Taxonomic Analysis of the Tortoise Mycoplasmas *Mycoplasma agassizii* and *Mycoplasma testudinis* by 16S rRNA Gene Sequence Comparison†

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The nucleotide sequences of the 16S rRNA genes of two mycoplasmas, *Mycoplasma agassizii* (proposed sp. nov.) and *Mycoplasma testudinis*, isolated from tortoises were determined and used for taxonomic comparisons. Signature nucleotide sequence motifs and overall sequence similarities to other mollicutes positioned these mycoplasmas in the *M. hyorhinis* and *M. pneumoniae* phylogenetic groups, respectively. A third, previously unrecognized tortoise mycoplasma was detected by 16S rRNA gene amplification and sequence analysis and was positioned in the *M. fermentans* phylogenetic group. The 16S rRNA gene of *Acholeplasma laidlawii* was similarly detected in a tortoise isolate, showing that diverse mollicutes can share the same family of reptilian host.

Two well-characterized mycoplasmal species have been isolated from tortoises: *Mycoplasma agassizii* (proposed sp. nov.), isolated from the upper respiratory tract of *Gopherus agassizii* and *Gopherus polyphemus* tortoises (4), and *Mycoplasma testudinis*, isolated from the cloaca but not the nasopharynx of a *Testudo graeca* tortoise (7). *M. testudinis* ferments glucose and grows readily in conventional mycoplasma media at 20 to 35°C. It is not a recognized tortoise pathogen. *M. agassizii*, a newly recognized species, ferments glucose and grows slowly (3 to 6 weeks for primary isolation) in SP4 broth (13) at an optimal temperature of 30°C. It is an etiological agent of a chronic upper respiratory tract disease of tortoises characterized by a nasal mucous discharge and degeneration of the airway epithelium (4). Upper respiratory tract disease is a significant health problem in captive and certain wild populations of tortoises (8). The causes and diagnosis of disease are important considerations in the management of legally protected populations of endangered tortoises.

There was no cross-reaction of tortoise antimycoplasma antibodies with *M. testudinis* antigens in an *M. agassizii*-specific enzyme-linked immunosorbent assay (12). This suggested that, although they share the same family of reptilian host, the two mycoplasmas are not closely related. Taxonomies of mollicute species have been constructed recently on the basis of 16S rRNA gene nucleotide sequences, which reflect their phylogenetic relationships (6, 9, 15). We determined the 16S rRNA gene nucleotide sequences of *M. agassizii* and *M. testudinis* for comparison with previously published 16S rRNA sequences to position the tortoise mycoplasmas in the taxonomy of mollicutes.

MATERIALS AND METHODS

Culture. *M. agassizii* PS6 was isolated from the nares of a *G. agassizii* tortoise with upper respiratory tract disease (3). A pure culture was derived from a single colony. The *M. testudinis* strain used was ATCC 43263. Cells were cultured at 30°C in SP4 broth (13) supplemented with glucose and 20% fetal bovine serum.

PCR amplification and sequencing. A crude proteinase K digest of 2 µl of a

culture in the mid-log growth phase, as indicated by acidification of the medium, was the template for PCR amplification of the 16S rRNA gene. Amplification primers were complementary to terminal sequences (sense strand nucleotides [nt] 11 to 30 5'-AGAGTTTGATCCTGGCTCAGGA-3'; antisense strand nt 1512 to 1493, 5'-GGTAGGGATACCTTGTTCAGACT-3') highly conserved in mollicutes (11). The optimum magnesium chloride concentration was 2.0 mM with 1 µM each primer and 200 µM deoxynucleoside triphosphates in a 50-µl volume. There were 35 cycles, each consisting of template denaturation for 45 s at 94°C, primer annealing for 1 min at 59°C, and polymerization for 2 min at 72°C. The sensitivity, determined by visualization of the 1.5-kb PCR product electrophoresed through ethidium bromide-stained agarose gels, was 5 pg of template DNA, or about 10³ cells. The PCR product was purified by gel filtration spin chromatography and quantitated by spectrophotometry before automated fluorescent dideoxy nucleotide sequence determination (Applied Biosystems, Inc.; model 373A, version 1.2.1) without molecular cloning. Both strands of each product were sequenced completely by primer walking.

Sequence analysis. Nucleotide sequences were compared by using VAX computer version 1.4 of the basic local alignment search tool of the National Center for Biotechnology Information, National Institutes of Health (1, 5). The conventional nucleotide numbering system based on the 16S rRNA gene of *Escherichia coli* was used for reference, although sequence length polymorphisms were observed among species. Pairwise nucleotide sequence similarity scores were computed with default gap and gap length weights. A score of 0 indicated that two sequences had only random sequence similarity, and a score of 1.0 indicated that two sequences were identical.

Nucleotide sequence accession numbers. The nucleotide sequences described here have been released to GenBank of the National Center for Biotechnology Information (2), to the European Molecular Biology Laboratory, and to the DNA Data Bank of Japan with accession numbers U09786 (*M. agassizii*), U09788 (*M. testudinis*), and U19768 (isolate H3110).

RESULTS AND DISCUSSION

A characteristic nucleotide sequence motif at nt 907 to 915, 5'-AAACTTAAA-3', including thymine at nt 912, placed *M. agassizii* in the *M. hominis* phylogenetic group described by Weisburg et al. on the basis of 16S rRNA gene sequence comparisons (15). That sequence is otherwise characteristic of archaeobacteria and eukaryotes and is rarely present in eubacteria. The *M. hominis* group described by Weisburg et al. (15) has been further subdivided by refinement of the 16S rRNA gene comparison methods by Maniloff (9) into *M. hominis*, *M. fermentans*, and *M. hyorhinis* groups. Overall nucleotide sequence similarity placed *M. agassizii* in the *M. hyorhinis* group, in which its closest relative (similarity score = 0.89) was *M. mobile*, which was originally isolated from the gills of a fish. Other members of that group are *M. hyorhinis*, *M. hyopneumoniae*, *M. neurolyticum*, and *M. sualvi*. For perspective, the

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† Journal series article R-04321 of the Florida Agricultural Experiment Station, University of Florida, Gainesville.

AGASSIZII H3110 TESTUDINIS	ACATGCATGT ACATGCATGT ATATGCAATG V1 *****	CGAGCGGAAC CGAGCGAAAG CGATCGGGTG *****	TAGTTTACTA TA .CTTG .TA TAÇC . .AATA *****	GTTTAGCGGC CTTTAGCGGC CACCAGAGCG *****	AA .TGGGTGA AAATGGGTGA GAACGGGTGA *****
AGASSIZII H3110 TESTUDINIS	GTAACACGTA GTAACACGTA GTAACACGTA	CCTAATCTAC CTCAACCTAC TCCAATCTGC	CTTAAGATC CTCACAGACT CCTATAGCGG	GGAACAACAA GGAATAACCA GGGACAACCTA	TGGGAAACCG AGGGAAACTI GTGCAAGAT
AGASSIZII H3110 TESTUDINIS	TTGCTAATAC TGGCTAATGC TAGCTAATAC	CGGATATACC CGGATACACC CGCATAAACA	TTTGAAAA . TTAATCTC . GTAACCTTATC	GCATTTTMTA GCATGAAGAT GCATGAGAAT	AAGCGAAAG AAGGTTAAAG TACTTTAAAG *****
AGASSIZII H3110 TESTUDINIS	GAGCAACTGC GAACTATTGT GTCCGTTTGG *****	TTCACTTTGA TTCACTGGGA ACCACTATAG *****	GATGAGGGTG GATGGGGGTG GATGAGGGTG *****	CGTTACATTA CGTTACATTA CGGCATATCA	GTTAGTTGGC GTTAGTTGGC GCTAGTTGGT *****
AGASSIZII H3110 TESTUDINIS	AGGGTAATGG ACGTGTAATG GAGGTAAACG	CCTACCAAGA ACCACCAAGA CCCACCAAGG	CGATGATGTA CGATGATGTA CTATGACGTG	TAGCCGAGTT TAGCCGAGTT TAGTTATGCT	GAGAGACTGA GAGAGACCGA GAGAGGTAGA *****
AGASSIZII H3110 TESTUDINIS	TCGGCCACAC ACGGCCACAC ATAACCACAA	TGGGACTGAG TGGGACTGAG TGGGACTGAG	ATACGGCCCA ATACGGCCCA ACACGGCCCA	GACTCCTACG GACTTCTACG TACTCCTACG	GGAGGCAGCA GGAGGCAGCA GGAGGCAGCA *****
AGASSIZII H3110 TESTUDINIS	GTAGGGAATA GTAGGGAATA GTAGGGAAT	TTCCACAATG TTCCACAATG TTTCCACAATG	GCGCGAAGCC GCGCGAAGCC GACGAAAGTC	TGATGGAGCG TGATGGAGCG TGATGGAGCA	ACACAGCGTG ACACAGCGTG ATGCCCGCTG V3 ***
AGASSIZII H3110 TESTUDINIS	CAGGATGAAG CAGGATGAAG AACGATGAAG	GTCTTC . .GG GTCTTC . .GG GTCTTTATGG	ATCGTAAACT ATCGTAAACT ATTGTAAAGT	GCTGTTATAC GCTGTTATAC TCCTTTATTT	AGAAAGAAAA AGAAAGAAAA GGGATGAATG *****
AGASSIZII H3110 TESTUDINIS	GATATAGGAG GATAAGGGAG ATTAGCAAAAG	TGGAAAGCCT TGGAAAGCCC GAAATGTGTG	ATATTATGAC TTATTATGAC .GAAATGTGTG	GGTACTGTAT GGTACTGTAT CTAGTTTGAC	CAGAAAGTGA CAGAAAGTGA CGTACCATT
AGASSIZII H3110 TESTUDINIS	CGGCAAACTA CGGCAAACTA CGACTAACTA	TGTGCCAGCA TGTGCCAGCA TGTGCCAGCA	GCCGCGGTAA GCCGCGGTAA GTCGCGGTAA	TACATAGGTC TACATAGGTC TACATAGGTT	ACAAGCGTTA ACAAGCGTTA GCAAGCGTTA *****
AGASSIZII H3110 TESTUDINIS	TCCGAAATTA TCCGAAATTA TCCGGATTTA	TTGGGCGTAA TTGGGCGTAA TTGGGCGTAA	AGCGTCCGTA AGCGTCCGTA AACAGCGCA	GGTTGTTTGT GGTTGTTTGT GGCGGATTAG	TAAGTCTGGA TAAGTCTGGA AAAGTCTGGT *****
AGASSIZII H3110 TESTUDINIS	GTAAAAAGCC GTCAAAAACC GTTAAATCAT	GCTGCTCAAC GAAGCCCAAC ACTGCTTAAC	GCCGGCAGCG TTCGGCTCGC GTTGAATGC	TTTAGATACT TTCAGATACT GTTGAATACT	GGCAAACTAG GGCAAACTAG CTAGTCTAG *****
AGASSIZII H3110 TESTUDINIS	AGTTATAAAG AGTTATAAAG AGTATGCTAG	AGGTAAGTGG AGGTTAGTGG AGAGTTCTGG	AATTTTCATGT AATTTTCATGT AATCTCCATGT	GAAGCGGTGG GAAGCGGTGG GAAGCGGTGG	AATGCGGTAGA AATGCGGTAGA AATGCGGTAGA *****
AGASSIZII H3110 TESTUDINIS	TATATGAAAG TATATGAAAG TATATGGAAG	AACACCAATG AACACCAATG AACACCAATG	GCGAAGGCAG GCGAAGGCAG GCGAAGGCAG	CTTACTGGTT CTTACTGGTT GAACCTTAGGC	ATATACTGAC ATATACTGAC CACTACTGAC *****
AGASSIZII H3110 TESTUDINIS	ACTGAGGGGC ACTGAGGGAC GCTTAGGGCT	GAAAGCGTGG GAAAGCGTGG GAAAGTGTGG	GG GGGCGCAAGC GG	 AGCA GTGGGGAGCA AGCA	AACAGGATTA CACAGGATTA AATAGGATTA *****
AGASSIZII H3110 TESTUDINIS	GATACCCCTG GATACCCCTG GATACCCCTAG	TAGTCCACGC TAGTCCACGC TAGTCCACAC	CGTAAACGAT CGTAAACGAT TGTAACGAT	GATCATTAGT GATCATTAGT GGATGTTAG	TGGTGGGGGA TGGTGGGGGA TGTGCGGGCG V5 ** *****
AGASSIZII H3110 TESTUDINIS	ACC . .CTCG ATTCAC ATCACCTCGG	TAACGCAGCT CGACGCAGCT TGCTGAAGTT	AACGCATTAA AACGCATTAA AACACATTAA	ATGATCCGCC ATGATCCGCC ACATCCTGCC	TGAGTAGTAT TGAGTAGTAT TGAGTAGTAT *****
AGASSIZII H3110 TESTUDINIS	GCTCGCAAGA GCTCGCAAGA ATTCGCAAGA	GTGAAACTTA GTGAAACTTA ATGAAACTCA	AA .GGAATTG AA .GGAATTG AACGGAATTG	ACGGGGACCC ACGGGGACCC ACGGGGACCC	GCACAAGCGG GCACAAGCGG GCACAAGTGG *****
AGASSIZII H3110 TESTUDINIS	TGGAGCATGT TGGAGCATGT TGGAGCATGT	GGTTTAATTT GGTTTAATTT GGTTTAATTT	GACGATACGC GACGATACGC GACGATACGC	GTAGAACCTT GTAGAACCTT GAAAAACCTT	ACCCACTCTT ACCCACTCTT ACCTAGACTT *****
AGASSIZII H3110 TESTUDINIS	GACATCCCTT GACATCCCTG GACATCCCTG	GCAAAGCT . GCAAAGCT . GCAAAGCTCC	ATAGAGATAT ATAGAGATAT ATAGAAATAT	AGTGGAGGTT AGTGGAGGTT ACGGGAGGTT	AACAAGGTGA AACCAGATGA AACCAGATGA V6 *****
AGASSIZII H3110 TESTUDINIS	CAGATGGTGC CAGATGGTGC CAGGTGGTGC	ATGGTTGTGC ATGGTTGTGC ATGGTTGTGC	TCAGCTCGTG TCAGCTCGTG TCAGCTCGTG	TCGTGAGATG TCGTGAGATG TCGTGAGATG	TTGGTTAAG TTGGTTAAG TTGGTTAAG *****
AGASSIZII H3110 TESTUDINIS	TCCTGCAACG TCCTGCAACG TCCCGCAACG	AGCGCAACCC AGCGCAACCC AGCGCAACCC	CT .ATCCTTA CT .ATCCTTA CTTATTGCTA	TTTACCTAAC TTTACCTAAC GTTACTT . .	ATTAAGTTGA ATTAAGTTGA TG . *****
AGASSIZII H3110 TESTUDINIS	GAACATATAC GAACATATACTCTAGC	GATACTGCTC GATACTGCTC GAGACTGCGA	AGGTAAGTGG AGGTAAGTGG ACGTAAAGTTG	GAGGAAGGTG GAGGAAGGTG GAGGAAGGTG	GGGATGACGT GGGATGACGT GGGATGACGT *****
AGASSIZII H3110 TESTUDINIS	CAAAATCATCA CAAAATCATCA CAAAATCATCA	TGCTCTTTAC TGCTCTTTAC TGCTCTTTAC	GAGTGGGGCA GAGTGGGGCA GAGTGGGGCA	ACACACGTGC ACACACGTGC ACACACGTGC	TACAATGGCC TACAATGGCC TACAATGGCC V8 *****
AGASSIZII H3110 TESTUDINIS	GGTACAAAGA GGTACAAAGA ATTACAAACA	GACGCAATAT GACGCAATAT GTTGCCAAAC	GGTGACATGG GGTGACATGG CGTAAGGTGG	AGCAAATCTG AGCAAATCTG AGCTAATCTG	AAAAACCGGT AAAAACCGGT TAAAGATGGT *****
AGASSIZII H3110 TESTUDINIS	CTCAGTTCGG CTCAGTTCGG CTCAGTTCGG	ATTGAAGGCT ATTGAAGGCT ATTGAAGGCT	GCAATTGCGC GCAATTGCGC GCAATTGCGC	TTTATGAAGT TTTATGAAGT CTCATGAAGT	CGGAATCGCT CGGAATCGCT CGGAATCACT **
AGASSIZII H3110 TESTUDINIS	AGTAATCGTA AGTAATCGTA AGTAATCGCG	GATCAGCTAC GATCAGCTAC AATCAGCTAT	GCTACGGTGA GCTACGGTGA GTCGCGGTGA	ATACGTTCTC ATACGTTCTC ATACGTTCTC	GGGTCTTTGA GGGTCTTTGA GGGTCTTTGA *****
AGASSIZII H3110 TESTUDINIS	CACACCGCCC CACACCGCCC CACACCGCCC	GTACACCATAT GTACACCATAT GTCAAACTAT	GGGAGCGCGT GGGAGCGCGT GAGAGCTGAT	AATGCCCAAA AATGCCCAAA AGTGTCTAAA	GTGCGTTTAG GTGCGTTTAG ACCG .TGTTT V9 *****
AGASSIZII H3110 TESTUDINIS	CTAACTTC . CTAACTAC . CTAACCGTAA	GGAAGCGACT GGAAGCGACT GGAAGCGCAT	GCCTAAGGCA GCCTAAGGTA GTCTAGGCA	GGATTGGTGA GGGATG . . . AGATCGGTGA	CTGGG .TGAGTGA TTGGAGTTA *****

FIG. 1. Multiple alignment of 16S rRNA gene nucleotide sequences of tortoise mycoplasmas. Regions complementary to terminal primers are truncated because they were not independently confirmed in this study. Sequences begin at nt 53 and end at nt 1492 in the conventional numbering system based on the 16S rRNA gene of *E. coli*. Asterisks approximately indicate the variable regions of the gene; V4 is absent in prokaryotic 16S rRNAs. H3110 was an uncharacterized tortoise mycoplasma.

similarity score of *M. agassizii* with *Ureaplasma urealyticum* was 0.71, and that with *E. coli* was 0.45.

In contrast, a different sequence motif at nt 913 to 920, 5'-AAACGGAA-3', including cytosine at nt 916, placed *M. testudinis* in the distant *M. pneumoniae* group described by Weisburg et al. (15), in which *M. pirum* was its closest relative (similarity score = 0.88) on the basis of overall 16S rRNA gene sequence similarity. The cytosine residue at nt 916 is an insertion in a region of the 16S rRNA gene that is invariant in length among archaeobacteria, eukaryotes, and other eubacteria and that has a highly conserved sequence (15). The similarity score of *M. pneumoniae* with *M. hyorhinae* was 0.72. Maniloff (9) and Weisburg et al. (15) concluded that other members of the group are *M. pneumoniae*, *M. genitalium*, and *M. gallisepticum*. Positioning of *M. testudinis* in the *M. pneumoniae* group and of *M. agassizii* in the *M. hominis* group was consistent with the previously observed antigenic differences between *M. agassizii* and *M. testudinis*.

A segment of the 16S rRNA gene from clinical mycoplasma

isolates cultured from nasal lavages of 35 *G. agassizii* and *G. polyphemus* tortoises with symptoms of upper respiratory tract disease was amplified by using the *Mycoplasma* genus-specific primer antisense strand nt 1055 to 1031, 5'-TGCACCATCTGTCACCTCTGTTAACCTC-3' (14), paired with the sense strand terminal primer. A *Sau*96I restriction endonuclease recognition site present in the V2 variable region of the gene segment (10) of *M. testudinis* conveniently distinguished it from *M. agassizii*. No isolate displayed the *M. testudinis* restriction

fragment length polymorphism pattern for the gene segment. Although the tortoises were not examined for cloacal mycoplasma infection, this result suggests that the infection site (respiratory tract versus excretory tract), in addition to the class or family of the host, may influence the species of mycoplasma likely to be found.

The 16S rRNA gene nucleotide sequences of close relatives of *M. agassizii* (the glucose fermenters *M. hyorhinis*, *M. hyopneumoniae*, *M. neurolyticum*, *M. sualvi*, and *M. mobile*) and of distant relatives of *M. agassizii* (the arginine hydrolyzers *M. hominis*, *M. arthritis*, *M. arginini*, *M. orale*, and *M. salivarum*) were analyzed in an attempt to design an *M. agassizii*-specific amplification primer. The primer selected (sense strand nt 471 to 490, 5'-CCTATATTATGACGGTACTG-3') was in the V3 variable region of the gene and was used in combination with the above-described *Mycoplasma* genus-specific primer. The 576-bp nucleotide sequence amplified with this combination of primers was screened for restriction endonuclease recognition sites unique to *M. agassizii* which could be used as independent confirmation of the species specificity of an amplification product. Only 1 of 177 endonuclease recognition sequences checked, *Age*I, was unique to *M. agassizii* in the amplified segment when compared with 32 published mycoplasma 16S rRNA gene sequences.

When rescreened with *Age*I, 10 of the 35 isolates described above did not show the *M. agassizii* restriction fragment length polymorphism pattern for the gene. The primer indicated was therefore only semispecific, and amplification products required an independent test for conclusive species identification. The 16S rRNA gene of 1 of the 10 isolates, strain H3110, was completely sequenced as described above (Fig. 1). It was from a previously uncharacterized mycoplasma without an obvious close relative but with overall sequence similarity to members of the *M. fermentans* group: *M. fermentans*, *M. bovis genitalium*, *M. californicum*, *M. agalactiae*, *M. lipophilum*, and *M. pulmonis* (similarity scores, 0.81 to 0.83). For perspective, the similarity score of *M. fermentans* with *M. hyorhinis* was 0.85. Each of the 10 isolates had a rare *Nci*I endonuclease recognition site at nt 290 that was not present in any other member of the *M. fermentans* group, which suggested that the isolates were all the same species of mycoplasma. These 10 isolates were all from *G. agassizii* tortoises. The potential pathogenicity of these isolates has not been investigated. In addition, one other atypical nasal isolate from a moribund *G. polyphemus* tortoise with advanced symptoms of upper respiratory tract disease was identified by partial sequence analysis of the 16S rRNA gene as *Acholeplasma laidlawii*, probably a nonpathogenic commensal.

Conclusion. We have positioned *M. agassizii* and *M. testudinis* in the *M. hyorhinis* and *M. pneumoniae* phylogenetic groups, respectively, on the basis of 16S rRNA gene nucleotide sequence comparisons. Mollicute infections can be highly host specific, and informal groupings based upon the host from which the organisms were first or are frequently isolated can have clinical utility. Reptiles might provide unique niches or microenvironments for infection, in part related to poikilothermy or hibernation behavior, very different from better-characterized animal mycoplasma hosts. If specific microbial phenotypes were necessary to colonize such environments, mycoplasmas infecting reptiles might be predicted to belong to a

phylogenetically close group different from those infecting other animals. However our results show that diverse mollicutes of the *M. hyorhinis*, *M. pneumoniae*, *M. fermentans*, and *Acholeplasma* groups all can be detected in tortoises. The 16S rRNA gene of *M. agassizii* will be useful for rapid, sensitive detection and identification of this tortoise pathogen through PCR-based restriction fragment length polymorphism analysis. This is particularly important because cultural isolation is technically challenging and can require 3 to 6 weeks. A 16S rRNA gene PCR-based diagnostic test will complement enzyme-linked immunosorbent assay of host exposure to *M. agassizii* by revealing the current infection status of asymptomatic tortoises.

ACKNOWLEDGMENTS

Oligonucleotide synthesis, nucleotide sequence determination, and VAX computer access were provided by the Interdisciplinary Center for Biotechnology Research at the University of Florida.

This work was supported by grants from Walt Disney World Co. and the U.S. Army.

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