

# **Evolution in the dark: A study of cave crickets in Southern Appalachia**

**Mary Kathryn Harrison**

**A thesis submitted to the faculty of the University of the South in partial fulfillment of the requirements for honors in the Department of Biology**

**May 2, 2007**

**Certified by: \_\_\_\_\_, Thesis Advisor**

\_\_\_\_\_

\_\_\_\_\_

**Table of Contents**

	Page
Abstract.....	1
Introduction.....	2
Materials and Methods.....	10
Results.....	16
Discussion.....	25
Acknowledgements.....	27
Literature Cited.....	28

## Abstract

In 1978 Hubbell and Norton described nine species of crickets in the Tribe Hadenocini found in caves or forest litter in the Southern Appalachians. According to the model proposed by these authors new species formed during glaciation. Hubbell and Norton (1978) suggested that the crickets are thermophile relicts that dispersed in the area before and after glaciation but, during glaciation, formed new species in allopatric refugia of caves or forests south of their normal ranges. Our mitochondrial DNA sequence data—the first obtained for members of this group--tested several hypotheses: 1) The Tribe Hadenocini is a monophyletic clade when compared with outgroups from their sister Tribe Dolocopodini, 2) The two genera *Euhadenoecus* and *Hadenoecus* are both monophyletic, 3) The forest *Euhadenoecus* and the troglloxenic *Euhadenoecus* are each monophyletic, 4) The two troglloxenes from Kentucky form a single clade, and the three most cave-adapted troglloxenes in Tennessee form a single clade. Our preliminary results support all four of these hypotheses. Our data, however, suggest that the divergence between the two genera and between the two clades of *Hadenoecus* occurred well before the Pleistocene and that only speciation within the two *Hadenoecus* clades occurred in the mid to late Pleistocene as suggested by Hubbell and Norton (1978).

## Introduction

Hubbell and Norton (1978) described the nine species of crickets of the Tribe Hadenocini found only in caves or forest litter in the Southern Appalachians, with the exception of one species whose range extends into southern New York State. This tribe belongs to the family Rhaphidophoridae and subfamily Dolichopoda along with only one other tribe, Dolichopodini, in which all species are also forest litter or cave-dwelling crickets in Southern Europe. Hubbell and Norton (1978) propose that the Southern Appalachian tribe was isolated from the Southern European tribe in the middle Eocene epoch after which the last land bridge between Northern Europe and North America was submerged. The authors used cladistic analysis of physical characteristics of the crickets along with knowledge of the geological history of the region to propose a phylogeny of the group (Figure 1).

They hypothesized a climate relict model, (Figure 2A) to explain evolution of these nine species during glaciation of the Pleistocene epoch and Wisconsin Glacial Stage. Most studies of Appalachian cavernicoles suggest that the glacial relict hypothesis best explains speciation of this region (Culver 1982). Rivera et al. (2001) and others have also posed a second model for adaptation to life in caves that involves parapatric speciation by active colonization or adaptive shifts (Figure 2B).

Glacial relicts in North America are most often described as species that migrated from their normal ranges and retreated into caves during interglacial periods as the temperature warmed and the climate grew more arid, forming new species by allopatry. Less frequently described, thermophile relicts are remnants of more tropical fauna, and are intolerant of the cold temperatures.

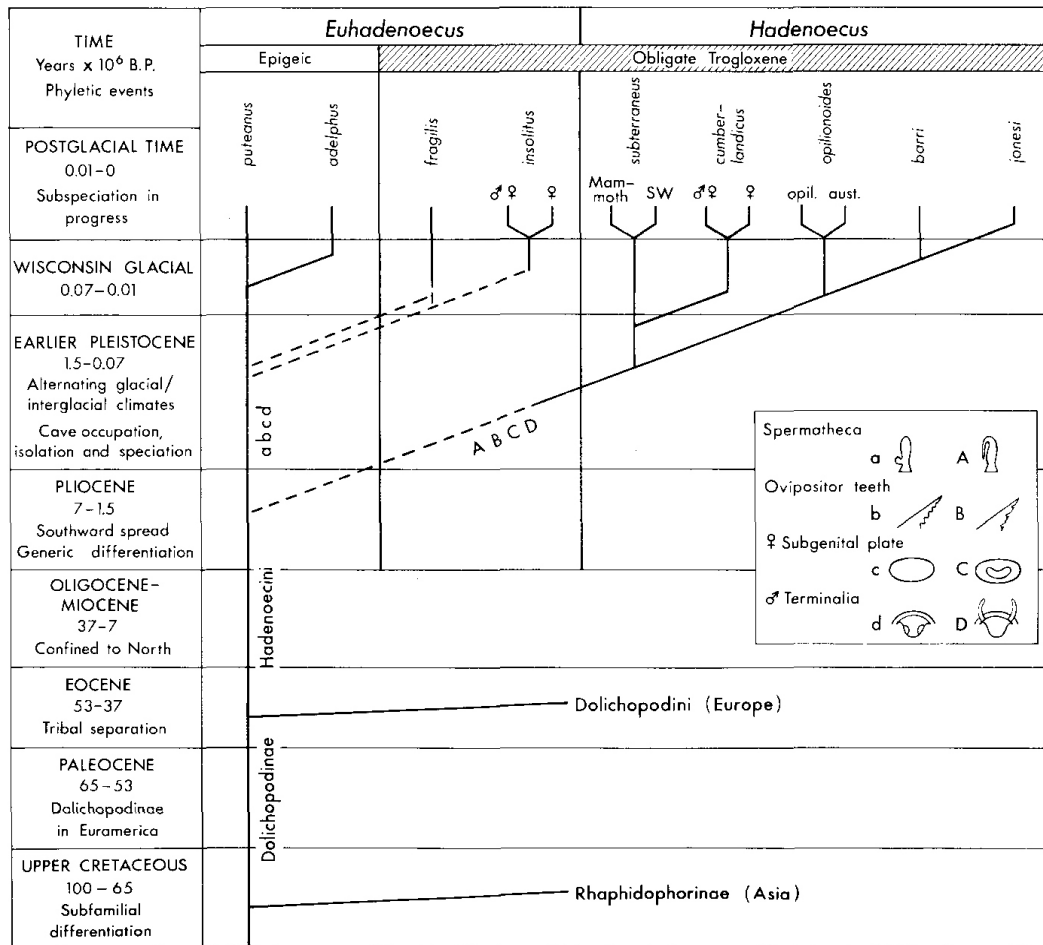


Figure 1. Hypothetical phylogeny of the Hadenocini (Hubbell and Norton 1978).

They are forced to retreat into caves during glaciation. According to both models, new species formed in allopatry, or isolation from one another. Hubbell and Norton (1978) suggest that the cavernicoles in tribe *Hadenocini* are thermophile relicts that dispersed in Southern Appalachia before and after glaciation, but during glaciation were isolated in caves or migrated southward out of their current ranges.

The tribe Hadenocini includes two genera—*Euhadenoecus* and *Hadenoecus*. According to Hubbell and Norton (1978) the two genera represent the earliest split in the tribe that occurred perhaps as early as the late Pliocene (ten million years ago).

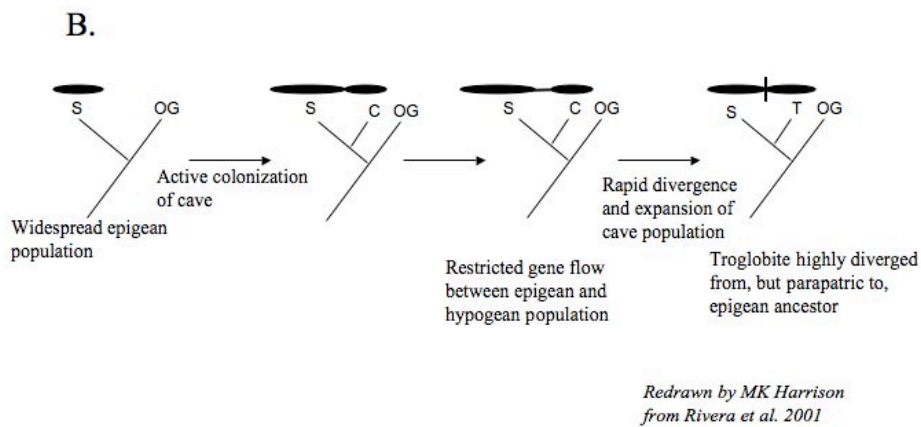
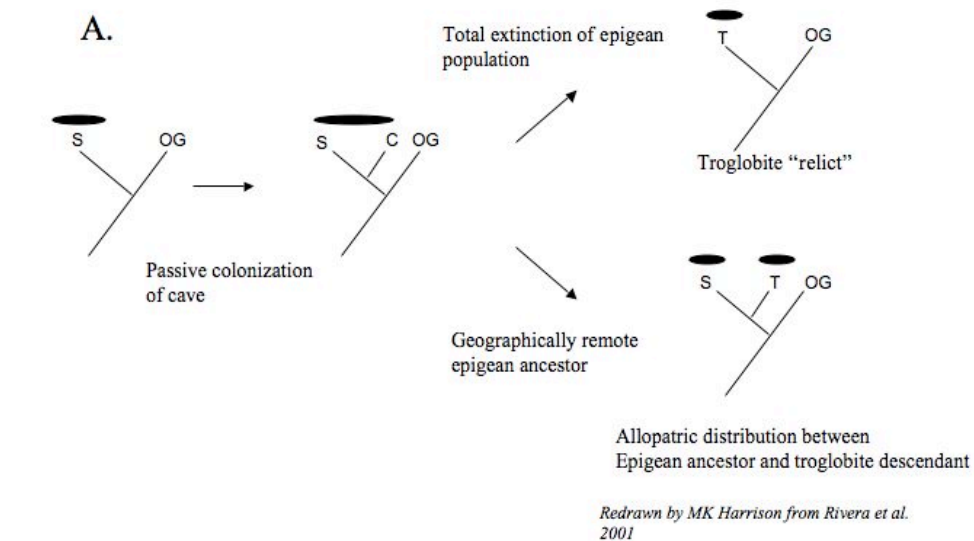


Figure 2. Phylogenetic prediction of speciation by the A) Climatic Relict Hypothesis suggesting allopatric speciation, B) Adaptive Shift Hypothesis suggesting parapatric speciation (Rivera *et al.* 2001). OG, outgroup; S, surface; C, cave; T, troglobite. Redrawn by M.K. Harrison.

The *Euhadenoecus* species are more ancestral in morphology than the highly troglomorphic *Hadenoecus* species. The four *Euhadenoecus* spp. include two forest-dwelling species, structurally adapted for life in the dark and two obligate troglaxenes that must reproduce in caves but also spend time in the forest. *E. insolitus* and *E. fragilis* are obligatory troglaxenes that Hubbell and Norton (1978) propose diverged from the forest dwelling *E. puteanus* and *E. adelphus* during the Pleistocene (1.5 million to 70,000 years ago). These authors propose that the most recent adaptation to caves occurred during the Wisconsin Glacial Stage 10-70 thousand years ago with some slight variations occurring as late as post-glacial periods.

There are five species in the tribe *Hadenoecini*; all are cavernicoles and occur in two clades according to Hubbell and Norton (1978). The least troglomorphic of these, *H. subterraneus*, is restricted to the huge cave systems of south-central Kentucky, which includes Mammoth Cave. Its closest congener, *H. Cumberlandicus*, also in Kentucky, is found east of these caves. The second clade includes the remaining three *Hadenoecus* species that display increasingly troglomorphic features along a north to south gradient on the Cumberland Plateau and the Eastern Highland Rim of Tennessee: *H. opilionoides* in north central Tennessee, *H. barri* in central Tennessee, and *H. jonesi* in southernmost Tennessee and northeastern Alabama, north of the Tennessee River. All ranges for the *Hadenoecus* species used in this study can be seen in Figure 3.

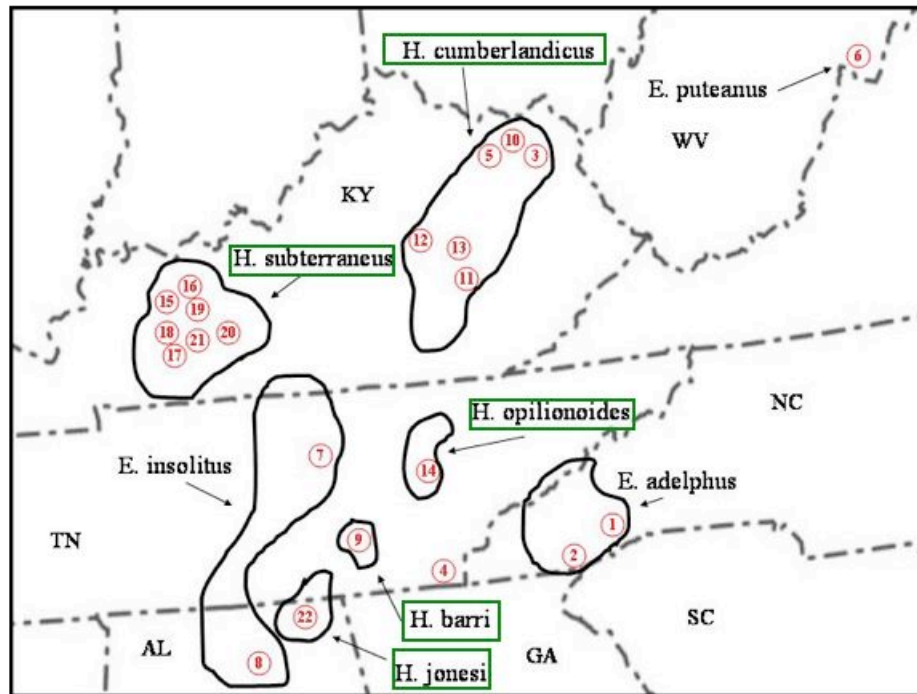


Figure 3. Map representing geographical location of twenty-two populations used in this study. 1)MON: Moonshiner's Cave, Henderson Co., NC 2)HIG: Highlands, Macon Co., NC 3)BAT: Carter Cave State Park, Carter Co., KY 4)FON: Fontana Dam, Swain Co., NC 5)LAU: Laurel Cave, Carter Cave State Park, Carter Co., KY 6)SMO: Smoke Hole Caverns, Grant Co., WV 7)IND: Indian Grave Point Cave, De Kalb Co., TN 8)ARG: Argo Cave, Jefferson Co., AL 9)WON: Wonder Cave, Grundy Co., TN 10)BAT: Bat Cave, Carter Cave State Park, Carter Co., KY 11)BAK: Baker Cave, Pulasky Co., KY 12)DBO: Daniel Boone Cave, Jessamine Co., KY 13)HIS: Hisel Cave Jackson Co., KY 14)BLF: Blind Fish Cave, White Co., TN 15)FNM: Mammoth Cave, Frozen Niagara, Edmonson Co., KY 16)GON: Great Onyx Cave, Edmonson Co., KY 17)JST: Jesse Stewart Cave, Butler Co., KY 18)MAM: Mammoth Cave, Marion Avenue, Edmonson Co., KY 19)PAR: Parkers Cave, Barren Co., KY 20)STH: Steep Hollow Cave, Warren Co., KY 21)WHE: Wheeler Cave, Logan Co., KY 22)GRE: Doug Green Cave, Jackson Co., AL

Adalgisa Caccone, who provided many of the crickets used in this study, and coworkers (1987a, b) published results that used two outdated molecular approaches to address the evolutionary history of crickets in the tribe Hadenocini. Their first approach compared allozyme variation among populations representing the nine species (Caccone *et al.* 1987a). This very low-resolution technique follows changes in the amino acid sequences of marker enzymes (allozymes) between populations. The evidence gathered from their approach supported Hubbell and Norton's morphological tree, seen in Figure

1, within each of the two genera; however, they were not able to produce one tree that included both *Euhadenoecus* and *Hadenoecus* genera. The approach taken in the second paper involved DNA-DNA hybridization between pairs of organisms. This technique relies on the fact that double-stranded DNA becomes single stranded when heated (Caccone *et al.* 1987b). Double-stranded DNA in which both strands come from the same or similar sources melts at a higher temperature than DNA in which the strands are from different sources. Hence, DNA hybrids with more DNA differences melt at lower temperatures, and this was used to construct a tree pairing species whose DNA hybrids melt at higher temperatures. The results of this assay support the Hubbell and Norton morphological tree as well, with the exception of the placement of *H. jonesi*. These data suggest the north to south migration of more troglomorphic crickets that was mentioned earlier. The patterns of branching obtained from DNA-DNA hybridization agree with the branching data from allozyme variation assays; there is less agreement between the hybridization data and Hubbell and Norton's morphology based patterns. For *Euhadenoecus*, the three methods appear congruous, suggesting two clades: the forest dwelling *E. puteanus* and *E. adelphus* in one, and *E. fragilis* and *E. insolitus* in the other. For the genus *Hadenoecus*, there are some discrepancies in the three data sets. Hubbell and Norton (1978) suggested that *H. subterraneus* and *H. cumberlandicus* are monophyletic; whereas, Caccone *et al.* (1987) suggest that *H. cumberlandicus* resides in a clade with *H. barri* and *H. opilionoides*, whereas Hubbell places *H. jonesi* in the clade with *H. barri*, and *H. opilionoides*. The allozyme data of Caccone *et al.* (1987a) place *H. jonesi* in a clade with the other crickets and DNA-DNA hybridization data (Caccone *et al.* 1987b) show *H. jonesi* as the most distant of all five species.

Caccone *et al.* (1987b) argue that the DNA-DNA data set is more reliable. However, while this method is higher resolution than the allozyme reaction assay, it may not accurately reflect evolutionary history. Using the high-resolution comparison of DNA sequences and cladistic analysis, we are able to generate a more truthful view of evolutionary history.

With the advent of the polymerase chain reaction in the late 1980's, it became possible to amplify DNA of particular genes in a mixture of total DNA from any organism and to sequence it using techniques that have now been replaced by automated DNA sequencing. Using mitochondrial (mt) DNA sequences that are maternally inherited and not diploid, these techniques have been used to resolve the phylogenies of many invertebrates (Danforth 1999; Frati *et al.* 1997). Analysis of the sequence of bases of particular gene(s) within a group of organisms allows one to construct trees based on many more characters than those previously constructed for this tribe. This allows construction of more accurate trees and calculation of the times of divergence. Unlike the work of Caccone *et al.* (1987a,b) this technique can test the times of divergence of organisms suggested by Hubbell and Norton (1978) by invoking a molecular clock that calibrates the rate of base changes per million years.

The work reported here is the first such mtDNA study of these crickets. Its goal is to elucidate some facets of the evolutionary history of the Tribe Hadenocini using analysis of mitochondrial DNA sequences. We planned to test four of Hubbell and Norton's hypotheses and their proposed dates of divergence. First, the tribe Hadenocini is monophyletic when compared with outgroups from the tribe Dolycopodini. Second, the two genera *Euhadenoecus* and *Hadenoecus* are each monophyletic and represent the first

split in the group. Third, the forest *Euhadenoecus* species and the troglloxenic *Euhadenoecus* species are each monophyletic. Fourth, within the genus *Hadenoecus*, *H. subterraneus* and *H. cumberlandicus* are monophyletic, and *H. opilionoides*, *H. barri*, and *H. jonesi* are monophyletic. We hope to determine whether these organisms evolved in the dark by the mechanism proposed by Hubbell and Norton (1978).

## Materials and Methods

### Specimen Collection:

We collected crickets of *H. jonesi* and *H. barri* species in two caves in the vicinity of Sewanee, Tennessee, specifically Dry Cave and White Cricket Cave. These specimens were stored at  $-70^{\circ}\text{C}$ . The other crickets in this study, gifts from Adalgisa Caccone, were collected in 1982 and identified by Russell Norton, co-author of the monograph on *Hadenoecini*. Their legs were stored at  $-20^{\circ}\text{C}$  until 2005 and at  $-70^{\circ}\text{C}$  thereafter. Five individuals from each of twenty-three populations seen on Table 1 representing nine species were used for this study.

Table 1. Samples of *Hadenoecus* and *Euhadenoecus* stored at  $20^{\circ}\text{C}$ , collected in 1982 and identified to species by Norton in 1982. Asterisk denotes a parthenogenic population. Crickets caught in Dry Cave and White Cricket Cave were collected and identified in 2005-2006.

Species	Cave	Symbol
<i>H. barri</i>	Wonder Cave	WON
<i>H. barri</i>	Dry Cave	
<i>H. barri</i>	White Cricket Cave	
<i>H. opilionoides</i>	Blind Fish Cave	BLF
<i>H. jonesi</i>	Doug Green Cave	GRE
<i>H. jonesi</i>	White Cricket Cave	
<i>H. cumberlandicus</i>	Hisel Cave	HIS
<i>H. cumberlandicus</i>	Baker Cave	BAK
<i>H. cumberlandicus</i>	BAT Cave	BAT
<i>H. cumberlandicus</i> *	Daniel Boone Cave	DBO
<i>H. subterraneus</i>	Mammoth Cave, Frozen Niagara	FNM
<i>H. subterraneus</i>	Mammoth Cave, Marion Avenue	MAM
<i>H. subterraneus</i>	Great Onyx Cave	GON
<i>H. subterraneus</i>	Wheeler Cave	WHE
<i>H. subterraneus</i>	Friendship Cave	FRI
<i>H. subterraneus</i>	Steep Hollow Cave	STH
<i>H. subterraneus</i>	Parker Cave	PAR
<i>H. subterraneus</i>	Jesse Stewart Cave	JST
<i>E. adelphus</i>	Highlands Cave	HIG
<i>E. adelphus</i>	Moonshiner Cave	MON
<i>E. puteanus</i>	Bat Cave	EBAT
<i>E. puteanus</i>	Fontana Dam	FON
<i>E. puteanus</i>	Smoke Hole Caverns	SMO
<i>E. puteanus</i>	Laurel Cave	LAU
<i>E. insolitus</i>	Argo Cave	ARG
<i>E. insolitus</i>	Indian Grave Point Cave	IND

Each cricket was assigned an accession number, and a Microsoft Excel database was maintained to record data about each. Details such as the dates collected, dates extracted, concentration of DNA extracted, concentrations of PCR products, and the primers used in amplification were recorded therein.

#### **DNA extraction and isolation:**

Tissue from a single hind leg was homogenized in a microcentrifuge tube with a cast fiberglass pestle in 180  $\mu$ L of 1x phosphate buffered saline (pH 7.0) and incubated with 2 $\mu$ L of 100 mM RNase A (Sigma) for two minutes at room temperature to digest RNA. Twenty  $\mu$ L of 600 mAU/mL Proteinase K (Qiagen, DNeasy® Kit) was added to digest all proteins during subsequent incubation at 70°C in SDS detergent solution for one to twenty-four hours until the tissue was fully digested. DNA was extracted from the homogenate according to the protocols of the Qiagen DNeasy® Tissue Kit for DNA extraction. The homogenate was added to a DNeasy spin column (Qiagen) collecting tube and centrifuged at 13,000 r.p.m. for one minute; the flow-through was discarded. The column was washed twice with 500 $\mu$ L aliquots of wash buffer in ethanol followed by centrifugation at the above rate. The DNA was eluted from the top of the column into a fresh collecting tube with two sequential 100  $\mu$ L aliquots of dilute Tris (Sambrook et al. 1989) buffer (.01M Tris, .001M EDTA, pH 8.0) followed by centrifugation. The DNA was stored in 1.5mL microcentrifuge tubes at -20°C.

Three  $\mu$ L of the purified DNA preps were run with a gel tracking dye on an 0.8% agarose gel containing ethidium bromide and was electrophoresed in Tris Acetate with EDTA (TAE) (Sambrook et al. 1989) buffer at 80mV. Once the gel was photographed on a BioRad Gel Doc™ system under UV light, values were assigned to signify the

quantity and purity of the DNA on a scale from - to +3 (- being no visible DNA, +3 being the cleanest, largest bands present on the gel).

### **Polymerase Chain Reaction:**

Samples of this DNA were amplified using the Polymerase Chain Reaction (PCR). Our study amplified the cytochrome oxidase I and II (CO) contiguous region and the large ribosomal subunit (16S) DNA of mtDNA. The primer sequences, their locations on the mt-DNA of *Drosophila yakuba*, and the sizes of the fragments amplified are given in Table 2 (Simon et al. 1994). We used forward primer C1-J-1718 (Ron) and reverse primer TK-N-3782 (Eva) that amplify 2,053 base pairs spanning most of the COI gene, the tRNA leu gene, all of the COII gene and a small portion of the tRNA lys gene. We used forward primer 16Sa and reverse 16Sb to amplify part of the gene coding for RNA of the large ribosomal subunit.

Table 2. Primers used for amplifying mtDNA regions.

<b>Name</b>	<b>Sequence</b>	<b>Location</b>	<b>Size (bp)</b>
16Sa	CGC CTG TTT AAC AAA AAC AT	13417N	551
16Sb	CCG GTC TGA ACT CAG ATC ACG T	12866J	
COI <sub>f</sub> (Ron)	GGA TCA CCT GAT ATA GCA TT(T/C) CC	C1-1729J	2053
COI <sub>r</sub> (Eva)	GAG ACC ATT ACT TGC TTT CAG TCA TC	TK-N-36782	

All amplification reactions were performed using Sigma's Taq SuperPack™ DNA Polymerase. Stock reagent concentrations, as well as the final reagent concentrations used in each PCR reaction in this study are found in Table 3. 50μL PCR reactions were run for each sample of DNA extracted.

Table 3. PCR reaction reagent concentrations.

	[Stock]	[Final]
PCR Buffer	10X	1X
MgCl <sub>2</sub>	25mM	2.0mM
dNTP	40mM	0.8mM
Forward Primer	10 $\mu$ M	0.25 $\mu$ M
Reverse Primer	10 $\mu$ M	0.25 $\mu$ M
Taq Polymerase	5u/ $\mu$ L	0.025u/ $\mu$ L

All amplifications were done on the MJ Research PTC 200 Thermal Cycler, in thin-walled 200  $\mu$ L PCR reaction tubes. Cycling conditions of the thermal cycler are listed in Table 4; the notation of 1-2 minutes for each extension is based on 1 minute per kilobase DNA being amplified, which was determined by the size of the fragment being amplified.

Table 4. Cycling conditions for PCR.

	Temperature ( $^{\circ}$ C)	Time (min)
Initial Melt	94 $^{\circ}$ C	2
5 cycles	94 $^{\circ}$ C	0.5
	45 $^{\circ}$ C	0.5
	72 $^{\circ}$ C	1-2
35 cycles	94 $^{\circ}$ C	0.5
	50 $^{\circ}$ C	0.5
	72 $^{\circ}$ C	1-2
Final Extension	72 $^{\circ}$ C	4
Hold	4 $^{\circ}$ C	$\infty$

Amplifications were run on 2 % agarose gels containing ethidium bromide at approximately 80mV in TAE buffer. A 1-kilobase ladder (Promega) was run on the gel for a size comparison standard.

#### **DNA Purification:**

The amplified DNA was purified by two methods. Qiagen's QIAquick<sup>®</sup> PCR Purification Kit was used to purify all samples suitable for sequencing. Five volumes of

buffer with ethanol were added to one volume of the PCR reaction and mixed. This mixture was placed in a QIAquick (Qiagen) spin column and placed into a 2mL collection tube and then centrifuged under the above conditions. The flow through from this centrifugation was discarded and the spin column was placed in a new 2mL collection tube and 0.75mL buffer with ethanol was added to the column and centrifuged to wash the DNA. The flow through was once again discarded and the column was placed in a new collection tube and re-centrifuged to remove all excess ethanol. The QIAquick column was placed in a clean 1.5mL microcentrifuge tube; 50µl elution buffer was added to the center of the QIAquick membrane and centrifuged to elute DNA from the column. Purified DNA amplifications were run on 1.5% agarose gels containing ethidium bromide along with 5ng, 10ng, and 20ng lambda DNA concentration standards.

Under conditions of unwanted multiple bands or DNA shearing, a gel extraction method was used to purify amplified DNA. If these circumstances occurred, the band containing the correct fragment was excised and weighed. Depending upon the weight, three volumes of buffer were added, the mixture was vortexed and incubated ten minutes at 50°C to melt the agarose. One gel volume of isopropyl alcohol was added to the mixture to precipitate the DNA. The flow through, containing the agarose, from centrifugation was discarded and the spin column containing the DNA was placed in a clean collection tube. At this point 0.5mL wash buffer was added and centrifuged. Again the flow through was discarded and the DNA was washed with an additional 0.5mL wash buffer and centrifuged twice more to remove excess agarose from the spin column. DNA was eluted as described above.

**DNA Sequencing:**

Purified samples (sufficient  $\mu\text{L}$  to yield 10 ng per 200 bp of PCR product), 1  $\mu\text{L}$  of 10 $\mu\text{M}$  of one of the two amplification primers, and water to yield a total volume of 18.0 $\mu\text{L}$  was prepared in 200 $\mu\text{l}$  PCR tubes. These were then shipped to the DNA Analysis Facility on Science Hill (DAFSH), at Yale University where all DNA sequencing was done.

**Data Analysis:**

The raw sequence data were received electronically and analyzed in Sequencher™ 4.2.2 (Gene Codes Corp., Ann Arbor, MI, USA). This program was used to view chromatograms of the eluate off the capillary gel of the sequencer and to assemble homologous sequences from different individuals. These were exported in nexus format to MacClade™ (Maddison, *et al.* 2005) to observe sequence variation between each individual in comparison to a “type” sequence at the top. Dots represent identical bases, and letters represent mutations from the “type” sequence at the top, in this case *Gryllus pennsylvanicus*, a species of field cricket. Modeltest 3.7 (Posada and Crandall 1998) analysis was used to determine the appropriate algorithm to use for phylogenetic analysis. The nexus file was then imported into Phylogenetic Analysis Using Parsimony\* (PAUP\*)™ (Swofford 2002) for phylogenetic analysis using the maximum likelihood algorithm in the Branch and Bound mode for the CO<sub>1</sub> data set and the heuristic mode for the CO<sub>2</sub> data set. Statistical analysis of these results was conducted using 100 to 1000 bootstrap replicates of the data.

## Results

High molecular weight DNA was needed from extraction methods for successful amplification. The results presented in Figure 4 show an effective DNA extraction with single bands near the top of the gel. This denotes high molecular weight DNA free from RNA contamination.

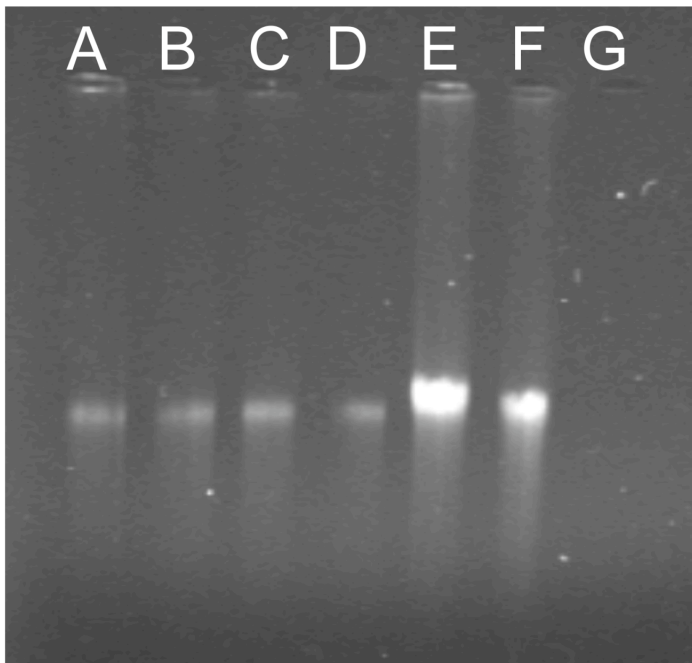


Figure 4. An 0.8% agarose gel containing ethidium bromide showing DNA preps in lanes A through G. On the scale of – to +3, lanes A-D were assigned +, lane E was assigned +3, lane F was assigned +2, lane G was assigned – due to the absence of DNA.

An 0.8% gel loaded with aliquots of PCR products from amplification with the COI primers Ron(COIf) and Eva(COIr) is shown in Figure 5 along with the 1-kilobase ladder (Promega) shown in lane 9. The amplified bands strongly visible in lanes 1, 5-8 indicate products of correct molecular weight (2053 base pairs between Ron and Eva primer) suitable for purification. Products seen in lanes 1, 5 and 7 were purified by the Qiaquick™ method and those of the same size, in lanes 6 and 8, by the gel-purification method prior to sequencing.

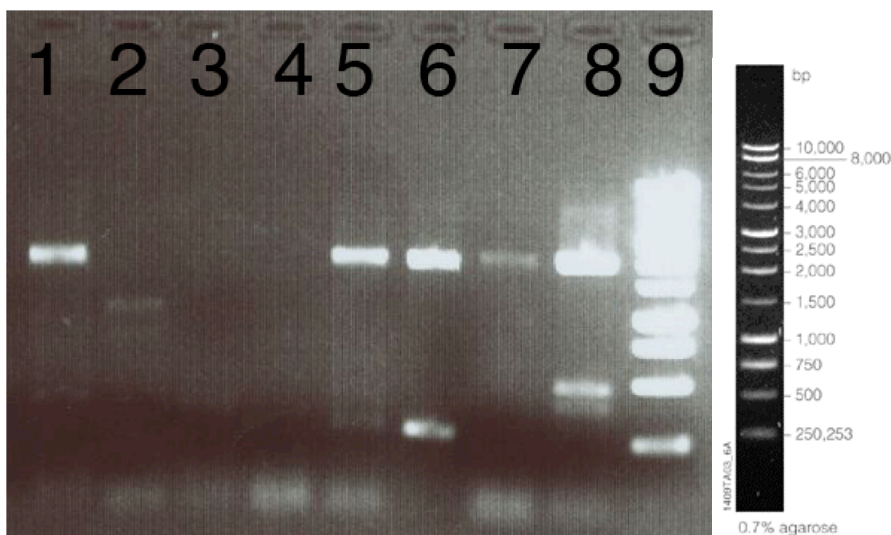


Figure 5. 2.0% agarose gel containing ethidium bromide showing PCR amplification products in lanes 1-8 and a 1kb ladder (Promega) in lane 9 and in the juxtaposed schematic.

A typical group of aligned sequences produced at DAFSH and compiled into comprehensive contiguous sequences (contigs) is seen in Figure 6.

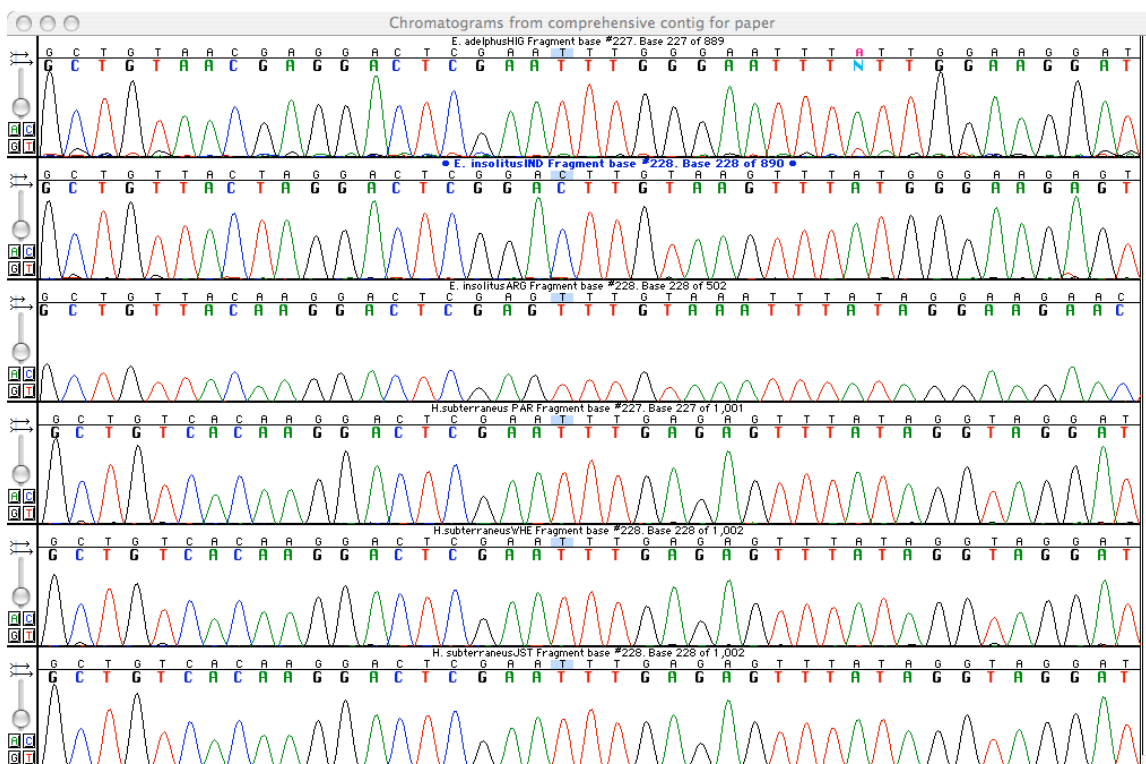


Figure 6. Chromatograms representing raw data, generated using Sequencher 4.2.2. Each nucleotide is assigned a specific color, confidence of assignment is denoted by strength of peak.

Each base is illustrated in a specific color; adenine is green, guanine is black, cytosine is blue and thymine is red. From these Sequencher chromatograms (Figure 6), any discrepancy in the computerized assignment of bases is resolved manually based on the strength of each color peak. Figure 7 shows an overview of the aligned sequences of the CO region reported in this paper. Those sequences depicted in green, on the left of the figure are sequenced from the Ron forward primer; the sequences shown in red are read from the Eva reverse primer.

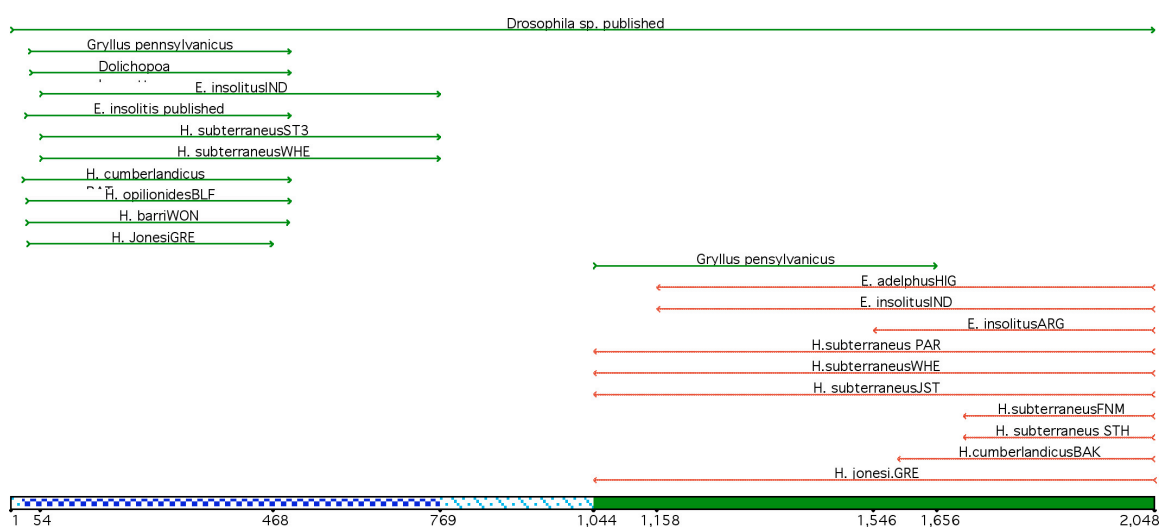


Figure 7. Comprehensive Contig generated by Sequencher 4.2.2. Green lines indicate sequence from forward primer, Ron; red lines indicate sequence from reverse primer, Eva. Total length of fragment is 2053 base pairs.

The lack of connectivity or overlap among the forward and reverse primers denotes the absence of sequence in the middle portion of this 2053 base pair fragment.

Aligning data in the nexus format of MacClade (Maddison *et al.* 2005), a sample of which is seen in Figure 8, makes visualizing similarities and differences of sequences among species highly graphic. Using these visualizations, we were able to compare individual sequences and draw conclusions based upon the sequence of the outgroup.

	281	290	300	310	315																															
<b>Gryllus pennsylvanicus</b>	T	G	T	T	G	A	G	C	A	G	T	T	G	G	G	A	T	A	C	A	G	C	T	T	T	C	T	A	T	T	A	T				
<b>Dolichopoa baccettee</b>	.	.	.	.	.	.	.	.	T	.	C	.	.	A	.	C	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	T	
<b>E. insolitusIND</b>	.	.	.	A	.	.	.	T	.	T	.	.	C	.	C	A	.	.	.	.	.	T	.	.	C	.	.	.	.	.	CC	.	T			
<b>E. insolitis (PUBLISHED)</b>	.	.	.	A	.	.	.	T	.	T	.	.	C	.	C	A	.	.	.	.	.	T	.	.	C	.	.	.	.	CC	.	T				
<b>H. subterraneus ST3</b>	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	CA	.	.	.	.	.	C	.	.	.	.	.	T	.	A	.	T	.	.	C	
<b>H. subterraneus WHE3</b>	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	CA	.	.	.	.	.	C	.	.	.	.	.	T	.	A	.	T	.	.	C	
<b>H. cumberlandicus BAT</b>	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	CA	.	.	.	.	.	C	.	.	.	.	.	.	.	C	.	A	.	T	C	.
<b>H. opilionides</b>	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	C
<b>H. barri</b>	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	C
<b>H. jonesi</b>	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	C

	316	320	330	340	350																													
<b>Gryllus pennsylvanicus</b>	T	A	T	T	A	T	C	A	C	T	A	C	C	A	G	T	T	T	A	G	C	T	G	G	T	G	C	T	A	T	T	A	C	A
<b>Dolichopoa baccettee</b>	.	.	C	.	.	.	.	T	.	.	.	.	T	.	.	.	.	.	C	.	T	.	A	.	A	.	A	.	.	.	.	.	.	T
<b>E. insolitusIND</b>	.	.	C	.	T	.	.	TT	.	.	.	.	T	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	.	.	C	.	T	
<b>E. insolitis (PUBLISHED)</b>	.	.	C	.	T	.	.	TT	.	.	.	.	T	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	.	.	C	.	T	
<b>H. subterraneus ST3</b>	.	.	C	.	T	.	.	C	.	T	.	.	T	.	.	.	.	.	.	A	.	.	.	.	C	.	A	.	A	.	.	.	T	
<b>H. subterraneus WHE3</b>	.	.	C	.	C	.	.	C	.	T	.	.	T	.	.	.	.	.	.	G	.	.	.	.	C	.	A	.	A	.	.	.	T	
<b>H. cumberlandicus BAT</b>	.	.	C	.	T	.	.	C	.	T	.	.	T	.	.	.	.	.	.	A	.	.	.	.	A	.	A	.	.	.	C	.	T	
<b>H. opilionides</b>	.	.	G	.	.	.	.	TT	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	A	.	A	.	.	.	.	.	
<b>H. barri</b>	.	.	G	.	.	.	.	TT	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	A	.	A	.	.	.	.		
<b>H. jonesi</b>	.	.	G	.	.	.	.	TT	.	.	.	.	T	.	.	.	.	.	.	.	A	.	.	.	.	A	.	A	.	.	.	.		

Figure 8. Sample of MacClade™ format representative aligned DNA sequences. Dots ‘.’ indicate identity to the first sequence and letters show sequence differences.

The sample in Figure 8 suggests the similarity of all Hadenocini and the greater similarity of *H. opilionoides*, *H. barri*, and *H. jonesi*. This can be seen by comparing the patterns of dots, representing identity to the outgroup, and letters, representing sequence differences.

We have obtained sufficient data for analysis from the CO gene but not from the 16S gene, so only the former is reported here. Because of the discontinuity of the CO regions amplified by Ron and Eva and because different individuals have been successfully sequenced in each region, their sequences have been analyzed separately. Outgroup sequences for Tribe Dolico podini in the subfamily Dolico poda are available from Genbank (Benson 2005) for the Ron data set, so they are used as outgroups for this data set. Sequences from the distantly related insect *Drosophila yakuba* have been used

for this purpose in the Eva data set. The phylogram in Figure 9 represents analysis of 414 base pairs of the Ron segment beginning at base pair 1729 of the COI sequence for seven individuals of the Tribe Hadenocini and five outgroups from Tribe Dolichopodini.

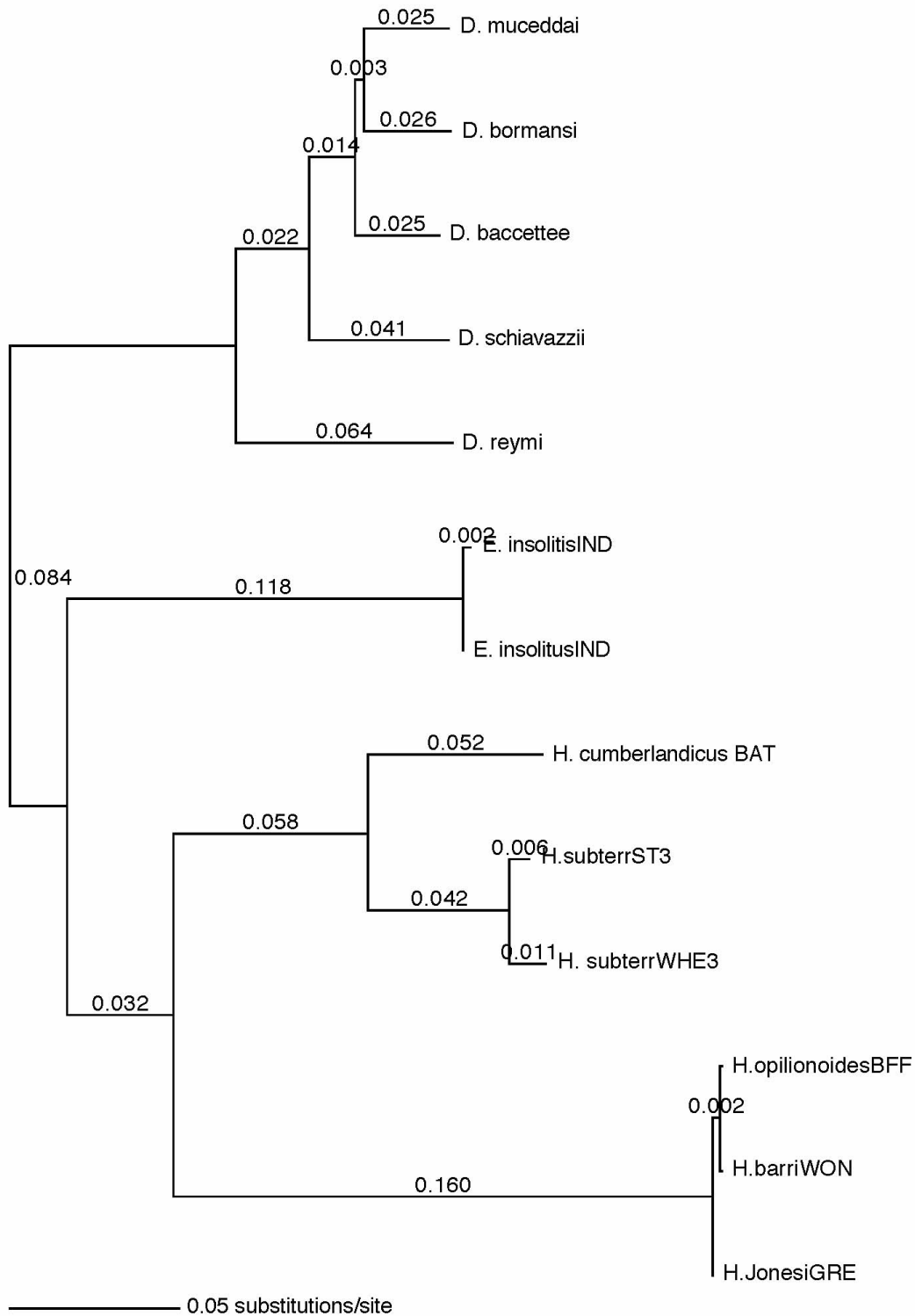


Figure 9. COI phylogram generated by PAUP showing average change along branches.

This analysis used a Maximum Likelihood algorithm in the Branch and Bound mode, but both the distance and parsimony algorithms yield the same clades. The amount of sequence divergence is proportional to the length of the branches in the phylogram and the distance along each branch is given by the numbers on the phylogram. Within the Hadenocini, the branches are much longer than in the Dolichopoda outgroups. Figure 10 shows the results of 1000 bootstrap replicates of the same data. The numbers on the tree reflect the percentage of replicates yielding the nodes on the tree, and values greater than 70 denote good support for a node (Felsenstein 1985).

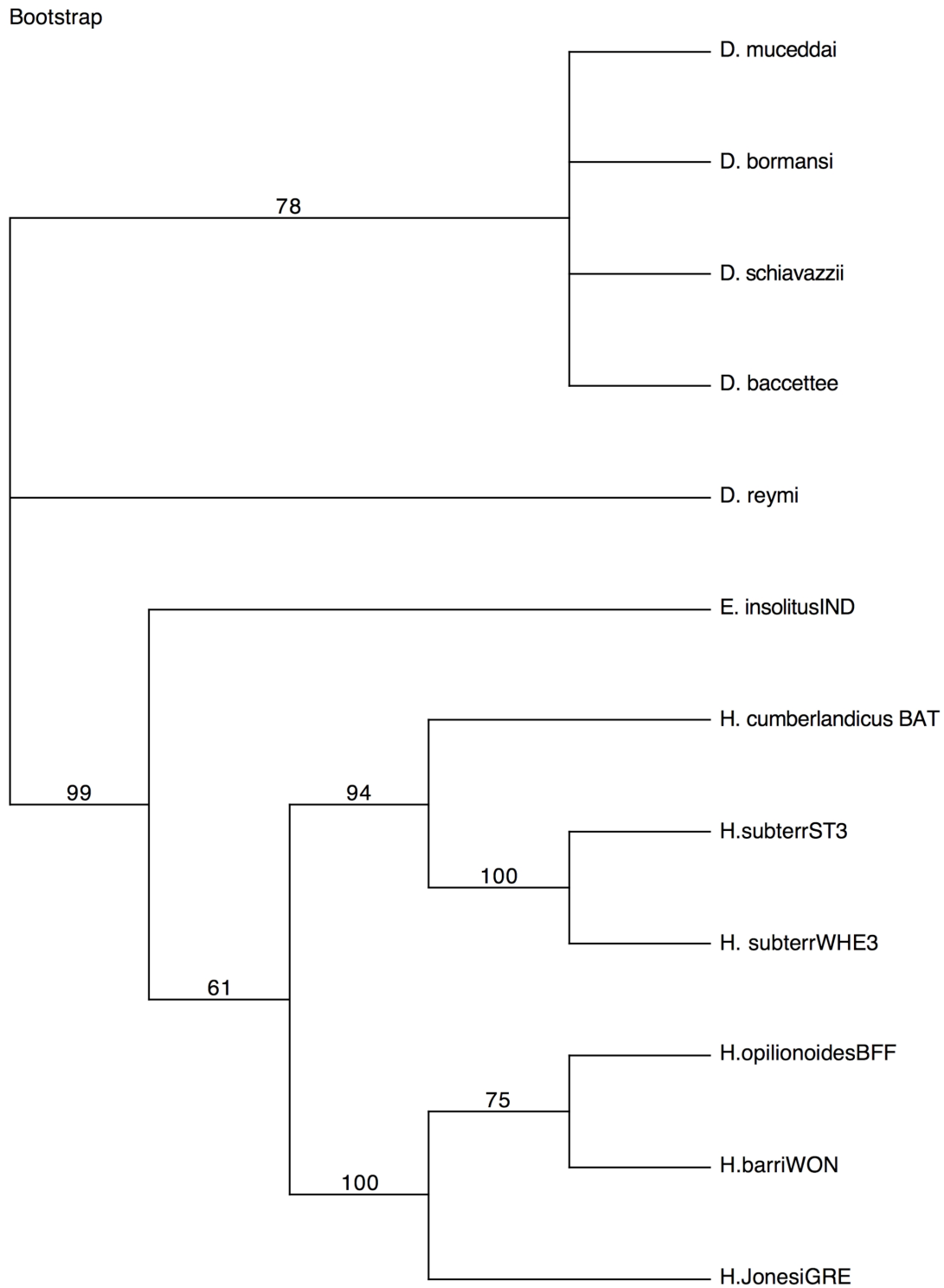


Figure 10. COron phylogram generated by PAUP showing percent support for each branch from 1000 bootstrap replicates. Branches supported by less than 50% have been collapsed.

The phylogram in Figure 11 represents similar analysis of 455 base pairs of the Eva segment of the CO data set and Figure 12, the results of 100 bootstrap replicates from the same data set.

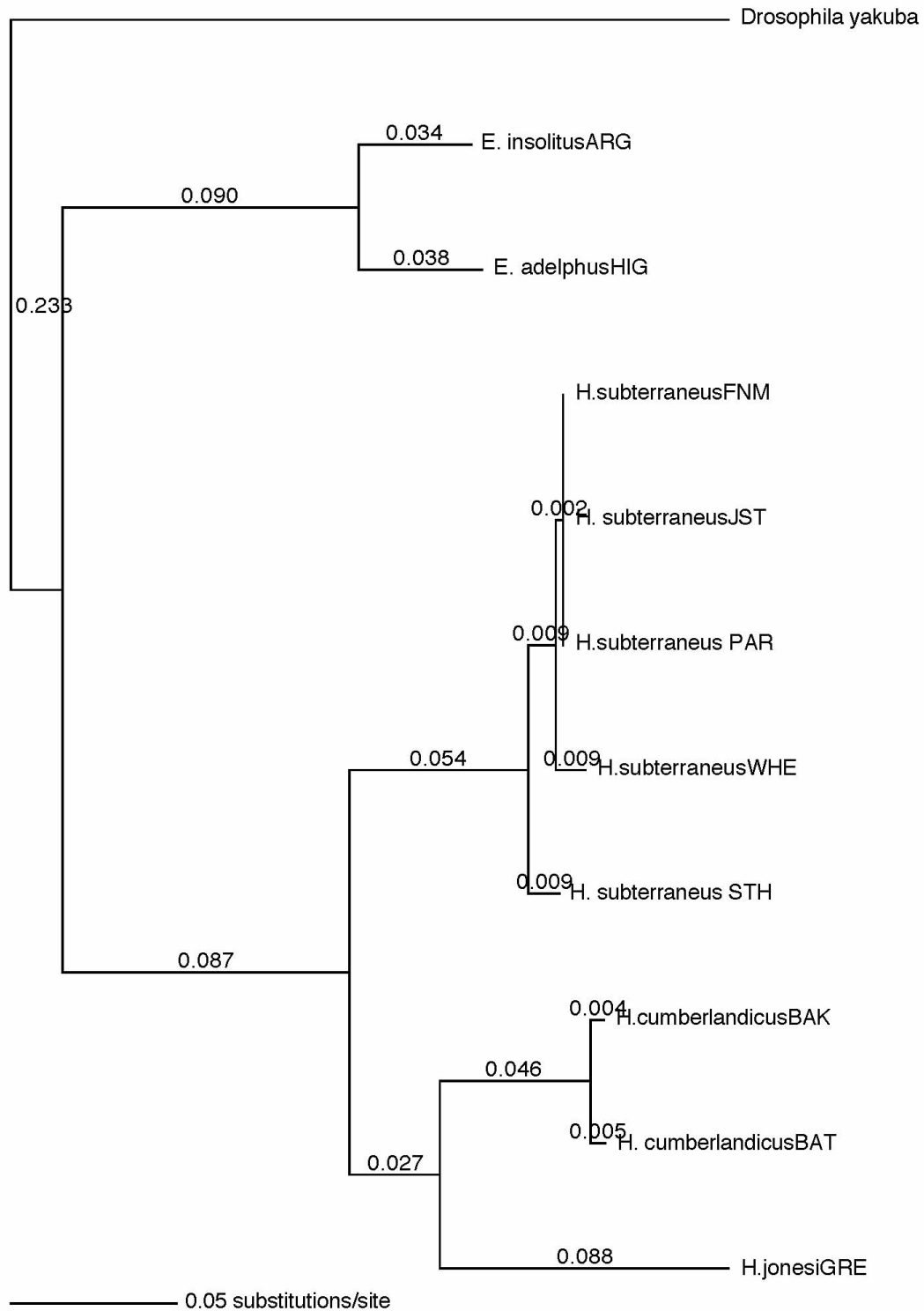


Figure 11. COEva phylogram generated by PAUP showing average change along branches.

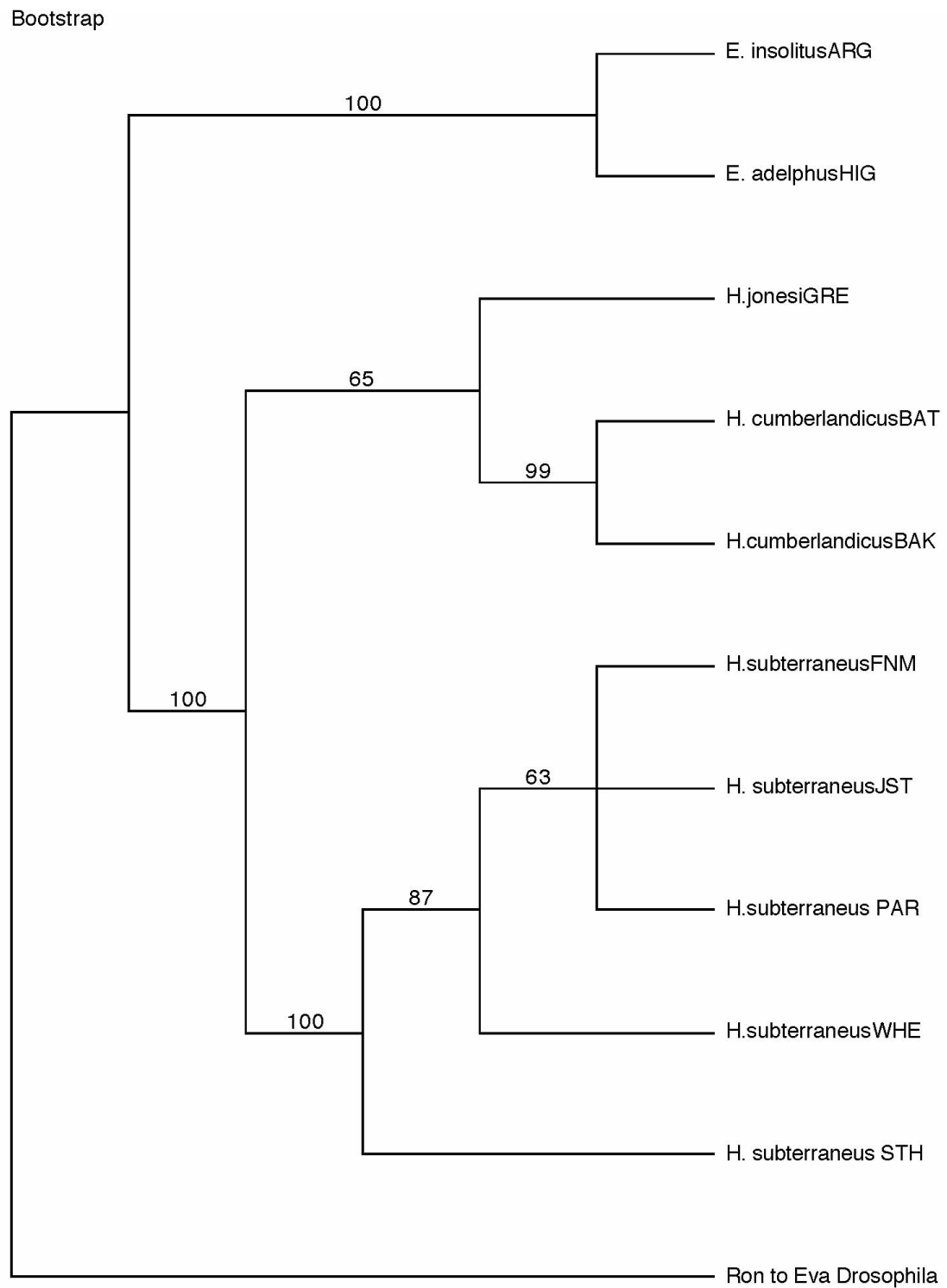


Figure 12. COeva phylogram generated by PAUP showing percent support for each branch from 100 bootstrap replicates. Branches supported by less than 50% have been collapsed.

## Discussion

These preliminary results support four of our original hypotheses. We failed to obtain sufficient data from *Euhadenoecus* spp. to address the hypothesis that the epigeal and troglonecic *Euhadenoecus* comprise separate monophyletic clades. The more complete Ron data support the hypotheses that the tribe Hadenocini is monophyletic when compared with outgroups from the tribe Dolycopodini; that the genus *Hadenoecus* is monophyletic and its separation from *Euhadenoecus insolitus* represents the first split in the group; and finally that, within the genus *Hadenoecus*, *H. subterraneus* and *H. cumberlandicus* are monophyletic, and *H. opilionoides*, *H. barri*, and *H. jonesi* are monophyletic. The less complete Eva data set does not support the last of these two hypotheses but does support the monophyly of the *Euhadenoecus* spp. We suspect that the sequence reported for *H. jonesi* may be that of a contaminant, because its distances from the other members of the genus are different from that seen in the Ron tree. All other distances are very similar in both trees. The agreement among sequences in the *jonesi*, *barri*, *opilionoides* clade suggests that they are the correct sequences, but we acknowledge that this evidence is shaky.

This discrepancy between the trees can only be resolved by obtaining more sequences from more individuals. So far the individuals in the presumed *H. jonesi* clade have been intractable to sequencing with the Eva primer, which makes it likely that the one sequence we have represents a contaminant. It has been equally problematic to obtain sequences for *Euhadanoecus* spp. with Ron. Both of these problems can be solved by designing primers that are perfect matches for sequences that we have that will better

match all sequences for amplifying and sequencing all members of the group. This task is currently underway.

If we invoke the conventional molecular clock for the rate of evolution of the CO gene region of 2% change per one million years (Brown *et al.* 1979, Allegrucci 2005), our data suggest that the extant members of this tribe represent ancient divergences. The pair wise differences among members of Tribe Hadenocini—up to 22%-indicate that they have been diverging from each other for as long as 11 million years and much longer than the Dolichopodini. Allegrucci *et al.* (2005) have conducted a parallel study on the latter tribe and suggest that all the members of this tribe diverged from a single ancestor 2.4 to 1.2 million years ago. Our data suggest that the divergence between *Euhadenoecus* and *Hadenoecus* as well as that between the two *Hadenoecus* clades occurred well before the Pleistocene, and that speciation within the two *Hadenoecus* clades occurred in the mid to late Pleistocene as suggested by Hubbell and Norton (1978). This is probably due to the fact that the Dolichopodini went through a bottleneck during glaciation (Hewitt 2003). It is well known that many species in North America were able to avoid such extinction during glaciation by migrating along the North to South mountain ranges (Hewitt 2003). Our data are simply too scanty to invoke a molecular clock at this time, but we are reasonably sure that at least three distinct ancestors of the extant species had already diverged well before glaciation. We haven't been able to discern how these species evolve in the dark, but our data should ultimately contribute to an understanding of that process.

### **Acknowledgements**

I gratefully acknowledge Dr. Henrietta Croom for her dedication, for many long hours of mentoring in the laboratory, in the classroom, and during her spare time. I also appreciate the work of Mallory Dorand, Lauren King, Dane Cooper, and Lauren Martin who offered a great deal to this study over the years. Thanks must also be extended to Dr. Kirk Zigler for his guidance in this project, and to Drs. Berner, Haskell, Lively, Palisano, and Zigler for their helpful comments and suggestions on this paper. Financial and grant support for this study was provided from the Department of Biology, the Center for Teaching and the Mellon Fund for Faculty Research of the University of the South.

### Literature Cited

- Allegrucci, G., V. Todisco, and V. Sbordoni. 2005. Molecular phylogeography of Dolichopoda cave crickets (Orthoptera, Rhaphidophoridae): A scenario suggested by mitochondrial DNA. *Molecular Phylogenetics and Evolution* 37, 153-164.
- Brown W.M., M. George, A.C. Wilson. 1979. Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences, USA*, 76, 1967–1971.
- Benson, D.A., I.K. Mizrachi, D.J. Lipman, J. Ostell, and D.L. Wheeler. 2005. Genbank. *Nucleic Acid Research* 33, D34-D38.
- Caccone, A. and V. Sbordoni. 1987a. Molecular Evolutionary Divergence Among North American Cave Crickets I. Allozyme Variation. *Evolution* 4, 1198-1214.
- Caccone, A. and J. Powell. 1987b. Molecular Evolutionary Divergence Among North American Cave Crickets II. DNA-DNA Hybridization. *Evolution* 4, 1215-1238.
- Culver, D.C. 1982. *Cave Life: Evolution and Ecology*. Harvard University Press, Cambridge, MA.
- Danforth, B.N. 1999. Phylogeny of the bee genus *Lasioglossum* (Hymenoptera: Halictidae) based on mitochondrial COI sequence data. *Systematic Entomology* 24 (4), 377–393.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783-791.
- Fрати, F., C. Simon, J. Sullivan, and D.L. Swofford. 1997. Evolution of the Mitochondrial Cytochrome Oxidase II Gene in Collembola. *Journal of Molecular Evolution* 44 (2), 145-158.
- Hewitt, GM. 2003. Ice Ages: their impact on species distributions and evolution. In “Evolution of Planet Earth”. Rothschild LJ & Lister AM, New York: Academic Press.
- Hubbell, T.H., and R.M. Norton. 1978. *The Systematics and Biology of the Cave-Crickets of the North American Tribe Hadenocini (Orthoptera Saltaoria: Ensifera: Rhaphidophoridae: Dolichopodinae)*. Ann Arbor, Michigan: Museum of Zoology, University of Michigan No. 156.
- Maddison, D.R. and W.P. Maddison. 2000. MacClade. Version 4. Sinauer Associates, Sunderland, MA.
- Posada, D. and K.A. Crandall. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* 14 (9): 817-818.

Rivera, M.A., F.G. Howarth, F. Taiti, G.K. Roderick, and F.H. Howarth. 2002. Evolution in Hawaiian Cave-Adapted Isopods (Oniscidea: Philosciidae) Vicariant Speciation or Adaptive Shifts? *Mol. Phylogenet. Evol.* 25, 1-9.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Sequencher 4.2.2. Gene Codes Co., Ann Arbor, Michigan.

Simon, C., F. Frati, A. Beckenback, B. Crespi, H. Liu, and P. Flook. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved PCR primers. *Annals Entomol. Soc. Am.* 87:651-701.

Swofford, D.L. 2001. PAUP\*: Phylogenetic analysis using parsimony (\*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.