A PRELIMINARY PHYLOGENOMIC ANALYSIS OF ADESMIINI (COLEOPTERA: TENEBRIONIDAE) AND STUDY OF PIMELIINAE HEAT SHOCK PROTEIN FUNCTIONAL GENOMICS

by

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I would like to dedicate this to my friends and family who have supported me from day one.

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ABSTRACT

Deserts, such as the Namib, Sonoran, and Saharan, are regions that are unsuitable habitats for many organisms. However, darkling beetles (Tenebrionidae), specifically Pimeliinae, have appeared to flourish in otherwise inhospitable environments. All organisms have heat shock proteins protecting cellular components from degradation due to environmental stress such as desert heat. Modifications to heat shock proteins may provide more efficient cellular protection allowing desert-dwelling beetles to survive in regions where few other organisms are found. I performed a study which analyzed heat shock protein 40 (Hsp40) homologs across Pimeliinae by using targeted enrichment and high-throughput sequencing of seven genetic loci from 142 taxa (25 tribes) to examine protein functionality and evolution. I determined that the critical J domain of Hsp40 is conserved across pimeliine taxa. Additionally, there were a variety of cysteine shifts within different pimeliine tribes throughout six of the seven homologs, indicating possible protein structure alterations. Maximum likelihood analyses of Hsp40 homologs determined that despite the relationships between the tribes shuffling, taxa remained within their respective tribes. In an effort to examine how Hsp40s may have evolved alongside other behaviors and life histories, the tribe Adesmiini (Tenebrionidae: Pimeliinae) was examined in detail.

Adesmiines thrive in the arid regions of sub-Saharan Africa, northern Africa, and the Palearctic. Despite having a few genera (i.e., *Onymacris*) which have been the subject of extensive life history analyses, Adesmiini has undergone few modern taxonomic studies. As a result, Adesmiini is a good candidate for phylogenetic investigation. To investigate evolutionary relationships, 510 targeted loci across 47 specimens (41 species, 10 genera) were used to produce a well-supported phylogeny. Current generic concepts were not in agreement with the resulting topology. In addition to producing a molecular phylogeny, two adesmiine traits of interest, activity time (diurnal/nocturnal) and substrate usage (psammophily), were also examined. Since Adesmiini is a predominantly diurnal tribe within a primarily nocturnal family, the activity time was mapped to the topology. From this study's tree, it was determined that there were at least three shifts from diurnal to nocturnal throughout Adesmiini. Several charismatic adesmiines occur on the dunes of southern Africa, so the shift to inhabiting sand hills (psammophily) devoid of vegetation was also investigated. Psammophily was determined to have arisen multiple times within Adesmiini, and the topology revealed no clear indication to a single radiation of adesmiine substrate usage. Finally,

a study was performed on Adesmiini using the same seven Hsp40 homologs as in the pimeliine functional genomics investigation. The resulting phylogenies indicate a correlation between Hsp40 modification and diurnal, psammophilous adesmiines.

CHAPTER 1. A PRELIMINARY PHYLOGENOMIC ANALYSIS OF ADESMIINI (COLEOPTERA: TENEBRIONIDAE)

1.1 Abstract

The darkling beetle tribe Adesmiini (Coleoptera: Tenebrionidae) includes over 200 species in 11 genera, primarily occurring in sub-Saharan southern Africa and the Palearctic region. However, generic diversity is much higher in southern Africa (all genera present) versus the Palearctic (only *Adesmia* represented). Adesmiini contains many conspicuous diurnal species, including the fog-basking beetle *Onymacris unguicularis*, a focal taxon for ecological research for decades. Despite interest in the tribe, there has been little phylogenetic research on Adesmiini, leaving generic concepts unexamined. In this study, the evolutionary history of Adesmiini was reconstructed using targeted enrichment plus high-throughput sequencing of 510 protein-coding genes to assess adesmiine generic taxonomy. Taxonomic and behavioral relationships between the current genera are discussed in the context of the resulting phylogeny.

1.2 Introduction

Adesmiini Lacordaire, 1859 (Tenebrionidae: Pimeliinae) is a charismatic tribe within the ecologically and morphologically diverse family Tenebrionidae, which currently contains over 20,000 species (Matthews et al., 2010). Adesmiini species occur across the arid and semi-arid regions of Africa and the Palearctic. However, only the genus *Adesmia* Fischer von Waldheim, 1822 occurs outside of sub-Saharan Africa. A few key characteristics of Adesmiini have made them the topic of ecological studies for decades. Many are diurnal and substrate specific (Penrith, 1979; Penrith, 1986; Lamb and Bond, 2013) and two species of *Onymacris* Allard, 1885 exhibit a behavior known as fog-basking to collect water in an otherwise arid landscape (Hamilton & Seely, 1976; Hauffe et al., 1988; Holm & Edney, 1973; Lamb & Bond, 2013; Mitchell et al., 2020; Nørgaard & Dacke, 2010). Despite interest in the ecology and behavior of Adesmiini taxa, the generic classification within the tribe has been poorly explored within a phylogenetic context (Penrith 1986; Lamb and Bond, 2013).

1.2.1 Taxonomy background

The tribe Adesmiini was originally described by Lacordaire in 1859 for the genera *Adesmia*, *Stenocara* Solier 1835, and *Metriopus* Solier, 1835 (Lacordaire, 1859). The genera *Alogenius* Gebien 1910, *Epiphysa* Blanchard 1845, *Eustolopus* Gebien 1938, *Onymacris*, and *Stenodesia* Reitter 1916 were added by various authors over the next ~100 years. In two papers, Koch (1944; 1948) described 55 African species across 10 genera and separated *Renatiella* Koch 1944 from *Adesmia* as its own genus (Koch 1944). The last major changes to the tribe were made when Penrith produced a series of publications (1979; 1984; 1986) focused on the southwestern African fauna.

Penrith's works involved critical examinations of morphology within the tribe, including the first cladistic analysis of Adesmiini genera, as well as a review of habitat information and biogeography. Within Penrith's revision of the tribe (1979), she moved the subgenus *Orientacara* Koch 1952 from *Stenodesia* to *Metriopus*. The genera *Ceradesmia* Gebien 1920 and *Coeladesmia* Reitter 1916, two *Stenodesia* species, and one *Stenocara* species were merged with *Metriopus*, the first two forming new subgenera. In Penrith's cladistic analysis of Adesmiini (1986) she made several more taxonomic changes, *Orientacara* was elevated from a subgenus in *Metriopus* to its own monotypic genus, while exploring generic relations and providing biogeographic hypotheses for the tribe. In Penrith (1986) the genera *Cauricara* Penrith 1979 and *Arenacara* Penrith 1979 were reinterpreted as subgenera of the widely distributed genus *Stenocara*. *Physosterna* Solier, 1837 was reduced to a subgenus of *Adesmia*, making it the fourth *Adesmia* subgenus in southern Africa. Overall, Penrith recognized 88 species (107 subspecies) 11 genera (1979; 1986) from sub-Saharan Africa, which are still considered valid today.

Penrith did not revise the Palearctic or north-central African members of the genus *Adesmia* in her work, a group that includes 59 species (117 subspecies) and eight subgenera not found in southern Africa. While Penrith hypothesized that the center of origin for the tribe was in the western area of southern Africa due to the number of genera present there (ten out of eleven), this hypothesis was not tested and the relationship of the northern *Adesmia* subgenera to the rest of the tribe was unclear.

1.2.2 Phylogenetic background

There has been some discrepancy in proposed sister tribes to Adesmiini. The sister tribe was thought to be Tentyriini Eschscholtz, 1831 (Penrith 1986) based on the enlarged mentum shared by both tribes. However, Erodiini Billberg, 1820, Asidini Fleming, 1821, Adelostomini Solier, 1834, and Zophosini Solier, 1834 all also share this character. Penrith considered Tentyriini to be sister to Adesmiini based on the presence of a mandibular process in the adesmiine genera *Alogenius* and *Epiphysa* and some Tentyriini species. These two adesmiine genera are also nocturnal, as are most Tentyriini. Based on their nocturnal behavior, unlike most Adesmiini taxa, and the presence of a mandibular process, Penrith hypothesized that *Alogenius* and *Epiphysa* were basal lineages within Adesmiini and supported the tribe's close relationship to Tentyriini (Penrith 1986).

More recently, in a study of the Zophosini, Steckel et al. (2013) proposed that Adesmiini was sister to Zophosini and Adelostomini based on a combined phylogenetic analysis of morphological and molecular (971 bp) data. However, this study only included individuals from Zophosini, Adelostomini, and Adesmiini, with *Pimelia* as the outgroup. To their credit, the main goal of Steckel et al. (2013) was to explore relationships within Zophosini and test the monophyly of this monogeneric tribe, with tribal relationships of the Namib Desert darkling beetles only mentioned briefly.

Cladistic analysis of generic relationships within Adesmiini by Penrith (1986) was based on 19 synapomorphic and apomorphic characters coded at genus level. Penrith mentions the difficulty in finding characters to separate Tentyriini and Adesmiini. The limited discriminating morphological traits were treated as catch-alls for genera despite species level morphology discrepancies, and Penrith admitted that the cladogram may not be stable for determining relationships between lineages. Despite this, Penrith's tribal level study of generic relationships was ahead of its time and provided the framework for further study of the tribe. Penrith's cladistic analysis supported *Alogenius* and *Epiphysa* as the earliest diverging lineages within Adesmiini, with *Onymacris*, *Eustolopus*, and *Physadesmia* Penrith, 1979 forming a clade sister to the remaining genera. The remaining genera formed the group *Renatiella* + (*Adesmia* + (*Stenocara* + (*Metriopus* + (*Orientacara* + *Stenodesia*))) as seen in Figure 1.1 (Penrith 1986). A significant issue with Penrith's analysis is that characters were coded at the generic level and do not allow for testing of whether the genera themselves are monophyletic. This is especially important as the

generic composition of the tribe has changed over time and several genera (*Adesmia*, *Alogenius*, *Metriopus*, and *Stenocara*) are further divided into subgenera.

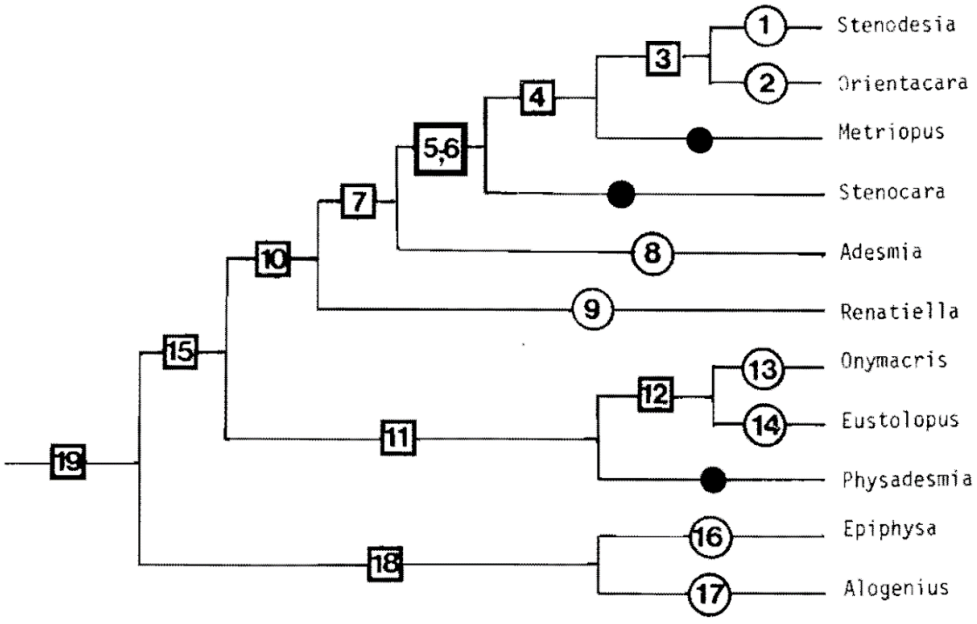

Figure 1.1. Cladogram from Penrith 1986 showing the relationships of the Adesmiini genera. Numbers on branches indicate the number of autapomorphic or synapomorphic traits identified for a clade.

Relationships within Adesmiini genera have rarely been examined, and primarily focus on the genus *Onymacris*. Penrith (1984) used 23 autapomorphic and synapomorphic traits to determine *Onymacris* species relationships. *Onymacris* species with white or black elytral coloration were mainly separated into two color-defined clades, with the exception of the black species *O. unguicularis* being sister to a white *Onymacris* clade. *Onymacris* was a monophyletic genus most closely related to *Eustolopus* and *Physadesmia*, in that order. Penrith also stated that *Onymacris* shares both enlarged and unequal tarsal claws and elongated tibial spurs with *Eustolopus*. Neither characteristic is seen in other adesmiine genera, hence Penrith (1979) had already hypothesized that they were closely related to each other. Lamb and Bond (2013) later reconstructed a molecular phylogeny for *Onymacris* and compared it to Penrith's cladogram. Lamb and Bond used six genetic loci, three mitochondrial and three nuclear, to determine the relationships of 12 of the 14 *Onymacris* species, 2 *Physadesmia* species, and one representative each of *Eustolopus*, *Renatiella*, *Physosterna*, *Stenocara*, and *Epiphysa*. The Adelostomini genus *Stips* Koch 1950 was used to root the phylogeny. They determined that while white and black

Onymacris lineages were monophyletic, the genus itself is not (Lamb and Bond 2013). Both results contradict Penrith (1986). In the phylogeny of Lamb and Bond (2013), *Physadesmia* was sister to the white *Onymacris* clade, with *Eustolopus* was sister to *Onymacris* and *Physadesmia globosa*. This follows Penrith's (1986) predictions of the three genera being closely related, based on Penrith's observation that all three genera are composed of psammophilous, long-legged, cursorial (running) species (Penrith 1979; 1986).

Although the Palearctic region contains 59 species (119 subspecies) of *Adesmia*, Palearctic *Adesmia* taxonomy has similarly had little attention. The only phylogenetic analysis of the group, Mas-Peinado (2015), looked at *Adesmia (Macradesmia) cancellata* Solier, 1835 haplotypes to determine species polymorphism and diversification. This study also provided insight into *Adesmia* and determined that the genus was paraphyletic, with northern African taxa grouping with southern African *Onymacris* species. Despite this, there has been no large-scale study of Palearctic Adesmiini taxonomy.

Despite decades of interest surrounding this Afrotropical and Palearctic tribe, no study has been performed using modern phylogenetic methods to assess the relationships between adesmiine genera, and thus help inform taxonomic decisions within Adesmiini. Understanding the evolutionary history of adesmiines is important as: 1. They are a subject of research interest for many researchers outside of systematics (ex. ecologists and engineers working on biomimicry); 2. The current genera may be based on characters heavily influenced by species ecology (ex. longer legs and eyes associated with diurnal life histories); and 3. A molecular phylogeny allows us to explore relationships without focusing on potentially plastic morphology to refine generic definitions and produce a stable taxonomy for future research. In this study, I analyzed 510 low-copy nuclear protein coding loci from 10 of the 11 Adesmiini genera (41 species, 47 operational taxonomic units (OTU)), excluding the monotypic genus *Orientacara*, to reconstruct evolutionary relationships within the tribe.

1.3 Methods

For this project, I used a combination of data previously collected by the Smith lab and newly collected data. All specimens were collected by members of the Smith Insect Biodiversity Lab (SIBL) or its collaborators. DNA extraction was performed on 60 Adesmiini specimens by disarticulating the head from the body and, in most cases, the coxa from the thorax for soft tissue

digestion. Large specimens (~15mm or longer in length) also had their thoracic cavity scraped for additional muscle tissue. Tissue digestion and DNA extraction were conducted using QIAGEN DNEasy Blood and Tissue kits (Qiagen). An Invitrogen Qubit dsDNA assay was used to determine the DNA concentration in extractions and those with a DNA mass of over 1,000 ng were suitable for sequencing. Lower concentrations were sent for sequencing to include representatives of taxa for which there were few specimens or higher concentrations were unattainable, resulting in 50 adesmiine extractions sequenced. Adesmiine samples, except for *Stenocara gracilis* and the outgroup taxa, were then sent to Arbor Biosciences for library preparation, targeted enrichment using custom MyBaits probes designed to capture 631 genetic loci from Pimeliinae, and DNA sequencing on a NovaSeq 6000 system for 150 bp paired end reads. Targeted enrichment probe design was described in Kanda (2017). Library preparation and targeted enrichment were done inhouse for Stenocara gracilis and outgroup species using NEBNext® Ultra™ DNA Library Prep Kits for Illumina and the same MyBaits custom probe kit was used to capture 631 loci. Inhouse libraries were sequenced at the University of Arizona Genomic and Technology Core Facility (UAGC) on an Illumina NextSeq 550 using 150bp paired end runs.

Read quality was assessed using FastQC v.0.11.9 (Andrews, 2010). Reads with an average sequence quality below 20, using sliding window approach, were removed from further analyses with Trimmomatic (Bolger et al., 2014). I mapped the reads using BWA and assembled them using SPAdes v.3.15.2 (Prjibelski et al., 2020) within the HybPiper v.1.3.1 (Johnson et al., 2016) bioinformatic pipeline using the bait probe markers for ortholog annotation. This pipeline was also used to extract introns using the intronerate.py script provided by HybPiper. Since the probeset was based on single copy nuclear protein coding genes, only exons were expected to be found. The retrieve_sequences.py script was then used to pull the supercontig sequences. Possible paralogs were reported with paralog_investigator.py script within the HybPiper pipeline. The pipeline was dependent on Biopython (Cock et al., 2009). I then aligned each of the loci by translating the nucleotide sequences to amino acids, aligned using MAFFT with the L-INS-I algorithm (Katoh et al., 2005) and then mapped the nucleotide sequences back to the aligned peptide sequences. Low-quality nucleotide sequence sites were masked using Gblocks while retaining codon positions (Castresana, 2002) and sequences with over 50% gaps per locus were trimmed out using trimAl (Capella-Gutiérrez et al., 2009). Amino acid sequences had both low yield sites and taxa trimmed for each locus using trimAl with the same parameters as nucleotide

sequences. Sequences were then concatenated by taxon using FASConCAT (Kuck, 2009) into one partitioned dataset. Low yielding loci (those with under eight taxa in the concatenated dataset) were manually discarded. After the trimming and discarding steps, a dataset of 510 loci spanning 170,710 amino acids or 512,130 bp for 47 OTUs was used for phylogenetic analyses.

ModelFinder (Kalyaanamoorthy et al., 2017), as implemented in IQ-TREE 2 (Minh, Schmidt, et al., 2020) was used to infer optimal substitution models for the dataset partitioned by locus as shown in Table 1.1. IQ-TREE 2 was then used to run maximum likelihood analyses using an edge-unlinked partition model (-sp), with the dataset partitioned by loci and the models for each locus applied from ModelFinder. Support for the resulting topology was assessed using 10,000 UltraFast Bootstrap (Hoang et al., 2018) iterations. IQ-TREE 2 was also used to infer 510 gene trees and reassess model selection for each. The 510 gene tree files were then input into ASTRAL-III v.5.7.7 (Zhang et al. 2018) to perform gene coalescence analyses. ASTRAL provided branch support as local posterior probabilities (PP) and the branch lengths correspond to coalescent units. From the output log, the final normalized quartet score, which is the proportion of loci tree quartets satisfied by the given species tree (Sayyari et al., 2016) for the coalescent analysis is 0.669. The closer the final normalized quartet score is to 1, the less discordant the gene trees are. The final optimization score is 57533861 quartets satisfying the species tree out of 86033982 possible gene tree quartets. All sequence assembly and phylogenetic analysis was performed on Purdue University's community cluster, Bell, within ITaP Research Computing (McCartney et al., 2014).

Table 1.1. A list of the partitioned substitution models used for the maximum likelihood analyses shown in Figure 1.2 as determined by ModelFinder in IQTREE 2. Ortholog numbers indicate genetic loci based on the *Tribolium* genome.

Model	**Ortholog**
Blosum62	ORTHOMCL4739, ORTHOMCL9604
cpREV	ORTHOMCL1818, ORTHOMCL3732, ORTHOMCL5026, ORTHOMCL5631, ORTHOMCL6797, ORTHOMCL7760, ORTHOMCL7822, ORTHOMCL7835, ORTHOMCL8075, ORTHOMCL8639, ORTHOMCL8918, ORTHOMCL8967, ORTHOMCL9062
Dayhoff	ORTHOMCL2656
DCMut	ORTHOMCL3337
FLU	ORTHOMCL2061, ORTHOMCL2348, ORTHOMCL3474, ORTHOMCL3486, ORTHOMCL3926, ORTHOMCL3937, ORTHOMCL4050, ORTHOMCL4272, ORTHOMCL4590, ORTHOMCL4769, ORTHOMCL4774, ORTHOMCL4808, ORTHOMCL4886, ORTHOMCL5051, ORTHOMCL5209, ORTHOMCL5434, ORTHOMCL5714, ORTHOMCL5824, ORTHOMCL5978, ORTHOMCL6008, ORTHOMCL6526, ORTHOMCL6527, ORTHOMCL6563, ORTHOMCL6804, ORTHOMCL7264, ORTHOMCL7404, ORTHOMCL7651, ORTHOMCL7790, ORTHOMCL7839, ORTHOMCL7853, ORTHOMCL7935, ORTHOMCL8001, ORTHOMCL8390, ORTHOMCL8429, ORTHOMCL8883
HIVb	ORTHOMCL1283, ORTHOMCL1583, ORTHOMCL2184, ORTHOMCL2567, ORTHOMCL3090, ORTHOMCL3175, ORTHOMCL3425, ORTHOMCL3499, ORTHOMCL3553, ORTHOMCL3679, ORTHOMCL3864, ORTHOMCL4168, ORTHOMCL4311, ORTHOMCL4557, ORTHOMCL4747, ORTHOMCL5112, ORTHOMCL5156, ORTHOMCL5170, ORTHOMCL5572, ORTHOMCL5784, ORTHOMCL5914, ORTHOMCL5988, ORTHOMCL6020, ORTHOMCL6147, ORTHOMCL6151, ORTHOMCL6248, ORTHOMCL6279, ORTHOMCL6584, ORTHOMCL6648, ORTHOMCL6658, ORTHOMCL6825, ORTHOMCL6892, ORTHOMCL6912, ORTHOMCL7057, ORTHOMCL7308, ORTHOMCL7315, ORTHOMCL7938, ORTHOMCL8119, ORTHOMCL8504, ORTHOMCL8601, ORTHOMCL8910
HIVbF	ORTHOMCL9173
HIVw	ORTHOMCL8859

Table 1.1 continued

JTT	ORTHOMCL1035, ORTHOMCL1303, ORTHOMCL1348, ORTHOMCL1364, ORTHOMCL1372, ORTHOMCL1432, ORTHOMCL1477, ORTHOMCL1508, ORTHOMCL1519, ORTHOMCL1696, ORTHOMCL1728, ORTHOMCL1785, ORTHOMCL1894, ORTHOMCL1949, ORTHOMCL1974, ORTHOMCL2010, ORTHOMCL2012, ORTHOMCL2075, ORTHOMCL2097, ORTHOMCL2141, ORTHOMCL2161, ORTHOMCL2222, ORTHOMCL2305, ORTHOMCL2318, ORTHOMCL2424, ORTHOMCL2452, ORTHOMCL2515, ORTHOMCL2535, ORTHOMCL2564, ORTHOMCL2616, ORTHOMCL2668, ORTHOMCL2748, ORTHOMCL2787, ORTHOMCL2920, ORTHOMCL2936, ORTHOMCL2938, ORTHOMCL3042, ORTHOMCL3105, ORTHOMCL3189, ORTHOMCL3302, ORTHOMCL3308, ORTHOMCL3433, ORTHOMCL3471, ORTHOMCL3514, ORTHOMCL3548, ORTHOMCL3593, ORTHOMCL3606, ORTHOMCL3671, ORTHOMCL3681, ORTHOMCL3691, ORTHOMCL3815, ORTHOMCL3834, ORTHOMCL3872, ORTHOMCL3891, ORTHOMCL3946, ORTHOMCL3952, ORTHOMCL4023, ORTHOMCL4039, ORTHOMCL4093, ORTHOMCL4128, ORTHOMCL4154, ORTHOMCL4171, ORTHOMCL4175, ORTHOMCL4214, ORTHOMCL4216, ORTHOMCL4283, ORTHOMCL4303, ORTHOMCL4315, ORTHOMCL4338, ORTHOMCL4469, ORTHOMCL4484, ORTHOMCL4501, ORTHOMCL4506, ORTHOMCL4539, ORTHOMCL4566, ORTHOMCL4595, ORTHOMCL4652, ORTHOMCL4715, ORTHOMCL4784, ORTHOMCL4789, ORTHOMCL4800, ORTHOMCL4846, ORTHOMCL4975, ORTHOMCL5012, ORTHOMCL5020, ORTHOMCL5023, ORTHOMCL5081, ORTHOMCL5103, ORTHOMCL5106, ORTHOMCL5117, ORTHOMCL5162, ORTHOMCL5195, ORTHOMCL5208, ORTHOMCL5269, ORTHOMCL5288, ORTHOMCL5290, ORTHOMCL5294, ORTHOMCL532, ORTHOMCL5339, ORTHOMCL5372, ORTHOMCL5383, ORTHOMCL5415, ORTHOMCL5478, ORTHOMCL5561, ORTHOMCL5570, ORTHOMCL5608, ORTHOMCL5670, ORTHOMCL5672, ORTHOMCL5718, ORTHOMCL5802, ORTHOMCL5913, ORTHOMCL5917, ORTHOMCL5920, ORTHOMCL5957, ORTHOMCL5966, ORTHOMCL6021, ORTHOMCL6022, ORTHOMCL6027, ORTHOMCL6053, ORTHOMCL6087, ORTHOMCL6090, ORTHOMCL6148, ORTHOMCL6160, ORTHOMCL6174, ORTHOMCL6237, ORTHOMCL6332, ORTHOMCL6366, ORTHOMCL6371, ORTHOMCL6375, ORTHOMCL6387, ORTHOMCL6472, ORTHOMCL6473, ORTHOMCL6477, ORTHOMCL6479, ORTHOMCL6509, ORTHOMCL6511, ORTHOMCL6572, ORTHOMCL659, ORTHOMCL6683, ORTHOMCL6698, ORTHOMCL6759, ORTHOMCL6903, ORTHOMCL6935, ORTHOMCL6955, ORTHOMCL6961, ORTHOMCL6976, ORTHOMCL6978, ORTHOMCL6979, ORTHOMCL7047, ORTHOMCL7080, ORTHOMCL7152, ORTHOMCL7212, ORTHOMCL7232, ORTHOMCL7265, ORTHOMCL7304, ORTHOMCL7317, ORTHOMCL7321, ORTHOMCL7349, ORTHOMCL7383, ORTHOMCL7388, ORTHOMCL7419 ,ORTHOMCL7495, ORTHOMCL7522, ORTHOMCL7545, ORTHOMCL7583, ORTHOMCL7586, ORTHOMCL7594, ORTHOMCL7641, ORTHOMCL7649, ORTHOMCL7681, ORTHOMCL7737, ORTHOMCL7740, ORTHOMCL7748, ORTHOMCL7788, ORTHOMCL7799, ORTHOMCL7850, ORTHOMCL7858, ORTHOMCL7921, ORTHOMCL7943, ORTHOMCL7968, ORTHOMCL7985, ORTHOMCL7989, ORTHOMCL8003, ORTHOMCL8004, ORTHOMCL8013, ORTHOMCL8060, ORTHOMCL8064, ORTHOMCL8090, ORTHOMCL8092, ORTHOMCL8099, ORTHOMCL8100, ORTHOMCL8102, ORTHOMCL8128, ORTHOMCL8189, ORTHOMCL8218, ORTHOMCL8255, ORTHOMCL8276, ORTHOMCL8281, ORTHOMCL8305, ORTHOMCL8393, ORTHOMCL8396, ORTHOMCL8422, ORTHOMCL8479, ORTHOMCL8497, ORTHOMCL8534, ORTHOMCL8561, ORTHOMCL8607, ORTHOMCL8631, ORTHOMCL8634, ORTHOMCL8665, ORTHOMCL8669, ORTHOMCL8713, ORTHOMCL8738, ORTHOMCL8912, ORTHOMCL8941, ORTHOMCL8958, ORTHOMCL8987, ORTHOMCL8988, ORTHOMCL8991, ORTHOMCL9045, ORTHOMCL9085, ORTHOMCL9166, ORTHOMCL9236, ORTHOMCL9371, ORTHOMCL9391, ORTHOMCL9506, ORTHOMCL9509, ORTHOMCL9532, ORTHOMCL9539, ORTHOMCL9610, ORTHOMCL9631, ORTHOMCL9668, ORTHOMCL9675

Table 1.1 continued

JTTDCMut	ORTHOMCL1891, ORTHOMCL1990, ORTHOMCL2487, ORTHOMCL2504, ORTHOMCL2791, ORTHOMCL2839, ORTHOMCL3134, ORTHOMCL3209, ORTHOMCL3306, ORTHOMCL3863, ORTHOMCL4073, ORTHOMCL4088, ORTHOMCL4184, ORTHOMCL4211, ORTHOMCL4215, ORTHOMCL4220, ORTHOMCL4264, ORTHOMCL4318, ORTHOMCL4374, ORTHOMCL4640, ORTHOMCL4735, ORTHOMCL4842, ORTHOMCL4854, ORTHOMCL4896, ORTHOMCL4994, ORTHOMCL5096, ORTHOMCL5134, ORTHOMCL5200, ORTHOMCL5376, ORTHOMCL5394, ORTHOMCL5435, ORTHOMCL5466, ORTHOMCL5476, ORTHOMCL5488, ORTHOMCL5506, ORTHOMCL5629, ORTHOMCL5821, ORTHOMCL5837, ORTHOMCL6107, ORTHOMCL6258, ORTHOMCL6394, ORTHOMCL6775, ORTHOMCL6865, ORTHOMCL6880, ORTHOMCL6926, ORTHOMCL6977, ORTHOMCL7188, ORTHOMCL7322, ORTHOMCL7356, ORTHOMCL7384, ORTHOMCL7409, ORTHOMCL7414, ORTHOMCL7512, ORTHOMCL7552, ORTHOMCL7584, ORTHOMCL7617, ORTHOMCL7759, ORTHOMCL8059, ORTHOMCL8312, ORTHOMCL8400, ORTHOMCL8425, ORTHOMCL8486, ORTHOMCL8498, ORTHOMCL8642, ORTHOMCL8960, ORTHOMCL9072, ORTHOMCL9196, ORTHOMCL9367, ORTHOMCL9430, ORTHOMCL9606, ORTHOMCL9622
JTTDCMutF	ORTHOMCL3874
JTTF	ORTHOMCL3684, ORTHOMCL3896, ORTHOMCL4081, ORTHOMCL6470, ORTHOMCL7795, ORTHOMCL9014, ORTHOMCL9501, ORTHOMCL9576
LG	ArgK, ORTHOMCL1375, ORTHOMCL2017, ORTHOMCL3123, ORTHOMCL3342, ORTHOMCL3343, ORTHOMCL3461, ORTHOMCL3516, ORTHOMCL3754, ORTHOMCL4439, ORTHOMCL4491, ORTHOMCL4517, ORTHOMCL4724, ORTHOMCL4765, ORTHOMCL4814, ORTHOMCL5151, ORTHOMCL5194, ORTHOMCL5489, ORTHOMCL5586, ORTHOMCL5693, ORTHOMCL5706, ORTHOMCL5786, ORTHOMCL5936, ORTHOMCL5953, ORTHOMCL6402, ORTHOMCL6421, ORTHOMCL6484, ORTHOMCL6520, ORTHOMCL6686, ORTHOMCL6767, ORTHOMCL6782, ORTHOMCL6929, ORTHOMCL7172, ORTHOMCL7288, ORTHOMCL7695, ORTHOMCL7702, ORTHOMCL8039, ORTHOMCL8182, ORTHOMCL8222, ORTHOMCL8259, ORTHOMCL8440, ORTHOMCL8559, ORTHOMCL8627, ORTHOMCL8659, ORTHOMCL8687, ORTHOMCL8712, ORTHOMCL8809, ORTHOMCL8860, ORTHOMCL8894, ORTHOMCL9012, ORTHOMCL9028, ORTHOMCL9075, ORTHOMCL9244, ORTHOMCL9389, ORTHOMCL9398, ORTHOMCL9443, ORTHOMCL9514, ORTHOMCL9550, ORTHOMCL9559, ORTHOMCL964, ORTHOMCL9660, ORTHOMCL9683
mtMAMF	ORTHOMCL9108
mtZOA	ORTHOMCL3045, ORTHOMCL4863, ORTHOMCL5425, ORTHOMCL6360, ORTHOMCL7194, ORTHOMCL8661
PMB	ORTHOMCL2678, ORTHOMCL3411, ORTHOMCL4523, ORTHOMCL8163
rtREV	ORTHOMCL4472, ORTHOMCL4524, ORTHOMCL5038, ORTHOMCL9390
rtREVF	ORTHOMCL8992
VT	CAD, ORTHOMCL2153, ORTHOMCL2605, ORTHOMCL3276, ORTHOMCL3501, ORTHOMCL3728, ORTHOMCL4812, ORTHOMCL5592, ORTHOMCL6137, ORTHOMCL6398, ORTHOMCL6714, ORTHOMCL6896, ORTHOMCL6945, ORTHOMCL7687, ORTHOMCL781, ORTHOMCL8443, ORTHOMCL8972, ORTHOMCL9065, ORTHOMCL9126
WAG	ORTHOMCL1202, ORTHOMCL8833, ORTHOMCL8847, ORTHOMCL8938

Phylogenies were rooted with Erodiini as they had been determined to be relatively distant from Adesmiini in an analysis of relationships between tribes in a larger dataset from the Smith lab (Smith et al., unpublished). The resulting topologies from concatenated and coalescent analyses were edited in Inkscape, with taxa and branches colored by time of activity and substrate usage (Inkscape Project, 2020).

1.4 Results

DNA from a total of 47 Adesmiini taxa, spanning 10 of the 11 adesmiine genera and 41 species (Table 1.2), were extracted, enriched, and analyzed in this study. Included in the dataset are generic type species from six of the ten genera: *Epiphysa*, *Alogenius*, *Renatiella*, *Stenocara*, *Metriopus*, and *Stenodesia* (Penrith, 1979). These taxa are mainly from southern Africa, but two Palearctic *Adesmia* representatives from Iran were also included.

Table 1.2. A breakdown of the Adesmiini taxa used in this study describing the substrate each species is commonly found on.

Genus	Specimen	Substrate (verbatim)	Time of Activity	Reference
Adesmia	*Adesmia (Physosterna) armatipes* Koch 1951	Under stones and shrubs, plains	Nocturnal/crepuscular	(Penrith, 1979)
	Adesmia (Physosterna) cribripes Haag 1875	Usually under trees or shrubs.	Nocturnal/crepuscular	(Penrith, 1979)
	Adesmia (Physosterna) goryi Solier 1835		Diurnal	(Penrith, 1979)
	Adesmia (Physosterna) porcata Solier 1835	Under plants on incipient and vegetated dunes; mainly on coastal dunes.	Diurnal	(Penrith, 1979)
	Adesmia (Physosterna) torulosa Pallas 1781	sand under shrubs (loose sand with sparse vegetation)	Nocturnal/crepuscular	(Penrith, 1979)
	Adesmia (Zambesmia) detrita Kuntzen 1914-16	Loose sand with sparse vegetation	Nocturnal/crepuscular	(Penrith, 1979)
	Adesmia (Zambesmia) seineri Kuntzen 1914-16	Loose sand with sparse vegetation	Nocturnal/crepuscular	(Penrith, 1979)
	Adesmia (Macradesmia) cancellata Solier 1835	sandy; grassy plain	Diurnal	(Broza et al. 1983; Mas-Peinado et al. 2015)
	Adesmia (Adesmia). montana Klug 1830	Sandy plains	Diurnal	(Koch, 1949)
Alogenius	*Alogenius (Alogenius) favosus* Erichson 1843	Rocky	Nocturnal/crepuscular	(Penrith, 1979)
	Alogenius (Alogenius) cavifrons robinsoni Koch 1951	Rocky	Nocturnal/crepuscular	(Penrith, 1979)
Epiphysa	*Epiphysa flavicollis* Fabricius 1794	Loose sand with vegetation, loose sand with sparse vegetation; sandy areas	Nocturnal/crepuscular	(Penrith, 1979)
	Epiphysa latisterna Koch 1951	Rocky outcrops	Nocturnal/crepuscular	(Penrith, 1979)
Eustolopus	*Eustolopus octoseriatus* Gebien 1938	sand/gravel plains	Diurnal	(Penrith, 1979)
Metriopus	*Metriopus (Metriopus) depressus* Haag 1875	compact sand, compact sand with stones, plains. sand plains, interdune valleys	Diurnal	(Penrith, 1979)
	Metriopus (Metriopus) hoffmannseggi Solier 1835	compact sand, loose sand with vegetation, loose sand with sparse vegetation	Diurnal	(Penrith, 1979)
	Metriopus (Metriopus) ruficornis Solier 1835	plains and vegetated sand dunes	Diurnal	(Penrith, 1979)

Table 1.2 continued

Onymacris	*Onymacris boschimana* Péringuey 1886	Loose sand with sparse vegetation, compact sand, loose sand with vegetation	Diurnal	(Penrith, 1979)
	Onymacris laeviceps Gebien 1938	Dune/Loose sand with sparse vegetation	Diurnal	(Hauffe et al., 1988; Holm and Edney, 1973; Koch 1951; Penrith, 1979)
	Onymacris marginipennis Brême 1840	Loose sand with sparse vegetation	Diurnal	(Lamb and Bond, 2013; Penrith 1979)
	Onymacris paiva Haag 1875	Loose sand with sparse vegetation	Diurnal	(Penrith, 1979)
	Onymacris plana Péringuey 1886	Dune/Loose sand with sparse vegetation	Diurnal	(Hauffe et al., 1988; Holm and Edney, 1973; Penrith, 1979)
	Onymacris rugatipennis Haag 1875	Dune/Loose sand with sparse vegetation	Diurnal	(Lamb and Bond, 2013; Penrith 1979)
Physadesmia	*Physadesmia bullata* Péringuey 1888	Sandy plains with small shrubs; also found on rocky hills and in dry river courses.	Diurnal	(Penrith, 1979)
	Physadesmia globosa Haag 1875	Sandy areas, usually under trees and bus often in dry river courses and on vegetated d where bushes large enough to provide shade available.	Diurnal	(Penrith, 1979)
Renatiella	*Renatiella reticulata* Gerstaecker 1854	Under stones on hard soil/compact sand	Nocturnal/crepuscular	(Penrith, 1979)
	Renatiella scrobipennis Haag 1875	under shrubs and trees in arid plains	Nocturnal/crepuscular	(Penrith, 1979)
Stenocara	*Stenocara (Arenacara) brunnipes* Haag 1877	loose sand with sparse vegetation	Diurnal	(Penrith, 1979)
	Stenocara (Cauricara) eburnea Pascoe 1866	gravel plains	Diurnal	(Penrith, 1979)
	Stenocara aenescens Haag 1875	rocky outcrops, compact sand, compact sand with stones, loose sand with vegetation	Diurnal	(Penrith, 1979)
	Stenocara cf. namaqua Péringuey 1899	Plains, compact sand with stones	Diurnal	(Penrith, 1979)
	Stenocara dentata Fabricius 1792	plains	Diurnal	(Penrith, 1979)
	Stenocara dentata rotundata Solier 1835	plains	Diurnal	(Penrith, 1979)
	Stenocara dilaticornis Koch 1951	gravel	Diurnal	(Penrith, 1979)

Table 1.2 continued

Stenocara	*Stenocara gracilipes* Solier 1835	Variety of habitats, but mainly rocky	Diurnal	(Penrith, 1979)
	Stenocara longipes Olivier 1795	sandy plains	Diurnal	(Penrith, 1979)
	Stenocara namaquensis Gebien 1910	Variety of habitats	Diurnal	(Penrith, 1979)
Stenodesia	*Stenodesia globulum* Haag 1875	gravel	Diurnal	(Penrith, 1979)
	Stenodesia serrata Fabricius 1781	gravel	Diurnal	(Penrith, 1979)

The resulting topology from the partitioned ML analysis (Figure 1.2) in IQ-TREE 2 uncovered multiple clades that do not support the current generic classification of Adesmiini. The genera *Stenodesia*, *Renatiella*, *Alogenius*, and *Epiphysa* (each represented by two species in this study) were recovered as monophyletic. The monophyly of *Eustolopus*, a genus with only two described species, could not be tested as only *Eustolopus octoseritus* was included in analyses. The remaining five genera included in this study were not recovered as monophyletic.

Eustolopus was recovered as a sister to a clade containing all *Onymacris* taxa in the analysis and *Physadesmia globosa*. *Physadesmia globosa* was recovered within *Onymacris* species, thus rendering *Onymacris* paraphyletic. The long-legged diurnal runners, *Onymacris*, *Eustolopus*, and *P. globosa*, are confirmed as a monophyletic group.

The genus *Adesmia* was recovered as polyphyletic with respect to *Renatiella*, *Physadesmia*, *Onymacris*, and *Eustolopus*. The subgenus *Physosterna* was non-monophyletic with *A.* (*Physosterna*) *cribripes* sister to *Physadesmia bullata* and these two taxa do not group with the rest of the *A.* (*Physosterna*) taxa included in the dataset. The two *Adesmia* (*Zambesmia*) species are grouped together but are separated from the rest of the genus by being sister to *Renatiella*. The Palearctic *Adesmia* representatives, *A.* (*Macradesmia*) *cancellata* and *A.* (*Macropoda*) *montana*, are grouped together and sister to the clade containing *Onymacris*, *Physadesmia globosa*, *Eustolopus*, and the African *Adesmia* subgenera.

Stenocara was recovered as polyphyletic with three separate clades, marked as A, B, and C in Figure 1.2. Clade A, including the type species for the genus (*Stenocara longipes*), also contains both *Stenodesia* species included in this analysis. Clade B was recovered within a clade containing all sampled *Metriopus* species, thus rendering *Metriopus* paraphyletic. While *Epiphysa* and *Alogenius* were recovered as sister genera, they were not placed as the basal lineage of Adesmiini with respect to the outgroup taxa. Instead *Stenocara* clade C was recovered as sister to the rest of the tribe. From the sampled outgroups, the tribe Zophosini was strongly supported as the sister tribe to Adesmiini.

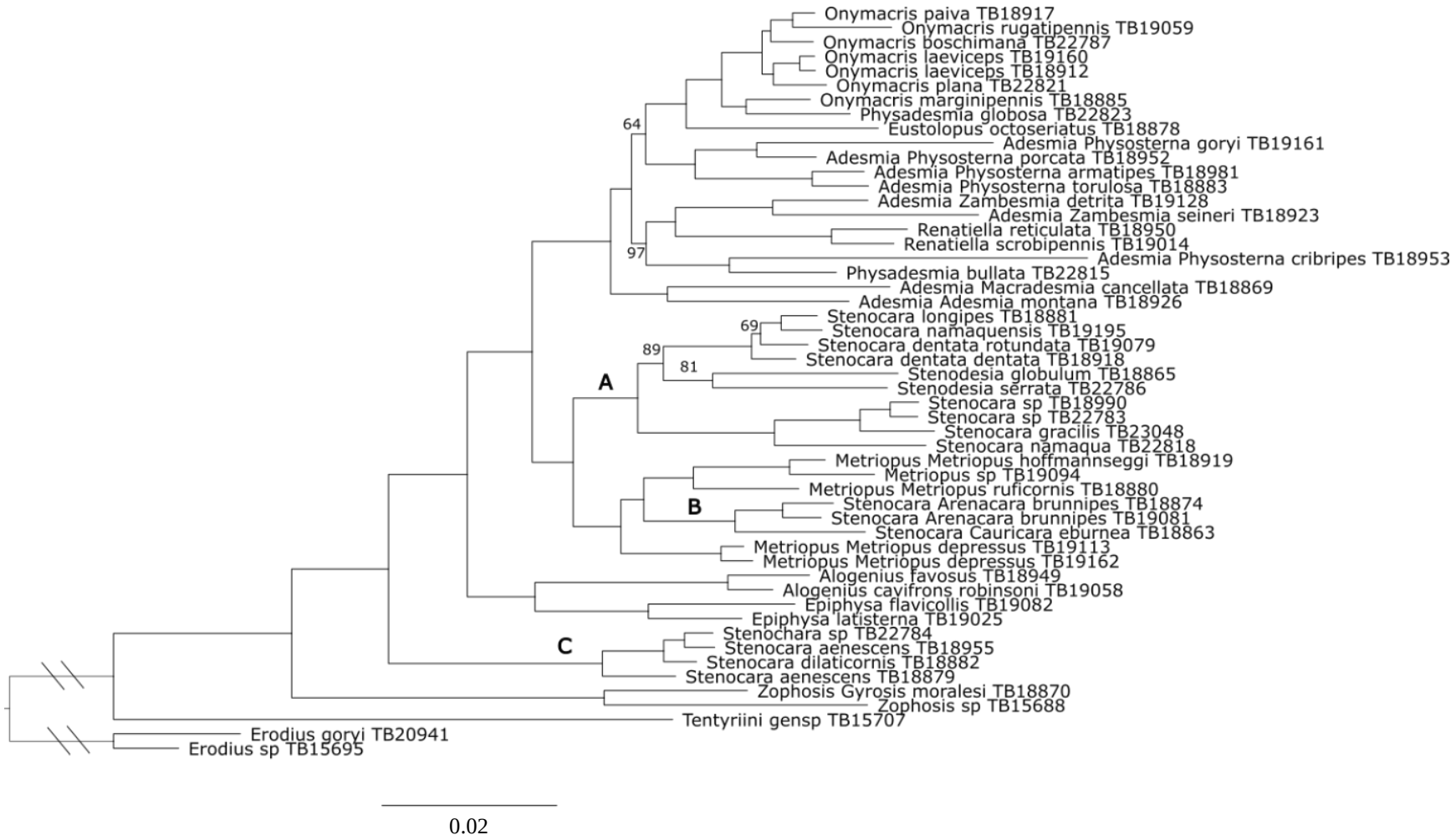

Figure 1.2. Maximum likelihood phylogenetic analysis of Adesmiini based on 510 amino acid loci from 47 OTUs from IQ-TREE 2. Support was assessed using 10,000 UF bootstrap replicates. Bootstrap values < 100 are depicted, branches with no values indicate a bootstrap value of 100. Letters on branches identify *Stenocara* clades.

The ASTRAL-III analysis (Figure 1.3), using a gene coalescence approach was largely concordant with the concatenated analysis run in IQ-TREE 2. However, *Adesmia* (*Physosterna*) *torulosa* and *armatipes* were recovered as sister to the *Onymacris*, *Physadesmia globosa*, and *Eustolopus* clade. This group is collectively sister to *Adesmia* (*Physosterna*) *cribripes* and *Physadesmia bullata*. Rather than *Adesmia* (*Physosterna*) *torulosa* and *armatipes* being sister to *Onymacris*, *Physadesmia*, *Eustolopus*, *Renatiella*, and the other southern African *Adesmia* species, *Adesmia* (*Physosterna*) *goryi* and *porcata* are instead. Finally, *Stenodesia* is recovered as polyphyletic.

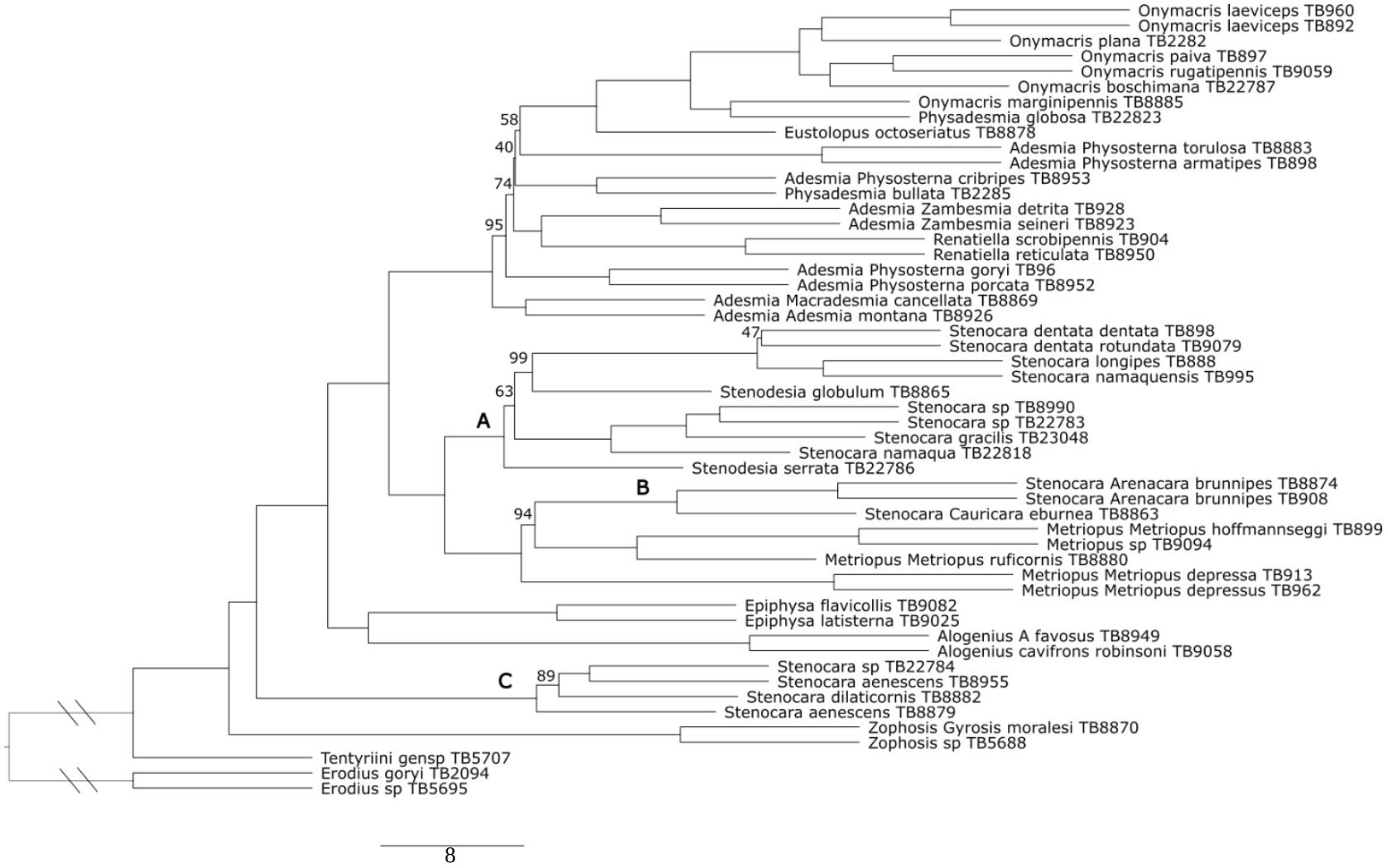

Figure 1.3. ASTRAL-III gene coalescence species tree inferred from 510 Adesmiini genetic loci where branch support is local PP and branch length is coalescent units. Branches with no values indicate a PP of 1.

1.5 Discussion

While Penrith's revisionary work on the southern African Adesmiini was thorough and is still extremely useful, her cladistic analysis based on limited external morphological characters did not reflect the evolutionary history of the lineages accurately based on the results of this study. The genera *Onymacris*, *Physadesmia*, *Adesmia*, *Metriopus*, and *Stenocara* were all recovered as para- or polyphyletic with strong support. This highlights the need for a generic-level revision of the Adesmiini to align their taxonomy to the recovered evolutionary history of the lineages.

Besides the limited number of morphological characters used by Penrith, it is likely that her analysis was further hindered by the nature of the characters themselves. Many are directly tied to species life history, particularly species activity cycles (diurnal versus nocturnal or crepuscular), substrate types (unvegetated sand dunes versus gravel plains or the semi-desert) and shifts in morphology adaptive to these habitats. These shifts include eye size, leg length, and tarsal claw length. Adesmiines have two general eye shapes, large ovoid or narrow, which tend to relate to their activity time as those with large eyes are mainly diurnal and those with narrow eyes are generally nocturnal. Adesmiine legs also have lengths ranging from short to long (Penrith, 1979). Based on my topology, these traits are not conserved with the generic relationships given by Penrith (1986). Therefore, life histories of species are not optimal for adesmiine analysis, affecting the generic topology provided by Penrith (1986).

In terms of generic relationships, we can confirm the claim that *Physadesmia globosa* should be transferred to *Onymacris*, and *Eustolopus* is sister to this clade, as stated by Lamb and Bond (2013). Therefore, the assumption from Penrith (1986) and Lamb and Bond (2013) that the three genera, *Onymacris*, *Physadesmia*, and *Eustolopus* are closely related to each other compared to other Adesmiini taxa is supported by this phylogeny. *Physadesmia* is shown to be paraphyletic, with *Physadesmia bullata* sister to *Adesmia cribripes* and *P. globose* sister to *Onymacris*; therefore, the genus still needs revision.

Alogenius and *Epiphysa*, as the two adesmiine genera with mandibular processes, were confirmed to be sister taxa, as per Penrith (1986). However, *Epiphysa* and *Alogenius* were not the most basal genera of Adesmiini, counter to Penrith's hypothesis (1986). Instead, *Stenocara* clade C was recovered as sister to the rest of the Adesmiini, followed by a clade containing the two nocturnal genera *Epiphysa* and *Alogenius* as sister to the rest of the sampled adesmiines. *Onymacris* is also shown to be deeply nested within the tribe with *Physadesmia* and *Eustolopus*

following in order. This is the opposite of the assumptions of Penrith (1986), whose analysis placed *Stenodesia*, *Orientacara*, *Metriopus*, and *Stenocara* as derived members of the tribe, while the psammophilous *Onymacris*, *Physadesmia*, and *Eustolopus*, were considered basal (Penrith 1979).

The Palearctic *Adesmia* species, *A. cancellata* and *A. montana*, are deeply nested within the southern African Adesmiini. They are sister to genera *Onymacris*, *Physadesmia*, *Renatiella*, and other *Adesmia* which confirms Mas-Peinado et al. (2015) statement that the genus is not monophyletic. Additional Palearctic species and subgenera are required to further study if the *Adesmia* northern range is the result of a single dispersal event or multiple ones to northern Africa and the Mediterranean.

Based on the topology recovered in this study (Figure 1.4), the ancestral state for Adesmiini activity patterns is likely diurnal. It is worth noting that Zophosini, the sister tribe to Adesmiini, are almost entirely diurnal as well. There appears to be at least three shifts to nocturnal activity patterns within the tribe; *Adesmia* (*Physosterna*) *armatipes* and *torulosa*, *Renatiella* and *Adesmia* (*Zambesmia*), and *Alogenius* and *Epiphysa* are the three nocturnal clades. The clades consisting of *Onymacris*, *Physadesmia globosa*, *Eustolopus*, *Stenocara*, *Stenodesia*, and *Metriopus* are fully diurnal.

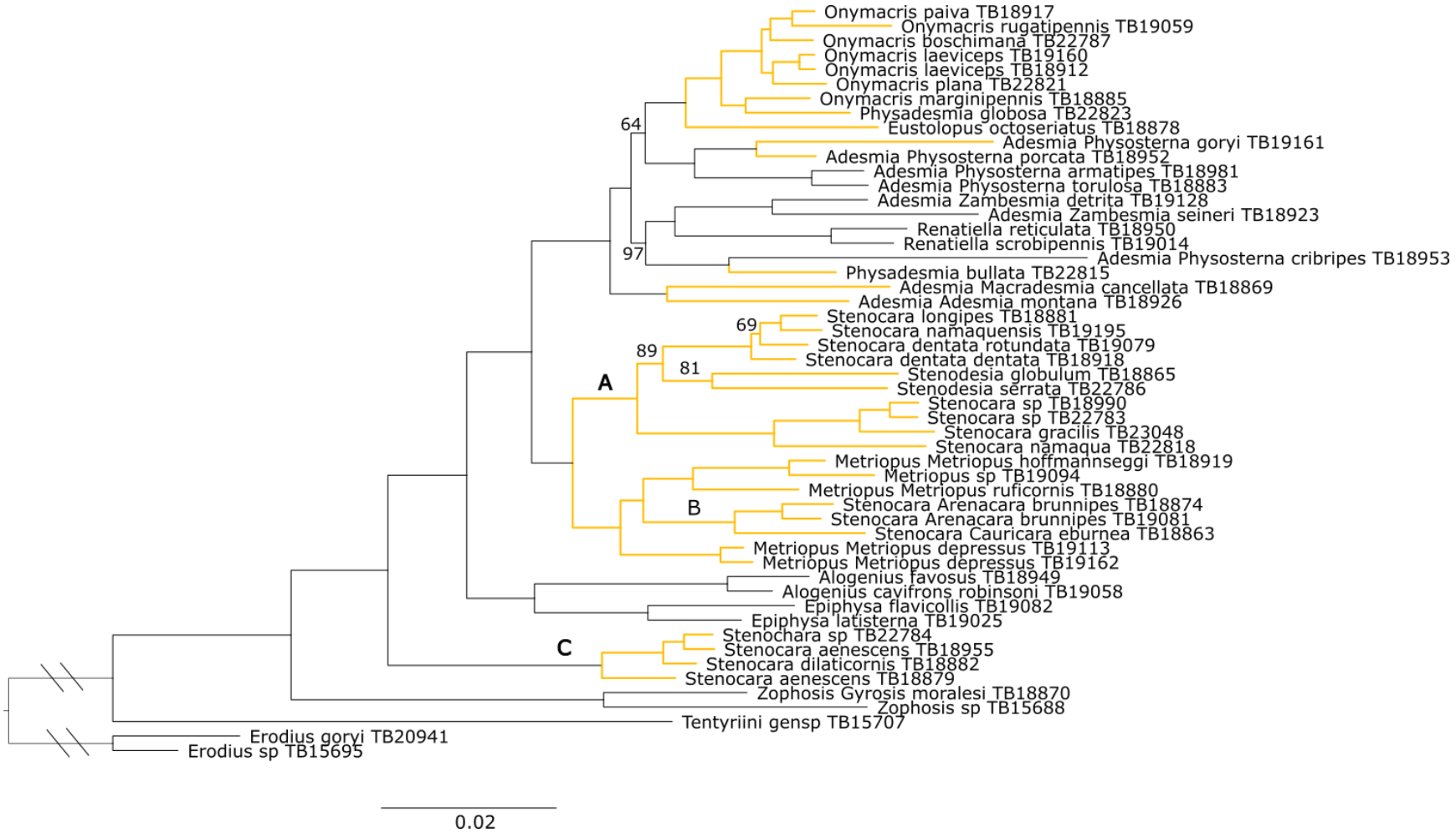

Figure 1.4. Maximum likelihood phylogeny from IQ-TREE 2 with branches colored species activity pattern. Yellow branches indicate diurnal species and uncolored branches showing crepuscular or nocturnal lineages. Outgroup color is not related to time of activity. Support was assessed using 10,000 UF bootstrap replicates. Bootstrap values < 100 are depicted, branches with no values indicate a bootstrap value of 100. Letters on branches identify Stenocara clades.

Based on the topology provided (Figure 1.5), the ancestral state of Adesmiini substrate usage is likely not psammophilous. Psammophily appears to arise multiple times throughout the tribe. The *Onymacris*, *Physadesmia globosa*, and *Eustolopus* clade all come out together as psammophilous. All analyzed *Adesmia* species, except for *Adesmia armatipes*, *A. cribripes*, and *A. torulosa*, are sand dwellers. However, they are broken up by *Renatiella*, which are found on non-sandy substrates. *Stenocara* is mainly non-sand dwelling, but there are two species which can be found in dune habitats the type species *Stenocara longipes* and *Stenocara brunnipes*. *Metriopus* has two psammophilous species, *M. depressus* and *M. hoffmannseggi*, and one species, *Metriopus ruficornis*, not found on dunes. *Alogenius* taxa and *Epiphysa latisterna* are found on other substrates, but *Epiphysa flavicollis* is psammophilous. While there seems to be a general accumulation of psammophily in the deeper adesmiine lineages, this is another example of a behavior which does not have a single evolution, but rather multiple evolution of the same trait.

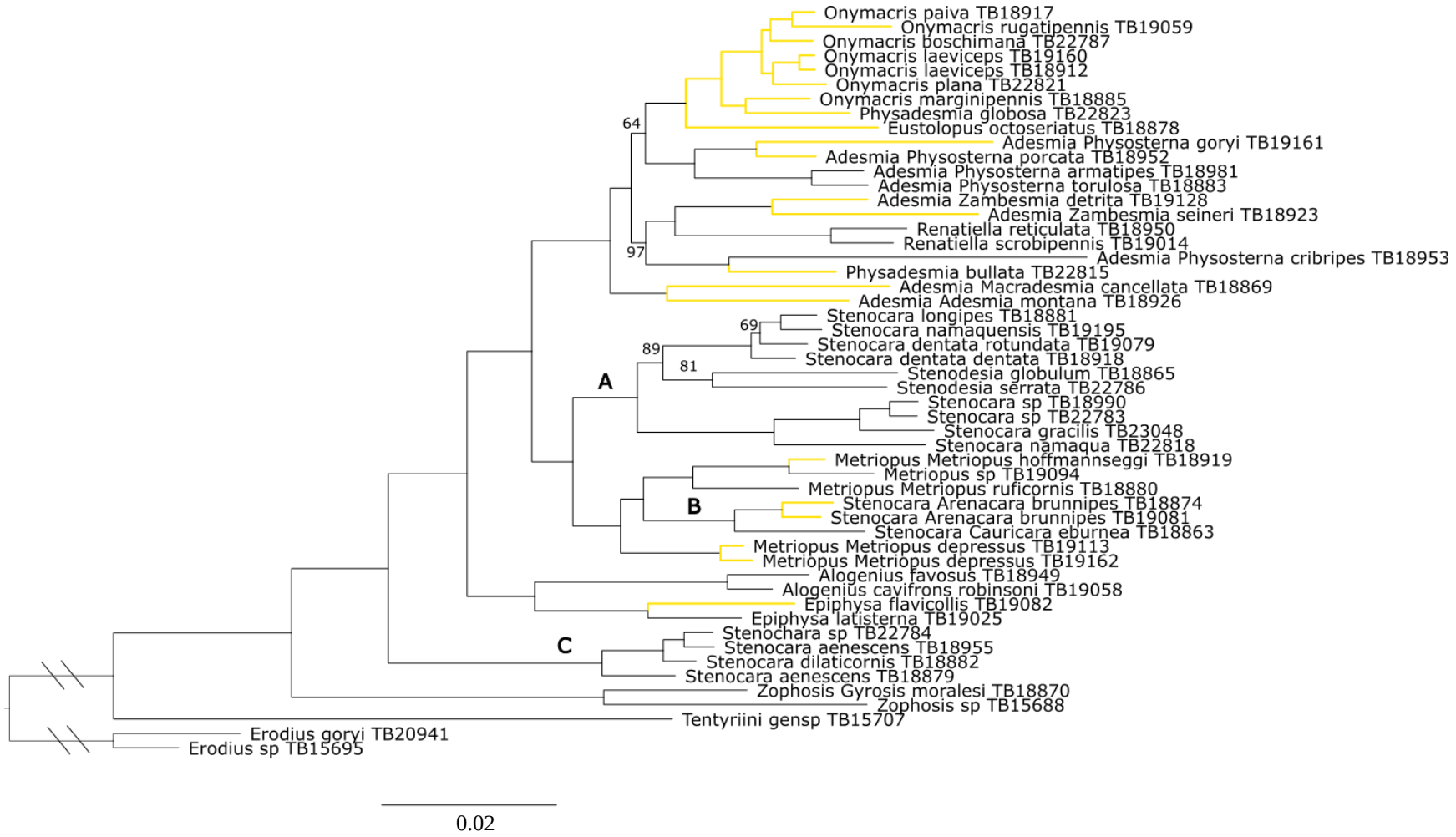

Figure 1.5. Maximum likelihood phylogeny from IQ-TREE 2 with branches colored by the substrate usage. Yellow branches depict psammophilous species and uncolored branches showing taxa found on other non-sand substrates. Outgroup color is not related to substrate use. Support was assessed using 10,000 UF bootstrap replicates. Bootstrap values < 100 are depicted, branches with no values indicate a bootstrap value of 100. Letters on branches identify Stenocara clades.

The results of this study show that the current taxonomy of Adesmiini is largely incongruent with the recovered evolutionary history of the tribe. A reanalysis of morphological data supporting genera, based on these results, is warranted. However, additional taxon sampling, especially from the Palearctic *Adesmia* subgenera and the monotypic *Orientacara*, should be included to help revise the tribe as a whole. This would allow for more detailed analyses of historical biogeography within the tribe, as well as morphological character shifts based on life histories.

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CHAPTER 2. THE FUNCTIONAL GENOMICS OF PIMELIINAE (COLEOPTERA: TENEBRIONIDAE) HEAT SHOCK PROTEIN 40 HOMOLOGS

2.1 Abstract

The subfamily Pimeliinae (Coleoptera: Tenebrionidae) is a highly diverse group of beetles with over 8,000 species primarily occurring in hot and arid deserts around the world. Many of these beetles have evolved morphological and behavioral strategies to survive harsh environments. All organisms have a variety of heat shock proteins which protect cellular components from degradation due to environmental stresses. However, variability in conserved proteins may result in a change of protein structure and therefore function. Modifications to heat shock proteins may allow desert-dwelling beetles to have more efficient cellular protection. Therefore, this study examined sequence changes of seven heat shock protein 40 (Hsp40) homologs and confirmed the conservation of functional Hsp40 domains using next generation sequencing and amino acid comparison. Phylogenetic analysis of Hsp40 homologs showed that pimeliine tribes were monophyletic, while the relationships between tribes shifted. A study of Adesmiini (Coleoptera: Tenebrionidae: Pimeliinae) Hsp40s was conducted for a more in-depth analysis of Hsp40 homologs' influence on ecology and species evolution. These results give a better understanding of how pimeliines use a cocktail of adaptations (morphological, behavioral, and cellular) to survive climates unsuitable, and sometimes inhospitable, for other organisms.

2.2 Introduction

Functional genomics is a growing field that seeks to address questions regarding morphology, physiology, and ecology through gene expression, i.e., how genotypes and epigenetic factors contribute to the expression of phenotypes. The rise of genomic studies in non-model organisms as the cost per genome has reduced is allowing researchers to study evolutionary and ecological questions in non-model groups using comparative genomics (Ekblom & Galindo, 2011; Seeb et al., 2011). Of particular interest are the genomic changes associated with living in variable, often inhospitable, climates, such as the ability of darkling beetles (Coleoptera: Tenebrionidae) to survive and thrive in arid desert habitats.

The use of taxa within Tenebrionidae for functional genomic studies is not a new concept. In fact, a widely used model organism from this family. *Tribolium castaneum* (Tenebrionidae: Tenebrioninae) has been used to study specific heat shock proteins (Mahroof et al., 2005; Xie et al., 2019; Xiong et al., 2018; Xu et al., 2009; 2010) and immune signaling and defensive proteins (Altincicek et al., 2008). This species has also been widely used as a model organism for insect development, genetic mapping, physiology, morphology, and evolution (Brown et al., 2009; Goodman et al., 2012; Wang et al., 2007). Other tenebrionid representatives with genomic studies includes *Anatolica polita* (Tenebrionidae: Pimeliinae) and *Tenebrio molitor* (Tenebrionidae: Tenebrioninae), among others (Li et al., 2020; Mao et al., 2019; XiaoXia et al., 2019). *Anatolica polita* has been used to study peptidoglycan recognition proteins in response to *Escherichia coli* (Mao et al., 2019; Xiao Xia et al., 2019) and the use of *A. polita* antifreeze proteins to cryopreserve sheep embryos (Li et al., 2020). While *T. molitor* has been studied for its biodegradation ability (Peng et al., 2019). *Tribolium castaneum*, *A. politica*, *T. molitor*, and a few other species have been analyzed as representatives of a family with over 20,000 species (Matthews et al., 2010). Which means this incredibly diverse family in the largest order of living animals is represented by few well-studied examples.

Pimeliinae (Coleoptera: Tenebrionidae) is an ideal group in which to study functional genomics due to their taxonomic (8,000+ species), behavioral, and morphological diversity. While pimeliine taxa occur in most terrestrial habitats around the world and thrive in a variety of environments, they mainly dwell in hot and arid deserts, like the Sonoran and Saharan Deserts (Hamilton & Seely, 1976; Koch, 1962; Rider, 2015). Desert-dwelling insects display a variety of morphological and behavioral traits to survive in hot dry habitats (Bousquet et al., 2018; Kergoat et al., 2014; Kramm & Kramm, 1972; Rider, 2015). These include, but are not limited to, crepuscular or nocturnal activity to avoid the hotter periods of the day and water-retention/desiccation prevention through elytral sealing mechanisms and waxy blooms (pruinescence) (Linz et al., 2016; Schilman et al., 2008). While behavioral and morphological alterations help beetles withstand severe environments, cellular defenses such as heat shock proteins are also likely to play key roles in their ability to survive.

Several cellular components are ubiquitous among all living organisms. Bacteria, archaea, and eukarya all have ribosomes, genetic material, and heat shock proteins (King & MacRae, 2015; Xu et al., 2010). These proteins are chaperones which prevent the denaturation of other cellular

proteins and whose expression is induced in response to environmental factors. Production of these proteins can be initiated by both biotic and abiotic stresses and are not limited to high heat (Zhao & Jones, 2012). There are multiple families of these stress-induced proteins, pointing to their significance, which are classified based on their molecular weight. This study will focus on heat shock protein 40 (Hsp40) homologs due to their inclusion in an existing genomic dataset composed primarily of low-copy nuclear genes collected for Pimeliinae (Smith et al., unpublished) and their conserved function, as seen in the conservation of Hsp40 J domains (Garbuz & Evgen'ev, 2017; Walsh et al., 2004). These two attributes make Hsp40 an optimal protein to study across Pimeliinae.

Hsp40 is a family of 40 kDa chaperone proteins which work as a coupling factor for Hsp70, a family of 70 kDa heat shock proteins. They work by stimulating ATP hydrolysis by Hsp70 (Kelley, 1998; Lee et al., 2000; Walsh et al., 2004) to stimulate the binding affinity of Hsp70 proteins for folding substrate polypeptides, disassembling other proteins, and moving proteins through organelle membranes (Walsh et al., 2004). Hsp40s are defined both by weight and the presence of a J domain. The J domain is a conserved Hsp40 binding site for the Hsp70 ATPase site (Kelley, 1998; Walsh et al., 2004). There are distinctive features within the J domain, including a conserved histidine-proline-aspartate (HPD) amino acid sequence and four clear helices (Kelley, 1998; Lee et al., 2000; Walsh et al., 2004).

The expression of heat shock proteins when exposed to stressors is variable for different taxa; therefore, the mortality rate of insects due to stress can vary depending on the specific heat shock protein and amount of each they produce (Garbuz & Evgen'ev, 2017; Nguyen et al., 2016; Walsh et al., 2004). Genetic variability in the genes that produce heat shock proteins can also alter their amino acid sequences, consequently their secondary/tertiary structures and ultimately their function. Therefore, evolutionary history and selection pressure to survive in stressful climates can result in differences within these proteins despite the high level of conservation seen in areas such as the J domain of Hsp40.

Hsp40 homologs have such a conserved region (the J domain) crucial for their functionality. However, sequence variability elsewhere within the homologs can alter the overall effectiveness of the proteins. One sequence variation with potential functional implications is the loss or insertion of cysteine residues between taxa. A cysteine site change can result in an adjustment of a protein's tertiary structure, resulting in functional alteration (Karnik, et al., 1988).

So, alterations in the amino acid sequences of Hsp40 homologs outside of the J domain could have a significant impact on beetle lineages' thermal tolerance and ability to survive (de Jong et al., 1993; A. D. Nguyen et al., 2016; Walsh et al., 2004).

The study of functional genetics within Pimeliinae is timely due to the sheer diversity of the subfamily and recent advancements to better understand the relationships between taxa and how they interact in their environments. Using homolog evolution instead of transcription and translation level analysis may be a way to determine the differences in Pimeliinae taxa and how they can survive harsh and arid environments. Despite the evolutionary changes of genes over time, there appears to be strong positive selection pressure to conserve the J domain region (de Jong et al., 1993; Garbuz & Evgen'ev, 2017; A. D. Nguyen et al., 2016). However, the remaining variable sections of the proteins may give insight into how taxa have adapted over time. In this study I explore the differences in seven Hsp40 homologs, representing the three Hsp40 subfamilies (A, B, and C), observing patterns in the evolution of these homologs within Pimeliinae as an introduction for further studies observing the effects these homolog changes may have on desert adaptations. This was done by (1) confirming the presence of key amino acid sequences, specifically within the J domain, which allow for functionality, (2) searching for cysteine shifts throughout the homologs, and (3) performing phylogenetic analyses on a dataset of seven Hsp's to compare the resulting topology to a Pimeliinae phylogeny based on 627 nuclear loci, one ribosomal, and one mitochondrial locus.

The Pimeliinae tribe Adesmiini is an appropriate tribe for a more in-depth discussion of heat shock proteins across species rather than throughout the entire subfamily. Adesmiini is an optimal tribe to further study heat shock proteins due to their taxonomic diversity (224 species across 11 genera) (Penrith 1979; 1986) and relatively well-known life histories. These life histories include diurnal and nocturnal taxa, which can be further divided into dune-dwelling diurnal species directly exposed to the daily high temperatures of western sub-Saharan Africa, where temperatures further climb as heat is radiated from the sand (Hauffe et al., 1988; Holm and Edney, 1973; Koch 1951; Penrith, 1979). Hence, changes in HSP40s may occur particularly between diurnal dune taxa and nocturnal taxa. Therefore, a final goal of this project is to observe if Hsp40 evolution reflects ecological evolution of adesmiine beetles.

2.3 Methods

2.3.1 Data acquisition

All specimens in this study were collected by members and collaborators of the Smith Insect Biodiversity Lab (Purdue University). The taxa include 142 pimeliines representing 25 tribes and 12 outgroup taxa. After disarticulating the head from the body and the coxa from the thorax, DNA extraction was performed, primarily using muscle tissue. Tissue lysis and DNA extraction were performed using Qiagen DNEasy Blood & Tissue kits as per the manufacturer's protocol. DNA libraries were prepared using the NEBNext® Ultra™ DNA Library Prep Kits for Illumina per the manufacturer's protocol. The resulting libraries were enriched using custom 120 bp MyBaits probes (Kanda, 2017) for 625 low-copy nuclear protein coding loci, one locus covering nuclear ribosomal DNA, and a locus including all mitochondrial protein coding genes. Low-copy protein coding genes were included in the MyBaits probe set based on the lack of identified paralogs and sequence variability of 10-20% within a set of 26 transcriptomes spanning the diversity of Pimeliinae and mapped to their gene identity using the *Tribolium* draft genome (T.cas 5.2) available on GenBank (Kanda, 2017; Schoch et al., 2020). Post hybridization captured libraries were sequenced using 150 base paired end runs on Illumina NextSeq 550 platforms at the University of Arizona's Genomic and Technology Core Facility (UAGC).

Within the HybPiper v.1.31.1 bioinformatic pipeline (Johnson et al., 2016), the nucleotide reads are mapped using BWA and assembled using SPAdes v.3.15.2 (Prjibelski et al., 2020). Introns were extracted from the assembled contigs using the intronerate.py script provided by HybPiper. The supercontig sequences were pulled using the retrieve_sequences.py script and any paralogs were reported using the paralog_investigator.py script. After assembly, stop codons were removed and nucleotide sequences were translated into amino acids. The translated sequences were aligned using MAFFT with the L-INS-i algorithm (Katoh et al., 2005) and then the nucleotides were mapped to the aligned amino acid sequences to produce both aligned nucleotide and amino acid datasets. Poorly aligned regions of the nucleotide sequences were masked using Gblocks v.0.91b (Castresana, 2002) and taxa which contained over 50% gaps in each sequence were excluded. This resulted in a dataset of over 750,000 base pairs across 627 targeted loci for 156 OTUs.

Genetic loci were identified as Hsp40 homologs based on gene identities mapped to the *Tribolium* genome and separated into a new dataset. This resulted in seven Hsp40 homologs represented in the larger dataset (Table 2.1): dnaj subfamily C members 2, 16, 17, and 25, dnaj subfamily A member 2, dnaj subfamily B member 14, and dnaj homolog dnaj-5. Alignments were checked in Mesquite v.3.61(Maddison & Maddison, 2019).

Table 2.1. A list of the proteins used in the study, their ortholog name from GenBank, their length in amino acid sequence number, and the number of taxa included in each dataset.

Protein name	**GenBank protein ID**	**Length (aa)**	**Number of taxa**
Hsp40 Subfamily C member 2	XP_008193970_1	608	153
Hsp40 Subfamily C member 16	XP_008197276_1	754	153
Hsp40 Subfamily C member 17	XP_974891_1	273	129
Hsp40 Subfamily C member 25	XP_971385_1	322	153
Hsp40 Subfamily A member 2	XP_970724_1	404	155
Hsp40 Subfamily B member 2	XP_972419_2	354	149
Dnaj-5	XP_971937_1	689	154

IQ-TREE 2 (Minh, Schmidt, et al., 2020) was used to perform both nucleotide and amino acid maximum likelihood analyses of the Hsp40 homologs and infer a well-supported species tree for Pimeliinae taxa. Substitution models were assigned using ModelFinder (Kalyaanamoorthy et al., 2017) as implemented in IQ-TREE 2 (Table 2.2). Maximum likelihood analyses were conducted on both the nucleotide and amino acid datasets using IQ-TREE 2 with support assessed using 10,000 UltraFast bootstrap iterations. Branches were collapsed to represent only tribal groupings with FigTree v.1.4.4 (FigTree, 2018).

Table 2.2. Substitution models for the following datasets: Pimeliinae nucleotide Hsp40, Pimeliinae amino acid Hsp40, Adesmiini nucleotide Hsp40, and Adesmiini amino acid Hsp40.

Sequence name	Pimeliinae amino acid substitution models	Pimeliinae nucleotide substitution models	Adesmiini amino acid substitution models	Adesmiini nucleotide substitution models
Hsp40 Subfamily C member 2	JTTDCMut+4	TIM3+F+R6	JTT+R2	TIM3+F+I+G4
Hsp40 Subfamily C member 16	JTT+R4	TIM3+F+R5	JTT+R2	TIM3e+I+G4
Hsp40 Subfamily C member 17	JTTDCMut+R4	TIM3+F+R5	JTT+G4	TN+F+G4
Hsp40 Subfamily C member 25	LG+I+G4	TIM3+F+R9	LG+R2	TIM3+F+I+G4
Hsp40 Subfamily A member 2	JTT+R4	SYM+R4	JTTDCMut+R2	TN+F+I+G4
Hsp40 Subfamily B member 2	JTT+R4	TIM3+F+R5	JTT+R2	TN+F+I+G4
dnaj-5	JTT+R4	TIM3e+R5	JTT+R2	TIM3+F+I+G4

2.3.2 J domain analysis

Four confirmed examples of J domains in Hsp40 homolog 1 were used to analyze J domains in the Pimeliinae dataset. J domains from *Tribolium castaneum* (XP_966855.1), *Scaptodrosophila lebanonensis* (XP_03037569), *Musca domestica* (XP_00584676), and *Anaplophora glabripenni* (XP_018569730) were used. Each Hsp40 homolog was aligned using MAFFT with the L-INS-i algorithm (Katoh et al., 2005) to the four confirmed J domains to approximate the location of this functional section. Once aligned, the presence of the HPD tripeptide was confirmed. This, along with the patterns mentioned above, indicates that the proteins are true Hsp40s. Conservation of the HPD tripeptide within the J domain was also confirmed using WebLogo (Crooks et al., 2004), which mapped amino acid frequency by alignment position (the more frequently an amino acid occurred at a site, the higher its letter). This is reported in "bits" and is a product of the amino acid equation in Crooks et al. (2014).

2.3.3 Cysteine shift analysis

Each of the seven Hsp40 homologs was analyzed manually at conserved cysteine residues for substitutions, insertions, or deletions of this structure-altering amino acid in MEGA v.11

(Tamura et al., 2021). Conserved cysteine sites were determined as ones where the majority of the taxa retained cysteine at a position. Taxa with changes in the conserved site, i.e., anything besides a cysteine, are noted as shown in Table 2.3.

Table 2.3. Sites of cysteine shifts in each of the seven included Hsp40 homologs.

Hsp40 homolog	**Site of conserved cysteines**	**Genera with cysteine shifts**	**Specimen identifier**	**Tribe**	**Site change if any**
dnaj-5	8	*Clamoris* Gozis, 1886	KKRNA00020	Phrenapatinae: Penetini	Phenylalanine
		Nyctopetus Guérin-Méneville, 1831	TB23034	Epitragini	Arginine
		Phloeodes	KKRNA00055	Zopheridae	Isoleucine
	252	*Adelostomini sp*	TB15727	Adelostomini	Deletion
		Branchus LeConte, 1862	TB18624	Branchini	Alanine
		Coniontis Eschscholtz, 1829	TB18627	Coniontini	Serine
		Cossyphodes Westwood, 1851	TB200002	Cossyphodini	Serine
		Craniotus LeConte, 1851	KKRNA00066	Asidini	Serine
		Diaperis Geoffroy, 1762	TB15742	Diaperinae: Diaperini	Serine
		Leptoderis Billberg, 1820	TB16842	Elenophorini	Serine
		Erodius Fabricius, 1775	TB20941	Erodiini	Glutamine
		Machla Herbst, 1799	TB17123	Asidini	Deletion
		Machla	TB17124	Asidini	Deletion
		Machla	TB20960	Asidini	Deletion
		Psammodes Kirby, 1819	TB16831	Sepidiini	Serine
	392	*Araeoschizus* LeConte, 1851	TB15689	Stenosini	Phenylalanine
		Cnemodinus Cockerell, 1906	AJRNA119	Cnemodini	Phenylalanine
		Pimelia Fabricius, 1775	KKDNA0300	Pimeliini	Leucine
	411	*Adelostomoid*	TB15727	Adeslostomini	Arginine
		Araeoschizus	TB15689	Stenosini	Arginine
		Arthroconus Solier, 1851	TB23007	Edrotini	Arginine
		Asbolus LeConte, 1851	TB15694	Cryptoglossini	Arginine
		Auchmobius LeConte, 1851	TB23009	Edrotini	Arginine

Table 2.3 continued

		Batuliomorpha Doyen, 1987	KKRNA00063	Akidini	Arginine
		Batulius LeConte, 1851	KKRNA00052	Akidini	Arginine
dnaj-5	411	*Chilometopon* Horn, 1874	TB22988	Edrotini	Arginine
		Cnemodinus	AJRNA119	Cnemodini	Arginine
		Cryptadius LeConte, 1851	TB22997	Edrotini	Arginine
		Edrotes LeConte, 1851	TB15737	Edrotini	Arginine
		Eleodes Eschscholtz, 1829	KKRNA00086	Blaptinae: Amphidorini	Glycine
		Emmenastrichus Horn, 1894	TB22990	Edrotini	Arginine
		Emmenides Casey, 1907	TB23001	Edrotini	Arginine
	411	*Erodius*	TB20941	Erodiini	Arginine
		Erodius	TB15695	Erodiini	Arginine
		Eulabis Eschscholtz, 1829	KKRNA00037	Tenebrioninae: Eulabini	Valine
		Eurychora Thunberg, 1789	AJRNA113	Adelostomini	Arginine
		Gyriosomus Guérin-Méneville, 1834	KKDNA0137	Nycteliini	Arginine
		Himatismus Erichson, 1843	KKDNA0064	Tentyriini	Arginine
		Hylithis Guérin-Méneville, 1834	TB23008	Edrotini	Arginine
		Hylocrinus Casey, 1907	TB23003	Edrotini	Arginine
		Machla	TB17124	Asidini	Arginine
		Machlamorpha	TB23040	Asidini	Glycine
		Melanastus Casey, 1907	TB22989	Edrotini	Arginine
		Mesabatodes Casey, 1907	TB23002	Edrotini	Arginine
		Metopoloba Casey, 1907	TB15697	Epitragini	Arginine
		Metoponium Casey, 1907	TB15732	Edrotini	Arginine
		Omopheres Casey, 1907	TB18630	Epitragini	Arginine
		Oxygonodera Casey, 1907	TB23011	Edrotini	Arginine
		Pectinepitragus Pic, 1927	TB18629	Epitragini	Arginine
		Pescennius Champion, 1884	TB22993	Edrotini	Arginine
		Phaleromela Reitter, 1916	KKRNA00034	Diaperinae: Phaleriini	Leucine
		Posides Champion, 1884	TB15465	Edrotini	Arginine

Table 2.3 continued

		Salax Guérin-Méneville, 1834	TB15690	Trilobocarini	Arginine
		Stenocara Solier, 1835	TB23048	Adesmiini	Arginine
		Stibia Horn, 1870	TB22987	Edrotini	Arginine
		Stictodera Casey, 1907	TB22986	Edrotini	Arginine
dnaj-5	411	*Telabis* Casey, 1890	TB23006	Edrotini	Arginine
		Tentyriini sp	TB15707	Tentyriini	Arginine
		Thinobatis Eschscholtz, 1831	TB15691	Thinobatini	Arginine
		Trichiotes Casey, 1907	TB16355	Edrotini	Arginine
		Trimytis LeConte, 1851	TB22996	Edrotini	Arginine
		Triorophus LeConte, 1851	TB15692	Edrotini	Arginine
		Uloma Dejean, 1821	KKRNA00042	Tenebrioninae: Ulomini	Valine
		Zophosis Latreille, 1802	TB15688	Zophosini	Arginine
Subfamily A member 2	183	*Adelostomini sp*	TB15693	Adelostomini	Isoleucine
	208	*Cryptochile* Latreille, 1828	AJRNA111	Cryptochilini	Valine
		Moluris Latrielle, 1802	TB16340	Sepidiini	Lysine
Subfamily B member 14	91	*Emmenides* Casey, 1907	TB23001	Edrotini	Serine
Subfamily C member 16	11	*Anchomma* LeConte, 1858	KKDNA0133	Stenosini	Arginine
		Araeoschizus	TB15689	Stenosini	Arginine
		Cardigenius Solier, 1836	TB22575	Asidini	Serine
		Conisattus Casey, 1895	TB18626	Coniontini	Tyrosine
		Diaperis	TB15742	Diaperinae: Diaperini	Tryptophan
		Physogaster Lacordaire, 1830	TB17045	Physogasterini	Serine
		Scotinus Kirby, 1819	TB22574	Asidini	Serine
		Scotinus	TB22573	Asidini	Serine
		Telabis	TB23006	Edrotini	Tryptophan
	17	*Stenomorpha* Solier, 1836	TB23044	Asidini	Serine
		Craniotus	KKRNA00066	Asidini	Phenylalanine
		Gyriosomus	KKDNA0137	Nycteliini	Serine
		Heterasida Casey, 1912	TB23044	Asidini	Phenylalanine
		Thinobatis	TB15691	Thinobatini	Tyrosine
	210	*Cossyphodes*	TB20002	Cossyphodini	Tyrosine
		Synhimba Koch, 1952	TB22644	Sepidiini	Tyrosine
		Uloma	KKRNA00042	Tenebrioninae: Ulomini	Tyrosine
	363	*Hypomelina sp* Koch, 1955	TB23058	Sepidiini	Isoleucine
	365	*Hypomelina sp*	TB23058	Sepidiini	Aspartate
	375	*Hypomelina sp*	TB23058	Sepidiini	Methionine
	422	*Strongylium* Kirby, 1819	KKRNA00075	Stenochiinae: Stenochiini	Insertion

Table 2.3 continued

	508	*Clamoris*	KKRNA00020	Phrenapatinae: Penetini	Insertion
	679	*Moluris*	TB16340	Sepidiini	Lysine
Subfamily C member 17	37	*Clamoris*	KKRNA00020	Phrenapatinae: Penetini	Threonine
Subfamily C member 2	13	*Asbolus*	TB15694	Cryptoglossini	Histidine
	346	*Metopoloba*	TB15697	Epitragini	Serine

2.3.4 Adesmiini analysis

Analyses of Adesmiini Hsp40 homologs followed methodology for the Pimeliinae phylogenetic analyses. IQ-TREE 2 (Minh, Schmidt, et al., 2020) was used to perform amino acid and nucleotide maximum likelihood analyses of the seven Hsp40 homologs and infer a species tree for the 47 Adesmiini taxa (41 species). Substitution models were determined using ModelFinder (Kalyaanamoorthy et al., 2017) as implemented in IQ-TREE 2 (Table 2.2). Maximum likelihood analyses were conducted on both the nucleotide and amino acid datasets using IQ-TREE 2 with support assessed using 10,000 UltraFast bootstrap iterations.

2.4 Results

2.4.1 Analysis of Pimeliinae Hsp40 homologs

When comparing the Pimeliinae phylogeny based on 627 loci (Figure 2.1) with the one produced using a nucleotide dataset of seven Hsp40 homologs (Figure 2.2), some discordance was found. Adelostomini is sister to Cryptoglossini and more closely related to Nyctoporini, and Anepsiini instead of being sister to Sepidiini. *Cossyphodes* Westwood, 1851 (Cossyphodini) is now sister to the clade which includes Caenocryptocini, Erodiini, Zophosini, Adesmiini, Tentyriini, Edrotini, Epitragini, and others, instead of being at the base of Asidini, Coniontini, Praocini clade. In the 627 loci tree, Edrotini is not monophyletic, and this phylogeny reflects that as well, but which taxa is intermingled within it and where is slightly different. Epitragini is now sister to the Edrotini clade instead of Thinobatini and *Salax* Guérin-Méneville, 1834

(Trilobocarini). *Morica* Solier, 1836 (Akidini) is now further integrated in the tree among other Pimeliinae tribes instead of being more basal than the non-Pimeliinae outgroups. And finally, *Cryptochile* Latreille, 1829 (Cryptochilini) is sister to all other tenebrionid taxa in the Hsp40 phylogeny instead of being sister to Pimeliini Latreille, 1802 and *Idisia* Pascoe, 1866 (Idisiini), as in the 629 loci phylogeny. Despite these differences, all tribes except Edrotini were still recovered as monophyletic.

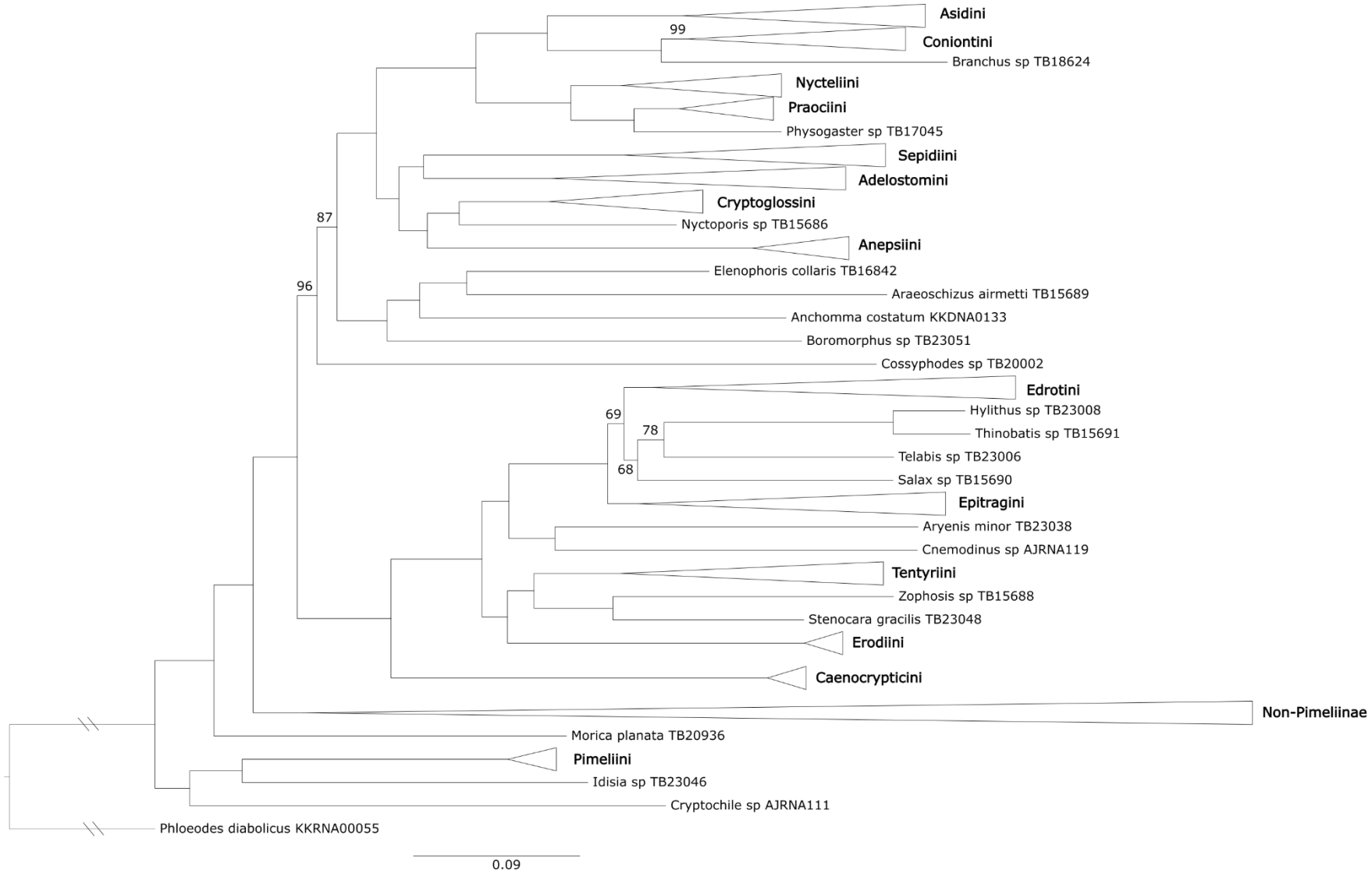


Figure 2.1. IQ-TREE 2 ML analysis of Pimeliinae using 627 nucleotide loci. Support was assessed using 1,000 bootstrap replicates. Bootstrap values <100 are depicted, branches with no value indicate a bootstrap value of 100.

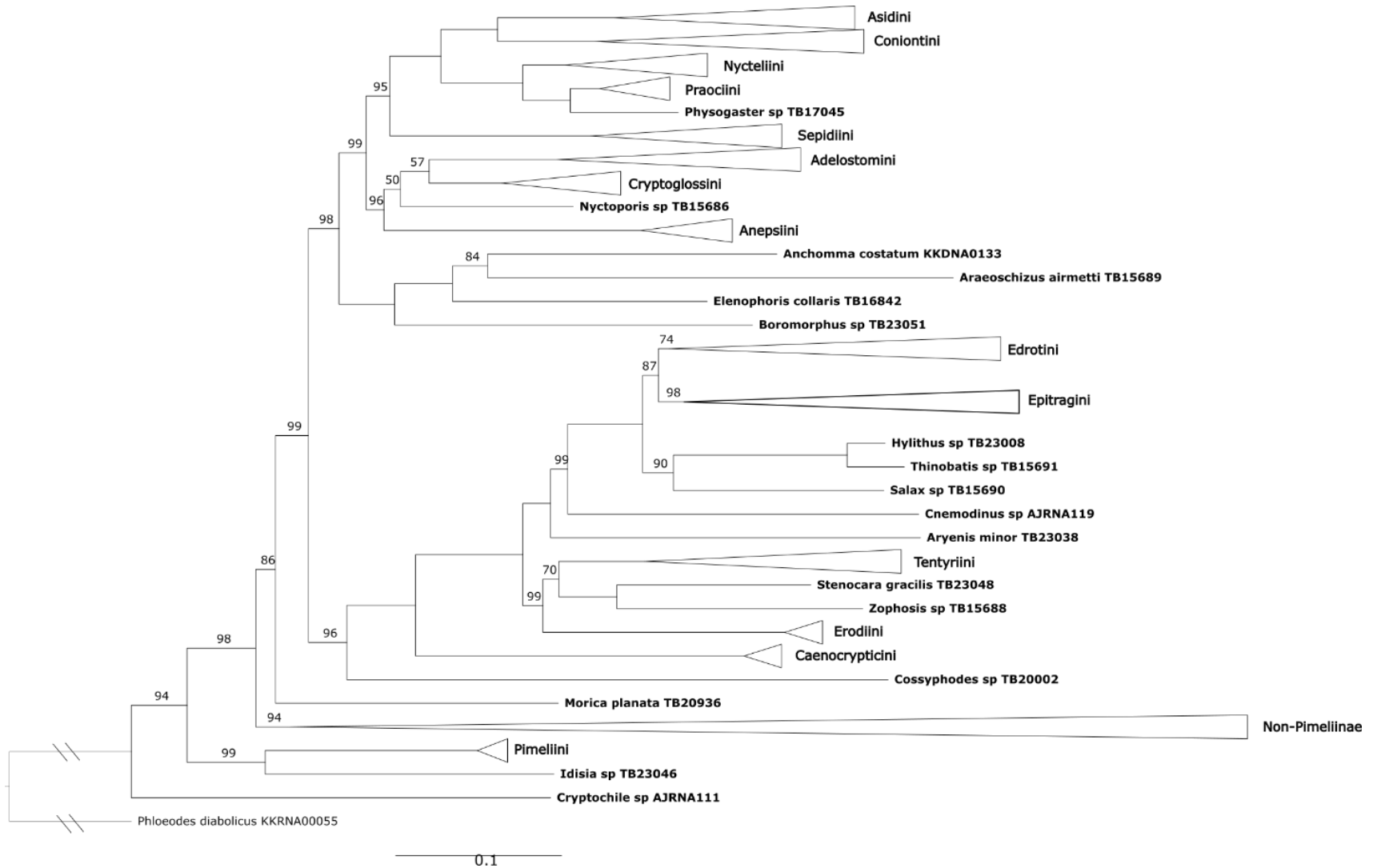


Figure 2.2. IQTREE 2 ML analysis of the Pimeliinae Hsp40 homologs using nucleotides. Support was assessed using 10,000 UF bootstrap replicates. Bootstrap values < 100 are depicted, branches with no values indicate a bootstrap value of 100.

There are a few differences between the phylogeny inferred from the Hsp40 amino acid sequences (Figure 2.3), the Hsp40 nucleotide sequences (Figure 2.2), and the 627 loci Pimeliinae tree (Figure 2.1). When comparing the amino acid Hsp40 phylogeny to the 627 loci phylogeny, the relationships between several tribes changed. In the amino acid Hsp40 topology, Adelostomini is sister to Anepsiini instead of Sepidiini, while Sepidiini is sister to Praocini, Nycteliini, Coniontini, and Asidini. The genus *Araeoschizus* LeConte, 1851 (Stenosini) is sister to *Anchomma* LeConte, 1858 (Anepsiini) instead of *Leptoderis* (Elenophorini). The Edrotini genus *Telabis* Casey, 1890 is placed within Epitragini. Of particular note, non-Pimeliinae outgroups, consisting of six other Tenebrionidae subfamilies, is sister to all Pimeliinae instead of Pimeliini, *Idisia* (Idisiini), *Morica* (Akidini), and *Cryptochile* (Cryptochilini) forming a clade that renders Pimeliinae polyphyletic.

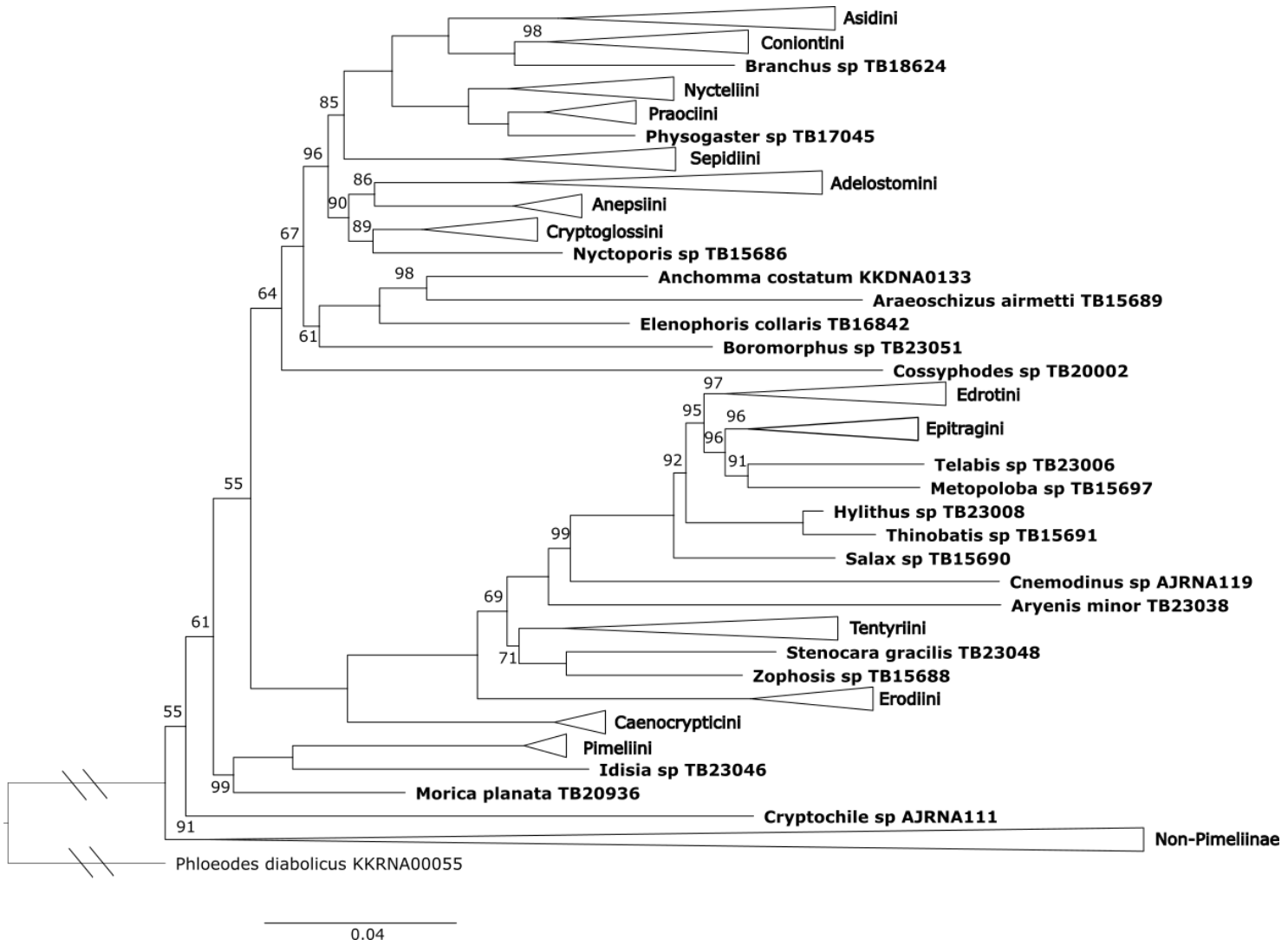
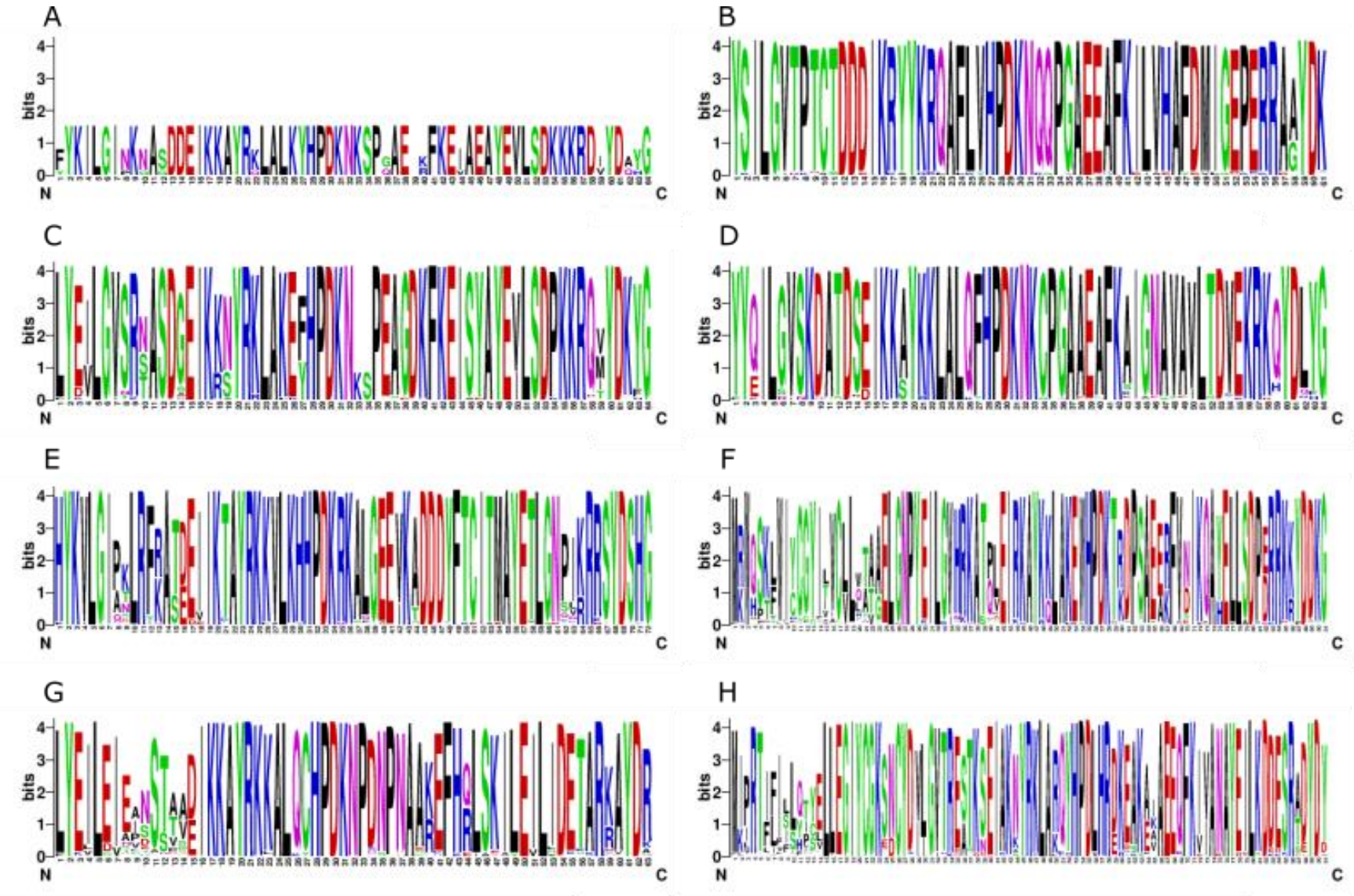


Figure 2.3. IQTREE 2 ML analysis of the Pimeliinae Hsp40 homologs using amino acid sequences. Bootstrap values <100 are depicted (10,000 UltraFast bootstrap replicates) and branches with no values indicate a bootstrap value of 100.

2.4.2 Functional J domain analysis

The functional J domains of Hsp40 homologs were found to be quite conserved for each homolog (Figure 2.4). The crucial HPD tripeptide was confirmed in each putative J domain. The level of conservation and the presence of the tripeptide support the assumption that these sections of the proteins are indeed J domains. Since they are highly conserved across the sampled Pimeliinae taxa, the 10-20% genetic variation threshold used for inclusion in the targeted enrichment probe set design was not found in the J domain of the Hsp40 homologs and indicating that the functionality of the J domain is not altered by genetic variation. Therefore, the variation must be found elsewhere in the Hsp40 homologs.

Figure 2.4: Aligned putative J domains with the height of the letters (X axis) indicating the frequency (bits) the amino acid appears in that position (Y axis) from the following protein fragments: (A) Reference J domains from NCBI (B) dnaj-5 homolog (C) Subfamily C member 2 (D) Subfamily C member 16 (E) Subfamily C member 17 (F) Subfamily C member 25 (G) Subfamily A member 2 (H) Subfamily B member 14.


A
B
C
D
E
F
G
H
bits
N
C

2.4.3 Cysteine shifts

Shifts from a conserved cysteine to any other amino acid or a deletion for Hsp40 homologs dnaj-5, subfamily A member 2, subfamily B member 14, subfamily C member 16, subfamily C member 17, subfamily C member 2, and subfamily C member 25 are noted in Table 2.3. Genera with amino acid changes are given along with their specimen identifier and the identified non-cysteine residue or deletion. Hsp40 subfamily A member 2 has fully conserved cysteines at positions 140, 143, 156, 159, 186, 199, 202, 277, and 322. Hsp40 subfamily B member 14 has its fully conserved cysteines at 11, 127, 311, and 345. Hsp40 subfamily C member 16 has four conserved cysteine positions: 164, 167, 174, and 664. Hsp40 subfamily C member 2 positions 130, 370, 575, and 584 are fully conserved across the included Pimeliinae taxa. Finally, the only two cysteine sites 21 and 26 in Hsp40 subfamily C member 25 are fully conserved.

There are a total of 81 cysteine shifts in the dataset across the pimeliine representatives. 57 of the changes are in dnaj-5 where Edrotini taxa show the highest number with 21 shifts. Asidini and Epitragini follow with six and four shifts, respectively. Adelostomini and Erodiini both have three cysteine shifts while Stenosini, Cnemodini, Akidini, and Tentyriini all have two. Branchini, Coniontini, Cossyphodini, Elenophorini, Sepidiini, Pimeliini, Nycteliini, Trilobocarini, Adesmiini, Thinobatini, Zophosini, and Cryptoglossini all have one dnaj-5 cysteine shift. In Subfamily A member 2, there were three cysteine shifts which were found in Adelostomini, Cryptochilini, and Sepidiini. Subfamily B member 14 only had one shift and it was in an Edrotini representative. Subfamily C member 16 had 18 shifts expressed by six Asidini and five Sepidiini shifts. Coniontini, Physogasterini, Nycteliini, Thinobatini, and Cossyphodini all have one cysteine shift. Finally, Subfamily C member 2 has two shifts, one in each of Cryptoglossini and Epitragini.

2.4.4 Adesmiini Hsp40 homolog analysis

Comparing the nucleotide analysis of the adesmiine Hsp40 homologs (Figure 2.5) to the 510 loci Adesmiini phylogeny in (Figure 2.2) (primarily composed of taxa not included in the Pimeliinae dataset) shows some differences in adesmiine relationships. *Stenocara namaqua* is now within the *Onymacris* clade. The *Adesmia cribripes* and *Physadesmia bullata* clade is sister to the *Onymacris*, *Physadesmia globosa*, *Eustolopus* clade rather than being sister to *Adesmia* (*Zambesmia)* and *Renatiella*. *Adesmia* (*Physosterna*) *goryi* and *Adesmia* (*Physosterna*) *porcata*

are now sister to the two Palearctic species, *Adesmia (Adesmia) montana* and *Adesmia (Macradesmia) cancellata* instead of directly related to *Adesmia armatipes* and *Adesmia torulosa*.

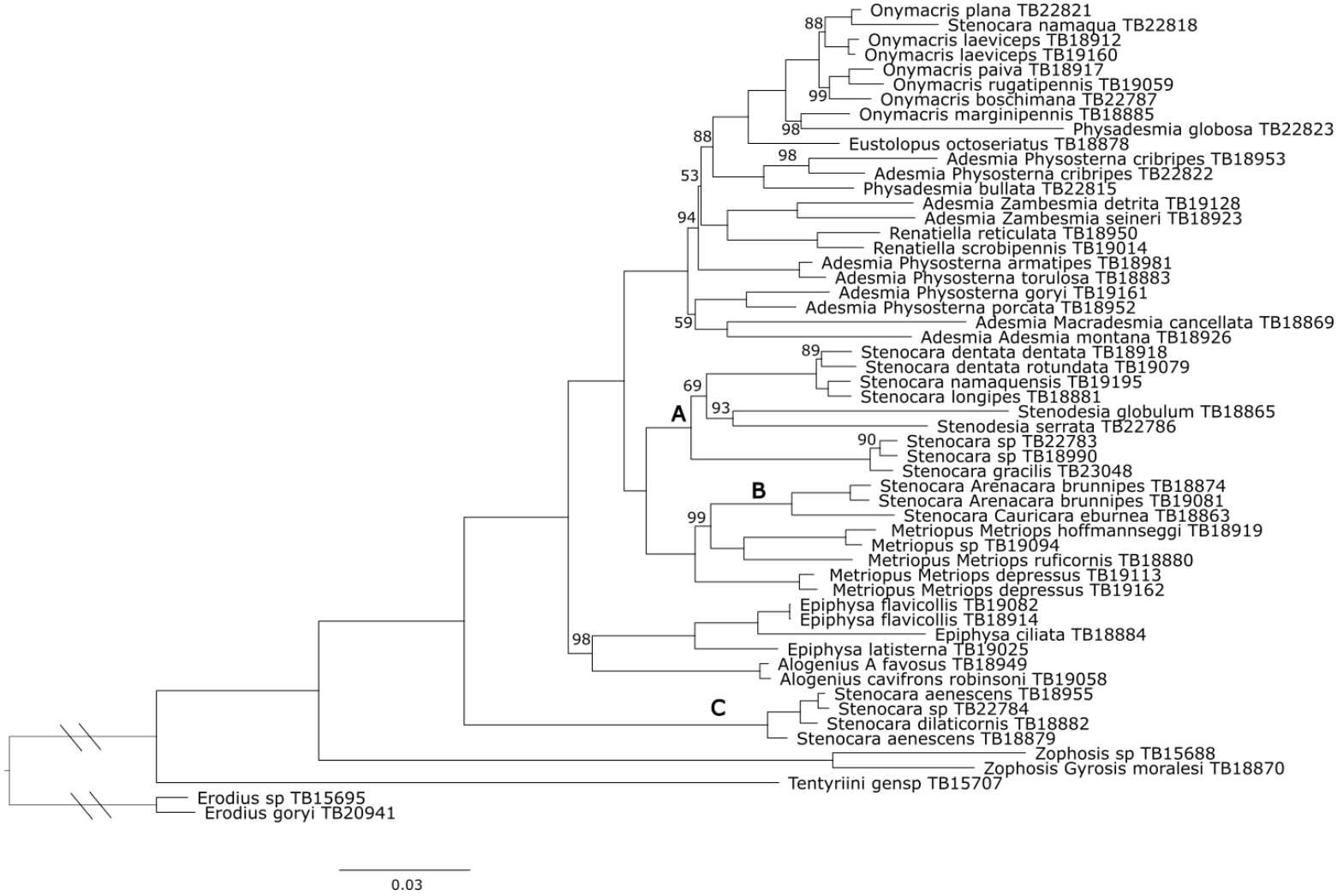


Figure 2.5. IQTREE 2 ML analysis of the Adesmiini Hsp40 homologs' nucleotide sequences. Support was assessed using 10,000 UF bootstrap replicates. Bootstrap values < 100 are depicted, branches with no values indicate a bootstrap value of 100. Letters on branches identify *Stenocara* clades.

The differences between the 510 loci Adesmiini phylogeny (Figure 1.2) and the amino acid maximum likelihood analysis (Figure 2.6) include *Stenocara* clade C sister to outgroup Zophosini taxa rather than to the rest of Adesmiini. Within the *Onymacris* clade, *Onymacris boschimana* is sister to *Onymacris plana* rather than *Onymacris paiva* and *Onymacris rugatipennis*. Similar to the nucleotide analysis, *Stenocara namaqua* is found within the *Onymacris* clade, although with low support. *Physadesmia bullata* and *Adesmia cribripes* fall sister to the *Onymacris*, *Physadesmia globosa*, *Eustolopus* clade rather than to *Adesmia* (*Zambesmia)* and *Renatiella*. The *Stenocara* clade B and *Metriopus* group is falling sister to the *Adesmia*, *Onymacris*, *Physadesmia*, *Eustolopus*, and *Renatiella* group rather than the clad B *Stenocara* and *Stenodesia* species. The two *Stenodesia* species are not sister to each other but separating into the two *Stenocara* groups within *Stenocara* clade A.

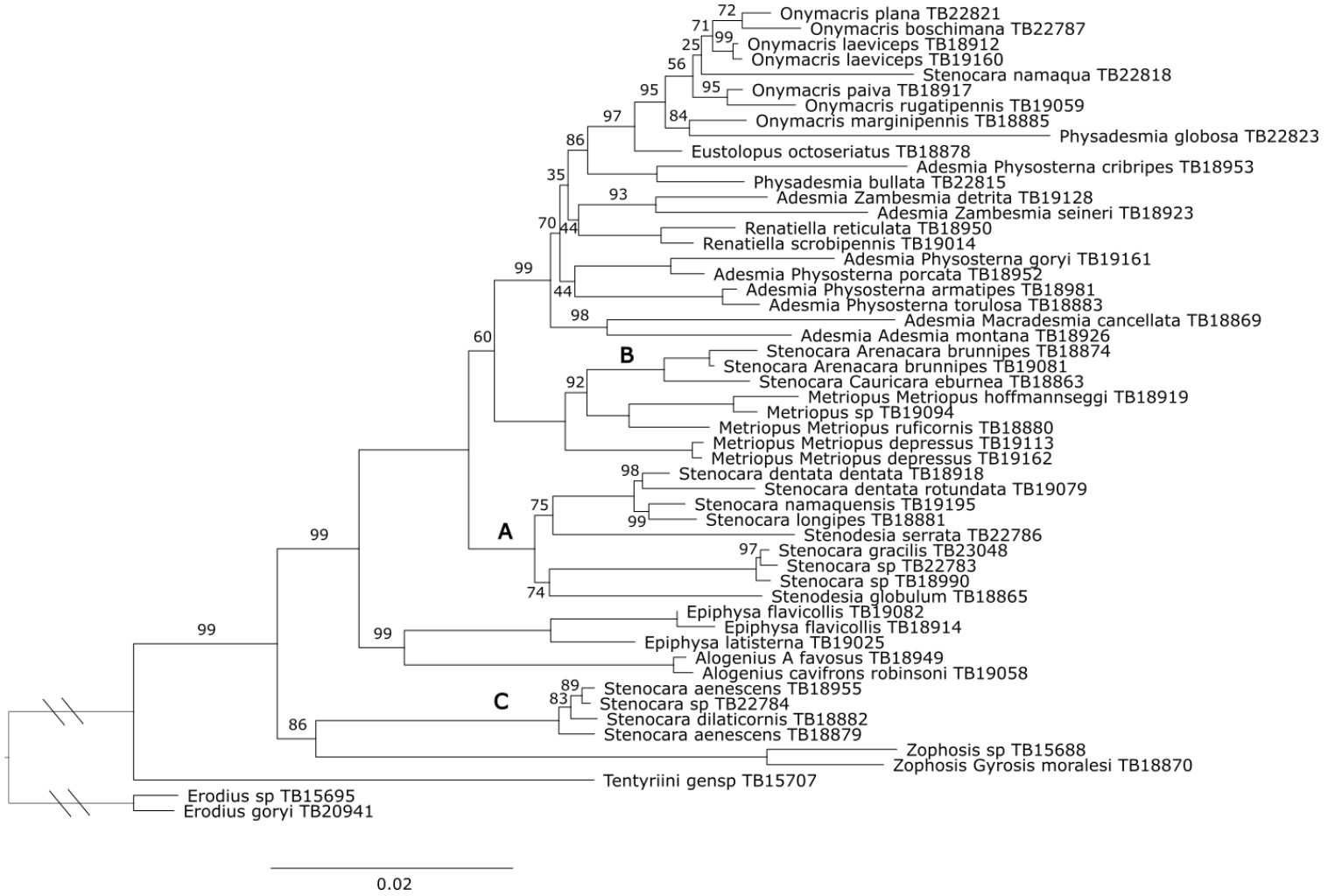


Figure 2.6. IQTREE 2 ML analysis of the Adesmiini Hsp40 homologs' amino acid sequences. Support was assessed using 10,000 UF bootstrap replicates. Bootstrap values < 100 are depicted, branches with no values indicate a bootstrap value of 100. Letters on branches identify *Stenocara* clade.

2.5 Discussion

While the amino acid and nucleotide Hsp40 analyses for Pimeliinae differ from the 627 loci phylogeny, they also differ from each other in both topology and overall support. The support from the 627 loci tree is much higher than both of the Hsp40 phylogenies, which each include seven loci. The Hsp40 ML analyses are similar to the 627 loci phylogeny when low support due to limited loci is considered. However, some topology differences do have an adequate level of support. Such as in the nucleotide analysis, *Cossyphodes* moves (BS=96), Epitragini and Edrotini are sister to each other (BS=87), *Morica* as sister to the other pimeliines (BS=86), and *Cryptochile* as at the base of the included tenebrionid taxa (BS=100). Changes with high support may be due to the evolution of Hsp40s. The nucleotide analysis generally has higher support than the amino acid analysis, which is especially seen in the inner nodes.

In regard to conserved cysteine position shifts, there are some interesting notes to mention. The tribe with the highest number of cysteine shifts, with 22 out of 81 total changes, is Edrotini, and Asidini follows with 12 total shifts. The fact that this increased number of shifts in comparison to other pimeliine tribes due to a denser sampling of taxa must be addressed. Edrotini is one of the higher sampled tribes with 19 out of the 142 total pimeliine OTUs while some other tribes have only one or very few representatives. However, a correlation between taxa number and cysteine shift number cannot be confirmed. In contrast to Edrotini, Asidini has 43 total taxa, over double the number of Edrotini taxa, while only having 12 cysteine shifts. Similarly, there are 35 Sepidiini taxa included in the study, but they only had seven total cysteine shifts in the Hsp40 homologs. The observation that while some tribes are more densely sampled with low numbers of cysteine shifts, some tribes, like Edrotini, do have a higher ratio of cysteine shifts to taxa examined. An increased number of cysteine shifts in this study may not be caused by an increased number of taxa examined. Therefore, these conserved amino acid positions may have a selective pressure influencing changes within some tribes (ex. Edrotini).

The contrast between both Hsp40 phylogenies and the Pimeliinae species tree demonstrate that while there may be relationship changes amongst tribes, the taxa within the tribes remain within their respective clades. However, variations of species habitat and life histories within the tribes were not assessed. Therefore, an analysis of pimeliine heat shock proteins including factors such as biogeography, ecology, and life history could provide more information on how selective pressures instigate changes in Hsp40's influence on ecology and species evolution.

After analyzing the Adesmiini topologies, there are some interesting differences between the 510 loci (Figure 1.2), Hsp40 nucleotide (Figure 2.5), and Hsp40 amino acid (Figure 2.6) phylogenies. In particular, *Stenocara* clade C is sister to *Zophosis* (Zophosini) species in the amino acid ML analysis. This level of conserved amino acid sequences could be due to both groups, *Stenocara* clade C and *Zophosis*, being diurnal taxa. Also, in both Hsp40 phylogenies, *Stenocara namaqua* is in the *Onymacris* clade. Both *Onymacris* species and *Stenocara namaqua* are long-legged, psammophilous taxa, indicating that they may have gone through convergent Hsp40 modifications due to, or resulting from, their shared diurnal, high intensity (cursorial), behaviors.

While there are differences in Hsp40s among pimeliine tribes, it is not the sole determiner of whether a group of beetles can survive in arid deserts. Overall, there is a basic assumption that insects survive in dry climates based on many factors which are a combination of molecular, behavioral, and morphological modifications. A more in-depth heat shock protein analysis across Pimeliinae, in association with life history, biogeographic, and morphological data, is needed to observe how these stress induced proteins vary across genera or species.

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