ABSTRACT

COEVOLUTIONARY ANALYSIS OF APPALACHIAN XYSTODESMID MILLIPEDES
AND THEIR SYMBIOTIC MESOSTIGMATID MITES

by

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Mites (Acari) form symbiotic relationships with many animal taxa including fish, amphibians, reptiles, birds, mammals, mollusks, and arthropods. They are frequently found living on millipedes, and it has often been speculated that these two groups of arthropods have, in some cases, undergone coevolution. However, this hypothesis has never been formally tested. Millipedes of the family Xystodesmidae Cook, 1985 (Diplopoda: Polydesmida) are often host to several symbiotic mite species, but very little work has been done to identify these acarines or to understand their relationship to the millipedes. In an attempt to better understand these associations, mites and their xystodesmid millipede hosts were collected in the broadleaf forests of the eastern United States. Mites in the genera *Stylochyrus* Canestrini and Canestrini, 1882 (Mesostigmata: Ologamasidae) and *Schwiebea* Oudemans, 1916 (Sarcoptiformes: Acaridae) were very prevalent among millipedes in the genera *Apheloria* Chamberlin, 1921*; Appalachioria* Marek and Bond, 2009; *Boraria* Chamberlin, 1943; *Brachoria* Chamberlin, 1939; *Dixioria* Chamberlin, 1947; *Nannaria* Chamberlin*,* 1918; *Pleuroloma* Rafinesque, 1820; *Prionogonus*

Shelley, 1982; *Rudiloria* Causey, 1955; and *Sigmoria* Chamberlin, 1939. Of the mite taxa collected, the species *Stylochyrus rarior* (Berlese, 1916) was found on the greatest number of sampled millipede taxa. The complete mitochondrial genome of *S. rarior* associated with an individual of the millipede genus *Apheloria* (Polydesmida: Xystodesmidae) was sequenced. The genome is 14,900 nucleotides in length, has all the typical genes of an arthropod mitochondrion, differs in gene arrangement from that of the ancestral arthropod, and has a gene order that is unique among mites and ticks. The major difference in *S. rarior* is the placement of the protein-coding gene *nad1*, which is positioned between the ribosomal RNA gene *12S* and the protein-coding gene *nad2* (transfer RNA genes and non-coding regions excluded). For use in coevolutionary analyses, the DNA from two mitochondrial regions (*16S/12S* and *cox1*) was sequenced for all collected xystodesmid millipede and *Stylochyrus* mite specimens. Phylogenetic trees were reconstructed for both of these millipede and mite taxa using Bayesian inference. Pairwise distance data was used in distance-based coevolutionary analyses, and reconstructed phylogenies were used in tree-based coevolutionary analyses. The phylogenetic analyses indicate *Stylochyrus* and xystodesmid millipede evolutionary history is incongruent. Moreover, the evolutionary relationships among mite individuals and populations have very low support values and indicate little to no geographic structuring. The coevolutionary analyses likewise detected no pattern of coevolution among these millipede and mite lineages. Unlike many arthropod species, *Stylochyrus* mites appear to be highly vagile.

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Angela Lynn Swafford

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CHAPTER 1: SYMBIOTIC MITES OF XYSTODESMID MILLIPEDES

1.1: Introduction

The order Mesostigmata, which contains approximately 12,000 known species, is a hyper-diverse arthropod group that comprises both free-living and symbiotic species of mites (Krantz and Walter, 2009). These mite species are commonly predatory, brown in color, and their first pair of legs is often used for sensory purposes in addition to walking (Hunter and Rosario, 1988). Mesostigmatid mites, as well as other acarine taxa, are probably poor dispersers as a consequence of their small size and lack of wings; therefore, phoresy is common practice among many groups of mites (Krantz and Walter, 2009). Phoresy is a short-term symbiotic relationship in which a small animal (the phoretic) increases its ability to disperse by attaching to a larger, more mobile animal (the carrier) (Farish and Axtell, 1971). A symbiotic relationship is an association between two or more organisms in which at least one of the organisms benefits; it can be mutual, commensal, or parasitic. Phoresy is considered to be a commensal type of symbiotic relationship because the phoretic benefits, but the carrier is usually not affected. Phoretic associations can be facultative or obligatory, and they can range from having only one very specific carrier to having a wide variety of taxa employed as carriers (Krantz and Walter, 2009). Animals in a phoretic stage often undergo an arrest of basic functions, such as development, reproduction, and feeding (Farish and Axtell, 1971). Once a new, suitable environment is reached, the mites can leave their carrier, and these halted functions can recommence. Phoresy commonly takes place in mites in the deutonymphal stage of development and in adults (Krantz and Walter, 2009). Despite the interesting life history characteristics of species attributed to this group and the close evolutionary associations of many of its members

with totally unrelated arthropod and other animal species, relatively few studies have focused on mesostigmatid mites.

It is now commonplace to employ very large sets of molecular data in evolutionary systematic studies, including multiple genes sampled across both nuclear and organellar genomes and sequence data comparisons based upon entire genomes (Boore *et al.*, 2005). Complete mitochondrial genome DNA sequences are often used because they are easy to isolate from nuclear DNA and tend to have a constant number of genes across most animal groups (Boore *et al.*, 2005). The circular mitochondrial genome of animals typically consists of one or two non-coding control regions and 37 genes: 13 protein-coding genes, 2 ribosomal RNA (rRNA) genes, and 22 transfer RNA (tRNA) genes. Complete mitochondrial genomes have been sequenced for over 200 arthropod species. Of these, 23 belong to the subclass Acari (mites and ticks). Mitochondrial genomes of acarines range in size from 13,103 nucleotides in *Tetranychus urticae* Koch, 1836 (Tetranychoidea) (Van Leeuwen *et al.*, 2008) to 24,961 nucleotides in *Metaseiulus occidentalis* (Nesbitt, 1951) (Phytoseiidae) (Jeyaprakash and Hoy, 2007). The ancestral arthropod gene order is considered to be the arrangement found in the horseshoe crab *Limulus polyphemus* Linnaeus, 1758 (Lavrov *et al.*, 2000). Some arthropods, including a number of mites, deviate from this arrangement. A few (such as *M. occidentalis*) even differ in the number of mitochondrial genes as a result of duplications or deletions (Fahrein *et al.*, 2007; Jeyaprakash and Hoy, 2007). The vast amount of differences seen among mite mitochondrial genomes indicates that there remains considerable work if we are to understand acarine systematics.

Generally speaking, we know very little about the genomes of mesostigmatid mites. The mitochondrial genome has been completely sequenced for only two species, and the synteny and size of their genomes are very different (Evans and Lopez, 2002; Navajas *et al.*, 2002;

Jeyaprakash and Hoy, 2007). *Metaseiulus occidentalis* has a surprisingly large mitochondrial genome that is very divergent from the ancestral arthropod condition (Jeyaprakash and Hoy, 2007). It contains both duplicated and triplicated regions, has short transfer RNAs, and may be lacking the two protein-coding genes *nad3* and *nad6* (Jeyaprakash and Hoy, 2007). The entire mitochondrial genome of *Varroa destructor* Anderson and Trueman, 2000 (Varroidae) has been sequenced twice with slightly different results (Evans and Lopez, 2002; Navajas *et al.*, 2002). Evans and Lopez (2002) concluded that the mitochondrial genome of *V. destructor* is 15,218 nucleotides in length, whereas Navajas *et al*. (2002) estimated the size to be 16,477 nucleotides. It was discovered that the protein-coding genes and ribosomal RNA genes of *V. destructor* are located at the same relative positions as in ancestral arthropods (Evans and Lopez, 2002; Navajas *et al.*, 2002).

Neither *M. occidentalis* nor *V. destructor* belong to the family Ologamasidae, which is a large, widespread family of soil-dwelling, predaceous mites that typically eat small invertebrates and their eggs (Krantz and Walter, 2009). Most ologamasid mite taxa are not phoretic; however, deutonymphs of a few genera have been found associated with mammal nests, carabid beetles, dipterans, and other arthropods (Krantz and Walter, 2009). *Stylochyrus rarior* (Berlese, 1916) is an ologamasid mite commonly found in moist, deciduous forests and is sometimes found associated with millipedes, small mammals, and birds (Kethley, 1983). Only juveniles in the deutonymphal stage have been found to form symbiotic relationships, while adults are usually free-living and collected in leaf litter on the forest floor (Kethley, 1983). It is therefore believed that *S. rarior* only forms temporary or phoretic symbiotic associations for dispersal purposes (Kethley, 1983). Deutonymphs have a distal hyaline extension on one of their cheliceral digits, which is often associated with mites that practice phoresy (Kethley, 1983). However, very little

work has been done to understand the phoretic relationships of *S. rarior* or to determine how many taxa these mites use as carriers.

The objectives of this study are: 1) to document the prevalence of symbiotic mites on xystodesmid millipede species of the Appalachian Mountains and 2) to examine the common mite *S. rarior* and sequence its entire mitochondrial genome. This work will lay the foundation for a future genetic study of the coevolution of these millipedes and their associated mites.

1.2: Methods

1.2.1: Sampling and Collecting

Xystodesmid millipedes were collected in October 2007, May to July 2008, and May to June 2009. Most collecting took place at the known localities of the millipede genus *Appalachioria* (Marek and Bond, 2006) in order to collect mite and millipede specimens for a future coevolutionary analysis. All xystodesmid millipedes found were examined for mites in the field. The millipedes that harbored mites were placed in individual collecting vials, so that there was no opportunity for the transfer of mites between millipedes. To ensure that few mites were lost during transport, mites that were visible on the millipedes were removed with soft forceps and placed in RNAlater (Qiagen Inc., Valencia, CA) in the field. Both millipedes and mites were transported back to the lab for identification and study. In the lab, millipedes were again examined for mites. If mites were found, they were placed in RNAlater and stored at -80˚C. Mites stored in RNAlater can be readily used for DNA extraction and sequencing. Millipede species were identified by morphology of male genitalia or by comparing the region of their mitochondrial DNA sequence spanning the 12S and 16S rRNA genes to those of the previously identified millipedes with Genbank accession numbers DQ4900648 through DQ4900700 (Marek

and Bond, 2006). For identification, mites were cleared in lactic acid or lactophenol and then mounted on microscope slides using Hoyer's mounting medium. Alcohol-preserved xystodesmid specimens from the collection at East Carolina University (ECU) were also examined for the presence of mites. Both the millipede specimens and the alcohol content of their vials were inspected for mites. All mites and millipedes collected as part of this study have been assigned unique voucher numbers and are currently stored in the collections at ECU (to be deposited in the Field Museum of Natural History collection).

1.2.2: Molecular Protocols

A modified DNA extraction method using a Qiagen DNeasy Tissue Kit (Valencia, CA) was used to isolate genomic DNA from one individual of *Stylochyrus rarior*. The purpose of changing this extraction protocol was to ensure that the mite was not destroyed and could later be mounted on a microscope slide for identification purposes. The first modifications to the normal protocol consisted of leaving the mite in the digestion mix at 55˚C for 24 hours followed by -40˚C for another 24 hours. After thawing at room temperature, all the liquid was removed and transferred to a new tube so that the mite could be recovered. The digestion mix was never vortexed while it contained the mite specimen. Instead, it was mixed gently by tapping the side of the tube. To complete the extraction procedure, 100 µL of buffer AE (10mM Tris Cl and 0.5mM ethylenediametetraacetic acid; warmed to 55˚C) was added to the sample and centrifuged. Then 100 µL of room temperature buffer AE was added to the sample and centrifugation was repeated to produce approximately 200 µL of genomic DNA. The extracted DNA from this single mite was used to sequence the entire genome of the mitochondrion.

First a region of the 16S ribosomal gene was amplified and sequenced using the universal

primers LR-J-12887 (5' CCGGTCTGAACTCAGA TCACGT 3') and LR-N-13398 (5' CGCCTG TTTATCAAAAACAT 3'). A 50 µL reaction was prepared comprising the following PCR mixture: 25.75 µL ultra pure water, 5 µL 2.5mM deoxyribonucleotide triphosphate (dNTP) mixture, 5 µL 10X Taq buffer, 5 µL of each 2.5 µM or 10 µM primer, 1 µL dimethyl sulfoxide (DMSO), 1 µL bovine serum albumin (BSA), 0.25 µL Takara Ex Taq DNA polymerase, and 2 µL genomic DNA. The following thermal cycle parameters were used: initial denaturation at 95°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 2 min. The reagent ExoSAP-IT (USB Corporation, Cleveland, OH) and its corresponding protocol were used to remove excess dNTPs and primers from the PCR product. This product was then used in a 10 µL sequencing reaction with the following components: 4.35 µL ultra pure water, 2 µL 5X sequencing buffer, 1 µL BigDye Terminator, 0.65 µL 2.5 µM or 10 µM primer, and 2 µL PCR product. The following thermal cycle program was used: 26 cycles of 96°C for 10 sec, 50°C for 15 sec, and 60°C for 4 min. This short DNA sequence was used to create primers specific to this individual mite in the 16S region that could then be used to amplify the entire mitochondrial genome according to the procedures of Hwang *et al*. (2001). The primers created were HPK16Sbb_mit91 (5' CATATTGATAAAATAGTTTGCGA CCTCGATGTT 3') and HPK16Saa_mit91 (5' TCAATACCTTCGCATAGTCAAAATACCAC GGC 3'). The following 50 µL PCR mixture was used: 24.5 µL ultra pure water, 8 µL 2.5mM dNTP mixture, 5 µL 10X LA PCR buffer, 5 µL of each 2.5 µM or 10 µM primer, 0.5 µL Takara LA Taq, and 2 µL genomic DNA. The thermal cycle parameters described by Hwang *et al.* (2001) were used, and this product was purified using the ExoSAP-IT procedure.

After the entire mitochondrial genome was amplified and cleaned (minus the short region

within 16S), an additional amplification was done using a Qiagen Repli-g Ultrafast Mini Kit to increase the amount of mitochondrial DNA. Next, two separate digestions were done using the restriction enzymes Rsa I (5' GTAC 3') and Alu I (5' AGCT 3'). These digested products were sorted on an agarose gel with a ladder, and fragments between 500 and 1500 nucleotides in size were excised from the gel and purified using a Qiagen MinElute Gel Extraction Kit. Using a Zero Blunt PCR Cloning Kit (Invitrogen, Carlsbad, CA), DNA was inserted into vectors and transformed into *E. coli* cells, which were then grown overnight on kanamycin agar plates. Isolated colonies, each containing a cloned *S. rarior* mitochondrial region, were amplified and sequenced using the primers M13 Reverse and T7 from the cloning kit. A 25 µL PCR reaction was done using the following mixture: 12.5 µL Promega GoTaq Green Master Mix (Promega, Madison, WI), 9.5 µL ultra pure water, 1.5 µL of each 2.5 µM or 10 µM primer, and 1 swab of an isolated colony. The following thermal cycle parameters were used: initial denaturation at 94°C for 10 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were cleaned up with ExoSAP-IT and sequenced using the same protocol described above. Sequences were viewed, edited, and assembled in the program Sequencher 4.8 (Gene Codes, Ann Arbor, MI).

In order to complete the whole mitochondrial genome sequence, primers pairs were designed using Primer3 (Rozen and Skaletsky, 2000) to fill in any gaps existing between the sequence fragments. Genomic DNA from *S. rarior* was amplified and sequenced using these designed primer pairs. Because all gaps were of unknown length, amplifications were done using Takara LA Taq and the corresponding 50 µL PCR mixture described above. The thermal cycle parameters for long amplification described in Hwang *et al*. (2001) were used but with an annealing temperature of 50°C. The same methods as described earlier were used to purify and

sequence the PCR products.

1.2.3: Genome Annotation

The tRNA genes were identified using tRNAscan-SE 1.21 (Lowe and Eddy, 1997); the cove cutoff score was set to 2 to detect as many potential genes as possible. The tRNA genes that were not found by this method were identified by comparison with known tRNAs of other arthropods and by looking for anticodons. Ribosomal RNA genes were annotated by alignment with available mitochondrial genomes of other Acari. Protein-coding genes were identified by using the NCBI ORF finder and by running a BLAST search against all mite DNA sequences available in Genbank. Regions that were not similar to known genes and did not have long open reading frames were considered to be non-coding control regions.

1.3: Results and Discussion

1.3.1: Prevalence of Mites on Millipede Hosts

A total of 136 xystodesmid millipedes (see Figure 1.1 for photos of representative xystodesmids) were collected in the Appalachian Mountains of North Carolina, Virginia, Kentucky, and Tennessee during the sampling period. Of these millipedes, 89 had symbiotic mites. Over 400 mites, representing the two orders Sarcoptiformes (including the genus *Schwiebea*) and Mesostigmata (including the genus *Stylochyrus*), were extracted from these millipedes. All *Stylochyrus* and *Schwiebea* individuals collected were in the deutonymphal stage of development. Over 100 *Stylochyrus rarior* deutonymphs (see Figure 1.1 for photos of *S. rarior*) were collected from 43 millipedes including the following genera: *Apheloria, Appalachioria, Brachoria, Dixioria, Nannaria, Pleuroloma, Prionogonus*, and *Sigmoria*. For those millipedes

that harbored this species of mite, the number of *S. rarior* per individual millipede ranged from 1 to 13 with an average of 2.49 (SD=2.58). About 51% (22 out of 43) of these millipedes had only a single individual of *S. rarior* associated with them. Deutonymphal mites of the genus *Schwiebea* were found on *Apheloria*, *Appalachioria*, *Brachoria*, *Boraria*, *Dixioria*, *Nannaria*, *Rudiloria*, and *Sigmoria*. Individuals of *Schwiebea* were collected from 57 xystodesmid millipedes and were generally more abundant per millipede than *Stylochyrus rarior*. The number of *Schwiebea* per millipede ranged from 1 to 26 with an average of 5 (SD=5.72). Although these mites were found in greater numbers on individual millipedes, they were not found on as many species of millipede as *S. rarior*. Fifteen millipedes were collected that had both *Schwiebea* and *Stylochyrus rarior*. A few unidentified mesostigmatid mites were also collected in very small numbers from xystodesmids.

Symbiotic mites were discovered on many different body parts of their millipede carriers. Some mites were found attached near the anterior end of a millipede, whereas others were found on the legs or near the bases of the legs. Others seemed to be actively moving along both the dorsal and ventral surfaces of the millipedes. It is not clear whether this activity is natural or whether it occurred because of human interference. *Stylochyrus* was typically active upon a millipede, while *Schwiebea* was usually inactive and attached under the head or near the legs of a millipede. This was as expected because *Schwiebea* deutonymphs have sucker-like attachment plates on their ventral surfaces and have been found tightly attached to other arthropods (Purrington and Drake, 2008). *Stylochyrus* deutonymphs lack attachment plates, so they must hang on to a carrier with their legs, claws, or chelicerae.

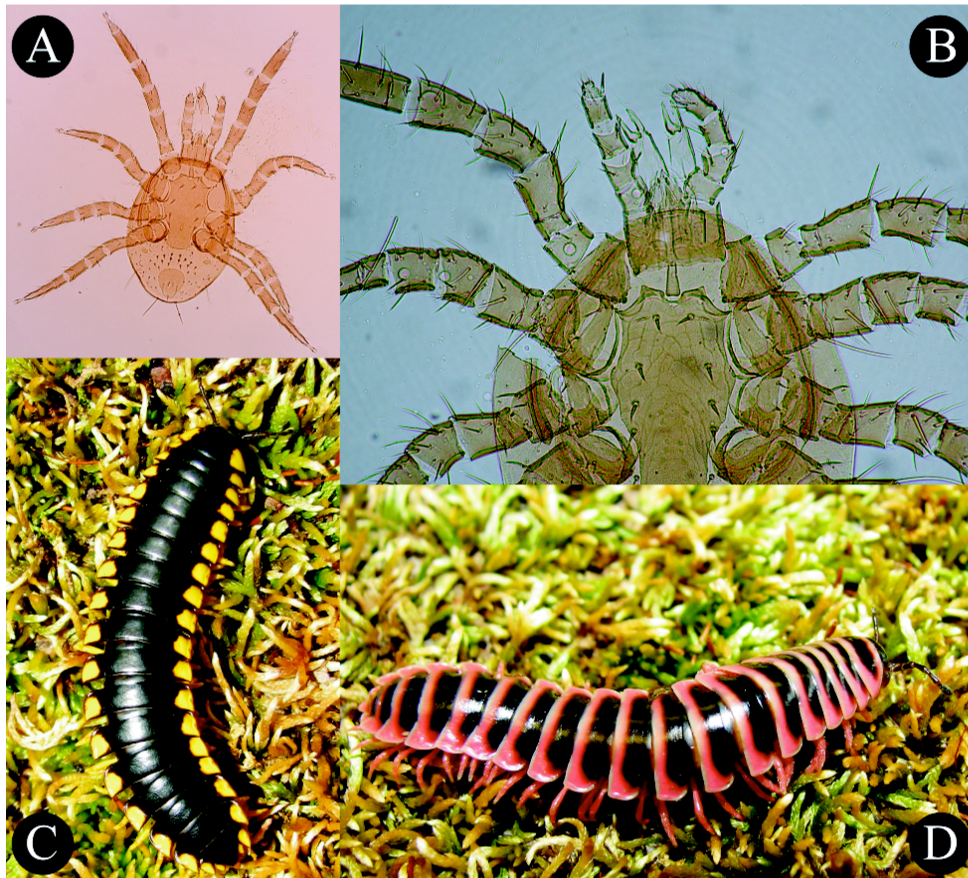

Figure 1.1: Photos of *Stylochyrus rarior* mites and xystodesmid millipedes. (A) Wholemount of *S. rarior* deutonymph in ventral aspect. (B) Anterior portion of *S. rarior* deutonymph in ventral aspect. (C) Live individual of *Apheloria* sp. from Kentucky. (D) Live individual of *Prionogonus* sp. from North Carolina.

Upon examination of the millipede collection at East Carolina University, more mite taxa were found to be associated with xystodesmids. In addition to *Schwiebea* and *Stylochyrus*, the following two other taxa were found: *Viedebanttia* Oudemans, 1929 (Sarcoptiformes: Acaridae) and Heterozerconidae (Mesostigmata). All the mites found were detached from their associate millipede specimen, which indicates that they fell off in storage. Mites from the ECU millipede collection could have been lost or even transferred between millipedes, which means that the

number and kinds of mites found may not be representative of natural populations. Mites were found associated with 119 preserved xystodesmid specimens. Of these millipedes, 72 were associated with *Stylochyrus rarior* (1 to 9 individuals per millipede), 34 with *Schwiebea* (1 to over 20 per millipede), 19 with *Viedebanttia* (1 to 7 per millipede), and 4 with heterozerconids (only 1 per millipede). Some of these mite taxa were found associated with the same individual millipede specimen. All individuals belonging to the genera *Stylochyrus*, *Schwiebea*, and *Viedebanttia* were juveniles in the deutonymphal stage. The 4 heterozerconid mites were adults. Heterozerconidae is an acarine family found primarily on juliform millipedes (Gerdeman *et al*., 2000). In the United States, heterozerconid mites are not known to be associated with any species of Polydesmida; therefore, this xystodesmid-associated heterozerconid may be an undescribed species.

1.3.2: S. rarior Taxonomy and Mitochondrial Genome Organization

Superorder **PARASITIFORMES**

Order **MESOSTIGMATA**

Suborder **MONOGYNASPIDA**

Superfamily **RHODACAROIDEA**

Family **OLOGAMASIDAE** Ryke, 1962

Genus ***Stylochyrus*** Canestrini and Canestrini, 1882

Type species: *Stylochyrus rovennensis* Cannestrini and Canestrini, 1882: 31-82

Stylochyrus rarior (Berlese, 1916)

Gamasiphis (*Epiphis*) *rarior* Berlese, 1916: 289-338

Epiphis rarior Berlese, 1916. – Vitzthum, 1942: 756; Kethley 1983: 2598.

Stylochyrus rarior (Berlese, 1916). – Lee, 1970: 94.

1.3.2.1: Diagnosis. All individuals of *S. rarior* collected are deutonymphs, are morphologically identical, and correspond to Kethley's (1983) descriptions. However, there were a few discrepancies between Kethley's (1983) illustrations and diagnosis of *S. rarior* deutonymphs, and they are clarified here. All dorsal setae are very short except for s4, Z3, and Z5, which are substantially longer (more than five times) in length. The fixed digit (not the movable digit) of the chelicera has an elongate, distal hyaline process and is longer in length than the movable digit.

1.3.2.2: DNA processing. Extracted genomic DNA from the *S. rarior* specimen MIT00091-4 was used to amplify and sequence the entire mitochondrial genome (GenBank accession number GQ927176). This mite was associated with a millipede of the genus *Apheloria* (SPC001176) from the Appalachian mountains of Tennessee. After both restriction enzyme digestions were completed and selected DNA fragments were cloned and sequenced, DNA sequences were assembled into 13 contigs in Sequencher 4.8. These contigs contained partial sequences of all the 13 protein-coding genes and the 2 ribosomal RNA genes normally present within a mitochondrial genome. Fourteen pairs of primers (Table 1.1) were designed to obtain DNA sequences from the missing regions between these 13 DNA fragments.

Table 1.1: Primer pairs and their locations.

Name	Primer Sequence	Location
Mit162R	5’-CTGTCAAATTAAACCCTCCAGC-3’	*nad1*
Mit289L	5’- GAAGATAATTCTTGCTCTAGATTGAAA-3'	*nad2*
Mit289R2	5’-CCAATTTACTTTTGACTTCCTCAAATA-3'	*nad2*
Mit194L2	5’-TTAGGGGATAACTAATACGGAGATAAA-3'	*nad2*
Mit194R2	5’-TTTATAGTAATACCAGCCATAATTGGG-3'	*cox1*
Mit160L2	5’-ATCTAATTCCTGCTAAGTGAAGTCTAA-3'	*cox1*
Mit160R	5’-CGACACCCCTATTCTTTGATCT-3'	*cox1*
Mit247L	5’-TATCGCCGAGGTATTCCTCTTA-3'	*cox1*
Mit247R	5’-AAACAAAACAAACTTTATATCAACCA-3'	*atp8*
Mit191L	5’-AGAAATTAATAGGTGGCCAGCA-3'	*atp6*
Mit191R	5’-TTCGACTTGGCTCTTGAATACA-3'	*nad3*
Mit291L	5’-GGAGTGTAAATTCAATCACGGA-3'	*nad5*
Mit291R	5’-CTCTTAAAAATGGAAACCCCG-3'	*nad5*
Mit199L	5’-GGTAGAATGTGGGGTTGGATAG-3'	*nad4*
Mit291R2	5’-CATCAAACACCCAAATAAAATAACC-3'	*nad5*
Mit199L2	5’-GTTAGCTGCAAATTTTGAGAGTGAT-3'	*nad4*
Mit199R	5’-ATTACCCAATCCTCCCCATAAT-3'	*nad4*
Mit209L	5’-GGTTATTATGGGTTAGTGGGGTG-3'	*cob*
Mit209R	5’-TTACCTTCATATCGGTCGAGGT-3'	*cob*
Mit175L	5’-AACAGCGAGTACAAAAGGAAGG-3'	*cob*
Mit175R	5’-CTAACACCCCTCCTCAAGAACA-3'	*cob*
Mit16SL	5’-AATAGTTTGCGACCTCGATGTT-3'	*16S*
Mit16SR	5’-TCCATTCTCTTAGCACCCAATT-3'	*16S*
Mit303L	5’-ATCAGGGGGCTTCAATAAAATT-3'	*16S*
Mit303R	5’-CAATTATTCATGAGAGCGACGG-3'	*16S*
Mit12SL	5’-TTTGGCGGTATTTCAATCTTTT-3'	*12S*
Mit12SR2	5’-AACCTTAAAAACAAAACTAAAACTGCC-3'	*12S*
MitND1L	5’-GGTAGTAATTTTAGCTGAAACAAATCG-3'	*nad1*

1.3.2.3: Genome organization. The mitochondrial genome of *Stylochyrus rarior* is circular, consists of 14,900 nucleotides, and contains 13 protein-coding genes, 22 tRNA genes, 2 rRNA genes, and 2 non-coding control regions (Figure 1.2, Table 1.2). Genes are encoded on both DNA strands (Table 1.2) as in other arthropods. The nucleotide composition of this mitochondrial genome consists of 38.3% adenine, 34.4% thymine, 17.8% cytosine, and 9.5% guanine. Approximately 72% of this entire mitochondrial genome sequence codes for proteins. Only 18 tRNA genes were identified and located by the program tRNAscan-SE. The other 4 (*trnL2*, *trnC*, *trnR*, *trnS1*) were located by comparison to known tRNA sequences of other arthropods and by determining their appropriate anticodons. There are 2 *trnL* genes and 2 *trnS* genes present. Several of the genes of the mitochondrial genome have short overlapping regions between them (Table 2).

The order of genes in the mitochondrion of *S. rarior* differs from the assumed ancestral arthropod synteny of *L. polyphemus*. This gene arrangement is also novel among acarines due to the placement of the protein-coding gene *nad1*, which is located between *12S* and *nad2* (tRNAs and control regions excluded). When compared to *L. polyphemus*, the positions of the transfer RNA genes *trnF* and *trnE* are swapped in *S. rarior*. The following two regions have also been transposed: *16S–trnV–12S* and *nad1–trnL1–trnL2*. There are two non-coding control regions, one on each side of the *nad1–trnL1–trnL2* region.

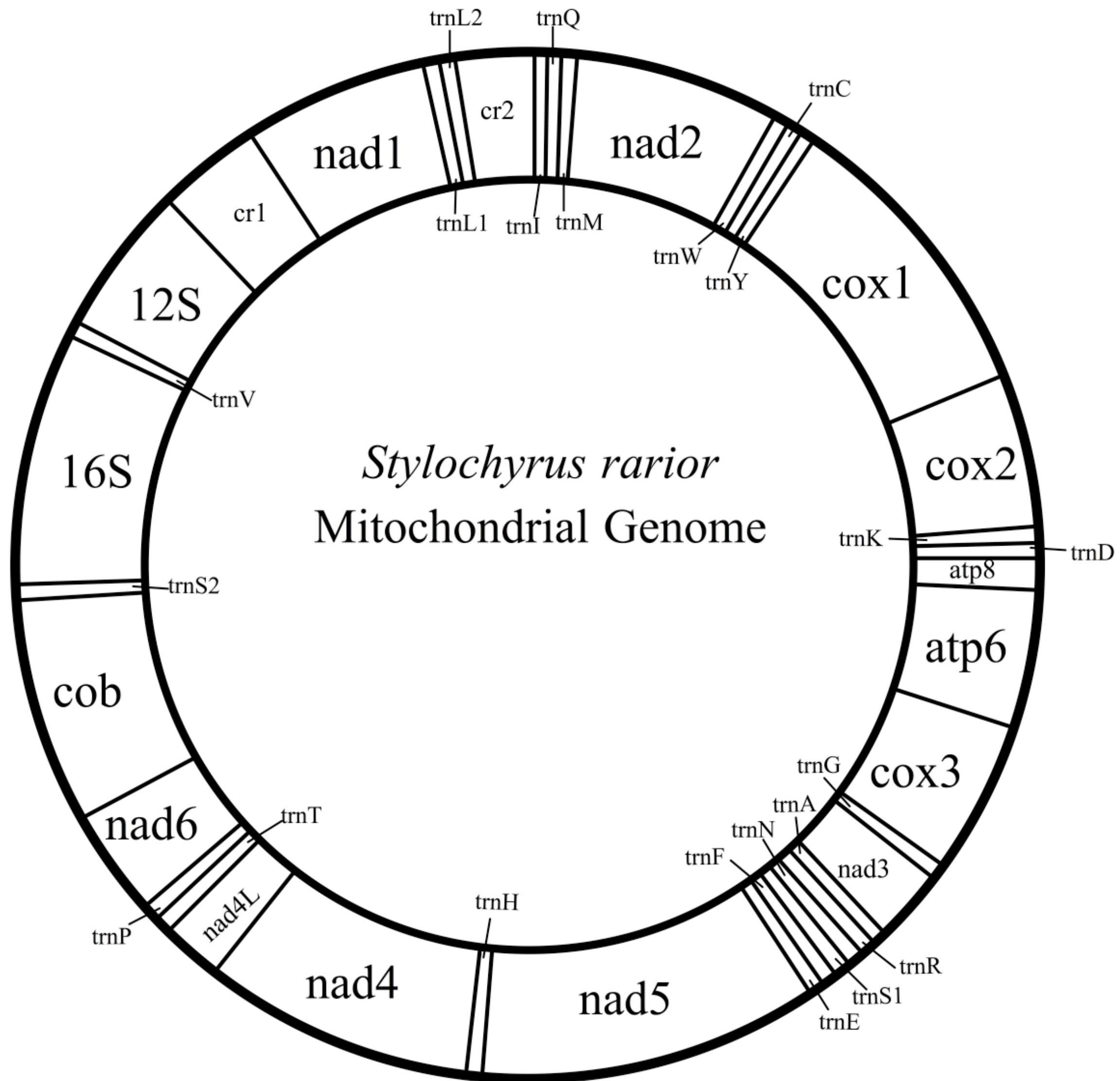

Figure 1.2: Mitochondrial genome map for *Stylochyrus rarior*.

Table 1.2: *Stylochyrus rarior* mitochondrial genome organization.

Gene	Gene Product	Position	Length	Strand
trnM	tRNA Methionine	1–63	63	+
nad2	NADH dehydrogenase subunit 2	64–1026	963	+
trnW	tRNA Tryptophan	1025–1085	61	+
trnC	tRNA Cysteine	1078–1140	63	–
trnY	tRNA Tyrosine	1129–1190	62	–
cox1	Cytochrome c oxidase subunit 1	1202–2725	1524	+
cox2	Cytochrome c oxidase subunit 2	2725–3400	676	+
trnK	tRNA Lysine	3401–3463	63	+
trnD	tRNA Aspartic Acid	3464–3524	61	+
atp8	ATP synthase F0 subunit 8	3525–3683	159	+
atp6	ATP synthase F0 subunit 6	3680–4345	666	+
cox3	Cytochrome c oxidase subunit 3	4345–5124	780	+
trnG	tRNA Glycine	5125–5187	63	+
nad3	NADH dehydrogenase subunit 3	5188–5526	339	+
trnA	tRNA Alanine	5526–5587	62	+
trnR	tRNA Arginine	5588–5646	59	+
trnN	tRNA Asparagine	5647–5707	61	+
trnS1	tRNA Serine 1	5708–5761	54	+
trnF	tRNA Phenylalanine	5762–5822	61	+
trnE	tRNA Glutamic Acid	5821–5880	60	–
nad5	NADH dehydrogenase subunit 5	5881–7549	1669	–
trnH	tRNA Histidine	7550–7611	62	–
nad4	NADH dehydrogenase subunit 4	7607–8935	1329	–
nad4L	NADH dehydrogenase subunit 4L	8937–9209	273	–
trnT	tRNA Threonine	9226–9285	60	+
trnP	tRNA Proline	9286–9348	63	–
nad6	NADH dehydrogenase subunit 6	9336–9782	447	+
cob	Apocytochrome b	9789–10899	1111	+
trnS2	tRNA Serine 2	10900–10962	63	+
16S	Large subunit ribosomal RNA	10963–12162	1200	–
trnV	tRNA Valine	12163–12234	72	–
12S	Small subunit ribosomal RNA	12235–12887	653	–
cr1	Control region 1	12888–13358	471	N/A
nad1	NADH dehydrogenase subunit 1	13359–14261	903	–
trnL1	tRNA Leucine 1	14262–14320	59	–
trnL2	tRNA Leucine 2	14314–14377	64	–
cr2	Control region 2	14378–14776	399	N/A
trnI	tRNA Isoleucine	14777–14837	61	+
trnQ	tRNA Glutamine	14838–14900	63	–

1.4: Conclusions

Stylochyrus rarior appears to be considerably more common among the xystodesmid millipede species than other mite taxa. This is one of the reasons why *S. rarior* was chosen for further study and use in a future coevolutionary analysis. It is also large in size and soft-bodied, which allows for easier DNA extractions. *S. rarior* is the first ologamasid and the first rhodacaroid mite to have its entire mitochondrial genome sequenced. This genome contains all 37 genes that are typical of the animal mitochondrion, and it also contains two non-coding regions. The mitochondrial gene order of *S. rarior* is different from the ancestral arthropod arrangement and is unique among the acarines. Acarine mitochondrial genomes often have gene rearrangements, but it is unclear whether these gene order differences have any phylogenetic significance because very few mitochondrial genomes of mites and ticks have been completely sequenced. *S. rarior* is only the third mesostigmatid mite for which the whole mitochondrial DNA sequence is known, and it may help to improve the understanding of acarine evolution.

There still remains much to learn about the ecology of phoretic mites and their millipede carriers. Phoresy could be beneficial to millipede-mites in ways other than just enhanced dispersal. For example, xystodesmid millipedes do not typically get eaten because they produce a hydrogen cyanide defense secretion (Marek and Bond, 2006). Perhaps the symbiotic mites of xystodesmid millipedes could also be protected from predation; it is unknown if phoretic mites are resistant to these cyanide secretions. Another uncertainty is how the millipedes respond to the association with their mite commensals; that is, do they benefit by the relationship, is there a cost, or is the association neutral?

CHAPTER 2: TESTING FOR MILLIPEDE-MITE CODIVERGENCE

2.1: Introduction

Coevolution is a fascinating evolutionary process that has interested biologists for decades. First coined by Ehrlich and Raven (1964), the term coevolution was only later explicitly defined by Janzen (1980) as the process in which two populations undergo evolutionary changes in response to one another. Lincoln *et al.* (1982) expanded upon this definition to further clarify that coevolution is the interdependent evolution of two or more ecologically related taxa. Coevolution usually occurs among taxa that have been closely associated for a long period of time (Ronquist, 1997).

Most commonly observed in organisms involved in symbiotic relationships (mutualism, commensalism, and parasitism), coevolution is thought to include five different types of processes: duplication, failure to speciate, sorting, host switching, and cospeciation. Duplication is a coevolutionary process in which one organism (the symbiont) speciates while the other organism (the host) does not (Johnson and Clayton, 2004). One host species will possess several symbiont species after a duplication event. The reverse of duplication is failure to speciate, a situation when a host speciates but its symbiont does not (Johnson and Clayton, 2004). In this scenario, one symbiont species may be found on several host species. Sorting occurs when a symbiont species becomes extinct or is lost from a host population such that a host species no longer has an associate (Johnson and Clayton, 2004). A host switch occurs when a symbiont species moves from one host species to another and can be categorized as either incomplete or complete. Incomplete host switches occur when individuals of one symbiont species associated with a single host species colonize a different host species (Johnson and Clayton, 2004). The symbiont species can then be found on two host species, or it can eventually be lost from the

original host species. Incomplete host switching does not involve speciation; however, complete host switching is usually associated with speciation. A complete host switch involves the duplication of a symbiont species on one host species followed by the shift of one of the symbiont species to a new host species (Ronquist, 1997; Charleston, 1998).

Finally, cospeciation is reciprocal speciation that is induced by a very close interaction between two species (Thompson, 1986) wherein hosts and their symbionts speciate simultaneously. The following three rules of cospeciation have been developed:

1. Farenholz's Rule (1913) states that phylogenetic trees of hosts and their symbionts tend to have identical topologies, indicating that cospeciation has occurred.
2. Szidat's Rule (1940) states that primitive hosts will have primitive symbionts, implying that derived hosts will possess derived symbionts.
3. Eichler's Rule (1942) states that a host taxon with many species will have a larger diversity of symbiont species than a host taxon with fewer species. In other words, the more host species there are, the more symbiont species expected.

Although it is often easier to define the five coevolutionary events in terms of species and speciation, any of these events may involve divergences occurring at levels other than species. The term codivergence is often used in place of cospeciation when the products of divergence are taxa other than species; therefore, cospeciation can be viewed as a particular type of codivergence (Light and Hafner, 2008). Among coevolving taxa, there are two categories of codivergence: strict and widespread. Strict codivergence occurs when there is a one to one ratio of host to symbiont taxa and the only events that explain their patterns of evolutionary history are

codivergences. Widespread codivergence among host and symbiont phylogenies occurs when codivergences are the most common events but not the only events that explain the observed associations. Although widespread codivergence among associated taxa is rarely found in nature, it is still much more common than strict codivergence (Hafner and Nadler, 1990).

Host and parasite associations are often the focus of widespread codivergence studies. Parasites, those organisms that have detrimental effects on their hosts, are often so intimately associated with their hosts that they may be very specialized and may only survive on one or a few host species (Thompson, 1994). However, coevolution can occur in other types of symbiotic relationships as well. This includes mutualism, in which both the host and the symbiont benefit, and commensalism, in which the symbiont benefits while the host is not affected.

Mites are known to develop symbiotic relationships with many groups of animals including fish, amphibians, reptiles, birds, mammals, and some invertebrates, such as mollusks and arthropods (Fain, 1994). Both parasitic and commensal associations can be formed between mites and other arthropods (Hunter and Rosario, 1988). Mites have the ability to adapt to many types of habitats and lifestyles and occupy an extraordinarily wide range of parasitic niches (Fain, 1994). Therefore, mites are some of the most suitable organisms in which to study coevolution, especially if they are parasites (Fain, 1994).

There are over fifteen known families (and three orders) of mites that form associations with myriapod taxa, and yet very little research has been done on millipede-mite associations (Gerdeman *et al.*, 2000). Depending on the species of millipedes and mites, the type of symbiotic relationship may vary from commensalism to obligate parasitism (Gerdeman *et al.*, 2000). Phoretic mites have been found on millipedes in Indonesia (Evans and Sheals, 1959), and other commensal millipede-mites have been reported from Africa (Tragardh, 1907). Most

millipede-mite studies conducted focus only on mites and usually comprise the description of new mite species, largely ignoring the millipedes that harbor them. That is, most studies simply report that mites are found living on millipedes, and there tends to be little or no information about the millipede or the nature of the putative symbiotic relationship. With few exceptions, such as *Narceus* Rafinesque, 1820 and *Tylobolus* Cook, 1904 (Kethley, 1978; Gerdeman *et al.*, 2000; Gerdeman and Klompen, 2003), the majority of millipede-mite studies have not been carried out in North America.

Myriapodologists have often speculated that millipedes and their associated mites are coevolving; however, this has never been tested. Kethley (1978) determined that three species in the millipede genus *Narceus* each harbor a different species of the mite genus *Narceolaelaps* Kethley 1978. The presence of one symbiont species on each host species raises the possibility of cospeciation between *Narceus* and *Narceolaelaps*. In another example, the mite family Heterozerconidae consists largely of species that live only on millipedes, indicating a very close symbiotic link between the heterozerconids and their hosts. These two mite taxa (the genus *Narceolaelaps* and the family Heterozerconidae) would be an ideal place to look for evidence of millipede-mite coevolution. However, the status of the genus *Narceus*, including how many species it comprises, is currently unclear (Walker *et al*., 2009) making it a difficult group to evaluate. Also, while there are not very many species of heterozerconid mites and their host millipedes, they are geographically widespread. An attempt to analyze this group would be costly and time consuming.

The focus of this study is on the millipede genus *Appalachioria* Marek and Bond 2006 (Polydesmida: Xystodesmidae), which has a manageable distribution and clearly defined species (and subspecies). Currently, there are four nominal species (two with supspecies) recognized: *A*.

eutypa eutypa Chamberlin, 1939); *A. eutypa ethotela* (Chamberlin, 1942); *A. falcifera* (Keeton, 1959); *A. separanda calcaria* (Keeton, 1959); *A. separanda hamata* (Keeton, 1959); *A. separanda separanda* (Chamberlin, 1947); *A. separanda versicolor* (Hoffman, 1963); and *A. turneri* (Keeton, 1959). Additionally, there are two undescribed species (Marek and Bond, 2006, 2007). These millipedes live in moist leaf litter from broadleaf forests in the eastern United States, and like most millipedes, are important decomposers (Marek and Bond, 2006). They are not very vagile and tend to live in isolated populations within mountainous regions (Marek and Bond, 2006); these two conditions are ripe for coevolution. Commonly found associated with individuals of *Appalachioria*, the mite genus *Stylochyrus* Canestrini and Canestrini, 1882 (Mesostigmata: Ologamasidae) will be the focal mite of this study. Mites in the genus *Stylochyrus* are soft-bodied and relatively large in size, making them easier with which to work. The goal of this study is to test the hypothesis that mites of the genus *Stylochyrus* and their associated xystodesmid millipedes, specifically those in the genus *Appalachioria*, have codiverged.

2.2: Methods

2.2.1: Sampling and Collecting

Xystodesmid millipedes and their associated mites were collected in the broadleaf forests of Kentucky, North Carolina, Tennessee, and Virginia. The methods used for collecting and transporting live specimens and for identifying mites and millipedes are described in Chapter 1 (Swafford and Bond, 2009). Several xystodesmid genera have overlapping ranges (Marek and Bond, 2006). Therefore, when possible, millipede specimens belonging to different species and genera were collected at a single locality. In addition, multiple specimens of a single millipede

species were collected in the same area when possible. This allowed for the comparison of individual mites from several millipede species occurring in the same locality and the comparison of individual mites from a single millipede species. This thoroughness permitted us to ascertain if the observed patterns of millipede and mite associations are due to coevolutionary interactions or simply geography.

2.2.2: Molecular Protocols

When compared with nuclear DNA, mitochondrial DNA (mtDNA) has a much lower rate of recombination, a simpler genetic structure, and faster rates of evolution (Rubinoff and Holland, 2005). These features render mtDNA appropriate to use when studying the evolutionary relationships of closely related taxa (genus-level and below). A primer set for one mitochondrial region including portions of the large subunit ribosomal RNA (*16S*), the transfer RNA Valine (*trnV*), and the small subunit ribosomal RNA (*12S*) has already been developed for xystodesmid millipedes (Marek and Bond, 2006). According to Light and Hafner (2008), coevolutionary analyses that compare the same DNA regions in host and symbiont taxa are much more informative than those that use different genes. Also more types of coevolutionary analyses can be attempted when the same gene regions have been sequenced for both hosts and symbionts. For example, tree-based coevolutionary methods can be used on any host-symbiont system for which phylogenies are available, while distance-based methods can only be used when the same DNA region is available for both hosts and symbionts (Light and Hafner, 2008). Using the same gene also allows branch lengths of the host and symbiont gene trees to be compared, which can test if rates of molecular evolution are identical in the hosts and symbionts and if cospeciation events occurred simultaneously (Light and Hafner, 2008). For these reasons, only mtDNA

sequences were used for the phylogenetic and coevolutionary analyses in this study. For each xystodesmid millipede and *Stylochyrus* specimen, two mitochondrial regions were amplified and sequenced. The two regions chosen were the *16S/12S* region described earlier and a portion of the protein coding gene Cytochrome c oxidase subunit 1 (*cox1*). By sequencing the mitochondrial *16S/12S* and *cox1* regions for both the millipedes and mites collected for this study, the actual relationships between individual hosts and symbionts can be examined.

2.2.2.1: Primer design. All primers used in this study are listed in Table 2.1. The three millipede primers (LR-J-12887dip2, LR-J-APHE1, and SR-N-145XXdip2) for the *16S/12S* mitochondrial region were taken from Marek and Bond (2006). All other primers were designed specifically for this study. Using the complete mitochondrial genome sequence of *Stylochyrus rarior* (Berlese, 1916) (Swafford and Bond, 2009), the two end primers (Mit16S_mite and Mit12SL) for the *16S/12S* region of the mites were designed to encompass approximately the same region as the millipede *16S/12S* primers. The three internal primers (LR-J-MIT, LR-J-MIT2, and SR-N-MIT) for this region were designed in conserved regions after individuals of *Stylochyrus* were sequenced with just the end *16S/12S* primers. Primers for the *cox1* region of the mites (HCO2198_mit and LCO1490_mit) were created from the *S. rarior* mitochondrial genome sequence in the same places as the universal barcoding primers HCO2198 and LCO1490 (Folmer *et al*., 1994). The two *cox1* primers for the millipedes (HCO2198_APP and LCO1490_APP) were designed in the same manner using the complete mitochondrial genome sequence of *Appalachioria falcifera* (Brewer *et al*., in preparation).

Table 2.1: Mite and millipede primers.

Name	Sequence	Location	Purpose
Mit16S_mite	AAATTAAAATAAGGGGTCCTTTCG	*16S*	Amp, Seq
Mit12SL	TTTGGCGGTATTTCAATCTTTT	*12S*	Amp, Seq
LR-J-MIT	TCCATTCTCTTAGCACCCAATT	*16S*	Seq
LR-J-MIT2	GATTCATAGGGTCTTCTTGTCCCACT	*16S*	Seq
SR-N-MIT	GTACATATCGCCCGTCGCTCTCATG	*12S*	Seq
HCO2198_mit	TGAAGTTACGGTCAGTTAGGAGTATA	*cox1*	Amp, Seq
LCO1490_mit	TTTCTACTAATCACAAAGATATTGG	*cox1*	Amp, Seq
LR-J-12887_dip2	CCGGTCTGAACTCAGATCATGT	*16S*	Amp, Seq
SR-N-145XX_dip2	GGACGTCAAGTCAAGGTGCAG	*12S*	Amp, Seq
LR-J-APHE1	GTTTCACCTTCATACCAGC	*16S*	Seq
HCO2198_APP	TAAACCTCCGGGTGACCAAAAAACCA	*cox1*	Amp, Seq
LCO1490_APP	ACTCTACTAATCATAAGGATATTGG	*cox1*	Amp, Seq

The Purpose column indicates which primers were used for amplifications (Amp) and which ones were used for sequencing (Seq).

2.2.2.2: Millipede extraction and amplification. Genomic DNA was extracted from 3-5 legs from each millipede using the Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, CA). The mitochondrial *16S/12S* region was amplified using the polymerase chain reaction (PCR) and the primers LR-J-12887dip2 and SR-N-145XXdip2 from Marek and Bond (2006). For each extraction, either a 50 µL or a 25 µL reaction was prepared. The following PCR mixture was used for a 50 µL reaction: 26.75 µL ultra pure water, 5 µL 2.5mM dNTP mixture, 5 µL 10X Taq buffer, 5µL of each 2.5 µM or 10 µM primer, 1 µL dimethyl sulfoxide (DMSO), 1 µL bovine serum albumin (BSA), 0.25µL Ex Taq DNA polymerase, and 1 µL genomic DNA. The following PCR mixture was used for a 25 µL reaction: 12.785 µL ultra pure water, 2.5 µL 2.5mM dNTP mixture, 2.5 µL 10X Taq buffer, 2.5µL of each 2.5 µM or 10 µM primer, 0.5 µL DMSO, 0.5 µL BSA, 0.125µL Ex Taq DNA polymerase, and 1 µL genomic DNA. For all reactions, the following thermal cycle parameters were used: initial denaturation at 95˚C for 2 min; 30 cycles of denaturation at 94˚C for 30 sec, annealing at 52˚C for 30 sec, and extension at 72˚C for 2 min; and final extension at 72˚C for 2 min. Using the extracted DNA, the *cox1* region

was also amplified and sequenced using the primers HCO2198_APP and LCO1490_APP. For each extraction, a 25 µL reaction was prepared using the following PCR cocktail: 13.375 µL ultra pure water, 2.5 µL 2.5mM dNTP mixture, 2.5 µL 10X Taq buffer, 2.5µL of each 2.5 µM or 10 µM primer, 0.5 µL DMSO, 0.125µL Ex Taq DNA polymerase, and 1 µL genomic DNA. The following thermal cycle parameters were used: initial denaturation at 95˚C for 2 min; 30 cycles of denaturation at 94˚C for 1 min, annealing at 49˚C for 1 min, and extension at 72˚C for 1 min; and final extension at 72˚C for 2 min.

2.2.2.3: Mite extraction and amplification. A DNeasy Kit and the modified protocol described in Swafford and Bond (2009) was used to extract DNA from entire mites. The mitochondrial *16S/12S* region was amplified using the primers Mit16S_mite and Mit12SL and either a 50 µL or a 25 µL PCR reaction mixture. The following PCR mixture was used for a 50 µL reaction: 25.75 µL ultra pure water, 5 µL 2.5mM dNTP mixture, 5 µL 10X Taq buffer, 5µL of each 2.5 µM or 10 µM primer, 1 µL DMSO, 1 µL BSA, 0.25µL Ex Taq DNA polymerase, and 2 µL genomic DNA. The following PCR mixture was used for a 25 µL reaction: 12.875 µL ultra pure water, 2.5 µL 2.5mM dNTP mixture, 2.5 µL 10X Taq buffer, 2.5µL of each 2.5 µM or 10 µM primer, 0.5 µL DMSO, 0.5 µL BSA, 0.125µL Ex Taq DNA polymerase, and 1 µL genomic DNA. The following thermal cycle parameters were used: initial denaturation at 95˚C for 2 min; 30 cycles of denaturation at 94˚C for 1 min, annealing at 50˚C for 1 min, and extension at 72˚C for 2 min; and final extension at 72˚C for 2 min. The *cox1* mitochondrial region was also amplified for *S. rarior* using the primers HCO2198_mit and LCO1490_mit. Either 50 µL or 25 µL PCR reaction mixtures were made comprising the same components and amounts as in the mite *16S/12S* mixtures. The following thermal cycle parameters were used:

initial denaturation at 95˚C for 2 min; 30 cycles of denaturation at 94˚C for 1 min, annealing at 48˚C for 1 min, and extension at 72˚C for 1 min; and final extension at 72˚C for 2 min.

2.2.2.4: Sequencing. The following purification and sequencing procedures were carried out for both millipedes and mites and both gene regions. Excess dNTPs and primers were removed from the PCR products using ExoSAP-IT (USB Corporation, Cleveland, OH). Direct sequencing reactions were carried out using the amplification primers and internal primer sets when necessary (Table 1). Sequencing consisted of a 10 μL sequencing reaction with the following components: 4.35 μL ultra pure water, 2 μL 5X sequencing buffer, 1 μL BigDye Terminator, 0.65 μL 2.5 μM or 10 μM primer, and 2 μL purified PCR product. The following thermal cycle program was used: 26 cycles of 96°C for 10 sec, 50°C for 15 sec, and 60°C for 4 min. The products from the sequencing reactions were purified using a Sephadex (Sigma-Aldrich, St. Louis, MO) procedure and were then rehydrated and loaded on an ABI 3130 Genetic Analyzer. The sequences were viewed and edited in the program Sequencher 4.8 (Gene Codes, Ann Arbor, MI).

2.2.3: Sequence Alignment and Phylogenetic Analysis

The resulting sequences were organized into six data matrices or datasets (Table 2.2). Datasets I, II, and III comprise *Stylochyrus* sequences; Datasets IV, V, and VI comprise xystodesmid millipede sequences. The variation in number of individuals in each dataset is the consequence of individuals that do not have an associate (either mite or millipede) due to extraction, amplification, or sequencing issues. These individuals were later removed from the coevolutionary analyses. Table 2.3 gives a list of all millipede and mite specimens included in

the phylogenetic analyses. The DNA sequences within each dataset were aligned using the program Muscle 3.6 (Edgar, 2004) and the default parameters (iterations and gap open cost parameters). DNA sequences from the outgroup acarine taxon *Ixodes holocyclus* Neumann, 1899 (GenBank accession number AB075955) were added to Datasets I, II, and III before alignment for help in rooting the reconstructed phylogenies. Obvious corrections to the alignment were made in Mesquite 1.12 (Maddison and Maddison, 2006).

Table 2.2: Datasets of mitochondrial DNA sequences.

Dataset	Taxon	# Individuals	Gene Region	# Partitions	Nucleotides
I	*Stylochyrus*	68	*16S/12S*	3	1382
II	*Stylochyrus*	71	*cox1*	1	483
III	*Stylochyrus*	66	*16S/12S + cox1*	4	1855
IV	Xystodesmidae	43	*16S/12S*	3	1298
V	Xystodesmidae	41	*cox1*	1	510
VI	Xystodesmidae	41	*16S/12S + cox1*	4	1808

Table 2.3: Millipede and mite specimens used in the phylogenetic analyses.

Millipede ID #	Millipede Taxon	Associated Mite ID #
SPC001176	*Apheloria* sp.	MIT00091-1
	Apheloria sp.	MIT00091-4
SPC001177	*Apheloria* sp.	MIT00092-1
	Apheloria sp.	MIT00092-4[1]
SPC001192	*Dixioria* sp.	MIT00138-1
SPC001193	*Dixioria* sp.	MIT00139-1
SPC001205	*Dixioria* sp.	MIT00144-1
SPC001223	*Appalachioria turneri*	MIT00156-1
SPC001237	*Brachoria hoffmani*	MIT00157-1
	Brachoria hoffmani	MIT00157-2
SPC001238	*Brachoria hoffmani*	MIT00158-1
	Brachoria hoffmani	MIT00158-2
SPC001293	*Apheloria* sp.	MIT00173-1
SPC001296	*Apheloria* sp.	MIT00175-1
SPC001297	*Apheloria* sp.	MIT00176-1
SPC001298	*Appalachioria separanda calcaria*	MIT00177-1

	Appalachioria separanda calcaria	MIT00177-2
SPC001306	*Brachoria dentata*	MIT00183-1
	Brachoria dentata	MIT00183-3
	Brachoria dentata	MIT00183-4
SPC001307	*Pleuroloma flavipes*	MIT00184-1
SPC001308	*Brachoria dentata*	MIT00185-1
SPC001310	*Brachoria dentata*	MIT00187-2[2]
	Brachoria dentata	MIT00187-3
SPC001311	*Brachoria dentata*	MIT00188-1
	Brachoria dentata	MIT00188-3
	Brachoria dentata	MIT00188-4
	Brachoria dentata	MIT00188-5
SPC001317	*Appalachioria falcifera*	MIT00190-1
	Appalachioria falcifera	MIT00190-2
	Appalachioria falcifera	MIT00190-3
SPC001318	*Appalachioria falcifera*	MIT00191-1
	Appalachioria falcifera	MIT00191-2
SPC001319	*Appalachioria falcifera*	MIT00192-1
SPC001322	*Appalachioria falcifera*	MIT00193-1
	Appalachioria falcifera	MIT00193-2
	Appalachioria falcifera	MIT00193-3
	Appalachioria falcifera	MIT00193-4
	Appalachioria falcifera	MIT00193-5
	Appalachioria falcifera	MIT00193-7
SPC001323	*Appalachioria falcifera*	MIT00194-1
	Appalachioria falcifera	MIT00194-2
	Appalachioria falcifera	MIT00194-4
SPC001324	*Appalachioria separanda versicolor*	MIT00195-1
	Appalachioria separanda versicolor	MIT00195-2
SPC001325	*Appalachioria separanda versicolor*	MIT00196-1
SPC001327	*Appalachioria* sp.	MIT00197-1
SPC001333	*Appalachioria separanda hamata*	MIT00199-1
SPC001334	*Appalachioria separanda hamata*	MIT00200-1
SPC001335	*Appalachioria separanda hamata*	MIT00201-1[2]
SPC001336	*Appalachioria eutypa ethotela*	MIT00202-1[1]
SPC001337	*Appalachioria eutypa ethotela*	MIT00203-1
	Appalachioria eutypa ethotela	MIT00203-2
SPC001338[3]	*Nannaria* sp.	MIT00204-1
SPC001339	*Appalachioria separanda hamata*	MIT00205-2
SPC001340	*Appalachioria separanda hamata*	MIT00206-1
SPC001343	*Brachoria* sp.	MIT00207-3
SPC001345	*Brachoria* sp.	MIT00209-3
SPC001346[1]	*Brachoria* sp.	MIT00210-1[2]
SPC001347[1]	*Brachoria* sp.	MIT00211-1
	Brachoria sp.	MIT00211-2
	Brachoria sp.	MIT00211-3

	Brachoria sp.	MIT00211-4
SPC001348	*Brachoria dentata*	MIT00212-1
SPC001349	*Brachoria dentata*	MIT00213-1[2]
	Brachoria dentata	MIT00213-2[2]
SPC001354	*Dixioria sp.*	MIT00217-2[3]
SPC001355	*Brachoria mendota*	MIT00218-1
	Brachoria mendota	MIT00218-2
SPC001361	*Prionogonus divergens*	MIT00221-1
SPC001362	*Brachoria dentata*	MIT00222-1
	Brachoria dentata	MIT00222-2
	Brachoria dentata	MIT00222-3
SPC001364	*Brachoria dentata*	MIT00224-1

The superscripts indicate which specimens could not be sequenced for both gene regions. A 1 means that only *16S/12S* was sequenced, a 2 means that only *cox1* was sequenced, and a 3 means that sequencing was not possible for that specimen. If no superscript is present, then it can be assumed that both *16S/12S* and *cox1* were successfully sequenced.

The aligned DNA sequences for each of the datasets (I – VI) were used to reconstruct phylogenies using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). MrModeltest 2.3 (Nylander, 2004) was first used to find the appropriate DNA substitution models for each dataset using the hierarchical likelihood ratio test (hLRT). The datasets that included more than one gene region (I, III, IV, and VI) were partitioned, and each partition was separately analyzed by MrModeltest. In MrBayes, two analyses of four Markov Chain Monte Carlo (MCMC) chains were simultaneously run until they converged. Convergence was reached when the average standard deviation of split frequencies fell below 0.01. Each analysis was run for at least 1,000,000 generations, and one tree was sampled every 100 generations. If convergence had not yet occurred, then extra generations were added until the average standard deviation of split frequencies did fall below 0.01. Plots of generations versus parameter values for the two runs were viewed in Tracer 1.3 (Rambaut and Drummond, 2003) to help estimate burn-in. After discarding the trees before burn-in, the parameter values and likelihood scores for all topologies were averaged and a consensus tree containing posterior probabilities was produced. All

phylogenies were viewed and rooted in FigTree 1.2 (Rambaut, 2009). *Stylochyrus* trees were rooted using the outgroup *I. holocyclus*. Xystodesmid trees were rooted with the non-apheloriine xystodesmid *Pleuroloma flavipes* Rafinesque, 1820 because all the rest of the specimens included in the analyses belong to the tribe Apheloriini. The reconstructed molecular phylogenies and the data matrices of aligned DNA sequences from both the mites and millipedes were used in the coevolutionary analysis.

2.2.4: Coevolutionary Analysis

To determine if coevolution has occurred between xystodesmid millipedes and *Stylochyrus*, two methods were employed: a distance-based method and a tree-based method. These two methods have their various benefits and drawbacks. Advantages of tree-based methods are that characters of any kind (morphological, molecular, a combination of both, etc.) can be used to reconstruct phylogenies and all coevolutionary processes are weighted and evaluated. However, analyses using tree-based methods, can present a number of problems. For example, these methods require that phylogenies are completely resolved, do not account for phylogenetic uncertainty, and cannot account for symbionts associated with multiple hosts, and therefore, may underestimate host switching (Hughes *et al*., 2007). According to De Vienne *et al*. (2007), it may be better to avoid using only tree-based methods because they sometimes may predict a cospeciation event between a host and symbiont when it did not really occur (pseudocospeciation). For example, a host switch between closely related host species followed by speciation of the symbiont could result in identical tree topologies of hosts and symbionts (De Vienne *et al*., 2007). Alternatively, distance-based methods may be more accurate in estimating the actual number of codivergence events. They do not require phylogenetic trees, and they can

handle situations in which there is not a one to one ratio of symbionts to hosts. However, distance-based methods focus only on codivergence events and do not test for the presence of other coevolutionary processes. Also, when performing distance-based coevolutionary analyses, molecular data from the same (homologous) gene region in hosts and symbionts must be used.

Because both methods have their advantages and disadvantages, it is best to use both types of coevolutionary analyses. However, according to the protocol for studying codivergence suggested by Light and Hafner (2008), it is not necessary to construct phylogenies or even attempt a tree-based method of analysis when a distance-based method concludes that there is a random association between host and symbiont taxa. Because phylogenetic trees were produced in this study and to ensure completeness, both distance-based and tree-based analyses were still performed. Small subsets of data were initially used to test out these two approaches and evaluate for significant coevolution. If codivergence was detected, then the analyses were repeated with the full datasets and all gene regions. Computational analyses of coevolution were evaluated using a number of various software packages, including those for both distance-based and tree-based methods.

2.2.4.1: Distance-based method. The computer program ParaFit (Legendre *et al*., 2002) tests for the presence of codivergence (or cospeciation) among hosts and their symbionts; it requires the assembly of several data matrices and files. First, aligned sequences from host millipedes and symbiotic mites were edited and ordered in Mesquite 1.12. A matrix (matrix A) of host and symbiont associations was created, in which the number 1 was used to specify a host-symbiont link, and 0 was used to indicate that a symbiont was not present on a host. Uncorrected pairwise distances were calculated in PAUP* 4.0b10 (Swofford, 1998) for both millipede and mite

datasets. Principal coordinates were computed from these distance matrices using the program DistPCoA (Legendre and Anderson, 1998). Principle coordinate values were corrected using method 1 (Lingoes) in DistPCoA. The host principal coordinate matrix (matrix C) was transposed so that ParaFit could analyze it, and the symbiont principal coordinate matrix (matrix B) produced by DistPCoA was left unmodified. The ParaFit analysis creates a fourth matrix, randomizes host-symbiont associations a total of 9999 times, and compares these permutations to the observed data. To determine if there is significant widespread codivergence, the ParaFitGlobal (PFGlobal) statistic was calculated. By removing individual host-symbiont links one at a time, ParaFit was also used to test how likely it is that a particular association is due to a codivergence event. The PFGlobal statistic should decrease if an association that contributes greatly to the overall host-symbiont relationship is removed. If a removal increases the global test statistic or does not affect it, then that individual association cannot be due to a codivergence event. The values of the two statistics ParaFitLink1 (PFLink1) and ParaFitLink2 (PFLink2) were calculated.

The procedure described above was carried out with six different subsets of the aforementioned datasets. Table 2.4 lists the species of millipede host, the specimen numbers of the hosts and symbionts, and the genes used in the six analyses. Four of these analyses were done using only the *16S/12S* dataset. One analysis used only information from the *cox1* region, and one analysis combined the *16S/12S* and *cox1* datasets. The *16S/12S* DNA region is not protein coding and is more variable between individuals than the *cox1* protein-coding region. It was therefore used in the majority of the ParaFit analyses. Table 3 also includes the number of host-symbiont associations or links (H-S) used in each analysis.

2.2.4.2: Tree-based method. TreeMap (Page, 1994; Charleston, 1998) determines which coevolutionary events best explain the associations between hosts and symbionts. The algorithm assigns costs to four coevolutionary processes (duplication, complete host switching, lineage sorting, and codivergence) and uses a jungle method to show all possible ways that a symbiont phylogeny can be mapped onto a host phylogeny; then it chooses the route that minimizes the overall cost of these events. TreeMap also includes a test of significance that determines if the host and symbiont trees are identical as a consequence of random chance. The symbiont tree is randomized 100 times, and then it is determined how many of these trees fit the suggested numbers and types of coevolutionary events of a reconstruction as well as the original symbiont tree.

The input for TreeMap consists of a tanglegram text file. A tanglegram includes both the host and symbiont phylogenies and the individual host-symbiont associations. The analyses completed in TreeMap 2.0 (Table 2.4) consisted of building a jungle, determining the best reconstruction of events, and significance testing. The default event costs were used and are as follows: codivergence = 0, duplication = 2, lineage sorting (loss of symbiont) = 1, and complete host switching = 1. The default parameters for jungle making and significance testing were also used. All analyses used molecular phylogenies produced from the *16S/12S* gene region only. To make the associations in the tanglegrams easier to visualize and read, TreeMap 3.0b was used to untangle the tanglegrams. By performing a heuristic search to find the least number of tanglegram links that cross, nodes of the symbiont or host tree were rotated (without changing their topologies) to minimize these crossings.

Table 2.4: Coevolutionary analyses performed in ParaFit and TreeMap.

Analysis ID	H-S	Gene	Host Species	Host ID	Associated Symbiont ID
PF6_16S	6	*16S/12S*	*Apheloria* sp.	SPC001176	MIT00091-1
			Appalachioria eutypa ethotela	SPC001336	MIT00202-1
			Brachoria mendota	SPC001355	MIT00218-2
			Dixioria sp.	SPC001205	MIT00144-1
			Pleuroloma flavipes	SPC001307	MIT00184-1
			Prionogonus divergens	SPC001361	MIT00221-1
TM6_16S	6	*16S/12S*	Used the same host and symbiont specimens as PF6_16S		
PF7AB_16S	8	*16S/12S*	*Appalachioria eutypa ethotela*	SPC001336	MIT00202-1
			Appalachioria falcifera	SPC001319	MIT00192-1
			Appalachioria separanda calcaria	SPC001298	MIT00177-1, MIT00177-2
			Appalachioria separanda hamata	SPC001334	MIT00200-1
			Appalachioria separanda versicolor	SPC001325	MIT00196-1
			Appalachioria sp.	SPC001327	MIT00197-1
			Appalachioria turneri	SPC001223	MIT00156-1
TM7A_16S	7	*16S/12S*	Used the same host and symbiont specimens as PF7AB_16S, excluding MIT00177-2		
TM7B_16S	7	*16S/12S*	Used the same host and symbiont specimens as PF7AB_16S, excluding MIT00177-1		
PF7CD_16S	8	*16S/12S*	*Appalachioria eutypa ethotela*	SPC001336	MIT00202-1
			Appalachioria falcifera	SPC001319	MIT00192-1
			Appalachioria separanda calcaria	SPC001298	MIT00177-1, MIT00177-2
			Appalachioria separanda hamata	SPC001339	MIT00205-2
			Appalachioria separanda versicolor	SPC001325	MIT00196-1
			Appalachioria sp.	SPC001327	MIT00197-1
			Appalachioria turneri	SPC001223	MIT00156-1
TM7C_16S	7	*16S/12S*	Used the same host and symbiont specimens as PF7CD_16S, excluding MIT00177-2		
TM7D_16S	7	*16S/12S*	Used the same host and symbiont specimens as PF7CD_16S, excluding MIT00177-1		
PF12_16S	12	*16S/12S*	*Appalachioria eutypa ethotela*	SPC001336	MIT00202-1
			Appalachioria falcifera	SPC001318	MIT00191-1
			Appalachioria falcifera	SPC001319	MIT00192-1
			Appalachioria falcifera	SPC001322	MIT00193-1
			Appalachioria falcifera	SPC001323	MIT00194-1
			Appalachioria separanda calcaria	SPC001298	MIT00177-1
			Appalachioria separanda hamata	SPC001334	MIT00200-1
			Appalachioria separanda hamata	SPC001339	MIT00205-2
			Appalachioria separanda hamata	SPC001340	MIT00206-1
			Appalachioria separanda versicolor	SPC001325	MIT00196-1
			Appalachioria sp.	SPC001327	MIT00197-1
			Appalachioria turneri	SPC001223	MIT00156-1
TM12_16S	12	*16S/12S*	Used the same host and symbiont specimens as PF12_16S		

PF7AB_cox1	8	*cox1*	*Appalachioria eutypa ethotela*	SPC001337	MIT00203-1
			Appalachioria falcifera	SPC001319	MIT00192-1
			Appalachioria separanda calcaria	SPC001298	MIT00177-1, MIT00177-2
			Appalachioria separanda hamata	SPC001334	MIT00200-1
			Appalachioria separanda versicolor	SPC001325	MIT00196-1
			Appalachioria sp.	SPC001327	MIT00197-1
			Appalachioria turneri	SPC001223	MIT00156-1
PF7AB_16Scox1	8	*16S*/12S+*cox1*	Used the same host and symbiont specimens as PF7AB_cox1		

The column H-S indicates the number of host-symbiont links. All symbionts are individuals belonging to the mite species *Stylochyrus rarior*.

2.3: Results

2.3.1: Xystodesmid Millipede Sampling

Individuals of the following xystodesmid genera were associated with *Stylochyrus rarior* and were successfully collected: *Apheloria* Chamberlin, 1921, *Appalachioria*, *Brachoria* Chamberlin, 1939, *Dixioria* Chamberlin, 1947, *Pleuroloma* Rafinesque, 1820, and *Prionogonus* Shelley, 1982. This also includes the following species and subspecies of *Appalachioria*: *A. eutypa ethotela*, *A. falcifera*, *A. separanda calcaria*, *A. separanda hamata*, *A. separanda versicolor*, *A. turneri*, and one undescribed species. See Swafford and Bond (2009) and their accompanying online accessory material for specific millipede-mite associations and geographic data.

2.3.2: Sequence Alignment and Phylogenetic Analysis

Among the *Stylochyrus* datasets (I, II, and III) and the xystodesmid datasets (IV, V, and VI), the *16S/12S* regions were found to be more variable than the *cox1* regions. The number of variable characters for each of the datasets are as follows: I: 213, II: 79, III: 229, IV: 349, V: 140, VI: 489. The substitution models inferred under the hLRT for each gene region from *Stylochyrus* are

HKY+I+G (*16S*), HKY (*trnV*), HKY+G (*12S*), and HKY+I+G (*cox1*). The models chosen for the xystodesmids are GTR+I+G (*16S*), HKY (*trnV*), HKY+I+G (*12S*), and GTR+I+G (*cox1*). The total generations run in MrBayes, number of trees discarded as burn-in, and average likelihood values are given for each dataset in Table 2.5. After 8 million generations, the two runs of Dataset II were nowhere near converging, so the analysis was discontinued. Consequently, only five of the six data sets were used to reconstruct phylogenies for this study (Figures 2.1 to 2.5). The thickened black branches indicate posterior probabilities equal to 1.0, while the thickened gray branches denote posterior probabilities between 0.90 and 0.99. The two *Stylochyrus* phylogenies (Figure 2.1 and 2.2) differ in topology, and the majority of the posterior probabilities are very low (less than 0.90). Geographic data (county and state) are listed for each *S. rarior* specimen included in the phylogenies. The three xystodesmid phylogenies (Figures 2.3 to 2.5) differ slightly in topology, and most nodes are well supported (posterior probability greater than 0.90).

Table 2.5: Results from the phylogenetic analyses in MrBayes.

Dataset	Generations	Trees Discarded (Burn-in)	Mean Likelihood Values	
			Arithmetic	Harmonic
I	4,005,000	5000	-4694.23	-4777.36
II	8,000,000	N/A	N/A	N/A
III	5,673,800	5000	-6237.19	-6323.88
IV	4,000,000	5000	-5093.38	-5155.68
V	2,000,000	2500	-2577.88	-2628.41
VI	1,060,000	2500	-7673.68	-7725.5

There are no results for Dataset II because the MrBayes runs would not converge.

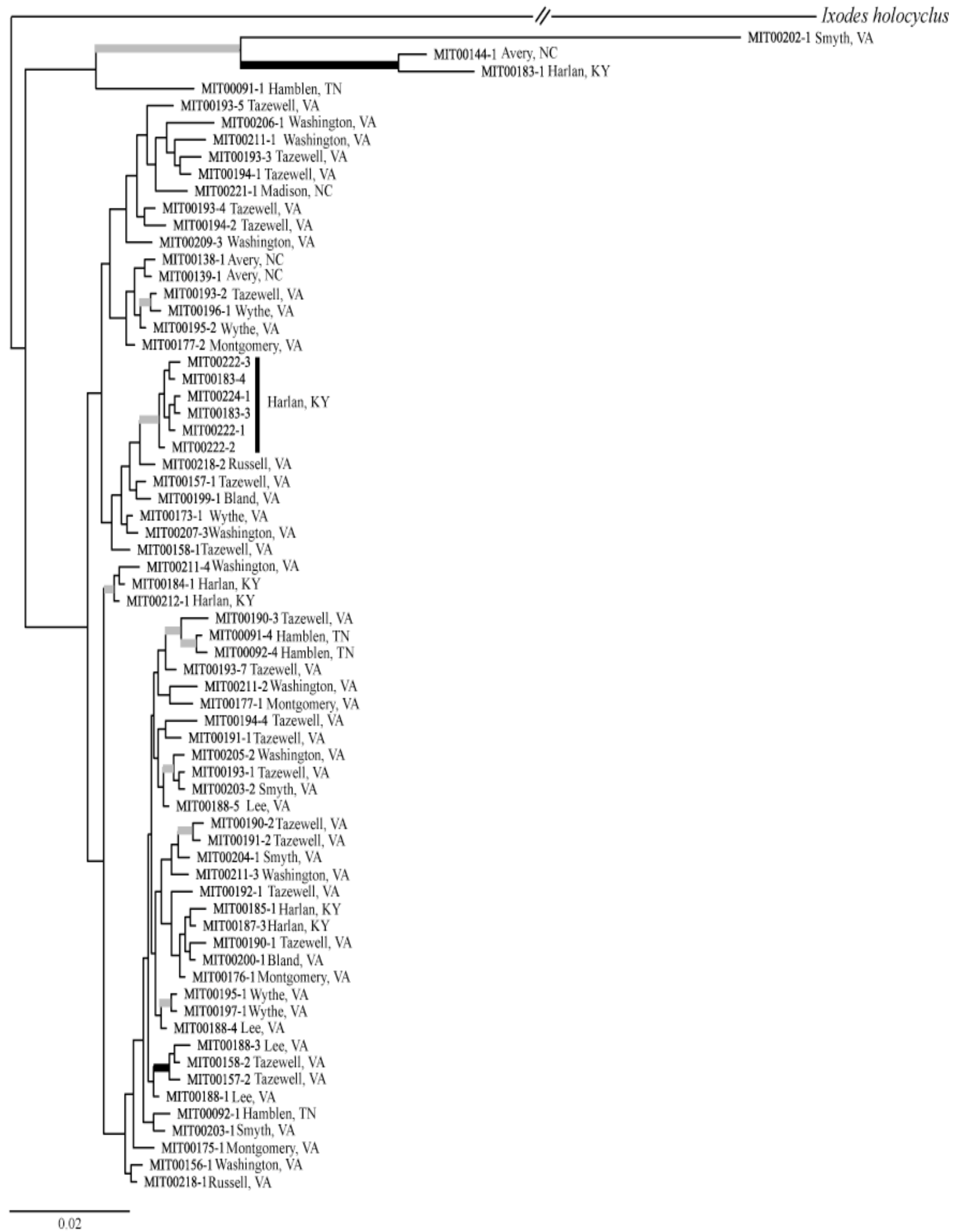

Figure 2.1: *Stylochyrus rarior 16S/12S* phylogeny. Phylogeny reconstructed using Bayesian inference for Dataset I (*Stylochyrus 16S/12S* data). The outgroup *Ixodes holocyclus* branch is abbreviated for ease of illustration (actual branch length is 0.9974).

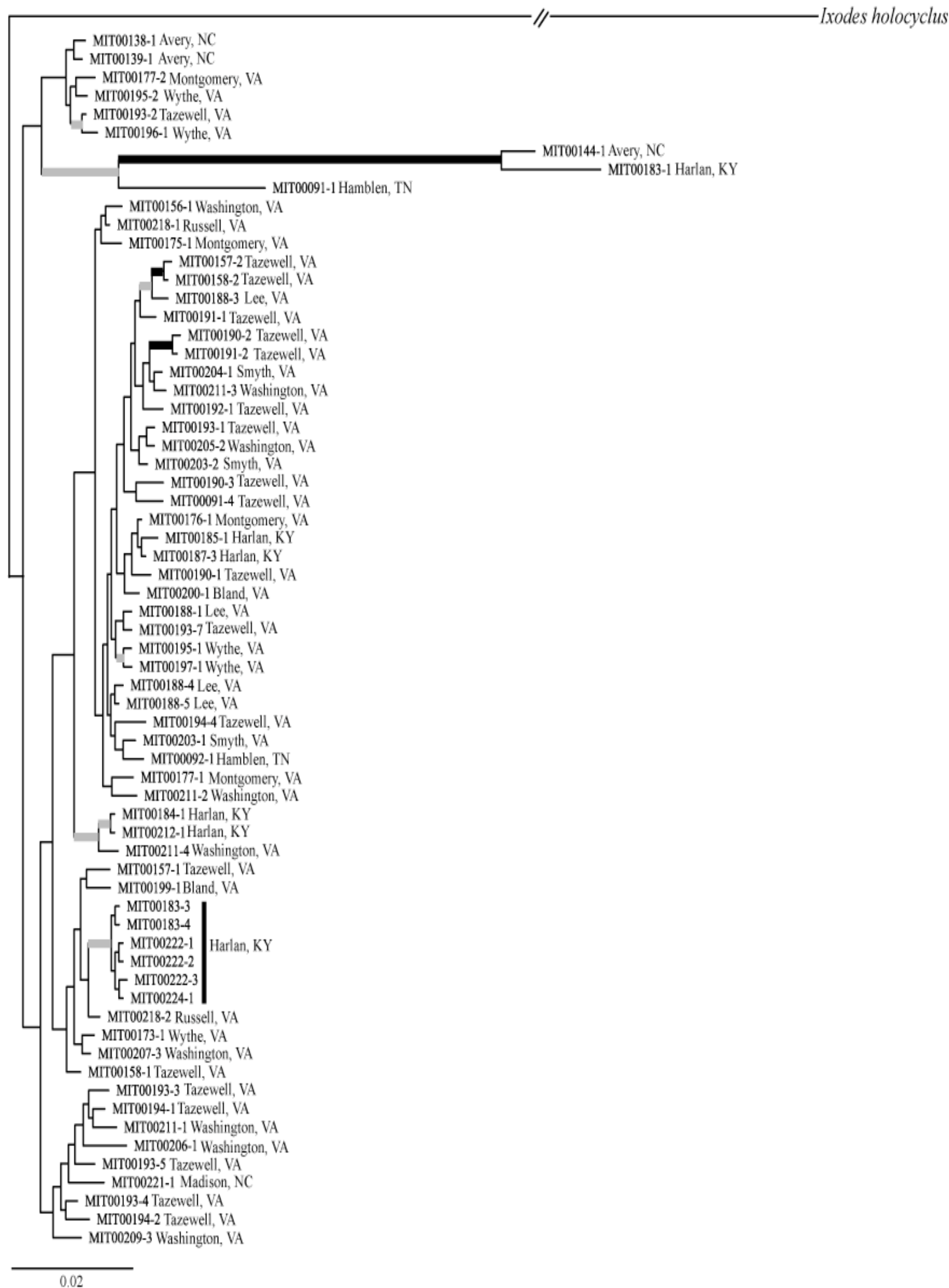

Figure 2.2: *Stylochyrus rarior 16S/12S + cox1* phylogeny. Phylogeny reconstructed using Bayesian inference for Dataset III (*Stylochyrus 16S/12S + cox1* data). The outgroup *Ixodes holocyclus* branch is abbreviated for ease of illustration (actual branch length is 1.5830).

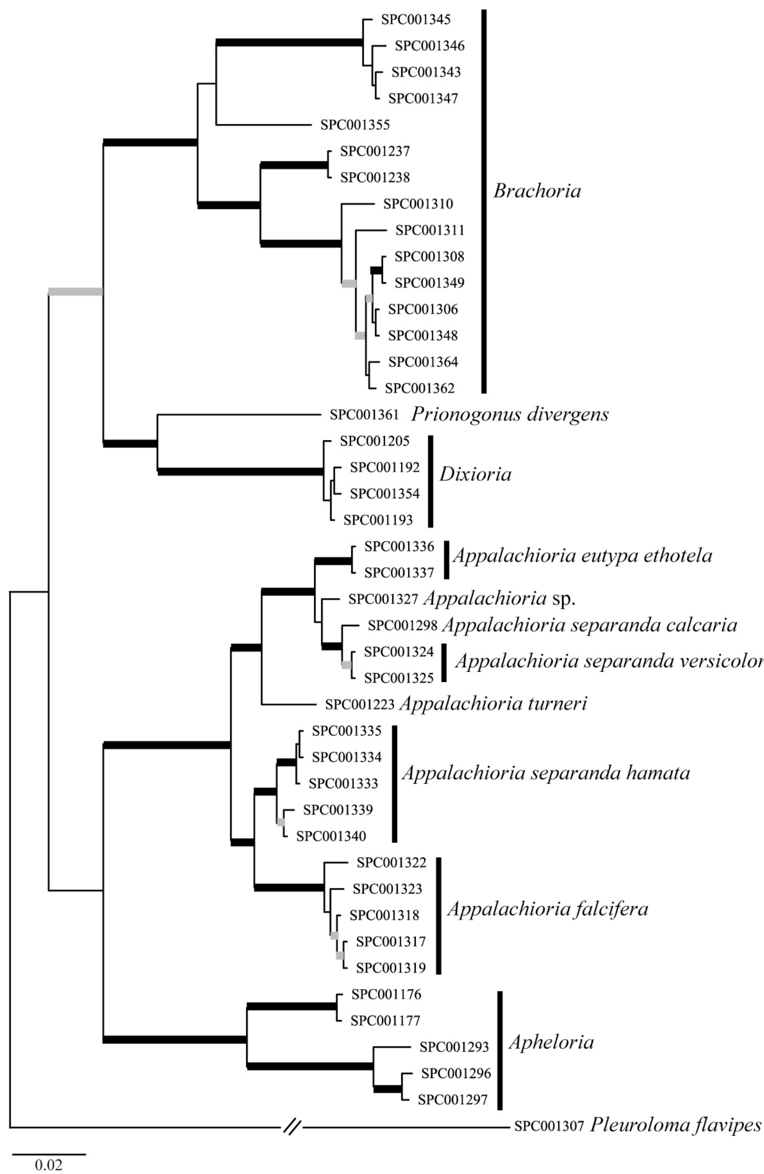

Figure 2.3: Xystodesmid *16S/12S* phylogeny. Phylogeny reconstructed using Bayesian inference for Dataset IV (xystodesmid *16S/12S* data). The outgroup *Pleuroloma flavipes* branch is abbreviated for ease of illustration (actual branch length is 0.18102).

Figure 2.4: Xystodesmid *cox1* phylogeny. Phylogeny reconstructed using Bayesian inference for Dataset V (xystodesmid *cox1* data).

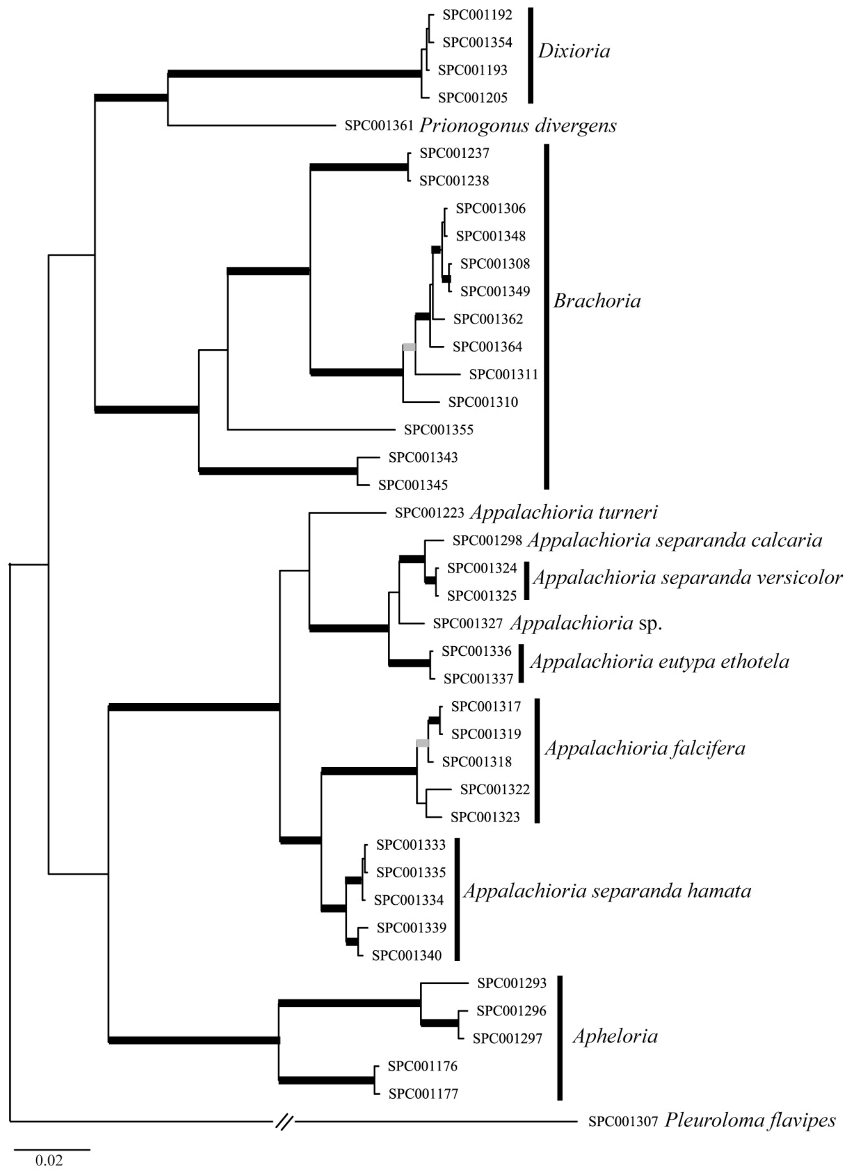

Figure 2.5: Xystodesmid *16S/12S + cox1* phylogeny. Phylogeny reconstructed using Bayesian inference for the xystodesmid *16S/12S + cox1* dataset (Dataset VI). The outgroup *Pleuroloma flavipes* branch is abbreviated for ease of illustration (actual branch length is 0.19799).

2.3.3: Coevolutionary Analysis

The data generally showed no pattern of codivergence among the millipede and mite lineages. All six ParaFit analyses had a PFGlobal statistic equal to zero, and all P-values were greater than 0.05; thus none of the analyses found any significant codivergence. All PFLink1 statistics had a value of zero. The values of PFLink2 were 0.13 and below (some even had negative values). Table 2.6 summarizes the results for each of the ParaFit analyses.

Table 2.6: Results from the six ParaFit analyses.

Analysis ID	PFGlobal	P-value	Host ID	Symbiont ID	PFLink1	P-value	PFLink2	P-value
PF6_16S	0.00002	0.45310	SPC001176	MIT00091-1	0.00000	0.68030	0.00767	0.67620
			SPC001336	MIT00202-1	0.00002	0.23860	0.13301	0.26630
			SPC001355	MIT00218-2	0.00000	0.67380	0.00065	0.67350
			SPC001205	MIT00144-1	0.00000	0.46460	0.03360	0.48280
			SPC001307	MIT00184-1	0.00000	0.20750	0.03068	0.22460
			SPC001361	MIT00221-1	0.00000	0.70400	0.00060	0.70380
PF7AB_16S	0.00000	0.58830	SPC001336	MIT00202-1	0.00000	0.53360	0.04501	0.53660
			SPC001319	MIT00192-1	0.00000	0.26330	0.00908	0.27410
			SPC001298	MIT00177-1	0.00000	0.80610	-0.00589	0.80430
			SPC001298	MIT00177-2	0.00000	0.79060	-0.00813	0.79180
			SPC001334	MIT00200-1	0.00000	0.18130	0.01203	0.19230
			SPC001325	MIT00196-1	0.00000	0.68730	-0.00523	0.69110
			SPC001327	MIT00197-1	0.00000	0.71970	-0.00509	0.72270
			SPC001223	MIT00156-1	0.00000	0.49930	0.00229	0.49960
PF7CD_16S	0.00000	0.59350	SPC001336	MIT00202-1	0.00000	0.54320	0.04280	0.54470
			SPC001319	MIT00192-1	0.00000	0.26970	0.00879	0.28240
			SPC001298	MIT00177-1	0.00000	0.79340	-0.00560	0.79460
			SPC001298	MIT00177-2	0.00000	0.77160	-0.00745	0.77360
			SPC001339	MIT00205-2	0.00000	0.19690	0.01035	0.20460
			SPC001325	MIT00196-1	0.00000	0.67940	-0.00505	0.68210
			SPC001327	MIT00197-1	0.00000	0.70280	-0.00480	0.70620
			SPC001223	MIT00156-1	0.00000	0.48750	0.00268	0.48820
PF12_16S	0.00000	0.12660	SPC001336	MIT00202-1	0.00000	0.09240	0.12757	0.09630
			SPC001318	MIT00191-1	0.00000	0.17510	0.00926	0.16260
			SPC001319	MIT00192-1	0.00000	0.14990	0.00987	0.13870
			SPC001322	MIT00193-1	0.00000	0.07800	0.00918	0.07410
			SPC001323	MIT00194-1	0.00000	0.17920	0.01420	0.16810
			SPC001298	MIT00177-1	0.00000	0.97900	-0.01048	0.97920
			SPC001334	MIT00200-1	0.00000	0.21000	0.00845	0.19760
			SPC001339	MIT00205-2	0.00000	0.28070	0.00611	0.26480
			SPC001340	MIT00206-1	0.00000	0.35210	0.01161	0.33860
			SPC001325	MIT00196-1	0.00000	0.96100	-0.01917	0.96600

			SPC001327	MIT00197-1	0.00000	0.91100	-0.01256	0.92150
			SPC001223	MIT00156-1	0.00000	0.70840	-0.00440	0.71570
PF7AB_cox1	0.00000	0.27290	SPC001337	MIT00203-1	0.00000	0.76600	-0.00309	0.76860
			SPC001319	MIT00192-1	0.00000	0.01800	0.01060	0.01970
			SPC001298	MIT00177-1	0.00000	0.96010	-0.00850	0.95980
			SPC001298	MIT00177-2	0.00000	0.24560	0.02164	0.24650
			SPC001334	MIT00200-1	0.00000	0.23190	0.00824	0.23170
			SPC001325	MIT00196-1	0.00000	0.21410	0.01394	0.21460
			SPC001327	MIT00197-1	0.00000	0.85200	-0.00462	0.85370
			SPC001223	MIT00156-1	0.00000	0.14790	0.00815	0.15120
PF7AB_16Scox1	0.00000	0.31900	SPC001337	MIT00203-1	0.00000	0.76940	-99.00000	1.00000
			SPC001319	MIT00192-1	0.00000	0.01940	-99.00000	1.00000
			SPC001298	MIT00177-1	0.00000	0.94990	-99.00000	1.00000
			SPC001298	MIT00177-2	0.00000	0.29360	-99.00000	1.00000
			SPC001334	MIT00200-1	0.00000	0.15570	-99.00000	1.00000
			SPC001325	MIT00196-1	0.00000	0.28820	-99.00000	1.00000
			SPC001327	MIT00197-1	0.00000	0.8062	-99.00000	1.00000
			SPC001223	MIT00156-1	0.00000	0.2823	-99.00000	1.00000

Each P-value column is associated with the statistic column directly to its left.

The TreeMap results (Table 2.7) showed that there is neither strict nor widespread codivergence occurring among *Stylochyrus* and their millipede hosts. One of the analyses, TM12_16S, could not be completed because the associations of the tanglegram were too complicated to be evaluated by TreeMap. The tanglegrams for all completed TreeMap analyses are provided in Figures 2.6 to 2.10 where the *Appalachioria* host tree is on the left (in black), the *Stylochyrus* symbiont tree is on the right (in red), and the blue lines indicate the individual mite and millipede associations. Among the five completed analyses, a total of 55 different possible reconstructions were produced: 12 reconstructions from TM6_16S, 10 from TM7A_16S, 5 from TM7B_16S, 24 from TM7C_16S, and 4 from TM7D_16S. Only one of these reconstructions of events was found to be significant (P-value < 0.05). It was from analysis TM7B_16S and was the lowest cost (c = 6) reconstruction out of all 55. It comprised 8 codivergence events and 2 duplication events each followed by a complete host switch. Figures 2.11 to 2.20 depict the lowest cost coevolutionary event reconstructions for each of the five completed analyses.

Table 2.7: Results from the six TreeMap analyses.

Analysis ID	Reconstruction	P-value	Sampling Error (+/-)	Co	Sw	Du	Lo	Total Cost
TM6_16S	1	1	0.0071	2	4	4	0	12
	2	1	0.0071	2	4	4	0	12
	3	1	0.0071	2	4	4	0	12
	4	1	0.0071	2	4	4	0	12
	5	1	0.0071	2	4	4	0	12
	6	**0.29**	**0.045**	**6**	**2**	**2**	**1**	**7**
	7	1	0.0071	2	4	4	0	12
	8	1	0.0071	2	4	4	0	12
	9	0.32	0.0466	6	1	2	4	9
	10	0.29	0.045	6	0	2	8	12
	11	1	0.0071	2	4	4	0	12
	12	1	0.0071	2	4	4	0	12
TM7A_16S	1	0.43	0.0495	4	4	4	0	12
	2	0.44	0.0496	4	4	4	0	12
	3	**0.37**	**0.0483**	**6**	**3**	**3**	**1**	**10**
	4	0.18	0.0384	6	2	3	3	11
	5	**0.39**	**0.0488**	**6**	**3**	**3**	**1**	**10**
	6	0.33	0.047	6	3	3	1	10
	7	0.42	0.0494	4	4	4	0	12
	8	0.46	0.0498	4	4	4	0	12
	9	0.42	0.0494	6	1	3	8	15
	10	0.9	0.03	4	0	4	18	26
TM7B_16S	**1**	**0.01***	**0.0099**	**8**	**2**	**2**	**0**	**6**
	2	0.27	0.0444	6	1	3	7	14
	3	0.25	0.0433	6	1	3	7	14
	4	0.25	0.0433	6	1	3	7	14
	5	0.29	0.0454	6	0	3	12	18
TM7C_16S	1	1	0.0071	2	5	5	0	15
	2	1	0.0071	2	5	5	0	15
	3	1	0.0071	2	5	5	0	15
	4	1	0.0071	2	5	5	0	15
	5	1	0.0071	2	5	5	0	15
	6	1	0.0071	2	5	5	0	15
	7	**0.95**	**0.0218**	**4**	**4**	**4**	**1**	**13**
	8	**0.91**	**0.0286**	**4**	**4**	**4**	**1**	**13**
	9	0.74	0.0439	4	3	4	3	14
	10	0.79	0.0407	4	3	4	3	14
	11	**0.92**	**0.0271**	**4**	**4**	**4**	**1**	**13**
	12	**0.92**	**0.0271**	**4**	**4**	**4**	**1**	**13**
	13	1	0.0071	2	5	5	0	15
	14	1	0.0071	2	5	5	0	15
	15	1	0.0071	2	5	5	0	15

	16	1	0.0071	2	5	5	0	15
	17	1	0.0071	2	5	5	0	15
	18	**0.98**	**0.014**	**4**	**4**	**4**	**1**	**13**
	19	0.95	0.0218	4	2	4	8	18
	20	0.93	0.0255	4	2	4	8	18
	21	1	0.0071	2	5	5	0	15
	22	1	0.0071	2	1	5	16	27
	23	1	0.0071	2	1	5	16	27
	24	0.98	0.014	4	0	4	19	27
TM7D_16S	**1**	**0.12**	**0.0325**	**6**	**3**	**3**	**0**	**9**
	2	0.44	0.0496	6	2	3	4	12
	3	0.42	0.0494	6	1	3	8	15
	4	0.71	0.0454	4	0	4	15	23
TM12_16S	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

The Co column indicates the number of codivergence events. The Sw column indicates the number of complete host switches. The Du column indicates the number of duplication events. The Lo indicates the number of sorting events (losses). Reconstructions in bold indicate those with the lowest cost for an individual analysis. The asterisk indicates a P-value that is significant. TM12_16S has no results because the analysis could not be completed.

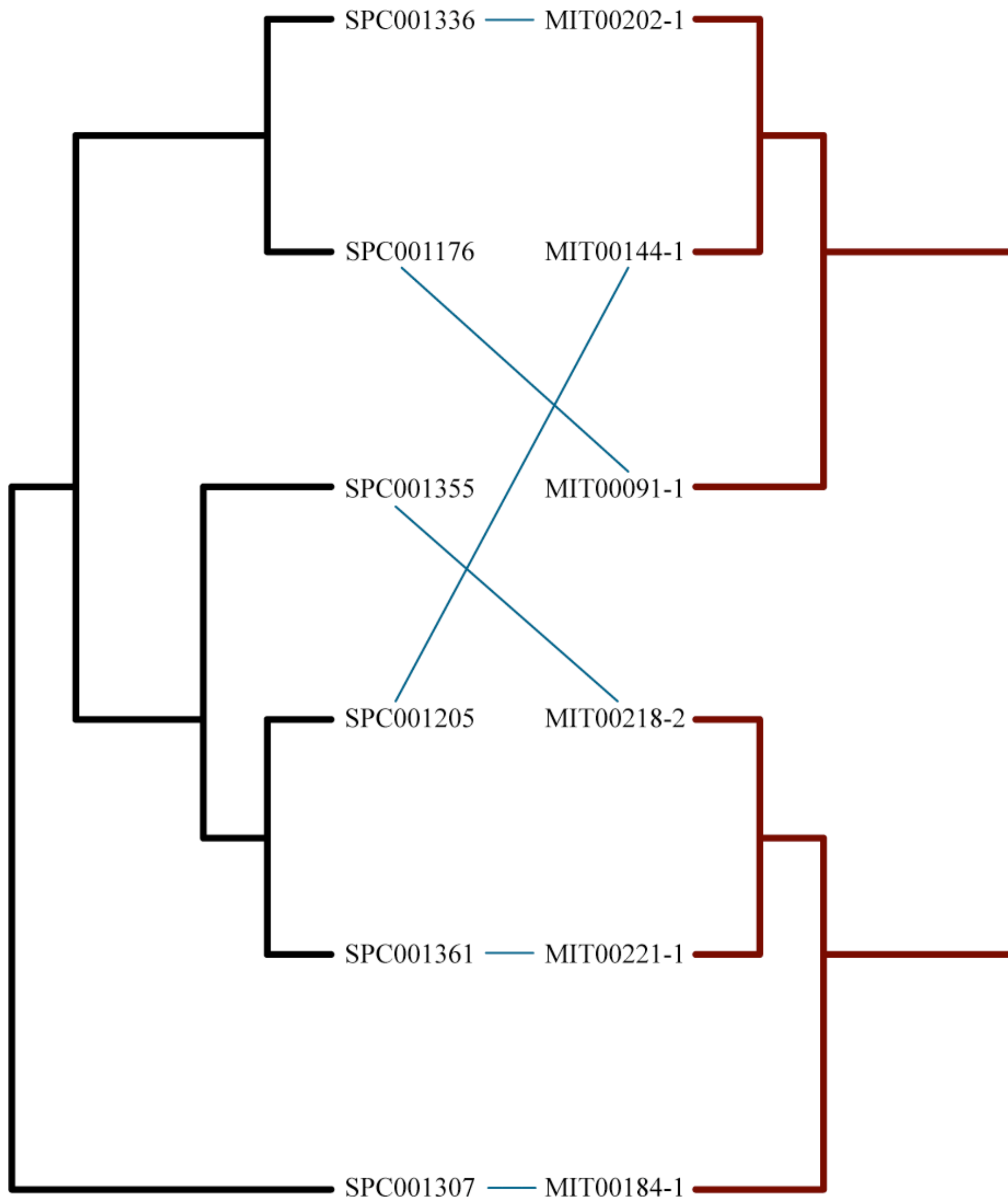

Figure 2.6: Tanglegram for analysis TM6_16S.

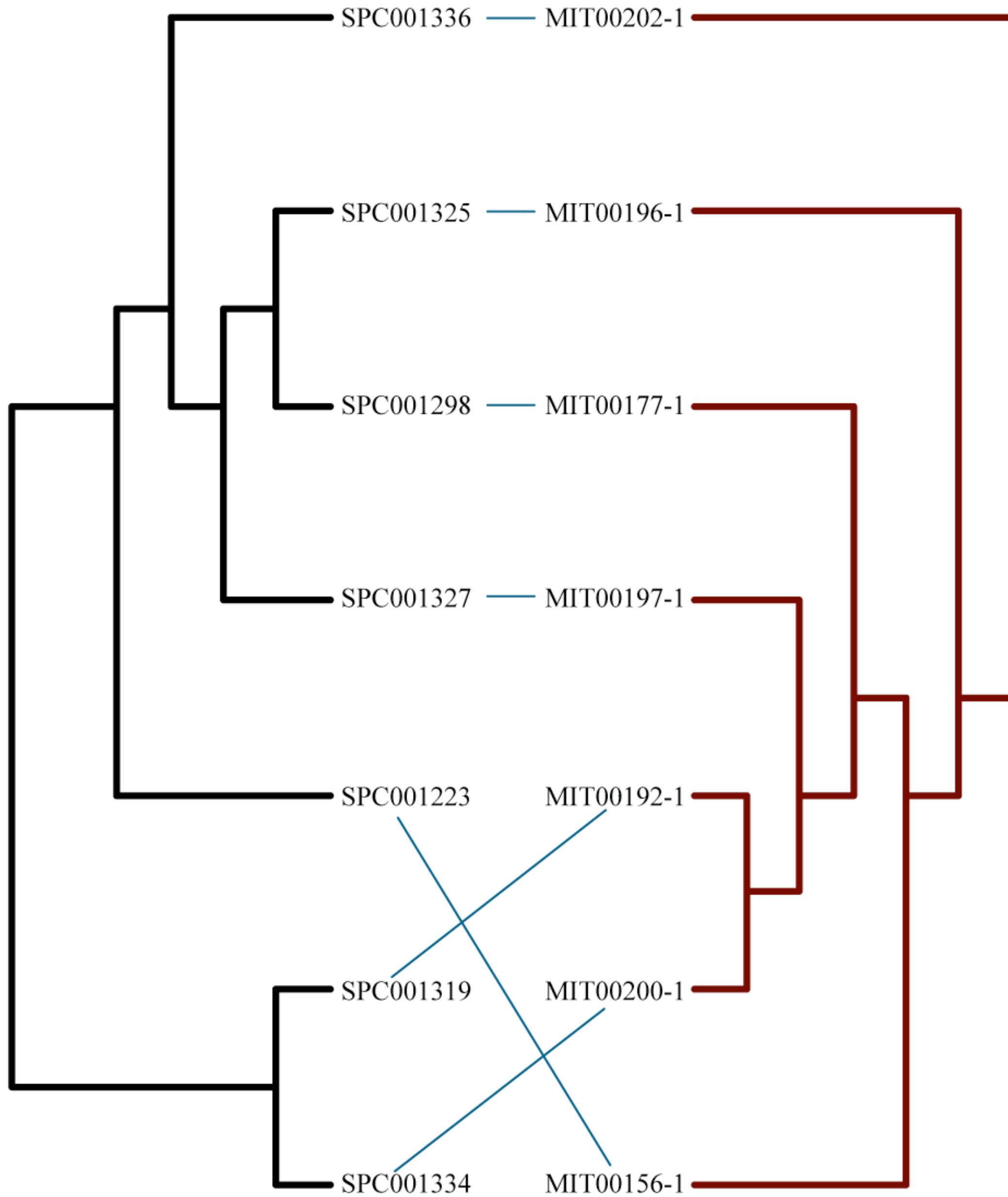

Figure 2.7: Tanglegram for analysis TM7A_16S.

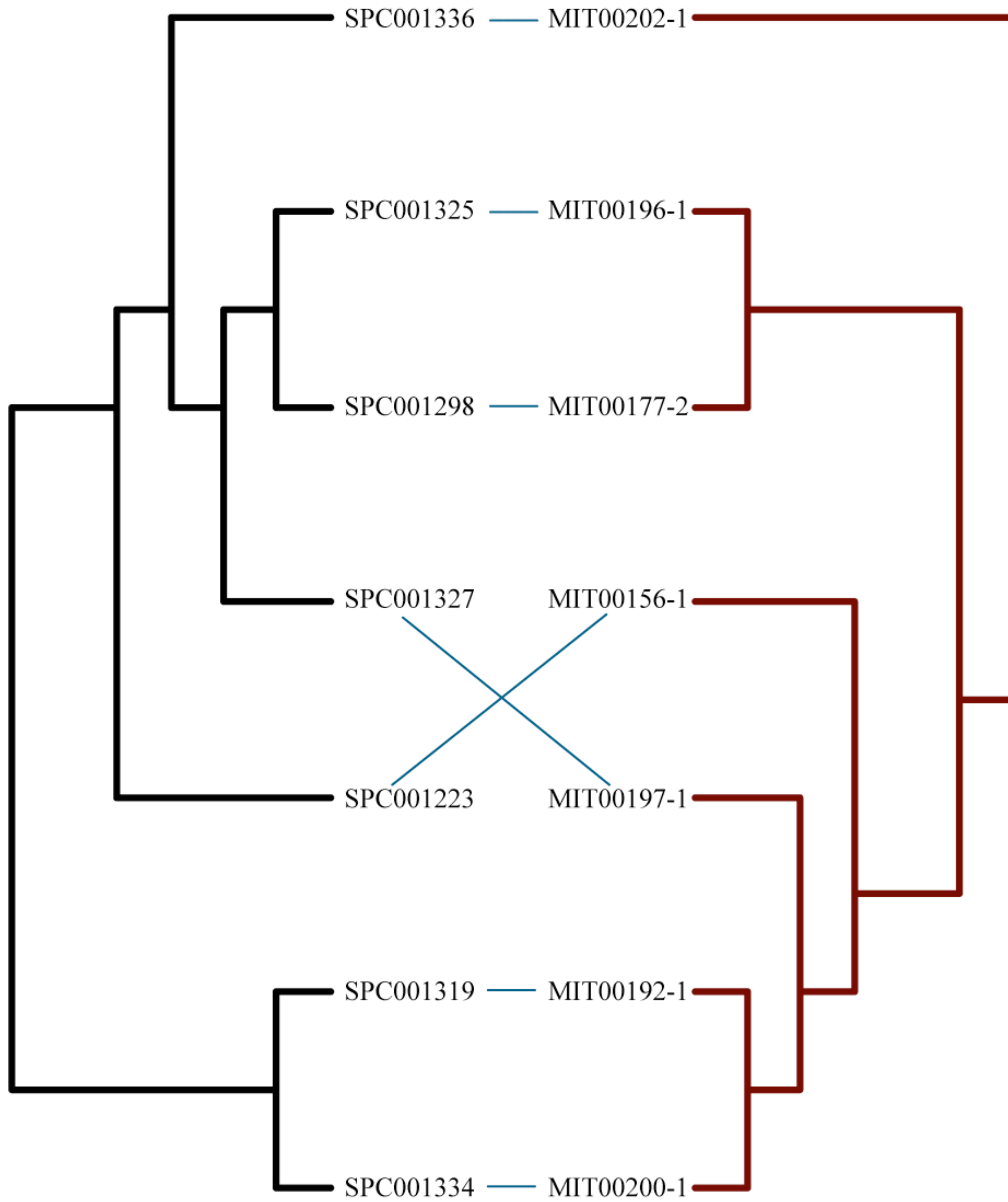

Figure 2.8: Tanglegram for analysis TM7B_16S.

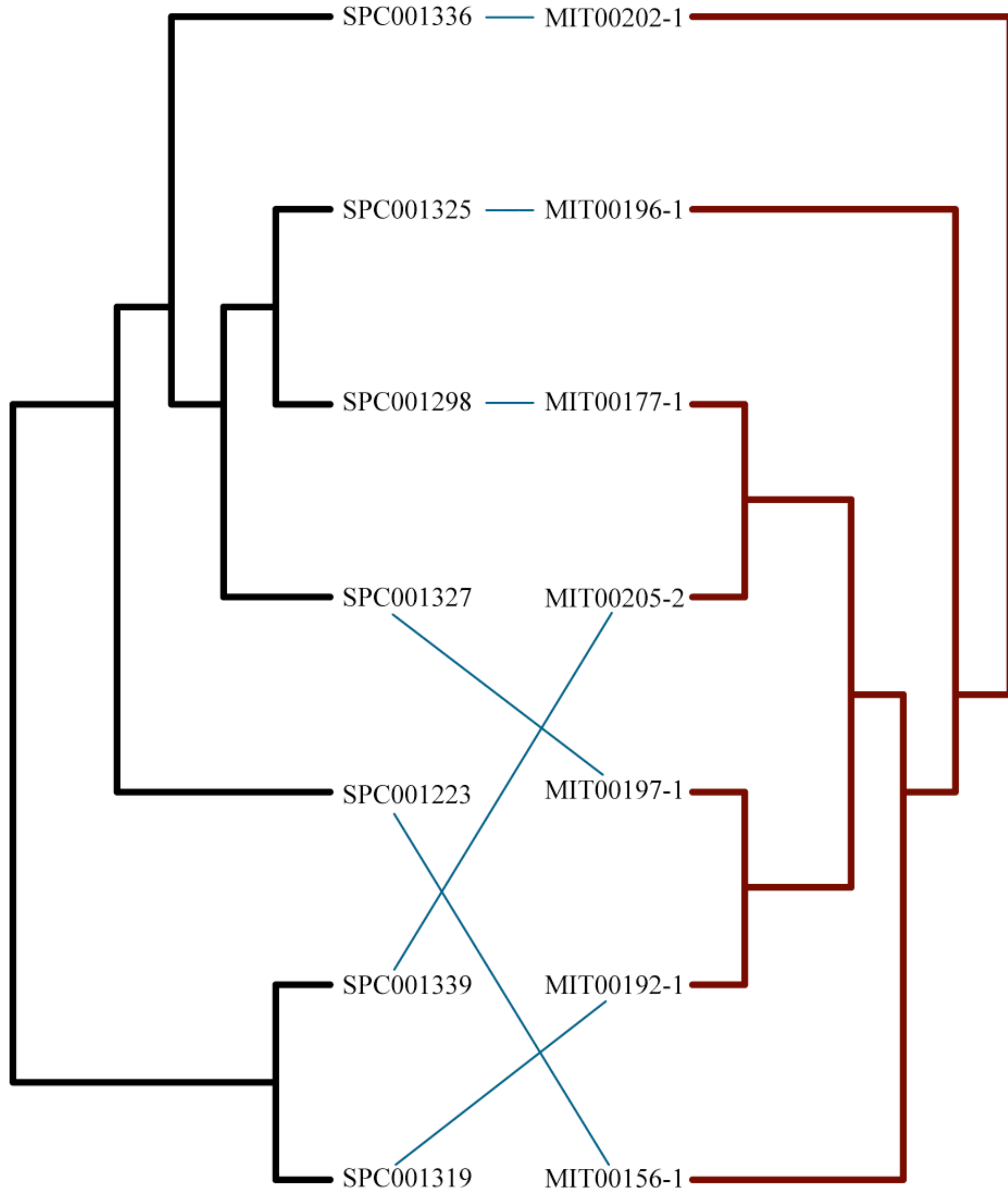

Figure 2.9: Tanglegram for analysis TM7C_16S.

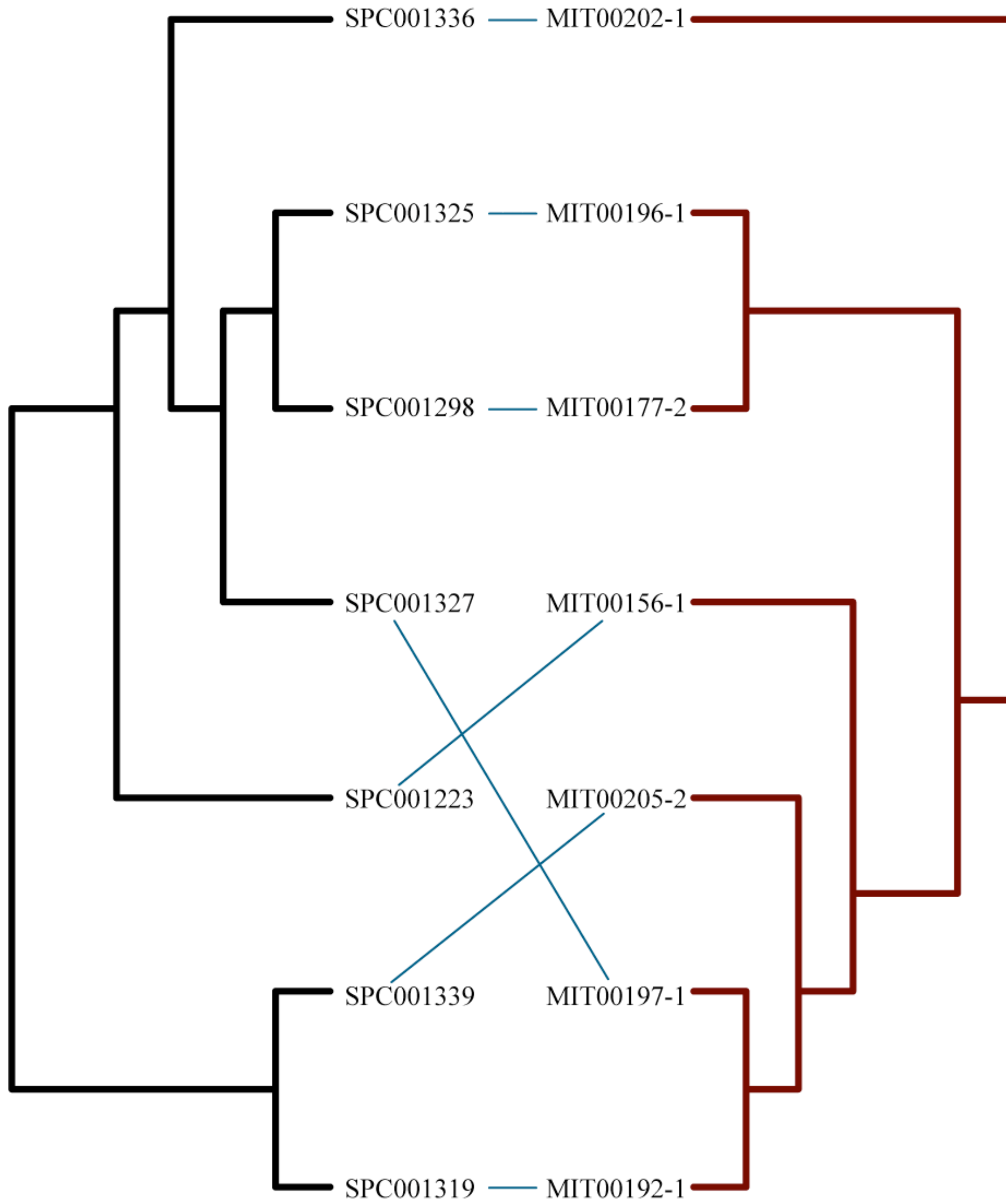

Figure 2.10: Tanglegram for analysis TM7D_16S.

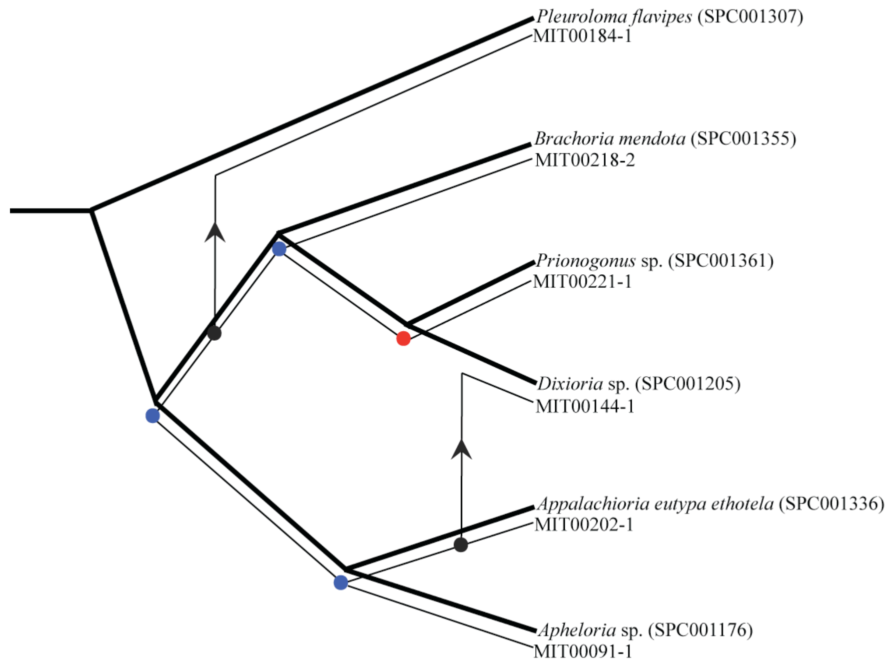

Figure 2.11: Lowest cost reconstruction (6) from analysis TM6_16S.

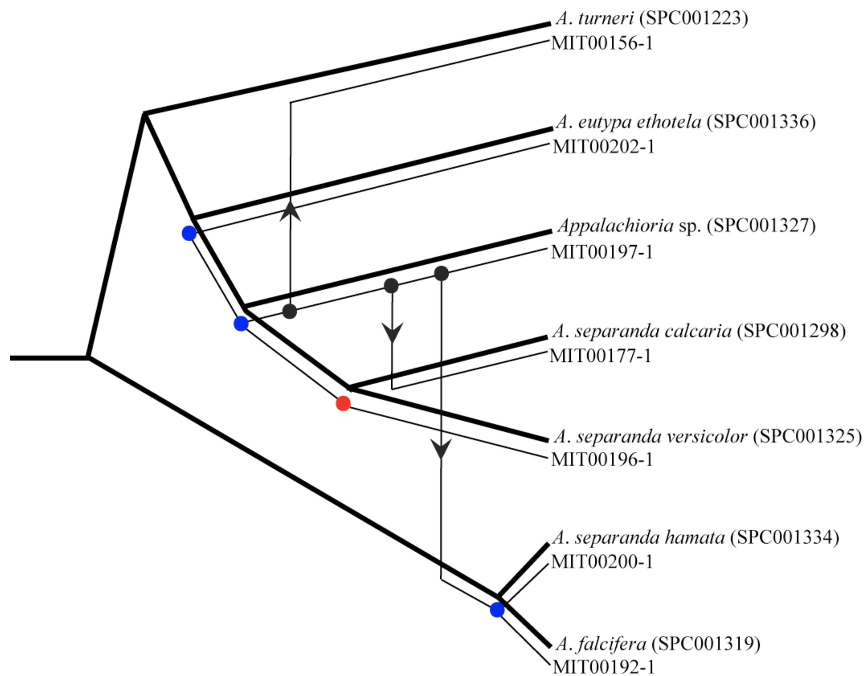

Figure 2.12: First lowest cost reconstruction (3) from analysis TM7A-16S.

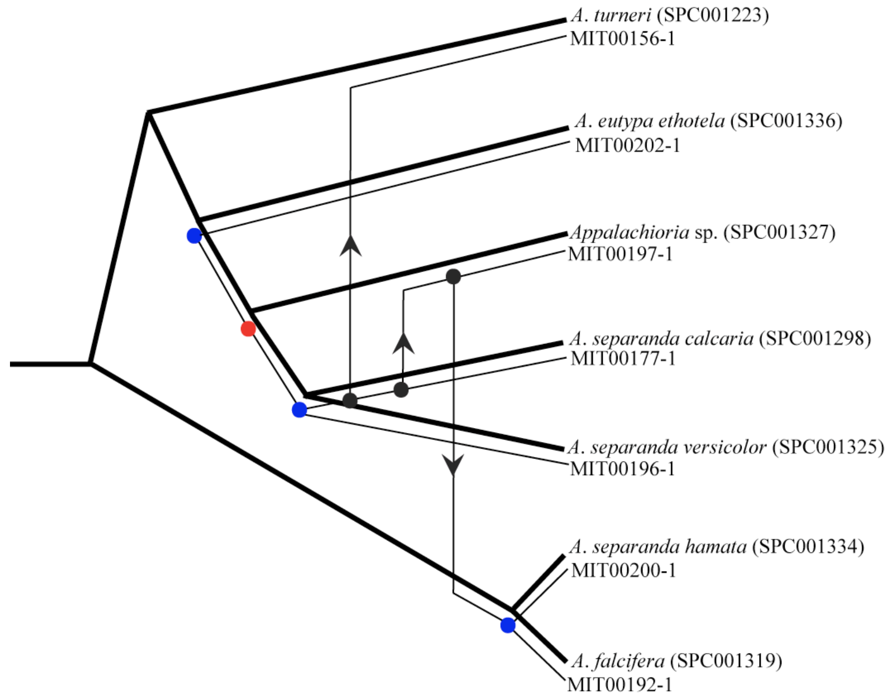

Figure 2.13: Second lowest cost reconstruction (5) from analysis TM7A-16S.

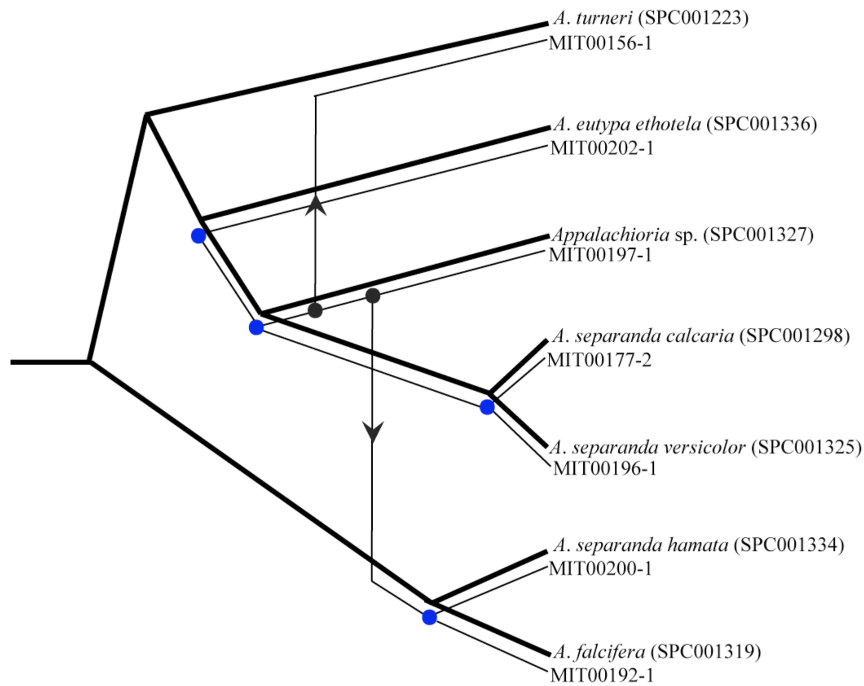

Figure 2.14: Lowest cost reconstruction (1) from analysis TM7B-16S.

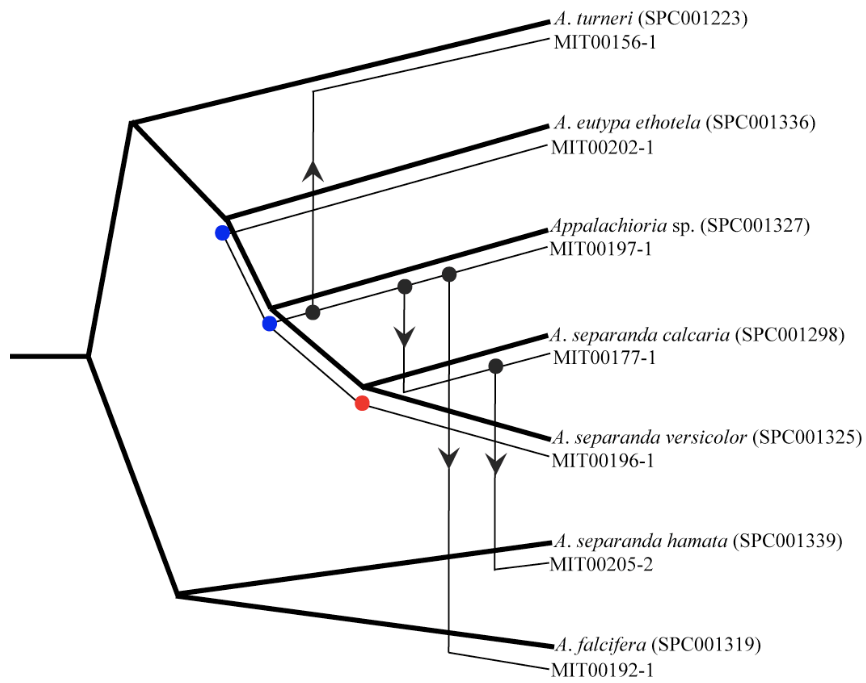

Figure 2.15: First lowest cost reconstruction (7) from analysis TM7C_16S.

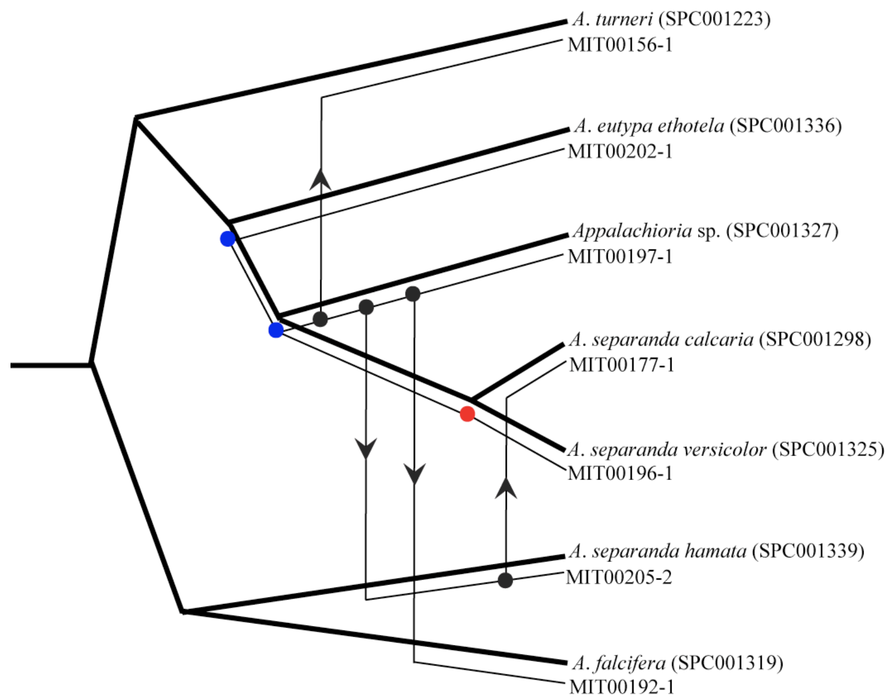

Figure 2.16: Second lowest cost reconstruction (8) from analysis TM7C_16S.

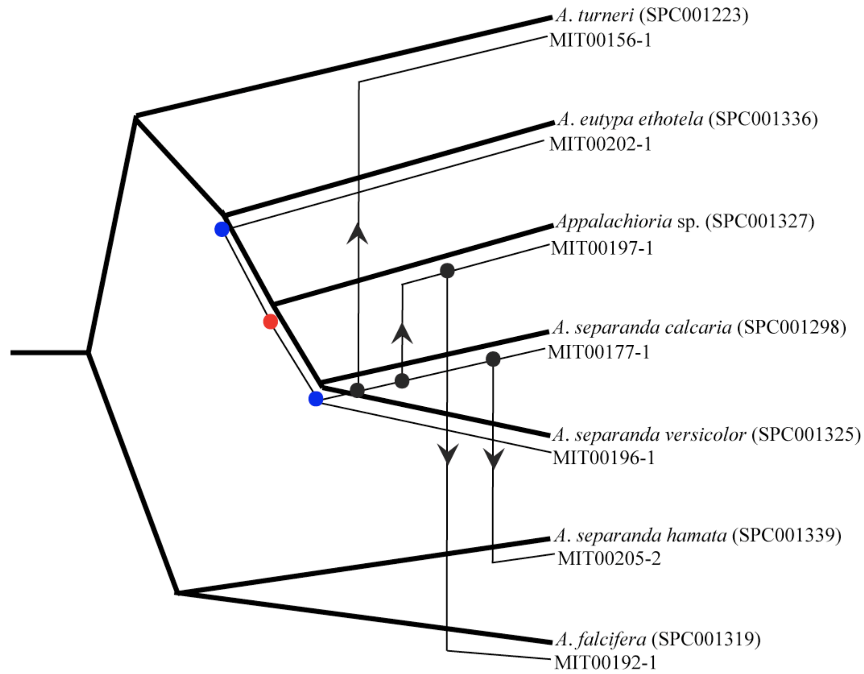

Figure 2.17: Third lowest cost reconstruction (11) from analysis TM7C_16S.

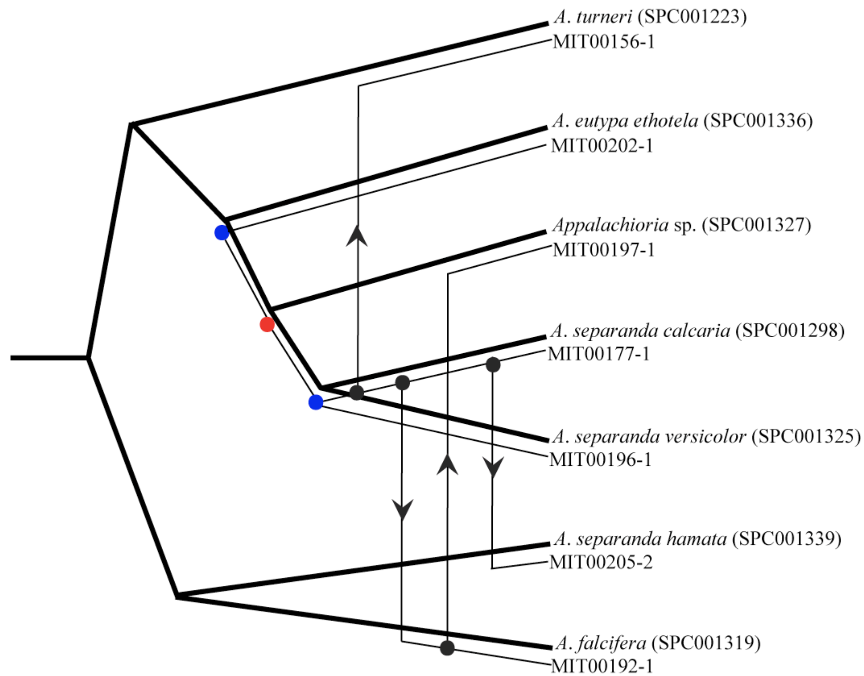

Figure 2.18: Fourth lowest cost reconstruction (12) from analysis TM7C_16S.

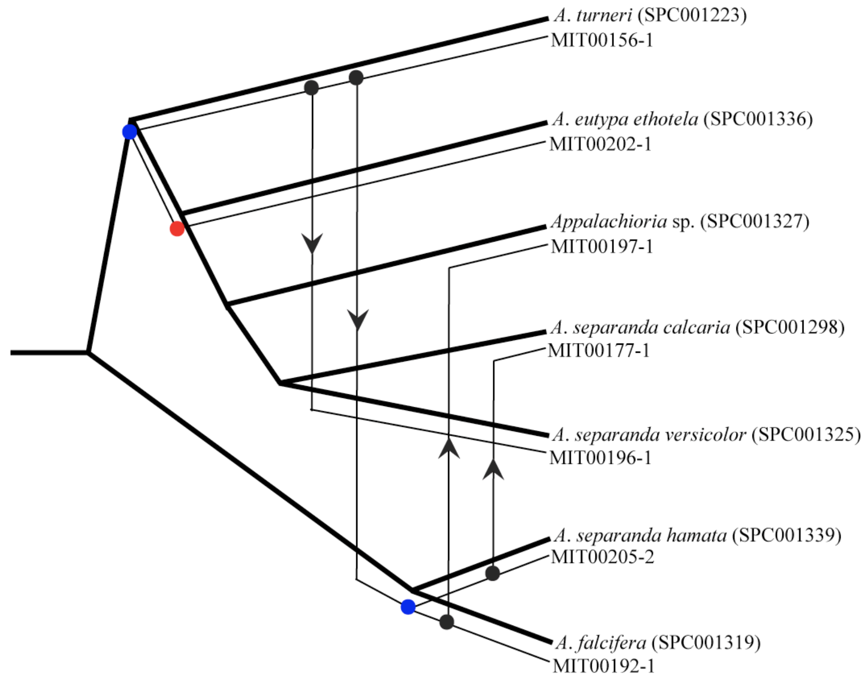

Figure 2.19: Fifth lowest cost reconstruction (18) from analysis TM7C_16S.

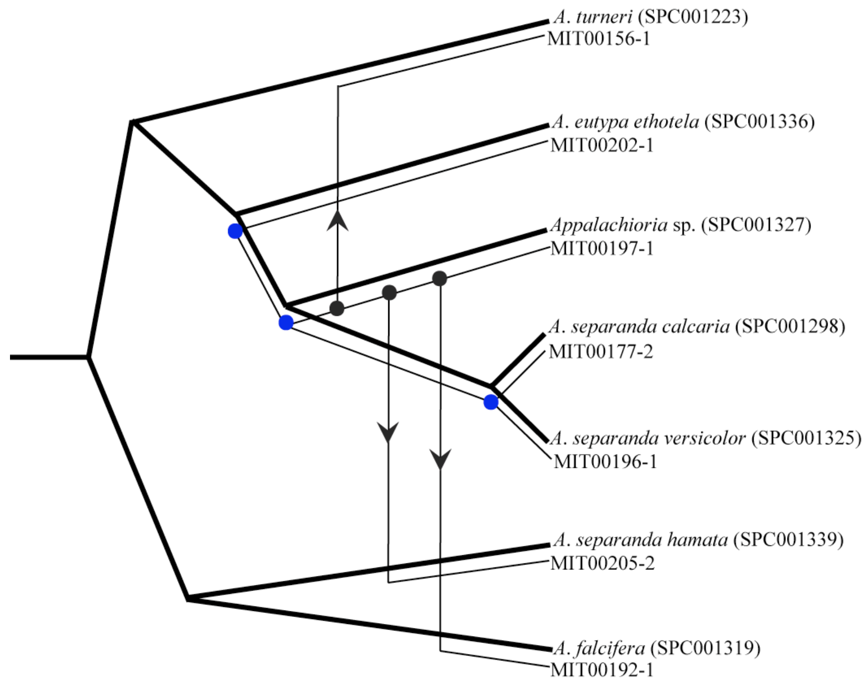

Figure 2.20: Lowest cost reconstruction (1) from analysis TM7D_16S.

2.4: Discussion

The results of both the phylogenetic and coevolutionary analyses demonstrate a complete lack of coevolutionary signal between *Appalachioria* and *Stylochyrus*. As expected, the millipedes are highly structured geographically; however, there is no corresponding structure among the mites. In addition, these millipede-mite associations are not consistent with the three rules of cospeciation. Host and symbiont phylogenies are incongruent, there is no pattern of association between primitive hosts and primitive symbionts, and there are not equal numbers of host and symbiont species. Consequently, our data fail to support the hypothesis of codivergence between *Stylochyrus* and *Appalachioria*.

Despite the lack of coevolutionary signal, the composition of the data used in this study potentially impacts the results of the Bayesian and coevolutionary analyses. For example, Dataset II consisted of sequences from the protein-coding region *cox1*, which is much slower evolving than the non-coding *16S/12S* region. There was very little variation in the *cox1* sequences from *Stylochyrus* (only 79 of the 483 characters were variable), which is likely why convergence of the simultaneous runs in MrBayes did not occur. The phylogeny produced for Dataset IV, is identical to the one estimated by Marek and Bond (2007), except for the placement of *Prionogonus divergens* (Chamberlin, 1939). It is placed as the sister taxon to the *Dixioria* species in this study, whereas, it is located within the *Sigmoria* Chamberlin, 1939 clade in Marek and Bond (2007). The phylogeny from Dataset V and the phylogeny from Marek and Bond (2007) have a few more discrepancies. It makes sense that the tree from Dataset IV is more similar to Marek and Bond's tree (2007) because the only molecular data they used was from the *16S/12S* mitochondrial region. The support values for the Dataset V phylogeny are lower, so it does not appear to be as reliable of an estimate as the other two reconstructed phylogenies in this

study or the one from Marek and Bond (2007), which included more taxa and both molecular and morphological characters.

If coevolution was occurring, then mites associated with species of *Appalachioria* should be more closely related to each other than mites from other xystodesmid genera. The phylogenies produced from Datasets I and III show that this is clearly not the case. Mites from the same locality and even the same millipede specimen do not appear to be closely related. For example, neither the mites from *A. separanda calcaria* specimen SPC001298 (MIT00177-1 and MIT00177-2) nor the mites from *A. falcifera* specimen SPC001317 (MIT00190-1, MIT00190-2, and MIT00190-3) are located on branches near each other on the phylogenies. The phylogenies from Datasets I and III have slightly different topologies; however, the latter has lower support values. The branch lengths on the millipede trees are much longer than those on the mite trees, which suggests that the millipede taxa diverged before the mite taxa.

Since no significant codivergence was found in the ParaFit analyses, it can be concluded that evolution is not occurring simultaneously in these millipede and mite taxa. In fact, the pattern of mite symbionts among the host millipedes is no different than random (PFGlobal = 0). Several associations from some of the analyses produced negative PFLink2 values. This indicates that widespread codivergence would have been more likely to occur with that link excluded. If coevolution had been found for the *16S/12S* region, then more analyses incorporating more individuals and including *cox1* and the concatenated datasets would have been warranted. Because a phylogeny for Dataset II could not be produced, *cox1* was excluded from the TreeMap analyses. Also, the support values (posterior probabilities) for the *Stylochyrus rarior* phylogenies are very low. Consequently, it is likely that the results from the TreeMap 2.0 analyses are not very reliable. In addition, TreeMap is not capable of evaluating tanglegrams

that include large numbers of taxa or complex associations due to many host switching events. Analysis TM12_16S consisted of only 12 associations but was still too convoluted for TreeMap to evaluate, which further indicates that codivergence is unlikely. The five completed TreeMap analyses produced different results, which is inconsistent with the presence of coevolution. If coevolution had occurred then it would be expected that just changing a few specimens would not affect the results of these analyses. Because the majority of the reconstructions were not found to be significant, the patterns and associations of *Stylochyrus* mites on xystodesmid millipedes are considered to be no different than if they were chosen at random.

In addition to the lack of coevolution with xystodesmid millipedes, these mites seem to have no apparent geographic structuring at the population level. *Stylochyrus* individuals present at a single locality do not always form an exclusive group. Support values in the mite tree are very low, which is likely because the mite mitochondrial sequences are just too similar for evolutionary relationships to be accurately resolved. As well as being genetically similar, individuals of *Stylochyrus* mites are morphologically similar across the range sampled in this study. There are many groups of other terrestrial arthropods, including pseudoscorpions, millipedes, and spiders, that show strong geographic structuring (Wilcox *et al*., 1997; Bond and Sierwald, 2002; Stockman and Bond, 2007). These highly structured arthropod groups tend to include many morphologically identical species (cryptic species) that are often not very vagile. For example, Stockman and Bond (2007) analyzed the California spider genus *Promyrmekiaphila* Schenkel, 1950 and discovered that this group shows extreme population structuring; almost all populations from separate localities have very divergent mtDNA sequences. The members of *Promyrmekiaphila* all look identical, but the amount of genetic variation present suggests that there are several cryptic species (Stockman and Bond, 2007).

Similar results were found in the Jamaican millipede species complex *Anadenobolus excisus* (Karsch 1881) (Bond and Sierwald, 2002) and the neotropical pseudoscorpion complex *Cordylochernes scorpioides* (Linnaeus, 1758) (Wilcox *et al*., 1997). Unlike these arthropod taxa, *S. rarior* appears to be highly vagile and has low population variation across its sampled range.

Many mite taxa cannot sufficiently disperse to new habitats on their own due to their small size and inability to fly. Therefore, phoresy, the formation of temporary symbiotic associations for dispersal, is commonly practiced among mites. The range of *S. rarior* is very large and encompasses a total of 11 states: Illinois, Indiana, Iowa, Kentucky, Missouri, New York, North Carolina, Ohio, Tennessee, Virginia, and West Virginia (Kethley, 1983; Swafford and Bond, 2009). *S. rarior* likely maintains this large range due to its phoretic dispersal capabilities (Kethley, 1983). However, even though xystodesmid millipedes have cyanide defense secretions that may provide protection for mites, millipedes are generally not very vagile, which makes them unlikely to be adequate mite carriers. From this we might infer that *S. rarior* uses other animals for dispersal purposes. In two other cases, *S. rarior* individuals were found associated with animals other than millipedes (Kethley, 1983); these include associations with a house sparrow and a single small mammal nest (Kethley, 1983). Other than these two vertebrate associations and the xystodesmid millipede associations, the majority of documented individuals of *S. rarior* are found in the absence of hosts and living mainly among oak leaf litter (Kethley, 1983). Alternatively, most phoretic mites are thought to be very specialized to a particular animal carrier (Bloszyk *et al*., 2006; Purrington and Drake, 2008). For example, there are two species of mites in Poland that are only found associated with a single species of centipede even though there are 30 species of centipedes in the area (Bloszyk *et al*., 2006). In

addition, many mites belonging to the suborder Astigmata attach to very specific places on their beetle carriers (Purrington and Drake, 2008). This suggests that coevolution between phoretics and their carrier is possible. However, this is not the case with *S. rarior* because it does not appear to be specific in its selection of carrier animals.

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