PHYLOGENY OF *ASTYLOPSIS* CASEY (COLEOPTERA: CERAMBYCIDAE) SPECIES AND PATTERNS OF HOST PLANT USE

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Entomology – Master of Science

2021

ABSTRACT

PHYLOGENY OF *ASTYLOPSIS* CASEY (COLEOPTERA: CERAMBYCIDAE) SPECIES AND PATTERNS OF HOST PLANT USE

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Cerambycid (longhorn) beetles are diverse in their morphology and life history traits, but all share the common trait of being larval plant-borers. The larvae bore into and develop inside various plant tissues thus they can potentially cause significant economic and ecological damage, especially when transported to non-native localities. There is little empirical data on cerambycid life history traits that are essential in understanding their ecological and economic effects. *Astylopsis* Casey (Lamiinae: Acanthocinini) is an eastern North American genus of six species. Host preference varies greatly among the species, including both angiosperms and gymnosperms. I used morphological characters and molecular data to reconstruct phylogenies of *Astylopsis* to test the hypothesis that host plant use among *Astylopsis* species is conserved. I constructed phylogenies using partial COI and CAD DNA sequences from *Astylopsis* species and outgroups using parsimony methods. *Astylopsis collaris*, *A. macula*, *A. sexguttata,* and *A. arcuata* were monophyletic in both COI and combined gene phylogenies, with the genus also exhibiting monophyly in the combined gene tree. Evidence of host shift from angiosperms to gymnosperms in some species was also observed. These results confirm current taxonomic separations among the four species and their outgroups and provide important host use information. No conclusions could be drawn regarding DNA variation in association with geographic locality. These findings will inform future studies expanding the molecular dataset for *Astylopsis* with additional genes (arginine kinase, 28S, and EF1-α) and species (*Astylopsis perplexa* and *A. fascipennis*).

ACKNOWLEDGMENTS

I would like to acknowledge the United States Department of Agriculture Animal and Plant Health Inspection Service for funding this project. I would like to thank my supervisor, Dr. Anthony Cognato, for his guidance in each stage of the process, as well as my committee members Dr. Henry Chung and Dr. Alan Prather for their valuable feedback and advice. I would also like to thank Dr. Patrick Gorring and Dr. Sarah Smith for assistance and direction in my project methodology. Lastly, I would like to express my appreciation for my family and friends for their encouragement and endless support during my degree, and my cats for their comfort and companionship while working from home.

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INTRODUCTION

Cerambycid (longhorn) beetles are diverse in their morphology and distribution, host plant selection, feeding, mating behavior, and life cycle ecology, but they all share the common trait of being larval plant-borers. Comprising over 36,000 species (Haack *et al*. 2017), members of Cerambycidae have radiated to all continents except for Antarctica. They thrive in habitats from sea level to an elevation of 4,200 meters in the mountains of China and Bolivia (Haack *et al.* 2017, Kariyanna *et al.* 2017, and Linsley 1959). Nearly all adults and larvae alike are phytophagous, selecting primarily woody plants and trees as their hosts, including both angiosperms and gymnosperms. The larvae of different lineages bore into and develop in different plant parts such as roots, stems, and branches; bark, sapwood, heartwood, and pith. Additionally, different genera attack trees at various growth stages (live, weakened, dying, dead), and can live in wet, damp, or dry wood (Haack *et al.* 2017, Kariyanna *et al.* 2017, and Linsley 1959).

Mating usually occurs on the host plant and cerambycids generally do not participate in any courtship behavior: the male will simply approach the female and attempt to mate (Kariyanna *et al.* 2017). Pheromones play a role in mate selection and host plant location for oviposition. The few cerambycid species that rely on long-range pheromones tend to have shorter antennae with increased surface area with serrate, lamellate, or pectinate forms, observed in some males in subfamily Prioninae. A few other species sensitive to long-range pheromones also have shorter antennae but exhibit less sexual dimorphism (Kariyanna *et al*. 2017). Female cerambycids will oviposit in, on, or near the host plant. Members of Lamiinae are unique in that they use their mouthparts and/or ovipositors to create an opening in the outermost tissues of the host tree and lay their eggs inside. Larvae later tunnel into the host's tissues, or the surrounding

soil if this was the oviposition site, and feed and develop for months to years. In many cases, pupation occurs in the same location as larval development and can last from days to months, depending on species and ambient temperature (Haack *et al.* 2017 and Kariyanna *et al.* 2017).

Given their habit of larval development inside plant tissues, cerambycids can potentially cause significant economic and ecological damage, especially when transported to non-native localities (Cocquempot and Lindelöw 2010 and Wu *et al.* 2017). There are cerambycid taxa with little empirical data on the life history traits that are essential in understanding their ecological and economic effects. *Astylopsis* Casey (Lamiinae: Acanthocinini) is a genus native to eastern North America with six species (*A. macula, A. collaris, A. sexguttata, A. arcuata, A. perplexa*, and *A. fascipennis*) about which little information is known regarding their host use. *Astylopsis* adults vary between 6-15 mm in length and generally bear reddish to brown integument pubescent with varying brown, black, grey, and white mottling. These elytral patterns are often used to diagnose the species. Their known forest habitats range from eastern coastal United States, north into Canada, south to Florida, and west to Kansas and Texas (Fig. 1). Host preference varies greatly among the species, which is unusual within cerambycid genera. *Astylopsis perplexa*, for example, bores into the stems of *Baccharis halimifolia,* the sea-myrtle shrub. In contrast, *A. sexguttata* and *A. arcuata* both prefer coniferous trees such as pine, spruce, and larch, while *A. collaris* and *A. macula* primarily select hardwood trees (Dillon 1956a, Linsley and Chemsak 1995, and Schiefer 2000). *Astylopsis fascipennis* is the most recently described species and its larval host is unknown, however, it was collected in hardwood-dominated forests and specifically collected from elm and dead sweetgum branches using beating sheets (Shiefer 2000).

Little is known of the evolutionary relationships among *Astylopsis* species and their associations with host plants. To address this knowledge gap, I reconstructed phylogenies for some *Astylopsis* species using DNA sequences of partial mitochondrial gene cytochrome oxidase subunit I (COI) and partial nuclear protein carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD) and morphological characters. Specimens were sourced from the Albert J. Cook Arthropod Research Collection or borrowed from other institutional collections in the states where these beetles are native. I used the most parsimonious trees to map host plant use, scored as broad categories such as conifers and hardwoods, on the resulting phylogenies to test the hypothesis that host plant use among *Astylopsis* species is conserved.

MATERIALS AND METHODS

In order to understand the evolutionary history of the genus *Astylopsis* and test the hypothesis that host plant use is conserved, I studied individuals of each species for variation in morphology, sequenced their DNA, and constructed phylogenetic trees with these data.

Specimens

Due to the COVID-19 pandemic in 2020-2021, I was unable to travel out of state to collect specimens for my project. Instead, *Astylopsis* specimens were sourced from the MSU A.J. Cook Arthropod Research Collection and borrowed from the institutes listed in Table 1. Additional specimens for molecular analysis were collected by hand and preserved directly into >90% ethanol or using flight interception traps through the US Forest Service Early Detection Rapid Response program (Rabaglia *et al*. 2019). Specimens of *Pseudastylopsis pini* Schaeffer*,* *Astylidius parvus* LeConte, *Astyleiopus variegatus* Haldeman, *Leptostylopsis planidorsus* LeConte, *Acanthocinus obsoletus* Olivier*,* and *Eupromus ruber* Dalman were included as outgroups.

Morphology

Astylopsis specimens were compared for interspecific and intraspecific variation in morphological characters. Ten specimens from each species (except eight specimens for *A. perplexa*) were examined with a Leica (Wetzlar, Germany) MZ6 compound microscope illuminated with two Ikea (Delft, Netherlands) JANSJÖ LED lamps. The individuals were scored for variation in external morphological characters and placed into a character matrix (Table 2). The matrix was scored according to the following list of characters and states.

1. *Cuticle color* (dorsal, anterior views, 18x): (0) Cinnamon Buff; (1) Cinnamon; (2) Clay Color; (3) Mikado Brown; (4) Sayal Brown; (5) Tawny Olive; (6) Verona Brown; (7) Snuff Brown

2. *Pubescence color* (dorsal view, 18x): (0) Pale Pinkish Buff; (1) Light Vinaceous-Cinnamon; (2) Light Pinkish Cinnamon

3. *Pubescence density,* % (dorsal, anterior views, 18x): (0) 70-79; (1) 80-89; (2) 90-100

4. *Ventral pubescence density,* % (ventral view, 18x): (0) 70-79; (1) 80-89; (2) 90-100

5. *Dorsoventral flatness*, mm (left lateral view, 7x): (0) 1.0-1.9; (1) 2.0-2.9; (2) 3.0-3.9

6. *Diameter of median callus,* mm (dorsal view, 7x): (0) 0.2-0.3; (1) 0.4-0.5; (2) 0.6

Characters 1 and 2 were scored corresponding to colors on plate 29 of a PDF file of Ridgway (1912) displayed on a 2014 Apple (Cupertino, California, United States) MacBook Pro Retina with Intel Iris 1536 MB graphics at 75% brightness with the Color LCD option selected. Characters 3 and 4 were scored as a percentage of the whole visible area that is covered by pubescence, and characters 5 and 6 were measured in millimeters with a micrometer in the stereoscope. Data was analyzed and used to construct a tree in PAUP* 4.0 b10 PPC (Swofford 2001) and the tree was edited for clarity in Inkscape v1.0.2.

DNA Sequencing

DNA was extracted from one leg (trochanter, femur, tibia, and tarsi) of each beetle using a Qiagen DNEasy blood and tissue kit (Hilden, Germany) with the protocol from the manufacturer. The purified DNA was used in a PCR of the partial mitochondrial gene cytochrome C oxidase subunit I (COI) and partial nuclear protein carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (CAD) using various primer pairs

(Table 3). I obtained PCR products with 12.5-µl ddH_2O, 2-µl 5x PCR Buffer (Qiagen), 2-µl $MgCl_2$ (Qiagen), 0.4-µl dNTP mix (Qiagen), 1-µl template DNA, 0.1-µl HotStar Taq DNA polymerase (Qiagen), and 1-µl each of forward and reverse primers. PCR was performed on a thermal cycler (PTC-2000, MJ Research, Waltham, Massachusetts, USA), or MyCycler Thermocycler (BioRad, Hercules, California, USA) under various conditions (Table 4). Products were then treated with Exo-SAP (USB Corp., Cleveland, Ohio, USA) and sequenced at the Michigan State University Research Technology Support Facility using BigDye Terminator v1.1 (Applied Biosystems, Foster City, California, USA). I visualized forward and reverse DNA sequences in Sequencher (Ann Arbor, Michigan, USA) to trim primer sequences, edit ambiguities, and create consensus sequences.

Phylogenetic Analyses

I constructed phylogenetic trees from the morphology matrix and DNA sequences as well as the publicly available barcode sequences archived in the Barcode of Life Data System (BOLD) (Table 5) (Ratnasingham and Hebert 2007). PAUP* with parsimony methods was used to generate the trees. Heuristic searches with 100 stepwise random additions with tree bisection-reconnection were performed and characters were unordered and equally weighted for the parsimony analysis. Bootstrap values under parsimony for the same parameters as above were calculated by performing 500 pseudoreplicates with simple additions in PAUP*. Nucleotide difference among sequences was measured as uncorrected p-distance. Bayesian confidence values for phylogenetic relationships were obtained with MRBAYES software (Huelsenbeck and Ronquist 2001). Tree files were edited for better visualization and clarity in FigTree v1.4.4 and Inkscape v1.0.2.

RESULTS

Preliminary Phylogeny

A preliminary phylogeny was constructed using barcoded COI sequences of *Astylopsis* specimens through BOLD to determine whether there was intraspecific variation based on locality differences as well as the evolutionary placement of each species in the overall genus clade. Sequences from the three most commonly encountered species, *Astylopsis sexguttata, A. collaris,* and *A. macula,* were available for this preliminary analysis (Fig. 2). This phylogeny exhibited monophyly for *A. collaris* and *A. macula*, and *A. sexguttata*. Intraspecific p-distance values (Table 6) were 4.3% or less. Interspecific p-distance was 14% between *A. macula* and *A. collaris*, and there was 17% nucleotide difference between *A. macula* and *A. sexguttata*, as well as between *A. collaris* and *A. sexguttata.*

Morphological Phylogeny

The phylogeny constructed in PAUP* of the morphology data (Fig. 3) showed no resolution among species, having had very little variation among individuals in the characters that I chose. Bootstrap values were not greater than 50%.

Molecular Phylogenies

Another tree was constructed with the COI sequences from the preliminary phylogeny and a set of newly generated COI sequences from *A. collaris, A. macula, A. sexguttata,* and *A. arcuata* (Fig. 4). Outgroups *Astyleiopis variegatus*, *Astylidius parvus*, *Pseudastylopsis pini, Leptostylopsis planidorsus, Acanthocinus obsoletus,* and *Eupromus ruber* were included in this tree. Each *Astylopsis* species was monophyletic. *Astylopsis collaris, A. sexguttata,* and *A. macula*

all exhibited bootstrap support of 100% and Bayesian posterior probabilities of 1.00. The *A. sexguttata* clade exhibits a sister group relationship with the one *A. arcuata* individual, having bootstrap support of 96% and Bayesian confidence of 1.00. Two barcoded sequences labeled *A. sexguttata* appeared in a separate clade with the outgroup *Astyleiopus variegatus.*

In a more comprehensive dataset including both mitochondrial and nuclear genes, the genus and all species were also monophyletic (Fig. 5). *Astylopsis* exhibited 99% bootstrap support and Bayesian confidence of 1.00. *Astylopsis sexguttata, A. collaris,* and *A. macula* each exhibited 100% bootstrap support and Bayesian confidence of 1.00. The *Astylopsis arcuata* individual formed a clade with *A. sexguttata* in this tree, with 98% bootstrap support and Bayesian confidence of 0.99.

DISCUSSION

Despite the diversity and pest importance of cerambycid beetles, data is still lacking on their evolutionary relationships and host use. This study further delineated this information in the lamiine genus *Astylopsis*, providing a view of morphological and molecular phylogenetic relationships. The morphological data examined showed no pattern among the six *Astylopsis* species, indicating that morphology is very conserved among individuals in each species, however it is not phylogenetically informative.

Examination of partial COI and CAD sequence data was more informative. There was no resolution at the intraspecific level for *Astylopsis sexguttata* in either of the strict consensus trees, which is expected as there are few character differences at this level. This was also supported with p-distance values found in a preliminary tree in that intraspecific nucleotide difference for COI were 2.5% for *A. macula*, 0.5% for *A. collaris*, and 1.4% for *A. sexguttata.* Interspecific variation for these three species was 14-17%, which is consistent with uncorrected pairwise distances measured among cerambycid species in previous studies (Farrell 2001 and Nearns 2013). In the COI tree, two *A. sexguttata* individuals from BOLD formed a clade with *Astyleiopus variegatus*, separate from the rest of the *Astylopsis* members. This is likely due to these individuals having been misidentified and correctly belonging to the outgroup, considering their bootstrap support and Bayesian confidence values of 100 and 1.00, respectively. COI phylogenetic data supported my hypothesis of monophyly for each *Astylopsis* species included (*A. collaris, A. macula, A. sexguttata,* and *A. arcuata*), confirming their current separations. The hypothesis of monophyly for each species, as well as the genus as a whole, was also supported in the combined tree. Monophyletic clades have been resolved in similar studies of cerambycid taxa at the subfamily, tribe, and genus level that are consistent with current separations (Farrell 2001,

Gutiérrez *et al*. 2020, Lee and Lee 2020, Li *et al.* 2016, Nakamine and Takeda 2008, Ohbayashi *et al*. 2009, Souza *et al*. 2020, and Toki and Kubota 2010). Despite varying localities among individuals in the COI phylogeny, no real conclusions could be formed on variation in DNA based on locality.

Both trees also exhibited informative relationships based on host plant use. *Astylopsis sexguttata* and *A. arcuata* are the conifer-feeding members of this genus, which is reflected in the clade that they formed with high bootstrap support and Bayesian posterior probability in both the COI and combined gene trees. Hardwood feeding species *A. collaris* and *A. macula* show earlier divergence than the conifer feeders, indicating hardwood trees represent the more ancestral host selected by *Astylopsis* beetles. Although conifers predate broad-leaved trees evolutionarily, a shift from angiosperms to derived gymnosperm feeding has been found in lamiine cerambycids in the tribe Lamiini (Toki and Kubota 2010), and also within subfamily Cerambycinae (Lee and Lee 2020). This pattern of host use may be more common, as it is also observed in scolytine bark beetles (Kirkandall *et al.* 2015). This host shift is not surprising, as the more ancestral ougroups use both angiosperm and gymnosperm hosts. Derived conifer-feeding *Astylopsis* species also may have acquired special adaptations to overcome conifer properties such as resin and lignin.

Overall, the relationships shown in these phylogenies confirm these four *Astylopsis* species and outgroups as being distinct taxa and provide another example of the host shift from angiosperms to gymnosperms. These results also confirm that our methods will continue to provide meaningful phylogenetic information on the relationships between *Astylopsis* species when incorporating more genes (*Wingless*, arginine kinase, 28S, and EF1-α) and other *Astylopsis* species (*A. perplexa* and *A. fascipennis*).

APPENDIX

APPENDIX

Table 1. Institutions from which *Astylopsis* specimens were borrowed.

Collection, Institution	Location	Acronym	# Loan Specimens	Corresponding Curator
Clemson University Arthropod Collection	Clemson, South Carolina	CUAC	59	Mike Ferro
Florida Department of Agriculture and Consumer Services, Florida State Collection of Arthropods	Gainesville, Florida	FDACS	8	Paul Skelley
Georgia Museum of Natural History, University of Georgia	Athens, Georgia	GMNH	40	E. Richard Hoebeke
Kansas University Biodiversity Institute	Lawrence, Kansas	KUBI	22	Zachary Falin
Albert J. Cook Arthropod Research Collection, Michigan State University	East Lansing, Michigan	MSUC	30	Gary Parsons
Mississippi Entomological Museum, Mississippi State	Mississippi State, Mississippi	MEM	92	Terence Schiefer
Texas A&M University Insect Collection	College Station, Texas	TAMU	163	Karen Wright
University of Texas Biodiversity Center	Austin, Texas	UTBC	4	Alex Wild
Wisconsin Insect Research Collection, University of Wisconsin-Madison	Madison, Wisconsin	WIRC	58	Craig Brabant

Table 2. Intraspecific character matrix for *Astylopsis* species *A. collaris, A. macula, A. sexguttata, A. arcuata, A. perplexa,* and *A. fascipennis.* Characters are ordered as mentioned in the text.

	ID	**1**	**2**	**3**	**4**	**5**	**6**
A. collaris	1	6	2	2	2	1	1
	2	7	2	2	2	1	1
	3	3	2	2	2	1	1
	4	3	2	0	1	1	1
	5	6	2	2	2	1	0
	6	6	2	2	2	1	1
	7	6	2	2	2	1	1
	8	4	2	2	2	1	0
	9	5	2	2	2	1	0
	10	4	1	2	2	1	1
A. macula	11	4	2	2	2	2	1
	12	3	2	2	2	1	1
	13	3	2	2	2	1	1
	14	2	2	2	1	1	1
	15	3	2	1	2	1	1
	16	4	2	2	1	0	0
	17	3	2	2	1	1	1
	18	0	2	2	1	1	0
	19	4	2	1	0	0	1
	20	6	2	2	1	1	1
A. sexguttata	21	6	2	2	2	0	1
	22	4	1	2	2	1	1
	23	4	2	2	2	1	1
	24	7	2	2	2	1	1
	25	6	2	2	2	0	1
	26	3	2	2	2	1	1
	27	1	1	2	2	1	1
	28	3	2	2	1	1	2
	29	6	1	2	2	1	1
	30	3	2	2	2	1	1
A. arcuata	31	3	0	2	2	1	1
	32	4	2	2	2	1	1
	33	5	0	2	2	1	1
	34	4	2	2	2	1	1

Table 2. (cont.)

	35	2	2	2	2	1	1
	36	7	2	1	1	1	1
	37	3	2	2	2	1	1
	38	3	2	2	2	2	1
	39	5	0	2	2	1	1
	40	4	2	2	2	1	1
A. perplexa	41	1	1	2	1	2	2
	42	7	2	2	2	2	1
	43	6	2	2	2	2	1
	44	1	1	2	2	2	2
	45	3	2	2	2	1	1
	46	4	1	2	2	1	1
	47	6	1	2	2	2	1
	48	4	1	2	2	1	1
A. fascipennis	49	3	2	1	2	0	1
	50	3	2	2	2	1	1
	51	6	2	2	2	1	1
	52	3	2	2	2	1	1
	53	6	2	2	2	0	1
	54	6	2	2	2	1	1
	55	6	1	2	2	1	1
	56	6	2	2	2	1	1
	57	1	1	2	2	1	0
	58	6	2	2	2	1	1

Table 3. Forward and reverse primers used for DNA sequencing of *Astylopsis* and outgroup beetles.

Gene	Primer	Sequence	Source
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Hebert *et al.* 2003
	HCO2198	TAAACTTCAGGGTGACCAAAAATCA	Hebert *et al.* 2003
CAD	CD338F	ATGAARTAYGGYAATCGTGGHCAYAA	Moulton and Wiegmann 2004
	CD668R	ACGACTTCATAYTCNACYTCYTTCCA	Wild and Maddison 2008

Table 4. Thermal cycler parameters for PCR of each gene. All temperatures are Celsius and all time is displayed as minutes : seconds. PCR was performed using tissue samples from specimens preserved in ethanol or dried specimens collected within the previous 20 years. The amplified PCR products were obtained in order to be sequenced and compared among species.

Gene	Initial Denaturation	Denaturation	Annealing	Elongation	Final Elongation	Hold
COI	95	94	50	72	72	12
	15:00	0:30	1:00	1:30	5:00	∞
		[	37 cycles	]		
CAD	94	94	50	72	72	12
	2:00	0:30	1:00	1:30	5:00	∞
		[	37 cycles	]		

Table 5. Sequence identifiers for sequences sourced from Barcode of Life Data System (BOLD). Sequence label corresponds with labels in Figure 2.

Sequence Label (as appears in Fig. 2)	BOLD Sequence ID
Astylopsis sexguttata1	BBCCA3340-12
Astylopsis sexguttata2	CERLF444-08
Astylopsis collaris1	CERLF432-08
Astylopsis collaris2	CERLF433-08
Astylopsis collaris3	CERLF435-08
Astylopsis collaris4	CERLF434-08
Astylopsis macula1	CERLF445-08
Astylopsis macula2	CERLF446-08
Astylopsis macula3	CERLF447-08
Astylopsis macula4	CERLF448-08
Astylopsis macula5	CERLF623-08
Astylopsis macula6	CERLF624-08
Astylopsis macula7	CERLF625-08
Astylopsis macula8	CERLF626-08
Astylopsis macula9	CNLMF980-14 KR123196
Astylopsis macula10	CNLMP2009-14 KR127106
Astylopsis macula11	CNLMR092-14 KR126679
Astylopsis macula12	CNPEP1441-14 KR130747
Astylopsis macula13	CNPER012-14 KR124596
Astylopsis	CNROS762-13 KR129993
Astylopsis macula14	CNSLI559-12 KM844100
Astylopsis macula15	CNTIE051-15 MF638667
Astylopsis macula16	OPPQE395-17
Astylopsis macula17	OPPQE399-17

Table 5. (cont.)

Astylopsis macula18	OPPQE400-17
Astylopsis macula19	OPPQI188-17
Astylopsis macula20	CERGL009-08
Astylopsis macula21	CERGL010-08
Astylopsis macula22	CNSLP1104-13 KM850268

Table 6. Interspecific and intraspecific p-distances between species in the preliminary phylogeny. P-distance measures represent percent difference between nucleotide sites. P-distance measures between sets of two individuals were calculated in PAUP* and then averaged to obtain the overall percent distance between nucleotide sites between the two species being compared.

Species 1	Species 2	P-Distance (%)
A. macula	*A. collaris*	14
A. macula	*A. sexguttata*	17
A. collaris	*A. sexguttata*	17
A. macula (clade 1)	*A. macula* (clade 2)	4.3
A. macula	*A. macula*	2.5
A. collaris	*A. collaris*	0.5
A. sexguttata	*A. sexguttata*	1.4

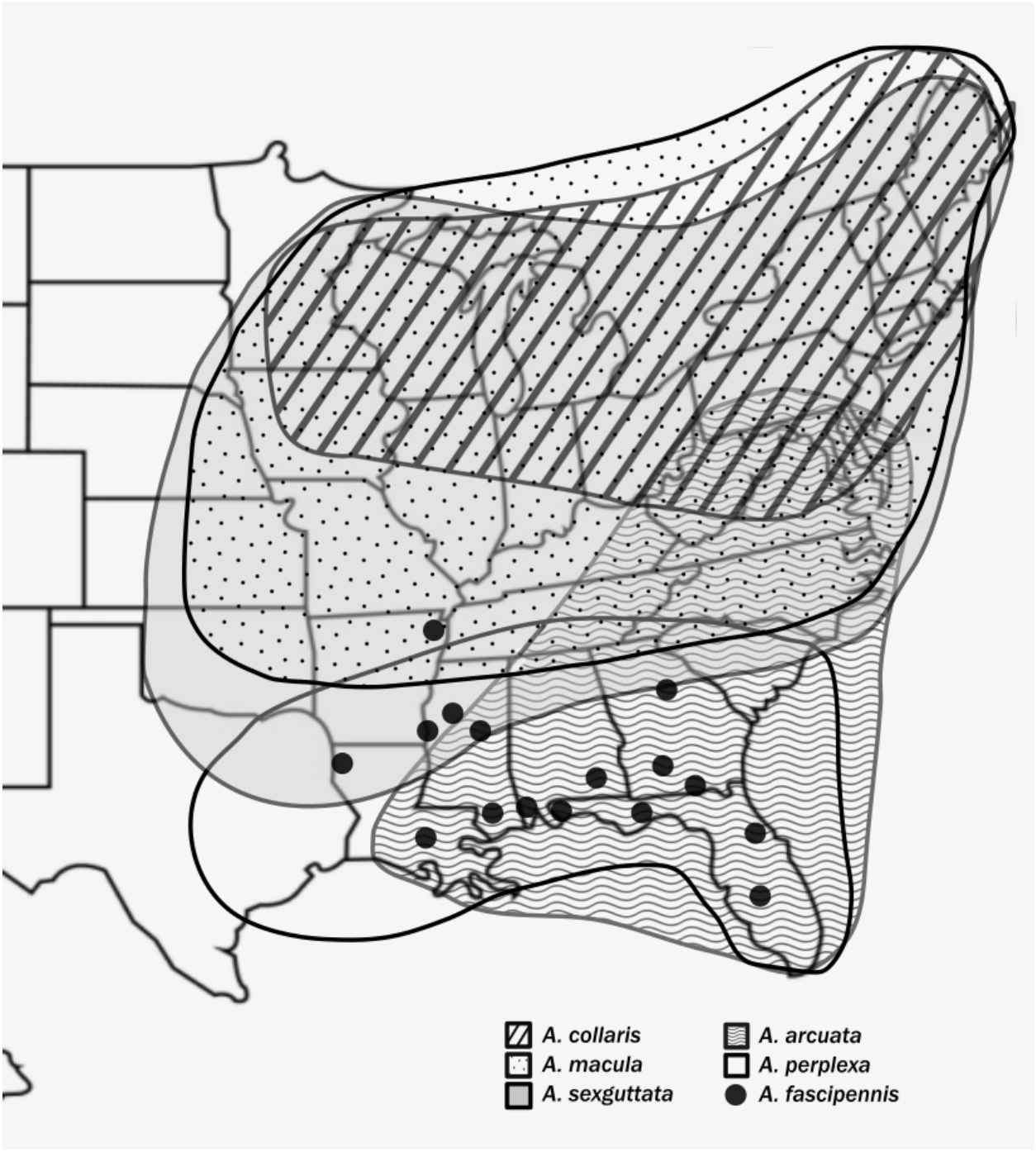

Figure 1. Map of the eastern United States depicting known geographic ranges of each *Astylopsis* species (Dillon 1956a, Linsley and Chemsak 1995, and Monné and Nearns 2020).

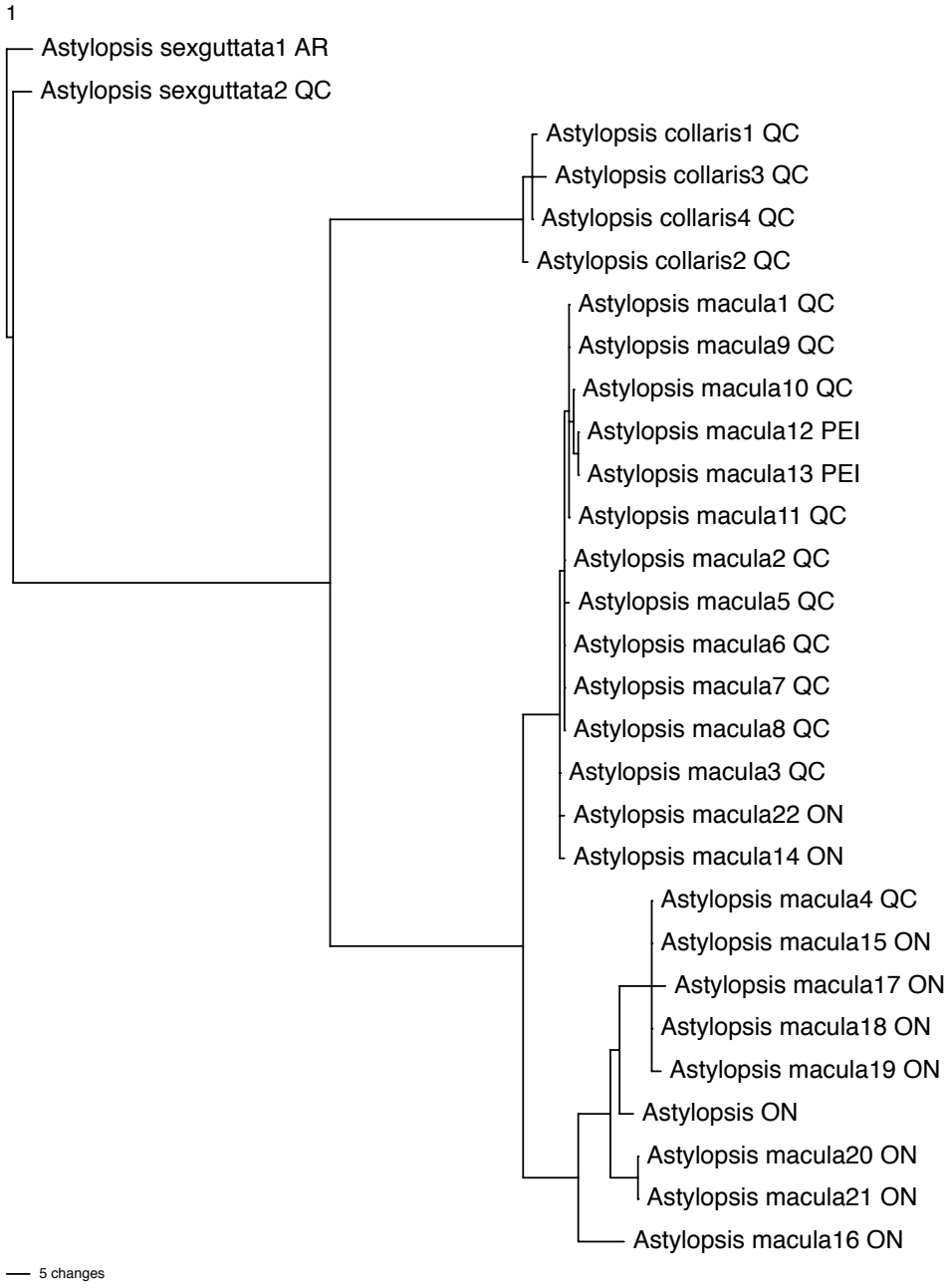

Figure 2. Preliminary phylogenetic tree using barcoded COI sequences for *Astylopsis sexguttata, A. collaris,* and *A. macula,* constructed in PAUP* using parsimony methods. Individuals are labeled with their species, serial numbers to differentiate individuals, and locality. Locality abbreviations: AR Arkansas, USA; QC Quebec, Canada; PEI Prince Edward Island, Canada; ON Ontario, Canada.

Figure 3. Phylogeny constructed from morphological data from 58 individuals (ten individuals each of *Astylopsis collaris, A. macula, A. sexguttata, A. arcuata,* and *A. fascipennis*, and eight *Astylopsis perplexa* individuals). This tree was constructed in PAUP* using a heuristic search with parsimony methods.

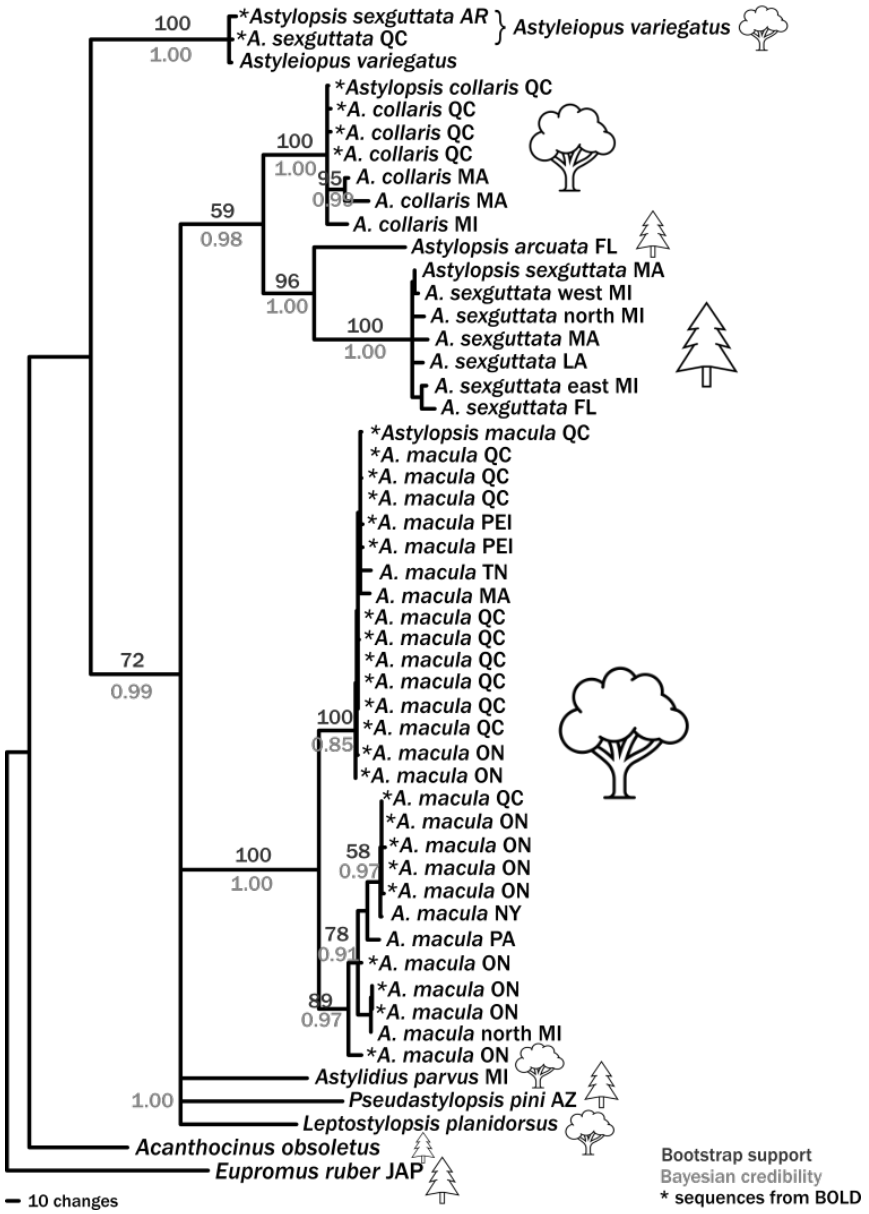

Figure 4. Phylogeny of *A. collaris, A. macula, A. sexguttata,* and *A. arcuata* individuals using barcoded COI sequences, indicated by an asterisk, and experimental COI sequences for the species. This tree was constructed in PAUP* using a heuristic search with parsimony methods. *Eupromus ruber, Acanthocinus obsoletus, Leptostylopsis planidorsus, Pseudastylopsis pini, Astylidius parvus,* and *Astyleiopus variegatus* were included as outgroups, rooted with *E. ruber.* Darker values above branch lines indicate bootstrap support for those relationships; lighter grey values below lines indicate Bayesian posterior probabilities. Tree icons indicate whether the species uses broad-leaved or coniferous hosts. Clades without bootstrap values were not found in the strict consensus of all parsimonious trees. Locality abbreviations: AR Arkansas, USA; QC Quebec, Canada; MA Massachusetts, USA; MI Michigan, USA; FL Florida, USA; LA Louisiana, USA; PEI Prince Edward Island, Canada; TN Tennessee, USA; ON Ontario, Canada; NY New York, USA; PA Pennsylvania, USA; AZ Arizona, USA; JAP Japan.

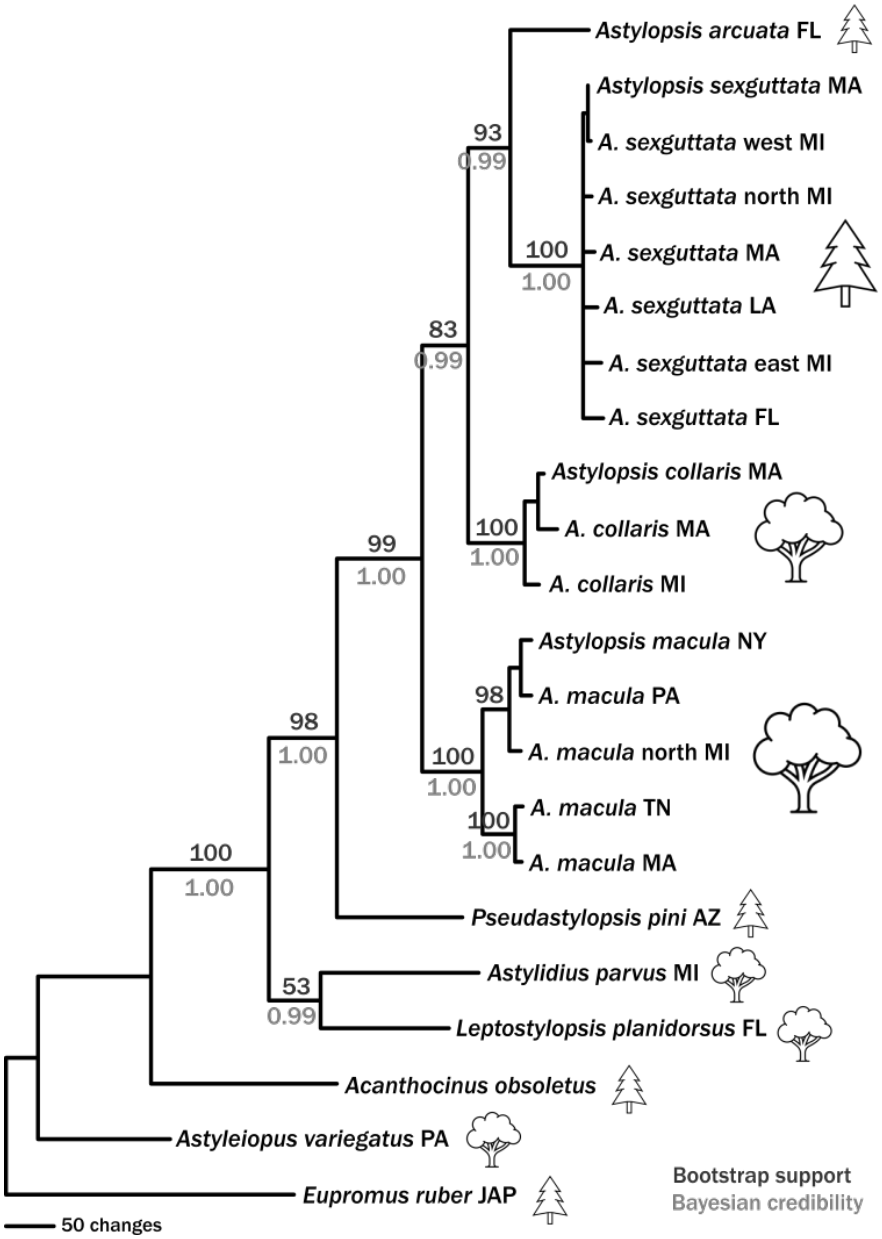

Figure 5. Phylogenetic tree visualization of *A. collaris, A. macula, A. sexguttata,* and *A. arcuata* individuals using experimental sequences of genes COI and CAD, represented by one of three parsimonious trees found in a heuristic search. *Eupromus ruber, Astyleiopus variegatus, Acanthocinus obsoletus, Leptostylopsis planidorsus, Astylidius parvus,* and *Pseudastylopsis pini* were included as outgroups, rooted with *E. ruber* Darker values above branch lines indicate bootstrap support for those relationships; lighter grey values below lines indicate Bayesian posterior probabilities. Tree icons indicate whether the species uses broad-leaved or coniferous hosts. Clades without bootstrap values were not found in the strict consensus of the three trees. Locality abbreviations: FL Florida, USA; MA Massachusetts, USA; MI Michigan, USA; LA Louisiana, USA; NY New York, USA; PA Pennsylvania, USA; TN Tennessee, USA; AZ Arizona, USA; JAP Japan.

REFERENCES

REFERENCES

Cocquempot, C. and A. Lindelöw. 2010. Longhorn beetles (Coleoptera, Cerambycidae) Chapter 8.1. In: Roques A *et al*. (Eds) Alien terrestrial arthropods of Europe. BioRisk*,* 4(1): 193–218.

Dillon, L. S. 1956*a*. The Nearctic components of the tribe Acanthocinini (Coleoptera: Cerambycidae). Part I. Annals of the Entomological Society of America 49:134–167.

Farrell, B. D. 2001. Evolutionary assembly of the milkweed fauna: Cytochrome oxidase I and the age of *Tetraopes* beetles. Molecular Phylogenetics and Evolution 18(3):467-578.

Gutiérrez, N., F. A. Noguera, S. Zaragoza-Caballero, and J. J. Morrone. 2020. Phylogenetic revision of the floricolous longhorn beetle genus *Strangalidium* Giesbert (Coleoptera: Cerambycidae). Systematics and Biodiversity 18(1):79-101.

Haack, R. A., M. A. Keena, and D. Eyre. 2017. Life history and population dynamics of cerambycids. In: Wang Q (ed) Cerambycidae of the world—biology and pest management. CRC Press, Boca Raton, 71–103.

Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. deWaard. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B 270:313–321

Huelsenbeck, J. P. and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17(8), 754–755.

Kariyanna, B., M. Mohan, and R. Gupta. 2017. Biology, ecology, and significance of longhorn beetles (Coleoptera: Cerambycidae). Journal of Entomology and Zoology Studies 5(4):1207-1212.

Kirkendall, L. R., P. H. W. Biedermann, and B. H. Jordal. 2015. Chapter 3. Evolution and diversity of bark and ambrosia beetles [pp. 85-156]. *In:* Bark Beetles (F. E. Vega and R. W. Hofstetter, editors). Academic Press.

Lee, S. and S. Lee. 2020. Multigene phylogeny uncovers oviposition-related evolutionary history of Cerambycinae (Coleoptera: Cerambycidae). Molecular Phylogenetics and Evolution 145 (2020) 106707.

Li, W., X. Yang, L. Quian, Y. An, and J. Fang. 2016. The complete mitochondrial genome of the citrus long-horned beetle, *Anoplophora chinensis* (Coleoptera: Cerambycidae). Mitochondrial DNA Part A 27(6):4665-4667.

Linsley, E. G. 1959. Ecology of Cerambycidae. Annual Review of Entomology 4:99-138.

Linsley, E. G., and J. A. Chemsak. 1995. The Cerambycidae of North America, Part VII, No. 2: Taxonomy and classification of the subfamily Lamiinae, tribes Acanthocinini through Hemilophini. University of California Publications in Entomology, Vol. 114. University of California Press, Berkeley, xii-292.

Monné, M. A. and E. H. Nearns. 2020. Catalogue of the Cerambycidae (Coleoptera) of Canada and United States of America. Part IV. Subfamily Lamiinae. Available from https://cerambycids.com/catalog/.

Moulton J. K. and B.M. Wiegmann. 2004. Evolution and phylogenetic utility of CAD (rudimentary) among Mesozoic-aged Eremoneuran Diptera (Insecta). Molecular Phylogenetics and Evolution 31(1):363-78.

Nakamine, H. and M. Takeda. 2008. Molecular phylogenetic relationships of flightless beetles belonging to the genus *Mesechthistatus* Breuning, (Coleoptera: Cerambycidae) inferred from mitochondrial COI gene sequences. Journal of Insect Science 8(70).

Nearns, E. H. 2013. Systematics of longhorned beetles (Insecta: Coleoptera: Cerambycidae). [Doctoral dissertation, University of New Mexico]. UNM Digital Repository.

Ohbayashi, N., J. Ogawa, and Z. Su. 2009. Phylogenetic analysis of the lamiine genus *Anoplophora* and its relatives (Coleoptera: Cerambycidae) based on the mitochondrial COI gene. Special Bulletin of the Japanese Society of Coleopterology 7:309-324.

Rabaglia, R. J., A. I. Cognato, E. R. Hoebeke, C. Johnson, J. R. Labonte, M. E. Carter, and J. Vlach. 2019. Early detection and rapid response: A 10-year summary of the USDA Forest Service program of surveillance for non-native bark and ambrosia beetles. American Entomologist 65(1), 29–42.

Ratnasingham, S. and P. D. N Hebert. 2007. BARCODING: Bold: The Barcode of Life Data System (http://www.barcodinglife.org): BARCODING. Molecular Ecology Notes 7(3), 355–364.

Ridgway, R. 1912. *Color standards and color nomenclature*. Washington, D. C: The author.

Shiefer, T. L. 2000. A new species of ASTYLOPSIS Casey (Coleoptera: Cerambycidae: Acanthocinini) from the Southeastern United States. The Coleopterists Bulletin 54(4):533-539.

Souza, D. d. S., L. Marinoni, M. L. Monné, and J. Gómez-Zurita. 2020. Molecular phylogenetic assessment of the tribal classification of Lamiinae (Coleoptera: Cerambycidae). Molecular Phylogenetics and Evolution 145 (2020) 106736.

Swofford, D. L. 2001. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.

Toki, W. and K. Kubota. 2010. Molecular phylogeny based on mitochondrial genes and evolution of host plant use in the long-horned beetle tribe Lamiini (Coleoptera: Cerambycidae) in Japan. Molecular Ecology and Evolution 39(4).

Wild, A. L. and D. R. Maddison. 2008. Evaluating nuclear protein-coding genes for phylogenetic utility in beetles. Molecular Phylogenetics and Evolution 48:877–891.

Wu, Y., N. F. Trepanowski, J. J. Molongowski, P. F. Reagel, S. W. Lingafelter, H. Nadel, S. W. Myers, and A. M. Ray. 2017. Identification of wood-boring beetles (Cerambycidae and Buprestidae) intercepted in trade-associated solid wood packaging material using DNA barcoding and morphology. Scientific Reports 7:40316.