, and three species. Interestingly, Australian GSU5485 separates with American TAAS-1, and American BARC2649 and Costa Rican GSU5367. This particular branch includes isolates from four species in three genera of two Tabanidae tribes from the U.S.A. and Australia. DF-1 (*Spiroplasma chrysopicola*) from Maryland, USA, however, separates alone.

GSU5603 screened positive for Group VIII, but short cells were never observed. In addition, GSU5603 screened positive for seven other Groups, untypical of Group VIII strains. Because of the reaction with other groups of spiroplasmas, GSU5603 probably should not have been chosen for this analysis. However, this strain was commonly isolated in temperate eastern Australia (21° S to 35° S), with 20 other isolates from 7 species, representing 3 genera, of two subfamilies of Tabanidae. None of these rapidly growing isolates exhibited short cell morphology. 126 bp of the 16S rDNA sequence for GSU5603 was only 91% identical as compared to the 100% identity for all of the other Group VIII strains. A complete analysis of the 16S gene is needed in order to determine

if GSU5603 fits into the Group VIII clades described by Dodge et al. (1998) and Gasparich et al. (2003).

To date, Group VIII *Spiroplasma* have only been distinguished based on detailed serological analyses, DNA-DNA reassociation data, and 16S rDNA sequence data (Whitcomb et al., 1999). The 16S rDNA sequence is conservative and does not separate the five strains from the U.S.A. (Dodge et al., 1998), however, these strains and others were phylogenetically separated in our study based on analysis of the 16S-23S rDNA intergenic sequence. Use of the 16S-23S spacer region has the potential to drastically affect strain classification for Group VIII and other closely related *Spiroplasma*. DNA sequence analysis would provide a relatively cheap, rapid alternative to differentiate these closely related strains.

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