PHYLOGENETIC ANALYSIS AS A MEANS OF SPECIES IDENTIFICATION WITHIN MYRMECOLACIDAE (STREPSIPTERA)

Halbert, N. R., L. D. Ross, J. Kathirithamby, J. B. Woolley, R. R. Saff & J. S. Johnston, 2001. Phylogenetic analysis as a means of species identification within Myrmecolacidae (Strepsiptera). – Tijdschrift voor Entomologie 144: 179-186, fig.1, tables 1-3. [ISSN 0040-7496]. Published 1 December 2001.

The relationship between males and females of the strepsipteran *Caenocholax fenyesi* Pierce (Myrmecolacidae) (hosts- males: *Solenopsis invicta* Buren (Formicidae), females: *Hapithus agitator* Uhler (Orthoptera)), as well as between *Stichotrema dallatorreanum* Hofeneder, and other unidentified myrmecolacid females from Papua New Guinea is examined. Methods were developed for extraction, amplification, and high-resolution sequencing of mitochondrial and nuclear DNA from alcohol-preserved Strepsiptera. Phylogenetic analyses were performed in the examination of myrmecolacid family structure based upon sequences from segments of the mitochondrial Cytochrome Oxidase Subunit I (COI, 300 bases) and the nuclear 18S rRNA (18S, 213 bases) genes. Sequence analysis of the COI and 18S regions showed 29% difference between the *C. fenyesi* male and putative female, 0.78% difference between two female Myrmecolacidae from West New Britain Province (WNB-A and WNB-B), and 0.95% difference between a different myrmecolacid female (WNB-C) from West New Britain Province, and a myrmecolacid female (POP) from Popondetta, Oro Province, Papua New Guinea. Comparison of levels of variation among myrmecolacid species indicates that the male *C. fenyesi* and the putative female are not members of the same species. Further, two females from West New Britain Province (WNB-A/B) form a new myrmecolacid species, while different females from West New Britain Province (WNB-C) and from Oro Province (POP) form a second new myrmecolacid species.

Correspondence: J. Spencer Johnston, Department of Entomology, Texas A&M University, College Station, TX 77843 USA

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Strepsiptera (Insecta), often referred to as 'stylops,' are curious entomophagous parasitoids of cosmopolitan distribution. They are curious not only for their biology but also for their host-parasitoid relationships. Kathirithamby (1989) reports that Strepsiptera are parasitic in members of seven insect orders (Thysanura, Blattodea, Mantodea, Orthoptera, Hemiptera, Hymenoptera and Diptera). The order Strepsiptera is divided into two suborders: Mengenillidia and Stylopodia (Kinzelbach 1971).

Strepsiptera have only two free-living stages: the small, active adult winged male and the 1st instar larva. The adult males have prominent compound eyes,

elegantly branched antennae, 'halter-like' forewings, and fan-shaped hind wings. They are short-lived (some live for only two hours), they do not feed, and a breeding flight is their sole mission. With the exception of the family Mengenillidae, the bizarre adult females are neotenic. Female Mengenillidae pupate outside their host, while female Stylopodia remain permanently endoparasitic; therefore, one must look for the host to find Stylopodia females. Strepsipteran females produce viviparous 1st instar larvae, which emerge via the brood canal opening/opening of the *apron* (Kathirithamby 2000) in the cephalothorax of the endoparasitic female. In endopterygote

hosts the 1st instars are carried back to the nests of the host (phoresy), where they enter and parasitise the eggs or larvae of the host (Kathirithamby 1989, Maceta et al. this issue). Adult male size varies between 1.6–6 mm, while neotenic females range between 2 mm–2.6 cm (Kathirithamby 1989).

Within the suborder Stylopidia, the family Myrmecolacidae is unique. Myrmecolacid males and females not only have contrasting morphology and development, but also parasitise different hosts. Myrmecolacid males parasitise Formicidae (ants) while females parasitise Orthoptera: Tettigoniidae, Gryllidae and Gryllotalpidae (grasshoppers, crickets) and Mantodea: Mantidae (mantids). As a result of the extreme sexual dimorphism of Strepsiptera and the dual hosts of the Myrmecolacidae, it is difficult to correctly match male and female Myrmecolacidae (Kathirithamby 1989, Kathirithamby & Hamilton 1992; Kathirithamby et al. 2001). Of the 98 species of Myrmecolacidae described, only five are of females. Of these females, only two have been identified and matched to their males. Eight species were described from males that have been found parasitic in formicid hosts, hence the hosts of only these species are known. The remaining 88 myrmecolacid species are based only on free-living males caught in light traps (Kathirithamby 1998, Kathirithamby et al. 2001). One approach used to pair males and females is to compare the respective 1st instar larvae found in the hosts (Luna de Carvalho 1967, 1973, Cook et al. 1998). However, on host entry first instars moult to apodous second instar larvae (Kathirithamby et al. 1984) so that comparisons of this nature are generally based on the shed cuticles of the first instar rather than the entire organism. This evaluation gives tenuous and uncertain relationships since first instars have few taxonomically useful characters, and comparisons of shed cuticles are even less reliable.

Caenocholax fenyesi Pierce

Pierce (1909) originally described *Caenocholax fenyesi* from four males collected in Mexico by Dr. A. Fenyés. Light trap collections of *C. fenyesi* in the United States show this species is found in 7 of the southern states. *C. fenyesi* was first recorded stylopizing *Solenopsis invicta* Buren (red imported fire ant) sexuals and workers by Kathirithamby and Johnston (1992) in College Station, Texas. Stylopization of *S. invicta* by pupal stages of *C. fenyesi* causes abnormal social behaviors such as isolation, unusual posture, and decreased aggressiveness (Cook 1996). The findings of Kathirithamby and Johnston (1992) and Cook (1996) suggest potential for biological control of *S. invicta* by *C. fenyesi*.

Two *Hapithus agitator* Uhler (Orthoptera: Gryllidae, collected by Dr. R. Wharton) were found parasitised by three strepsipteran females in College Station, Texas. The females were originally identified as *C. fenyesi* based on external morphological comparisons of the shed 1st instar cuticles of the male *C. fenyesi* and the 1st instar larvae found in the putative females (Cook 1996, Cook et al. 1998).

Stichotrema dallatorreanum Hofeneder

Hofeneder (1910) described the female and 1st instar larvae (1920) of *Stichotrema dallatorreanum* which parasitises Orthoptera: Tettigoniidae in Papua New Guinea (PNG). The hosts are *Segestidea novaeuguineae* (Brancsik), *Segestes decoratus* Redtenbacher and *Sexava nublia* Stål, of which the first two are economic pests of the oil palm (*Elaeis guineensis*), a significant cash crop in PNG (Kathirithamby et al. 1998; Solulu et al. 1998; Kathirithamby et al. this issue). *S. dallatorreanum* is primarily, if not exclusively, parthenogenic (Kathirithamby unpublished).

Unknown Species

Two species of Tettigoniidae from PNG, *Phyllophorella subinermis* Karny (Phyllophorinae) from West New Britain Province (WNB-A and WNB-B) and *Paracaedicia* sp. (Phaneropterinae) found in West New Britain Province (WNB-C) and Popondetta, Oro Province, Papua New Guinea (POP) were found to be parasitised by unknown female Myrmecolacidae (Kathirithamby et al. this issue). The unknown species and their hosts are summarized in table 1, along with the other species considered in this study.

Locus Choice and Analysis

Accurate and robust phylogenetic analysis requires the use of appropriately variable characters. For molecular data, this means choosing a marker that is neither hypervariable (leading to 'noisy' data) nor hypovariable (excess uninformative characters). Mitochondrial DNA sequences are commonly employed in phylogenetic studies because of their generally highly variable nature compared to nuclear loci (Vawter & Brown 1986), which allows for the detection of genetic variation between closely related species and even individuals of the same species (Simon et al. 1994). Strepsiptera (Elenchidae) are known from the fossil record to have been present in the Late Cretaceous period 146–65mya (Kukalova-Peck 1991), which necessitates the utilization of highly conserved loci. As such, Cytochrome Oxidase I (COI), which is relatively conserved among mitochondrial genes (Hillis et al. 1996), was first used as the molecular marker of choice in this study. To confirm and expand our data, we also sequenced sections of the mitochondrial 12S rRNA gene, a nuclear rRNA

Table 1. Summary of Strepsipteran species analyzed.

Strepsipteran Species (family), sex	Host Species (order)
<i>Stichotrema dallatorreanum</i> Hofeneder (Myrmecolacidae), female	<i>Segestidea novaeguineae</i> (Brancsik) (Orthoptera)
<i>Elenchus japonicus</i> ¹ Esaki & Hashimoto (Elenchidae), male and female	<i>Sogatella furcifera</i> Hováth, <i>Nilaparvata lugens</i> Stål (Homoptera)
<i>Caenocholax fenyesi</i> Pierce (Myrmecolacidae), putative female	<i>Hapithus agitator</i> Uhler (Orthoptera)
<i>Caenocholax fenyesi</i> Pierce (Myrmecolacidae), male	<i>Solenopsis invicta</i> Bruen (Hymenoptera)
Species from West New Britain-A&B (Myrmecolacidae), female	<i>Phyllophorella subinermis</i> Karny (Orthoptera)
Species from West New Britain-C (Myrmecolacidae), female	<i>Paracaedicia</i> sp. (Orthoptera)
Species from Popondetta (Myrmecolacidae), female	<i>Paracaedicia</i> sp. (Orthoptera)
<i>Xenos vesparum</i> Rossi ¹ Rossi (Stylopidae), female	<i>Polistes dominulus</i> Christ (Hymenoptera)

¹ *E. japonicus* and *X. vesparum* used as outgroups for phylogenetic analysis

internal transcribed spacer, 28S D2, and the nuclear 18S rRNA gene, all of which are generally presumed to be more highly variable between sequences of related taxa than COI.

The species examined in this study belong to the family Myrmecolacidae. To place the myrmecolacid sequences in a monophyletic ingroup and better determine the relationships of the species in question, *Elenchus japonicus* Esaki & Hashimoto (Elenchidae) and *Xenos vesparum* Rossi (Stylopidae) were chosen as outgroups for sequence analysis (see table 1). Both species serve exceptionally well as outgroups because they are taxonomically outside, but closely related to, the Myrmecolacidae. *E. japonicus* parasitises *Sogatella furcifera* Horváth and *Nilaparvata lugens* Stål (Homoptera: Delphacidae) which are pests of rice in South Asia and South East Asia, and is a member of the family Elenchidae. The classification of Elenchidae as a sister group to Myrmecolacidae is based on morphological characters such as in the antennal joints, number of tarsal joints, and wing venation (in the male), the shape of the cephalothorax, brood canal opening (in the female), and the host group parasitised (Homoptera) (Kathirithamby 1989).

X. vesparum parasitises *Polistes dominulus* Christ (Hymenoptera: Vespidae) in the Palaearctic region and belongs to the family Stylopidae, which is the largest of the strepsipteran families.

MATERIALS AND METHODS

Species Collection and DNA Extraction

S. invicta colonies from Bee Creek Park in College Station, Texas were screened for the presence of parasitic male *C. fenyesi* (June & July, 1998). The putative female *C. fenyesi* parasitic in *H. agitator* was captured

in a malaise trap in Lick Creek Park, College Station, Texas, as was the unparasitised *H. agitator*. The other female Myrmecolacidae were collected during JK's field trips to PNG. JK also collected *E. japonicus* in Matsue, Japan, and *X. vesparum* near Florence, Italy. Samples and hosts were preserved separately in 95% ethanol.

DNA extractions were performed using a QIAampTM Tissue Kit (QIAGEN[®]) with 25–40 mg tissue. The tissue protocol was used with the following exceptions and clarifications: samples were rinsed to eliminate excess ethanol and air dried in 1.5 mL microfuge tubes; Proteinase K digestion was performed overnight (minimum 12 hours); a 5 minute, 70°C incubation of the spin column loaded with 100 µL ddH₂O preheated to 70°C preceded elution; DNA was eluted twice with 100 µL ddH₂O preheated to 70°C (total elution volume of 200 µL); extracted DNA was stored at –20°C. DNA was easily extracted from female Strepsiptera, but DNA extractions from males proved more difficult, possibly due to their limited size and proteinaceous exoskeleton. Female strepsipteran are larger and composed of soft tissue, which more readily permits protein degradation and nucleic acid extraction. Despite conventional precautions, the DNA from strepsipteran samples tended to degrade faster than normal when compared to host tissues and other samples in the laboratory.

Locus Amplification and Sequencing

The primer names and sequences chosen in this study are given in table 2. Products were amplified using standard PCR techniques on a GeneAmp[®] PCR System 9700 (PE Applied Biosystems). All reactions were 50 µL total in volume as follows: 25 µL Qia-gen[®] Taq PCR Master Mix (1.25U Taq polymerase,

Table 2. Primer sequences.

Primer	Forward Sequence	Reverse Sequence	Expected Size
12S	5'TACTATGTTACGACTTAT	5'-AAACTAGGATTAGATACCC	400 bp
COI ¹	5'GGATCACCTGATATAGCATTCCC	5'CCCGGTAAAATTAAAATATAAACTTC	440 bp
D2	5'GAGTTCAAGAGTACGTGAAACCG	5'CCTTGGTCCGTGTTTCAAGAC	500 bp
18S	5'GCGTATACTAAAGTTGTTGCGG	5'GCCCTTCCGTCAATTCCTTTA	600 bp

¹ standardized names by Simon et al. (1994): COIF = C1-J-1751; COIR = C1-N-2191

0.5X PCR Buffer, 1.5 mM MgCl₂, 200 µM each dNTP), 0.5–1 µg DNA, 10–25 pmol each primer, 1.5mM MgCl₂, and ddH₂O to volume. Cycling conditions for COI were as follows: 1 cycle X 94°C 3 minutes; 40 cycles X 96°C 45 sec/46°C 45 sec/72°C 45 sec; 1 cycle X 72°C 10 min. Cycling conditions for 12S were the same as for COI, but with a 52°C annealing temperature. Cycling conditions for D2 and 18S were as follows: 1 cycle X 95°C 2 min; 40 cycles X 95°C 15 sec/52°C 15 sec/68°C 5 sec; 1 cycle X 68°C 5 min. PCR products were verified on 1.2–1.5% agarose gels stained with ethidium bromide. PCR products were purified using the QIAquick™ PCR Purification Kit (QIAGEN®).

ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Mix was used in the sequencing reaction. The sequencing reactions were as follows: 2 µL Big Dye™, 2 µL template DNA (20 ng/µL), and 2 µL primer (5 pmol/µL). The primers used for sequencing were the same as indicated for PCR. The cycling parameters followed those given for Big Dye™ sequencing reactions. Products of the sequencing reactions were ethanol precipitated and sequenced according to standard instructions for the ABI Prism® 377 Sequencer.

Data Analysis

Sequences were obtained for at least two members of each examined species in table 1. Forward and reverse sequences were aligned and corrected using Sequencher 3.0 (Gene Codes Corp. 1995), generating a consensus sequence for each sample. Consensus sequences were aligned with each other using CLUSTAL X (Higgins et al. 1992) with a gap opening of 10, a gap extension of 0.5, and a transition weight of 0.5. Phylogenetic Analysis Using Parsimony (PAUP* 4.0b2, Swofford 1999) was employed for parsimony analysis of the sequences. A maximum parsimony method was used, which minimizes the number of nucleotide substitutions necessary to explain the data set. Maximum parsimony solutions were found by a branch-and-bound search followed by bootstrapping to test the strength of relationships between taxa.

RESULTS

Though sequences were obtained for all four loci for most of the taxa in question, only COI and 18S proved to be reliable and conserved enough to use in this study. Sequences for the COI and 18S products can be located through GenBank accession numbers AY039094–AY039107. As expected, COI was the most conserved of the 4 loci. The 12S and D2 alignments contained a number of insertion/deletion events as well as single base changes. Further, the 12S PCR products were consistently difficult to sequence, possibly due to the high AT content of the amplified product leading to mispriming events during sequencing. Preliminary alignments and phylogenetic analyses for the 12S and D2 data were ambiguous and are consequently not included in this report.

Of the 440 bp amplified for the COI gene region, 300 bases were sequenced and analyzed for all species named in table 1 except WNB-C. Of these, there was only a single 6 bp insertion/deletion site between *E. japonicus*/X. *vesparum* (families Elenchidae/Stylopidae) and all other sequences (family Myrmecolacidae). Of the approximately 600 bp amplified for the 18S gene region, 213 bases were sequenced and analyzed for all species named in table 1 except *E. japonicus* male/female and *C. fenyesi* male. The 18S alignment contained several small gaps and more overall base differences between species than COI. When parsimony analysis was performed on either locus singly, the resulting trees were congruent with each other. That is, except for the missing taxa in each set, the data seem to have comparable phylogenetic signals. The COI+18S data set consistency was also tested using the partition homogeneity test across 1000 replicates in PAUP. The homogeneity test follows that described as the incongruence-length difference test by Farris et al. (1995). The results gave a p-value of 0.78, indicating that the data are relatively homogeneous and that the additional homoplasy created by combining the two sets is minimal. Combining the COI and 18S sequence data for this analysis also allows the inclusion of more taxa without confounding the results. Consequently, the following results were ob-

tained by concatenating the entire 513 bp data set (COI+18S) in PAUP.

Table 3 shows the total number of substitutions and percent sequence divergence between taxa for COI+18S. Distances could not be determined between the following due to missing taxa between the two loci: WNB-C and *C. fenyesei* male; WNB-C and *E. japonicus* male/female. The *E. japonicus* male/female and *X. vesparum* taxa were treated as paraphyletic outgroups to the myrmecolacid ingroup. Parsimony analysis using the branch-and-bound method produced one most parsimonious solution of total length 433, consistency index (CI) 0.7259, and retention index (RI) 0.7102 excluding uninformative substitutions (fig. 1). Bootstrapping support values were generated for 1000 replicates and are also shown in fig. 1.

DISCUSSION

Comparisons of total nucleotide variation and pairwise distances (table 3) reveal that the male *C. fenyesei* and the putative female are not the same species. The male *C. fenyesei* and putative female sequences vary at 87 of the 513 bases analyzed for COI and 18S (29% corrected difference). In addition, the male *C. fenyesei* and putative female do not appear as a monophyletic clade in the phylogeny (fig. 1). In fact, the putative female *C. fenyesei* shared 31 unambiguous substitutions with the other myrmecolacids not found in the *C. fenyesei* males. In comparison, the *E. japonicus* male and female consistently showed no differences within the 300 examined bases of COI, while WNB-A/B showed two nucleotide differences (versus 87 differences in COI between *C. fenyesei* male/putative female). The high amount of variation between the male *C. fenyesei* and the putative female is consistent with variation between other species in this study. For

instance, the nucleotide variation between *S. dallatorreanum* and WNB-A is 20%, which is of the same magnitude of variation observed between the male *C. fenyesei* and putative female. The phylogenetic analysis supports the hypothesis that the female in question is of the family Myrmecolacidae, since this taxa falls between known myrmecolacid taxa (*C. fenyesei* male and the WNB/POP/*S. dallatorreanum* clade).

The high p-value for the homogeneity test (0.78) and the high CI (0.7259) indicate that not only are the two data sets relatively homogenous, but they are also largely free of background noise. Further, the high bootstrapping values indicate the tree phylogeny is robust. As with all sequence analysis, the validity of this analysis is compromised by the possibility of contamination. In the case of parasitoid organisms, the most likely cause of contamination is the host. For this reason, DNA from hosts of *E. japonicus*, *C. fenyesei* male, *C. fenyesei* putative female, and *S. dallatorreanum* was amplified and sequenced. Comparisons of host and respective parasite partial sequences as well as GenBank BLAST searches indicated that host DNA did not contaminate the samples. Additionally, if the putative female *C. fenyesei* (or any other sample) were contaminated, presumably we would not have been able to root the 'ingroup' (theoretical composite containing a non-strepsipteran host) with a strepsipteran outgroup. At the very least, a host contaminant masquerading as a strepsipteran would be subtended on an unusually long branch. This did not occur (fig. 1). We are, therefore, confident that the sequences were not contaminated with host DNA.

As predicted by Kathirithamby (unpublished), WNB-A and WNB-B are the same species (*Stichotrema jeyasothiae* Kathirithamby, 2001, page 191 this issue). These taxa form a monophyletic group (fig. 1) and have a low level of sequence divergence (0.78%, table

Table 3. Base differences and total variation between taxa for COI and 18S (513 bases total) are shown. * Indicates that distances could not be calculated due to missing taxa between the two loci. Total number of substitutions is shown below the diagonal, while percent sequence divergence is shown above the diagonal.

	1	2	3	4	5	6	7	8	9	10
1 <i>S. dallatorreanum</i>	-	0.19048	0.39464	0.32775	0.20085	0.20507	0.07619	0.23326	0.42353	0.42353
2 POP	88	-	0.33333	0.30580	0.15339	0.15737	0.00948	0.17444	0.38929	0.38929
3 <i>C. fenyesei</i> male	103	97	-	0.34694	0.30667	0.31333	*****	0.29000	0.39100	0.39100
4 <i>X. vesparum</i>	137	137	102	-	0.29322	0.29978	0.15337	0.29075	0.25606	0.25606
5 WNB-A	95	77	92	134	-	0.00780	0.08531	0.17928	0.37716	0.37716
6 WNB-B	97	79	94	137	4	-	0.09479	0.18725	0.38062	0.38062
7 WNB-C	16	2	*	25	18	20	-	0.12871	*****	*****
8 <i>C. fenyesei</i> female	108	86	87	132	90	94	26	-	0.37024	0.37024
9 <i>E. japonicus</i> male	108	109	113	74	109	110	*	107	-	0.00000
10 <i>E. japonicus</i> female	108	109	113	74	109	110	*	107	0	-

Fig. 1. Cladogram for COI & 18S sequence data rooted with *X. vesparum* and *E. japonicus* outgroups. The total tree length is 433, consistency index is 0.7259, and retention index is 0.7102. Numbers in bold indicate unambiguous base substitutions, while those in italics indicate bootstrap values.

3) consistent with variation among conspecific individuals. Similarly, POP and WNB-C appear to be the same species (*Stichotrema waterhousi* Kathirithamby, 2001, page 195 this issue) (0.95% difference, table 3). *S. dallatorreanum*, the clade formed by WNB-A and WNB-B females, and the clade formed by POP and WNB-C females form three distinct lineages supported by 56, 38, and 25 unambiguous substitutions respectively (fig. 1). These results indicate that the three groups are separate species. As such, there are currently three new species of female Myrmecolacidae: a female which parasitises *H. agitator* in College Station, Texas (putative *C. fenyessi* female), *S. jeyasothieae* which parasitises *Phyllophorella subinermis* Karny (Tettigoniidae: Phyllophorinae) in West New Britain Province and *S. waterhousi* which parasitises *Paracaedicia* sp. (Tettigoniidae: Phaneropterinae) in West New Britain Province and Popondetta, Oro Province, Papua New Guinea (Kathirithamby et al. this issue). Molecular analysis of known myrmecolacid males (without matched females), and of the few known unmatched females will likely lead to the identification of species pairs. At least in the case of the *C. fenyessi* in Texas, further field collections will be necessary, since there are no potential pairs for either the male *C. fenyessi* or the putative female *C. fenyessi*.

The results of this study satisfactorily prove that, even within a family as divergent as Myrmecolacidae, phylogenetic analysis of molecular data can be reliably used as a method for determining relationships between and among species given the appropriate amount of character conservation. As such, we expect that such methods will be used in the future for the identification of new species as well as the association of unmatched males and females in Strepsiptera.

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