

Phylogenomic inference of the higher classification of velvet ants (Hymenoptera: Mutillidae)

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Abstract

The family Mutillidae (Hymenoptera) is a species-rich group of aculeate wasps that occur worldwide. The higher-level classification of the family has historically been controversial due, in part, to the extreme sexual dimorphism exhibited by these insects and their morphological similarity to other wasp taxa that also have apterous females. Modern hypotheses on the internal higher classification of Mutillidae have been exclusively based on morphology and, further, they include Myrmosinae as a mutillid subfamily. In contrast, several molecular-based family-level studies of Aculeata recovered Myrmosinae as a nonmutillid taxon. To test the validity of these morphology-based classifications and the phylogenetic placement of the controversial taxon Myrmosinae, a phylogenomic study of Mutillidae was conducted using ultraconserved elements (UCEs). All currently recognized subfamilies and tribes of Mutillidae were represented in this study using 140 ingroup taxa. The maximum likelihood criterion (ML) and the maximum parsimony criterion (MP) were used to infer the phylogenetic relationships within the family and related taxa using an aligned data set of 238,764 characters; the topologies of these respective analyses were largely congruent. The modern higher classification of Mutillidae, based on morphology, is largely congruent with the phylogenomic results of this study at the subfamily level, whereas the tribal classification is poorly supported. The subfamily Myrmosinae was recovered as sister to Sapygidae in the ML analysis and sister to Sapygidae + Pompilidae in the MP analysis; it is consequently raised to the family level, Myrmosidae, **stat.nov.** The two constituent tribes of Myrmosidae are raised to the subfamily level, Kudakrumiinae, **stat.nov.**, and Myrmosinae, **stat.nov.** All four recognized tribes of Mutillinae were found to be non-monophyletic; three additional mutilline clades were recovered in addition to Ctenotillini, Mutillini, Smicromyrmini, and Trogaspidiini sensu stricto. Three new tribes are erected for members of these clades: Pristomutillini Waldren, **trib.nov.**, Psammothermini Waldren, **trib.nov.**, and Zeugomutillini Waldren, **trib.nov.** All three recognized tribes of Sphaerophthalminae were found to be non-monophyletic; six additional sphaerophthalmine clades were recovered in addition to Dasymutillini, Pseudomethocini, and Sphaerophthalmini sensu stricto. The subtribe Ephutina of Mutillinae: Mutillini was found to be polyphyletic, with the *Ephuta* genus-group recovered within Sphaerophthalminae and the *Odontomutilla* genus-group recovered as sister to Myrmillinae + Mutillinae. Consequently, the subtribe Ephutina is transferred from Mutillinae: Mutillini and is raised to a tribe within Sphaerophthalminae, Ephutini, **stat.nov.** Further, the taxon Odontomutillinae, **stat.nov.**, is raised

from a synonym of Ephutina to the subfamily level. The sphaerophthalmine tribe Pseudomethocini was found to be polyphyletic, with the subtribe Euspinoliina recovered as a separate clade in Sphaerophthalminae; consequently, Euspinoliina is raised to a tribe, Euspinoliini, **stat.nov.**, in Sphaerophthalminae. The dasylabrine tribe Apteromutillini was recovered within Dasylabridae and is proposed as a new synonym of Dasylabridae. Finally, dating analyses were conducted to infer the ages of the Pompiloidea families (Mutillidae, Myrmosidae, Pompilidae, and Sapygidae) and the ages of the Mutillidae subfamilies and tribes.

KEYWORDS

Aculeata, Myrmosidae, parasitoid wasps, Pompiloidea, ultraconserved elements

INTRODUCTION

Wasps of the family Mutillidae (Hymenoptera: Aculeata), commonly known as velvet ants, are a conspicuous yet little-known component of the world's tropical and temperate ecosystems (Figure 1). Velvet ants are primarily solitary ectoparasitoids of ground-nesting bees and apoid wasps (Hymenoptera: Apoidea) with a few host records known for Coleoptera, Diptera, and Lepidoptera (Brothers et al., 2000; Luz et al., 2016). These insects exhibit extreme sexual dimorphism, with females being apterous and males typically being fully winged (Figure 1). The sexes have few shared characters, with most species and even many genera being known from only a single sex. Past researchers included within Mutillidae unrelated taxa that are now considered distinct families, or nonmutillid subfamilies, in part due to the superficial similarity of the apterous females; such taxa include Bradynobaenidae, Chyphotidae, Sapygidae: Fedtschenkiinae, Tiphidae: Brachycistidinae, and Thynnidae: Methochinae (André, 1902; Bischoff, 1920; Fox, 1899; Schuster, 1947, 1949; Waldren, 2021). Modern phylogenetic analyses have helped clarify the identity of these taxa as distinct from Mutillidae (Branstetter, Danforth, et al., 2017a; Brothers, 1975, 1999; Brothers & Carpenter, 1993; Pilgrim et al., 2008). However, there remains a taxon, the Myrmosinae, whose membership within Mutillidae remains controversial. Morphology-based phylogenetic analyses recovered myrmosines as a subfamily of Mutillidae (Brothers, 1975, 1999; Brothers & Carpenter, 1993; Lelej & Nemkov, 1997; Brothers & Lelej, 2017), while molecular-based phylogenetic analyses recovered myrmosines as their own family, the Myrmosidae (Branstetter, Danforth, et al., 2017a; Debevec et al., 2012; Pilgrim et al., 2008). Myrmosines are unique within Mutillidae as females have an articulating pronotal-mesonotal suture (Figure 1l), whereas in all other Mutillidae it is fused and immobile (Figure 1b–g, i, k).

The modern foundation for mutillid classification is based on Brothers (1975), who conducted the first cladistic study of Aculeata with an emphasis on Mutillidae at a global scale. He classified the family into seven subfamilies, four tribes, and four subtribes. Further, Brothers (1975) moved several subfamilies out of Mutillidae and into their own family, Bradynobaenidae (i.e., Apterogyninae, Bradynobaeninae, Chyphotinae, and Typhoctinae (including Eotillini)). Brothers and Carpenter (1993) and Brothers (1999) expanded upon the study of Aculeata by Brothers (1975) using a cladistic approach and reached similar results to the latter. The mutillid classification proposed by Brothers (1975) remained the sole hypothesis for more than two decades until Lelej and Nemkov (1997) conducted a cladistic analysis

that resulted in a slightly different classification. To resolve the differences between these two competing classifications, a joint cladistic study of Mutillidae based on morphology was undertaken by Brothers & Lelej (2017). This study is the most morphologically-comprehensive analysis of the family to date. Brothers and Lelej (2017) coded their terminals at the genus level and used 230 characters for their cladistic analysis. The higher taxa they proposed were delimited by synapomorphies discovered through their cladistic analyses, and their final classification for Mutillidae is visually summarized here in Figure 2a, b. Three new tribes and one subtribe were erected: Apteromutillini (Dasylabridae), Ctenotillini (Mutillinae), Dasymutillini (Sphaerophthalminae), and Euspinoliina (Sphaerophthalminae: Pseudomethocini). The family Mutillidae currently includes 4603 valid species among 220 genera and classified into 8 subfamilies (including Myrmosinae), 13 tribes, and 4 subtribes (Brothers & Lelej, 2017; Pagliano et al., 2020).

The phylogenetic placement of the mutillid subfamily Myrmosinae has historically been controversial, having either been considered its own family (Ashmead, 1899; Branstetter, Danforth, et al., 2017a; Pilgrim et al., 2008; Skorikov, 1935; Suárez, 1988), a subfamily or tribe of Mutillidae (André, 1902; Bischoff, 1920; Brothers, 1975; Brothers & Lelej, 2017; Fox, 1894; Lelej & Nemkov, 1997), or a subfamily of Tiphidae (Krombein, 1940). Modern cladistic analyses using morphology have supported Myrmosinae as the sister subfamily to the remaining Mutillidae (Brothers, 1975; Brothers & Lelej, 2017; Lelej & Nemkov, 1997), while molecular phylogenetic analyses have supported it having family-level status (Branstetter, Danforth, et al., 2017a; Debevec et al., 2012; Pilgrim et al., 2008). Pilgrim et al. (2008) and Debevec et al. (2012) found Myrmosinae to be sister to Sapygidae, while Branstetter, Danforth, et al. (2017a) found Myrmosinae to be sister to Mutillidae and it was considered its own family. These studies, however, used a limited number of mutillid samples as their focus was on Aculeata as a whole.

Considering the historical uncertainty regarding the placement of Myrmosinae and a lack of consensus among recent phylogenetic studies using different types of data and methodologies, a phylogenomic approach to resolve these differences is desirable. Further, a phylogenomic study testing the relationships of the higher taxa of Mutillidae is also desirable, as all previous classifications have been based on morphology. The only published molecular phylogenetic analysis dedicated to a supra-generic group of mutillids is that of Pitts et al. (2010), who investigated the Nearctic nocturnal Sphaerophthalminae using two ribosomal DNA internal transcribed spacer regions, ITS1 and ITS2.

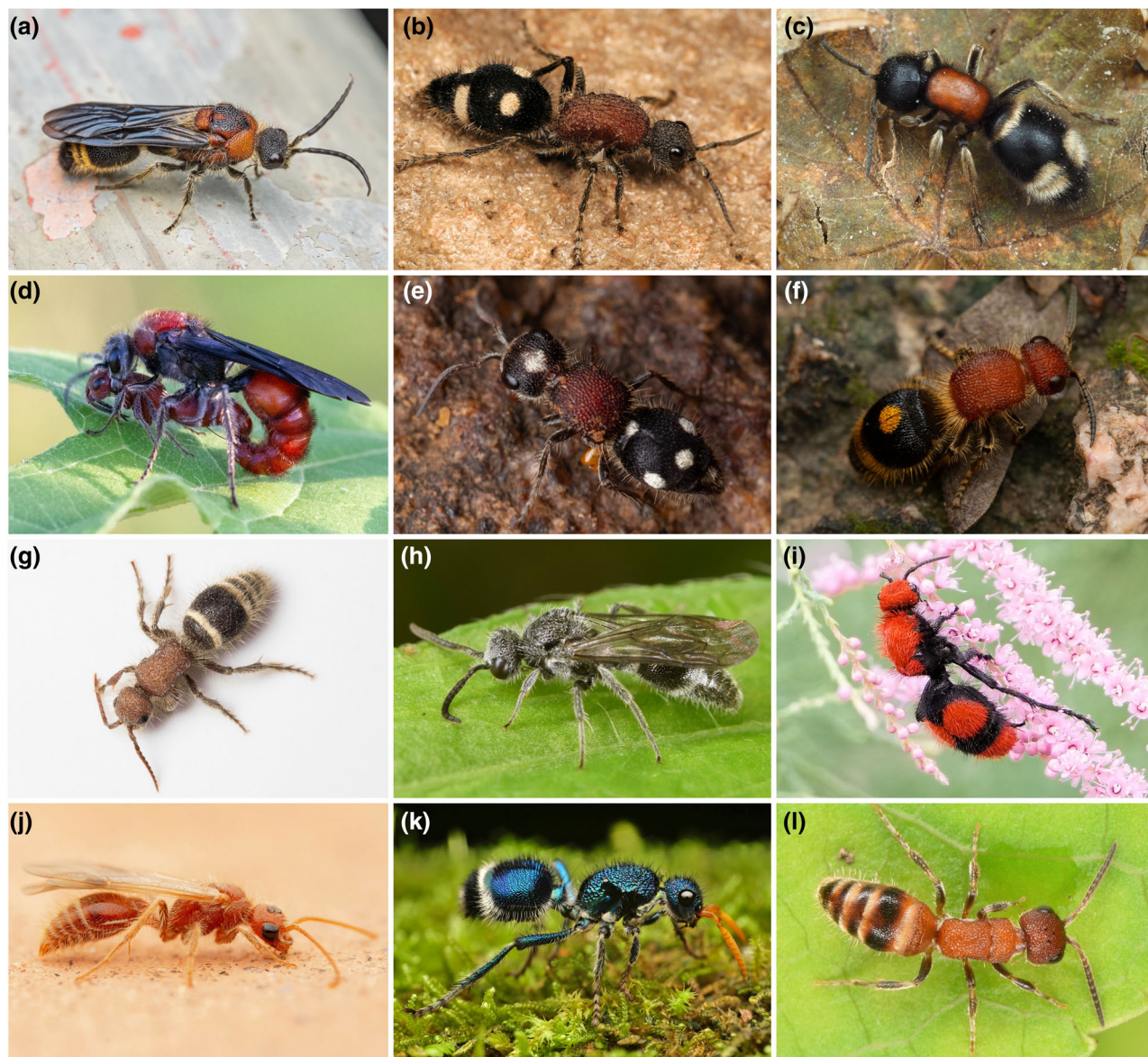


FIGURE 1 A few examples of the diversity of Mutillidae and Myrmosidae. (a) *Orientilla* sp. (Mutillidae: Dasylabrinae), photograph by Artur Tomaszek. (b) *Dolichomutilla* sp. (Mutillidae: Mutillinae: Mutillini), photograph by Wynand Uys. (c) *Mutilla marginata* Baer, 1848 (Mutillidae: Mutillinae: Mutillini), photograph by Karim Stroehriegel. (d) *Timulla vagans* (Fabricius, 1798) (Mutillidae: Mutillinae: Trogaspidiini), photograph by Jeff O'Connell. (e) *Spilomutilla* sp. (Mutillidae: Myrmillinae), photograph by Jithesh Pai. (f) *Odontomutilla uranioides* Mickel, 1933 (Mutillidae: Odontomutillinae), photograph by Lawrence Hylton. (g) *Pseudophotopsis syriaca* (André, 1900) (Mutillidae: Pseudophotopsidinae), photograph by Konstantinos Kalaentzis. (h) *Ephuta* sp. (Mutillidae: Sphaerophthalminae: Ephutini), photograph by E. Christina Butler. (i) *Dasymutilla occidentalis* (Linnaeus, 1758) (Mutillidae: Sphaerophthalminae: Dasymutillini), photograph by David Tibbetts. (j) *Odontophotopsis conifera* Schuster, 1958 (Mutillidae: Sphaerophthalminae: Sphaerophthalmini), photograph by Jake N. (k) *Ephutomorpha paradisiaca* Zavattari, 1914 (Mutillidae: Sphaerophthalminae: Clade 8I), photograph by Philipp Hoenle. (l) *Myrmosa* sp. (Myrmosidae: Myrmosinae), photograph by E. Christina Butler.

This contribution represents the first molecular phylogenetic analysis of Mutillidae at the family level. The goal of this study is to test the morphology-based higher classification of Mutillidae proposed by Brothers and Lelej (2017) and also the position of Myrmosinae in relation to the rest of Mutillidae with a phylogenomic approach using ultraconserved elements (UCEs). UCEs are highly conserved regions of the genome that are shared among distantly-related taxa. Each UCE is flanked by variable sites that

provide phylogenetic signal, and the UCE itself, while also informative, additionally provides a series of shared character states between taxa. The function of UCEs in the genome is unknown, but there is evidence that they are involved in gene regulation (Pennacchio et al., 2006) and development (Sandelin et al., 2004; Woolfe et al., 2004). The first general approach to using UCEs in a phylogenetic context was performed by Faircloth et al. (2012). UCEs have recently been used to infer the phylogeny of Aculeata

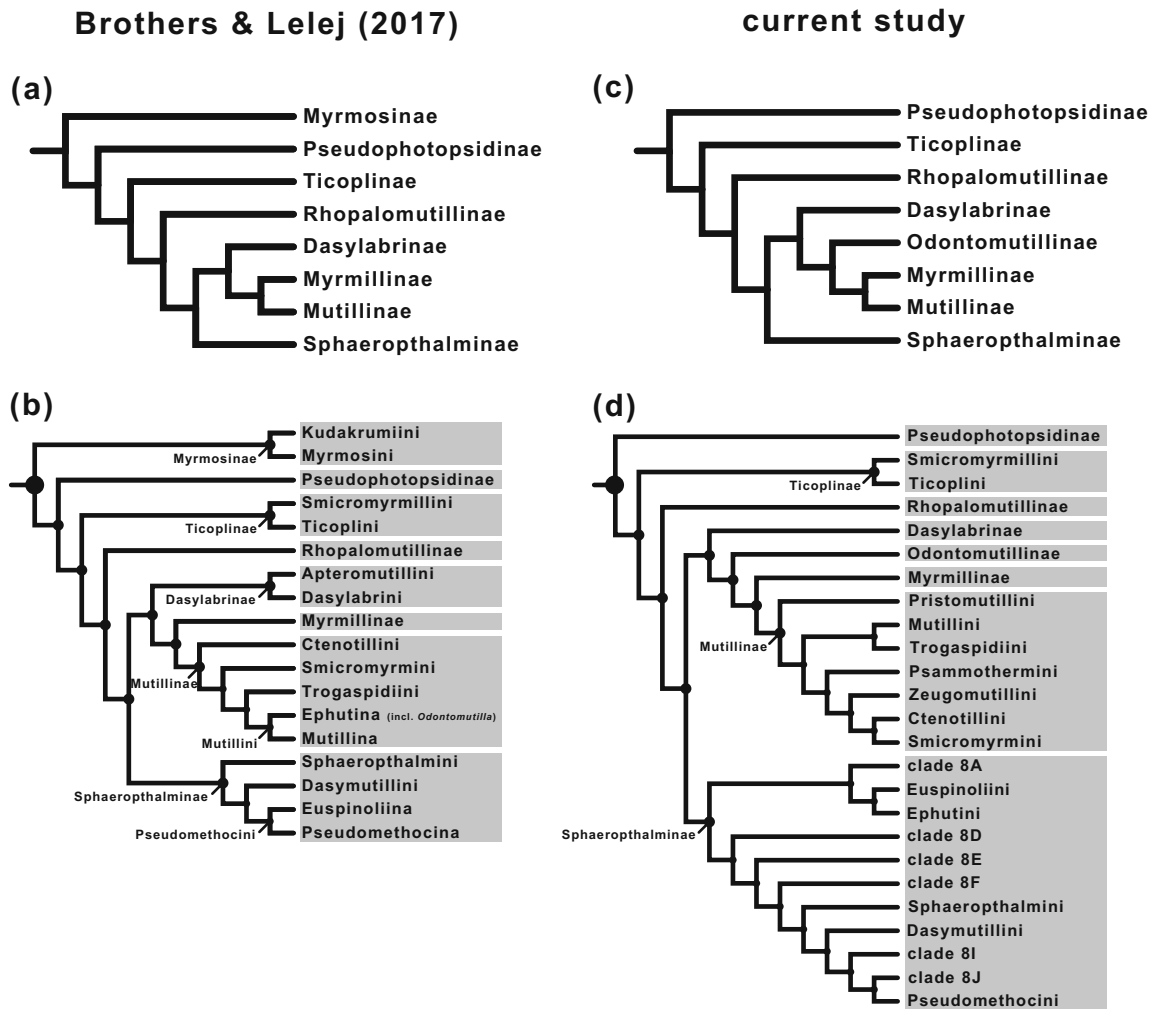


FIGURE 2 Higher classification hypotheses for Mutillidae. (a) Mutillidae subfamily classification proposed by Brothers & Lelej (2017). (b) Mutillidae tribal classification proposed by Brothers & Lelej (2017). (c) Mutillidae subfamily classification proposed herein. (d) Mutillidae tribal classification proposed herein. Myrmosinae is excluded from (c) and (d) as it is considered a separate family from Mutillidae in the current study.

(Branstetter, Danforth, et al., 2017a), and they are increasingly being used to infer phylogenies across a variety of taxa.

MATERIALS AND METHODS

Taxon sampling

Specimens representing 192 taxa were chosen for the study with 140 ingroup taxa (Mutillidae including Myrmosinae) and 52 outgroup taxa. All Mutillidae subfamilies and tribes recognized by Brothers and Lelej (2017) were represented, and terminals were treated at the species level rather than the genus level in contrast to Brothers and Lelej (2017). All samples were dried, pinned museum specimens of various ages collected within the last 50 years, with the oldest specimen collected in 1974. Each specimen was assigned a unique specimen identifier (USI) with the prefix MUT, TIM, EX, PS, or U depending on the taxon and the location where the laboratory work was conducted.

Outgroup data for 49 taxa were sourced from Branstetter, Danforth, et al. (2017a), Faircloth et al. (2015), and Sadler (2018) representing most families of Aculeata, and data for three taxa were sourced from an ongoing study of Pompilidae (Pitts unpub.). An additional 17 ingroup taxa were sourced from Sadler (2018).

Voucher specimens from which new molecular data were acquired for this study are deposited at the Entomological Museum of Utah State University (EMUS) (Logan, UT, U.S.A.). These specimens were identified to genus or species using Arnold (1956), Bartholomay et al. (2019), Bischoff (1920), Brothers (1971, 2015), Cambra and Quintero (1996, 1997, 2004), Cambra et al. (2016, 2017), Casal (1962a, 1962b, 1964a, 1968a, 1968b, 1970), József and Zoltán (2011), Krombein (1940, 1972), Lelej (1980, 1985, 1995a, 1995b, 1996, 2002, 2005), Lelej and Brothers (2008), Lelej et al. (2017), Luz and Williams (2014), Mickel (1935a, 1935b, 1936a, 1937b, 1938a, 1938b, 1938c, 1939, 1941, 1943, 1952, 1960, 1964), Mitchell & Brothers (1998, 2002), Nonveiller (1979, 1980, 1995, 1996, 1997), Nonveiller & Petersen (1995, 1996), O'Toole (1975), Pagliano & Strumia (2007),

Pitts (2000, 2003), Pitts & McHugh (2002), Sadler et al. (2017), Schuster (1949, 1951, 1958), Suárez (1962), Tanner et al. (2009), Tu et al. (2014), Turrisi et al. (2015), Waldren et al. (2020), Wasbauer (1973), Williams & Pitts (2008), Williams et al. (2019a, 2019b, 2019c), and Wilson & Pitts (2008).

Molecular data acquisition

Library preparation, UCE enrichment, and contig assembly were performed in two separate laboratories (Utah State University (EMUS), Logan, UT, U.S.A. and the National Museum of Natural History (NMNH), Washington, D.C., U.S.A.) and the methodologies used in both labs were mostly similar. Any methods that differ between the labs are noted as having been performed at either EMUS or NMNH.

DNA extraction was performed using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Indianapolis, IN, U.S.A.) at EMUS or with a standard phenol-chloroform method at Cornell University and later prepared at NMNH. Entire adult specimens were primarily used for extraction except for rare species in which a single mesoleg and metaleg were removed and partly crushed. The entry point for extraction material into the specimens was typically the resulting pin hole in the mesosoma after removing the pin. Following extraction, specimens were rinsed using 95% ethanol and remounted on pins to allow for future study. The extracted DNA was quantified using a Qubit 3.0 Fluorometer and was prepared for shearing at a target concentration of 50 ng/100 µL. The samples were then sheared into fragments ranging between 400 and 600 base pairs using a Qsonica Q800R2 sonicator at EMUS or a Qsonica Q800R sonicator at NMNH (Qsonica L.L.C., Newtown, CT, U.S.A.). Library preparation was performed using a KAPA HyperPrep Kit (Roche Sequencing and Life Science, Wilmington, MA, U.S.A.) and Illumina TruSeq-style adapters (Glenn et al., 2019). Libraries were pooled at equimolar ratios of 10 with up to 500 ng of DNA used for targeted UCE enrichment. Each pool was enriched using a standardized protocol based on myBaits Hybridization Capture for Targeted NGS Manual v.4.01 and Branstetter, Danforth et al. (2017a). Enrichment was performed using a custom Ant-Specific probe set at EMUS (myBaits UCE Hymenoptera 2.5Kv2A) which targets 2524 conserved loci (Branstetter, Longino et al., 2017b) or the Principal Hymenoptera probe set at NMNH (myBaits UCE Hymenoptera 2.5Kv2P) which targets 2590 conserved loci (Branstetter, Danforth et al., 2017a) (both available from Arbor Biosciences (Ann Arbor, MI, U.S.A.)). The probes were hybridized to the libraries at 65°C for an incubation period of 24 h. Enrichment success was determined via qPCR using a Bio-Rad CFX96 system. Each pool was quantified using the qPCR results and was further pooled into pools of 110 total libraries. The EMUS pools were sequenced using an Illumina HiSeq X Ten system at Novogene (Chula Vista, California, U.S.A.) and the NMNH pools were sequenced using an Illumina HiSeq 2500 system at the Cornell Institute of Biotechnology (BRC) (Ithaca, New York, U.S.A.).

Molecular data assembly, alignment, and processing

The software package PHYLUCE v.1.6.6 (Faircloth, 2016) was used for all post-sequence data processing and preparation for phylogenetic analysis. Raw FASTQ files were first demultiplexed using BMap and adapter sequences were removed using Illumiprocessor. Contigs were assembled using SPAdes (Bankevich et al., 2012) at EMUS or Trinity at NMNH. These contigs were then matched to the ant-specific probe set (myBaits UCE Hymenoptera 2.5Kv2A) using the PHYLUCE pipeline with the thresholds set to 60 and 80 for sequence identity and overlap, respectively. A master UCE sequence data file was generated using the FASTA information pulled from the match counts. The sequences were aligned using MAFFT v.7.407 (Kato & Standley, 2013). Poorly-aligned regions were cleaned and trimmed from the data set using Gblocks (Talavera & Castresana, 2007) with reduced stringency parameters (b1:0.5, b2:0.5, b3:12, b4:7). Alignments were filtered for missing data using a PHYLUCE script requiring that each alignment include data for ≥75% of taxa. The resulting data set was used for all subsequent phylogenomic analyses.

Phylogenomic analyses

The program IQ-TREE v.1.6.1 (Nguyen et al., 2015) was used for maximum likelihood inference (hereafter ‘ML’). The data set was partitioned by UCE loci with each partition allowed a different evolutionary speed (‘-spp’ option). ModelFinder (Kalyaanamoorthy et al., 2017) was used to find the best-fit model of sequence evolution per partition (Chernomor et al., 2016). The SH-like approximate likelihood ratio test (SH-aLRT, ‘-alrt’ option) (Guindon et al., 2010) and ultrafast bootstrap approximation (UFBoot, ‘-bb’ option) (Hoang et al., 2017) were used to evaluate branch support with each set to 1000 replicates. To test if tree space was adequately searched, four additional independent ML analyses were performed using the aforementioned settings. Finally, five additional independent ML analyses were performed using the aforementioned settings with IQ-TREE v.2.2.0.7 along with an option to reduce the potential overestimation of ultrafast bootstrap approximations due to the potential presence of severe model violations (UFBoot2, ‘-bnni’ option) (Hoang et al., 2017). The thresholds that were used to determine well-supported clades were ≥80% for SH-aLRT and ≥95% for UFBoot. The resulting phylogenetic tree from the first of 10 total IQ-TREE ML analyses (i.e., the regular analysis without the UFBoot2 option using v.1.6.1) was visualized in FigTree v.1.4.4 (Figures 3–5).

The program TNT v.1.5 (Goloboff & Catalano, 2016) was used for maximum parsimony inference (hereafter ‘MP’). Settings used include 100 cycles of Random Addition Sequence, 25 iterations of Drift, 25 iterations of Ratchet (Nixon, 1999), and branch-swapping with TBR. All characters were treated as unordered and equally weighted. Gaps were treated as missing data. Branch supports (Bremer, 1988, 1994; Brower, 2006) were calculated using 1000 suboptimal trees up to 10,000 additional steps longer; these suboptimal trees were then treated to TBR branch-swapping. The resulting cladogram was visualized in FigTree v.1.4.4 (Figure 3).

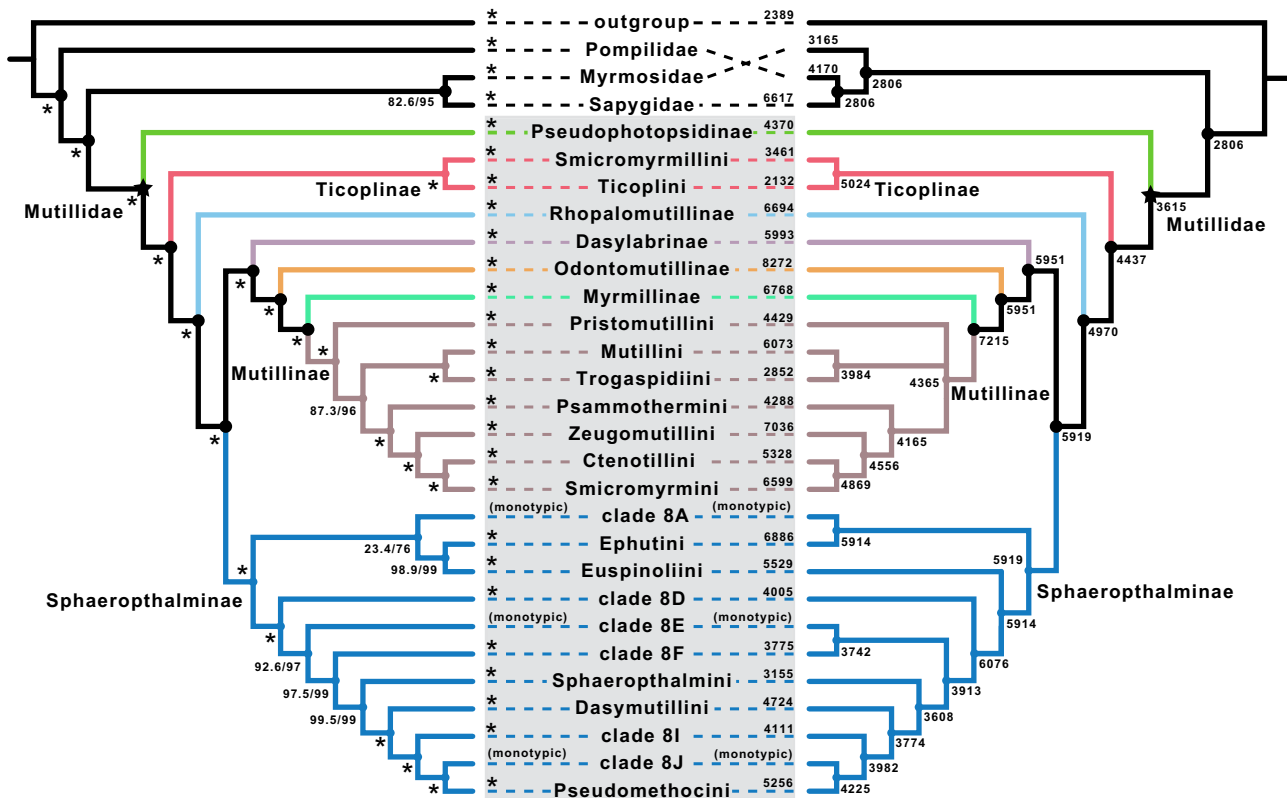


FIGURE 3 Comparison between the results of the maximum likelihood analysis (left) and maximum parsimony analysis (right). For the maximum likelihood (ML) tree, the asterisks indicate SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. For the maximum parsimony (MP) cladogram, the numerical values are branch supports and have no upper limit.

Dating analyses

The programs BEAST v.1.10.4 (Suchard et al., 2018) and IQ-TREE v.2.1.1 (Minh et al., 2020) were used to estimate ages for Pompiloidea with emphasis on Mutillidae. To reduce computational demands, 50 loci were randomly sourced from the master alignment used in the ML and MP analyses, and this data set was treated as a single partition. In addition, the ML tree was used as a fixed topology reference tree in all analyses. Estimated ages are reported herein in a split format, with the estimated age inferred using BEAST first and the estimated age inferred using IQ-TREE second (e.g., 23/21 Ma). Further, the final estimated ages were rounded to the nearest whole million years.

For the BEAST analyses, BEAUti v.1.10.4 was used to generate the XML file. The substitution model used was GTR + G. An uncorrelated relaxed clock with a lognormal distribution was used (Drummond et al., 2006). The tree prior used was Speciation: Birth-Death Process (Gernhard, 2008). The tree-generating operators were turned off (i.e., subtreeSlide, narrowExchange, wideExchange, and wilsonBalding). Priors used to calibrate the tree were derived from both primary fossil data and secondary previously published dating analyses. The estimated ages of Apocrita (settings: mean = 194 Ma, SD = 10, normal distribution) and Aculeata (settings: mean = 161 Ma, SD = 10, normal distribution) were sourced from Branstetter et al. (2017), specifically the median ages of their 50 random loci BEAST

analysis. Priors derived from fossil data were dated using the median of date ranges sourced from <http://fossilworks.org/>; the geologic time scale used herein was also derived from this website. Confirmed fossil Mutillidae are only known from Baltic amber specimens for the myrmosine tribe Kudakrumiini (Bischoff, 1916; Lelej, 1986), Dominican amber specimens for the sphaerophthalmine tribe Dasymutillini (Manley & Poinar, 1991, 1999, 2003), and a Dominican amber specimen for the mutilline subtribe Ephutina (Brothers, 2003). No fossil specimens were personally examined in this study, although the illustrations and/or photographs associated with their original descriptions allowed for accurate subfamilial or tribal placement; refer to the discussion section for more information regarding these fossils.

The myrmosine tribe Kudakrumiini was represented in the analysis by the genus *Protomutilla* Bischoff, 1916 from Baltic amber from the late Eocene (Priabonian Stage) dated 37.2–33.9 Ma (settings: mean = 35.6 Ma, SD = 1, lognormal distribution, mean in real space). The clade *Dasymutilla* Ashmead, 1899 + *Traumatomutilla* André, 1901 within Dasymutillini was represented by *Dasymutilla albifasciatus* Manley & Poinar, 1999. The clade *Ephumelia* Casal, 1968b + *Ephuchaya* Casal, 1968b + *Ephuta* Say, 1836 within Ephutina was represented by *Ephuta clavigera* Brothers, 2003. Both *D. albifasciatus* and *E. clavigera* are from Dominican amber from the early Miocene dated 20.4–13.7 Ma (settings: mean = 17.1 Ma, SD = 1, lognormal distribution, mean in real space). For the other two families of Pompiloidea,

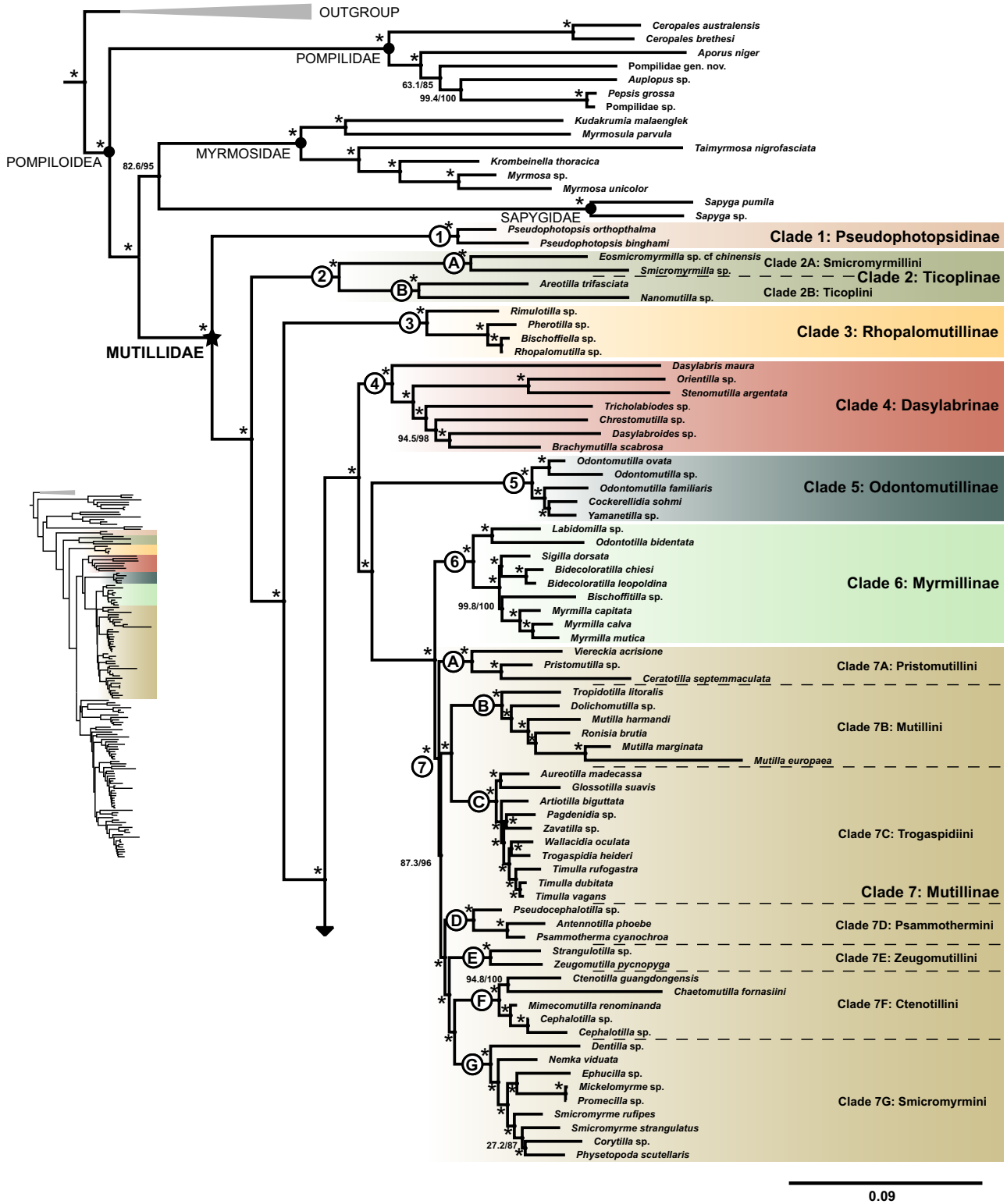


FIGURE 4 Higher classification of Mutillidae using the maximum likelihood tree topology, part 1; for part 2 see Figure 5. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Pompilidae and Sapygidae, only the oldest known fossil was included. The Sapygidae were represented by *Cretofedtschenkia santanensis* Osten, 2007 from the Crato Formation dated 122.46–112.6 Ma

(settings: mean = 117.5 Ma, SD = 3, lognormal distribution, mean in real space). The Pompilidae were represented by the pepsine species *Cryptocheluis leleji* Waichert, Rapoza & Rodriguez (Waichert et al., 2019) from

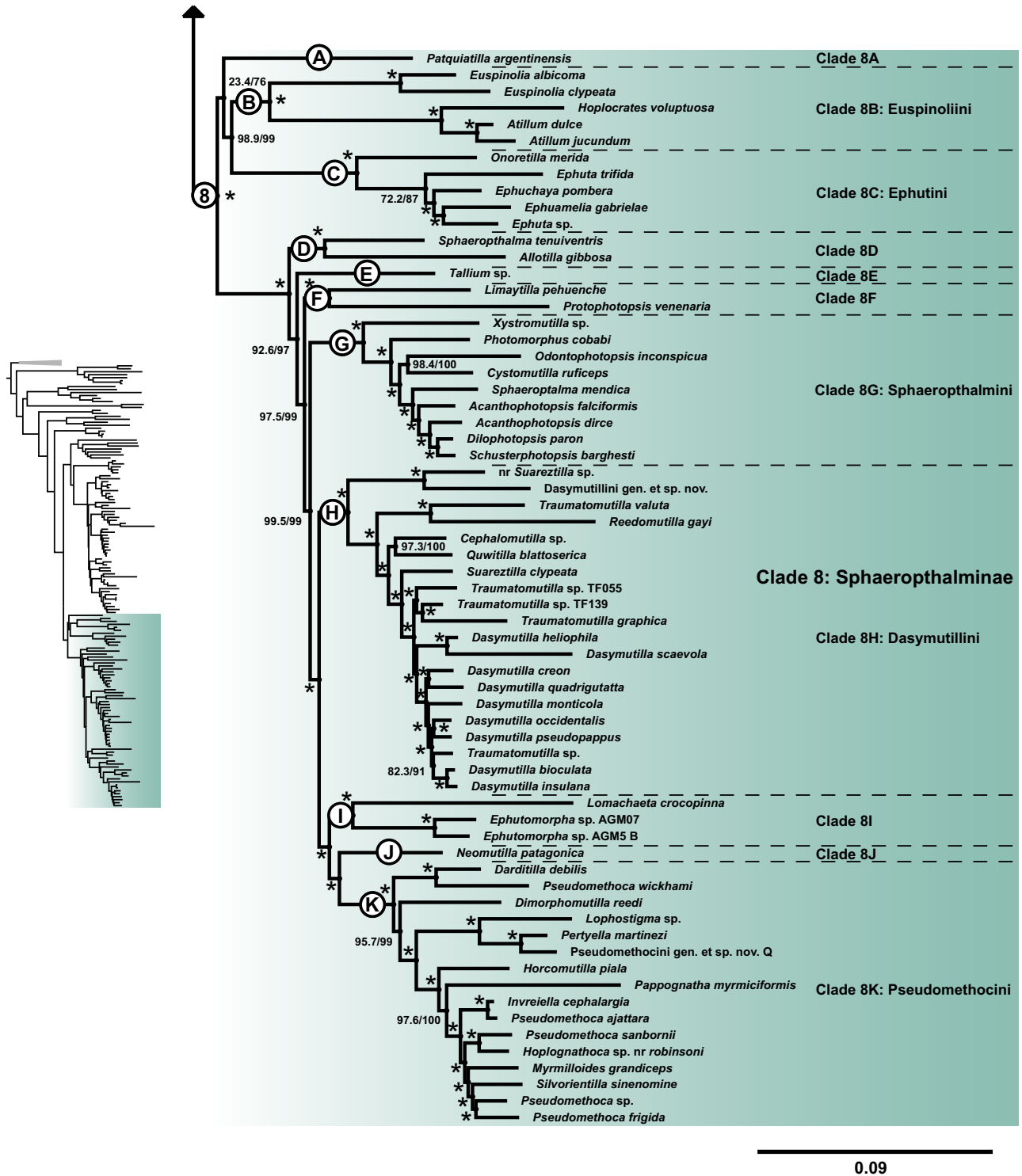


FIGURE 5 Higher classification of Mutillidae using the maximum likelihood tree topology, part 2; for part 1 see Figure 4. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

the Fur Formation dated 55.8–48.6 Ma (settings: mean = 52.2 Ma, SD = 2, lognormal distribution, mean in real space). The standard deviations used account for the currently estimated lower and upper age boundaries, 5% and 95% of their distribution, respectively. Last, the priors *uclcd.mean* and *uclcd.stdev* were set to 0.001672 and 0.381,

respectively, based on test analyses performed before the final analyses. Three independent Markov Chain Monte Carlo (MCMC) runs were performed with length of chain set to 300,000,000 and were logged every 2000 generations. The BEAST analyses were conducted using the CIPRES Science Gateway v.3.3 (Miller et al., 2010). Logs of the BEAST

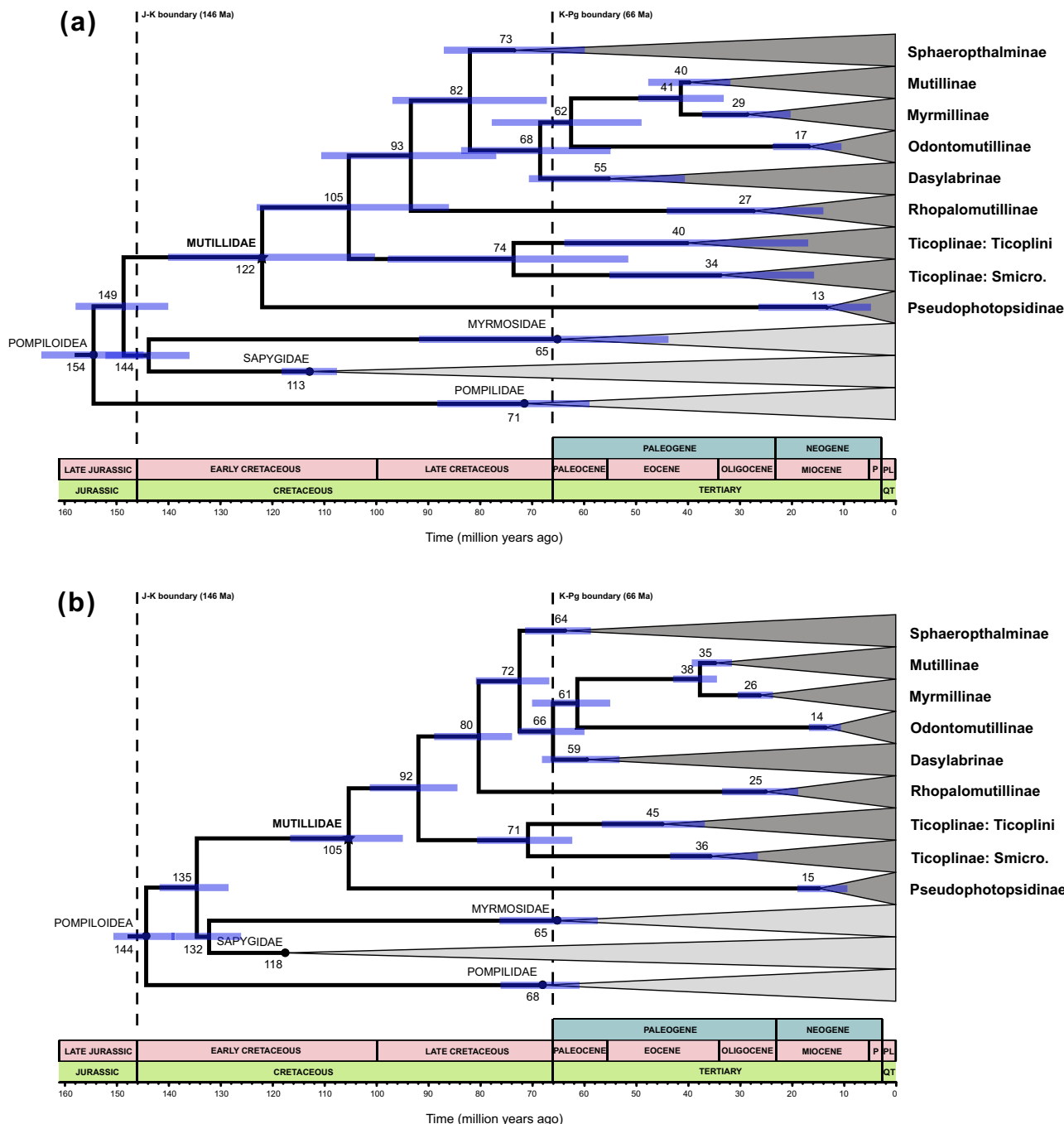


FIGURE 6 (a) Chronogram of Pompiloidea families and Mutillidae subfamilies using BEAST. The purple bars represent 95% HPD intervals. (b) Chronogram of Pompiloidea families and Mutillidae subfamilies using IQ-TREE v.2.1.1. The purple bars represent confidence intervals.

analyses were assessed in Tracer v.1.7.1 (Rambaut et al., 2018) to confirm convergence and adequate effective sample sizes (ESSs). The independent runs were combined in LogCombiner v.1.10.4. Ten percent of states were discarded as burn-in, and states were resampled at a frequency of 10,000. A maximum clade credibility tree was generated in TreeAnnotator v.1.10.4 and visualized in FigTree v.1.4.4 (Figure 6a).

For the IQ-TREE analysis using the least-squares criterion (To et al., 2016), the substitution model used was GTR + G, the root was dated as 194 Ma, the tips were dated as 0 Ma, and the outgroup of Aculeata was set as the taxon “Ichneumoninae_sp_EX481.” The mean

calibrations used in the BEAST analysis were also used in the IQ-TREE analysis. The resulting chronogram was visualized in FigTree v.1.4.4 (Figure 6b).

RESULTS AND DISCUSSION

UCE loci recovered among the 192 taxa used in this study ranged from 77 to 2009 (mean = 1277; median = 1230). The final alignment used in the analyses was composed of 238,764 base pairs with

19.03% total missing data. 54,610 characters were constant, 23,327 were parsimony-uninformative, and 160,827 were parsimony-informative. ModelFinder assigned substitution models for 663 partitions for use in the ML analysis. The five regular independent ML analyses resulted in trees with identical topologies and with most major nodes having both 100% SH-aLRT and UFBoot support values (Figures 3–5 and S1–S9 are the results of the first analysis using IQ-TREE v.1.6.1 without the ‘-bnni’ option). In addition, the five independent ML analyses using UFBoot2 (i.e., the ‘-bnni’ option) resulted in trees with identical topologies and with nodes having support values mostly similar to the former five regular ML analyses in which this option was not used (except that SH-aLRT values that were <100% increased and UFBoot values that were <100% decreased in relation to the regular analyses). The MP analysis resulted in two most parsimonious trees consisting of 1,781,022 steps (CI = 0.234; RI = 0.565); branch support values ranged from 2806 to 7215 (Figure 3 is the strict-consensus tree).

The ML and MP analyses resulted in similar topologies (Figure 3) and are identical with respect to subfamily interrelationships and composition within Mutillidae (excluding Myrmosinae). The superfamily Pompiloidea, to which Mutillidae, Pompilidae, and Sapygidae belong, was found to be monophyletic in both ML and MP analyses. The Myrmosinae were recovered as sister to Sapygidae in the ML analysis with relatively high support (82.6 SH-aLRT/95 UFBoot) (Figures 3, 4) while for the MP analysis the Myrmosinae were sister to Pompilidae + Sapygidae (branch support = 2806) (Figure 3). The results of the ML analysis with Myrmosinae as sister to Sapygidae are congruent with the results of Pilgrim et al. (2008). The relationships between the Mutillidae subfamilies are consistent between the analyses and overall are mostly congruent with the results of Brothers & Lelej (2017) (Figure 2). The exception is that the *Odontomutilla* genus-group, classified in Brothers & Lelej (2017) as a member of the Mutillini subtribe Ephutina, was recovered as sister to Myrmillinae + Mutillinae. The eight subfamilies recognized in the current study were recovered as Pseudophotopsidinae + (Ticopliinae + (Rhopalomutillinae + (Sphaerophthalminae + (Dasylabrinae + (Odontomutillinae + (Myrmillinae + Mutillinae)))))) (Figures 3–5). Subfamilies were numbered 1–8 beginning with the subfamily Pseudophotopsidinae, and tribes were given alphanumeric labels according to their subfamily membership (e.g., the subfamily Mutillinae is clade 7; the tribe Mutillini is clade 7B). The final classification is visually summarized in Figure 2c, d. The master reference for the alphanumeric naming of clades is the ML tree (Figures 4, 5). Based on these results, the monophyletic status of the subfamilies and tribes of Mutillidae are discussed on a taxon-by-taxon basis below and are highlighted in red in their respective section of the ML tree (Figures S1–S9).

The BEAST and IQ-TREE analyses for ancestral dating each resulted in a single chronogram, which were formatted to allow for comparisons at the family level and subfamily level (Figure 6), tribe level for Mutillinae (Figure S10), and tribe level for Sphaerophthalminae (Figure S11). These analyses more or less resulted in relatively similar age estimates despite the different models these programs use. The ages estimated using IQ-TREE tended to be younger than those estimated using BEAST. The difference between the ages inferred for

Mutillidae, 122/105 Ma, is notable, although both dates are within the same epoch (Early Cretaceous).

Myrmosidae Fox, 1894, stat.nov.

The subfamily Myrmosinae was recovered outside of Mutillidae, and depending on the analysis, as either sister to Sapygidae (ML, Figure 3, 82.6 SH-aLRT/95 UFBoot) or sister to Pompilidae + Sapygidae (MP, Figure 3, branch support = 2806). The various analyses of Brothers & Lelej (2017) consistently recovered Myrmosinae as sister to Mutillidae, and it was therein considered the subfamily sister to the remaining Mutillidae (Figure 2a, b). In contrast to the results of Brothers & Lelej (2017), three separate studies including the current one, which analysed different sets of molecular data, recovered Myrmosinae as sister to Sapygidae and not Mutillidae: (1) Pilgrim et al. (2008) used MP and Bayesian approaches with four nuclear genes and a final aligned data set of 2700 bp; (2) Debevec et al. (2012) used ML and Bayesian approaches and was based on an expanded data set of Pilgrim et al. (2008) with a final aligned data set of 4126 bp; (3) the current study was based on UCEs with an aligned data set of 238,764 bp.

Pilgrim et al. (2008) used a single representative for Myrmosinae (*Myrmosula* sp. nov.), and their results differed based on the analysis and the data set. Their Bayesian analysis with the molecular-only data set recovered Myrmosinae as sister to Sapygidae, whereas the combined molecular and morphological data set (the latter data set derived from Brothers 1999; Brothers & Carpenter 1993) resulted in Myrmosinae as sister to Mutillidae. Their MP analyses for both the molecular-only and combined data sets resulted in Myrmosinae as sister to Tiphidae. Only in the Bayesian combined analysis was Myrmosinae recovered as sister to Mutillidae; however, this relationship was not supported at the 0.95 PP level. Branstetter, Danforth et al. (2017a) recovered Myrmosinae as sister to Mutillidae with Sapygidae as sister to Pompilidae using UCEs. However, their data set only included a single representative for Myrmosinae (*Myrmosa* sp.) and nine for other Mutillidae. The UCE data set used for the current study was significantly expanded and included six myrmosine taxa and 134 mutillid taxa. Further, the data set herein includes the data from the same *Myrmosa* sp. used in Branstetter, Danforth et al. (2017a).

Given the well-supported results of the ML and MP analyses in the current study based on six Myrmosinae taxa representing both of its component tribes, an aligned data set of 238,764 characters, and the results of previous studies based on different sets of molecular data that resulted in Myrmosinae being treated as a distinct family (Debevec et al., 2012; Pilgrim et al., 2008), Myrmosidae, **stat.nov.**, are here raised to the family level. It has two subfamilies, both of which were recovered as monophyletic: Kudakrumiinae Krombein, 1979, **stat.nov.** and Myrmosinae Fox, 1894, **stat.nov.** The relationship between these former tribes of Myrmosinae (now subfamilies of Myrmosidae) as being sister taxa is congruent with Brothers & Lelej (2017).

A reappraisal of the morphological synapomorphies of Myrmosidae and Mutillidae is required in light of the results herein. Several recent morphological studies have yielded additional characters that support Myrmosidae and Mutillidae as distinct families. Kumpanenko et al. (2022) discovered that the morphology of the sting apparatuses of Myrmosidae (as Myrmosinae) and Mutillidae are functionally distinct from one another: Myrmosidae have a decurved sting shaft, whereas the mutillid subfamilies Dasylabrinae, Mutillinae, Myrmillinae, and Sphaerophthalminae have a coiled sting shaft. Further, the musculoskeletal systems that support the action of these two forms of sting shaft are necessarily different from each other (Hermann, 1968; Kumpanenko et al., 2022). The length of the sting also differs between these families, as myrmosids have a sting that is shorter than all of the mutillids that were examined in a study of the sting length of Aculeata by Sadler et al. (2018).

Mutillidae Latreille, 1802

The family Mutillidae (excluding Myrmosinae) was recovered as monophyletic in all analyses (100 SH-aLRT/100 UFBoot; branch support = 3615) and is here considered to include eight subfamilies: Pseudophotopsidinae + (Ticoplineae + (Rhopalomutillinae + (Sphaerophthalminae + (Dasylabrinae + (Odontomutillinae, **stat. nov.** + (Myrmillinae + Mutillinae)))))) (Figure 2c). Except for Myrmosidae, **stat.nov.** and Odontomutillinae, **stat.nov.**, the ML and MP topologies recovered here are similar to those of Brothers & Lelej (2017) (i.e., Myrmosinae + (Pseudophotopsidinae + (Ticoplineae + (Rhopalomutillinae + (Sphaerophthalminae + (Dasylabrinae + (Myrmillinae + Mutillinae)))))) (Figure 2a). The monophyly of the tribes proposed by Brothers & Lelej (2017), however, is unsupported as revealed by the molecular analyses herein (Figures 2d, S1–S9). Each subfamily and tribe is discussed below with comparisons given between the results of Brothers & Lelej (2017) and the current study.

Pseudophotopsidinae Bischoff, 1920

Clade 1

This subfamily, represented solely by the genus *Pseudophotopsis* André, 1896 (Figure 1g), is here considered the subfamily that is sister to the rest of Mutillidae (Figures 2c, 3, 4).

Ticoplineae Nagy, 1970

Clade 2

Both of the currently recognized tribes of Ticoplineae—Smicromyrmillini (clade 2A) and Ticoplini (clade 2B)—were found to be monophyletic and sister taxa (Figures 2d, 4). These results are congruent with those of Brothers & Lelej (2017). A cladistic analysis and genus-level

revision of Ticoplineae based on morphology was published by Mitchell & Brothers (2002).

Rhopalomutillinae Schuster, 1949

Clade 3

The ML and MP topologies of the four rhopalomutilline genera (*Bischoffiella* Brothers & Nonveiller, 2015, *Pherotilla* Brothers, 2015, *Rhopalomutilla* André, 1901, and *Rimulotilla* Brothers, 2015) are identical to that of Brothers & Lelej (2017) (Figure 4).

Dasylabrinae Invrea, 1964

Clade 4

The subfamily Dasylabrinae is currently composed of two tribes: Apteromutillini Brothers & Lelej, 2017 and Dasylabrini Invrea, 1964. Apteromutillini consists of three genera: *Apteromutilla* Ashmead, 1903, *Brachymutilla* André, 1901, and *Liotilla* Bischoff, 1920. Apteromutillines are notable in that males are entirely apterous in all three component genera (Brothers & Lelej, 2017). In both ML and MP analyses, the apteromutilline *Brachymutilla scabrosa* Bischoff, 1920 was found to be nested within Dasylabrini (Figures 4, S1), rendering the latter tribe paraphyletic. In several analyses, and notably in their preferred most-parsimonious tree (Figures 5, 11, and 12 in their study), Brothers & Lelej (2017) recovered a paraphyletic Apteromutillini as sister to Sphaerophthalminae + (Dasylabrinae + (Myrmillinae + Mutillinae)). None of the Brothers & Lelej (2017) analyses included Apteromutillini as a member of Dasylabrinae, and the tribe was repositioned post-analysis to be a member of Dasylabrinae. The results herein demonstrate that *Brachymutilla* is a dasylabrine and *Apteromutilla* likely is as well; the position of *Liotilla* is unclear. Suitable material for molecular work was only available for *Brachymutilla* and future analyses including *Apteromutilla* and/or *Liotilla* may support the reinstatement of Apteromutillini (minus *Brachymutilla*). Apteromutillini is here synonymized under Dasylabrini, **syn.nov.**, leaving a single subfamily, Dasylabrinae, without tribal division.

Odontomutillinae Lelej, 1983, stat.nov.

Clade 5

The mutilline subtribe Ephutina sensu Brothers & Lelej (2017), with its two apparent genus-groups, the *Ephuta* genus-group (Figure 1h) and *Odontomutilla* genus-group (Figure 1f), was recovered as polyphyletic (Figure S4). Neither genus-group was recovered as a member of Mutillinae or even as a sister taxon to the other. The *Ephuta* genus-group was nested within Sphaerophthalminae, and the *Odontomutilla* genus-group was recovered as sister to Myrmillinae + Mutillinae. The

Odontomutilla genus-group is herein raised to the subfamily level, Odontomutillinae, **stat.nov.** For further discussion, see the Mutillinae: Mutillini section.

Myrmillinae Bischoff, 1920

Clade 6 (= Myrmillinae sensu stricto), clade 7A (= Mutillinae: Pristomutillini, **trib.nov.**)

The subfamily Myrmillinae was found to be paraphyletic due to *Ceratotilla* Bischoff, 1920, *Viereckia* Ashmead, 1903, and the mutilline genus *Pristomutilla* Ashmead, 1903 being recovered as sister to the remaining Mutillinae (clade 7A; Figure S2); the relationship between these three genera was well-supported in the ML and MP analyses (100 SH-aLRT/100 UFBoot; branch support = 4429). In most of the analyses in Brothers & Lelej (2017), *Ceratotilla* and *Viereckia* were recovered as sister to the remaining Myrmillinae, and *Pristomutilla* was recovered as sister to the remaining Mutillinae. In their female-only analysis with additive characters and implied weighting, the single most-parsimonious tree resulted in *Ceratotilla*, *Pristomutilla*, and *Viereckia* being sister to Mutillinae which matches the results in this study. The two myrmilline genera, *Ceratotilla* and *Viereckia*, are transferred to Mutillinae and along with *Pristomutilla*, form the clade sister to the remaining Mutillinae. A new tribe, Pristomutillini, **trib.nov.**, is erected for these genera in the Mutillinae: Pristomutillini section below.

Mutillinae Latreille, 1802

Clade 7

The topologies of the ML and MP analyses were similar (Figure 3), except that a polytomy was formed between three lineages in the MP strict-consensus cladogram (i.e., clade 7A + (Mutillini + Trogaspidiini) + (clade 7D + (clade 7E + (Ctenotillini + Smicromyrmini))). The subfamily Mutillinae was rendered polyphyletic due to the subtribe Mutillini: Ephutina being recovered in two places in the topology well outside of Mutillinae. This is further discussed in the Mutillinae: Mutillini section below.

Mutillinae: Ctenotillini Brothers & Lelej, 2017

Clade 7F (= Ctenotillini sensu stricto), Clade 7A (= Pristomutillini, **trib.nov.**), Clade 7E (= Zeugomutillini, **trib.nov.**)

The mutilline tribe Ctenotillini was found to be nonmonophyletic in both ML and MP analyses, with members recovered in three separate lineages of Mutillinae (clades 7A, 7E, and 7F) (Figure S3). As mentioned in the Myrmillinae discussion, the ctenotilline genus *Pristomutilla*, along with the myrmilline genera *Ceratotilla* and *Viereckia*, were sister to the remaining Mutillinae (clade 7A). Second, the ctenotilline

genera *Strangulotilla* Nonveiller, 1979 and *Zeugomutilla* Chen, 1957 (clade 7E) were found to be sister to the remaining ctenotillines and Smicromyrmini. Clade 7F forms Ctenotillini sensu stricto, which includes the genera *Cephalotilla* Bischoff, 1920, *Chaetomutilla* Nonveiller, 1979, *Ctenotilla* Bischoff, 1920, and *Mimecomutilla* Ashmead, 1903. Two ctenotilline genera, *Arcuatotilla* Nonveiller, 1998 and *Lehritilla* Lelej, 2005, were not included in this study due to a lack of available material.

Ctenotillini sensu Brothers & Lelej (2017) is supported only by a single unambiguously-placed homoplasious synapomorphy: the first flagellomere is less than 0.6 times the length of the second flagellomere in males. This character is shared with many Smicromyrmini and nonmutilline taxa. Further, there are four ambiguously-placed homoplasious synapomorphies supporting Ctenotillini, two of which Brothers & Lelej (2017) considered significant: (1) the prementum has a posterior dome-like tubercle in the females, and (2) the posterodorsal margin of the propodeum has more than three spines in the females. Both of these characters are shared with other nonctenotilline taxa and some ctenotilline genera lack these characters (e.g., some *Pristomutilla* females lack a prementum tubercle, and *Arcuatotilla* and *Mimecomutilla* females lack propodeal spines (Nonveiller, 1995, 1997, 1998)). The group was relatively poorly supported in the Brothers & Lelej (2017) analyses. Two new tribes, Pristomutillini, **trib.nov.** and Zeugomutillini, **trib.nov.**, are erected for the genera recovered in clades 7A and clade 7E, respectively; refer to the sections dedicated to these new tribes for further discussion.

Mutillinae: Mutillini Latreille, 1802

Clade 7B (= Mutillini sensu stricto), Clade 5 (= Odontomutillinae, **stat.nov.**), Clade 8C (= Sphaerophthalminae: Ephutini, **stat.nov.**)

The tribe Mutillini is currently divided into two subtribes: Ephutina and Mutillina (Figure 1b, c). Further, the subtribe Ephutina is composed of two apparent lineages: the *Ephuta* genus-group (Figure 1h) and the *Odontomutilla* genus-group (Figure 1f). This tribe was rendered polyphyletic by the *Ephuta* genus-group being recovered as a member of Sphaerophthalminae (Figure S4). In addition, the *Odontomutilla* genus-group was recovered as sister to Myrmillinae + Mutillinae (Figure S4). These results support that neither genus-group is a genuine member of Mutillinae. The subtribe Ephutina sensu Brothers & Lelej (2017) was supported by a single unique and unambiguously-placed synapomorphy: the hypostomal carina is strong anterolaterally but is obsolete posteriorly in males. Another unique but ambiguously-placed synapomorphy is the second tergum has the felt line as a broad patch in the females. As noted by the authors, this character is questionable as a synapomorphy due to many species of the *Odontomutilla* genus-group having a simple, linear felt line on the second tergum, and most species of the *Ephuta* genus-group lack a felt line on this sclerite. Last, 15 unambiguously placed homoplasious synapomorphies are listed that are shared with a number of non-Ephutina taxa. In light

of the homoplastic nature of the synapomorphies that define *Ephutina* sensu Brothers & Lelej (2017) and the results of the ML and MP analyses herein, the *Ephuta* genus-group is now considered a tribe of Sphaerophthalminae, Ephutini, **stat.nov.**, and the *Odontomutilla* genus-group is now considered a subfamily, Odontomutillinae, **stat.nov.** (from synonymy with Ephutina).

The sister tribe of Mutillini, Trogaspidiini (clade 7C), was rendered polyphyletic by the genus *Dolichomutilla* Ashmead, 1899 (Figure 1b) being recovered within Mutillini (Figure S6). In the preferred most-parsimonious tree in Brothers & Lelej (2017), this genus is sister to the remaining Mutillini sensu Brothers & Lelej (2017), and their male-only analysis recovered *Dolichomutilla* as a member of Mutillina. In addition, they noted that Mutillina is not supported by any unique synapomorphies but rather three homoplasious synapomorphies: (1) the head is not broadened much but is long and rounded posteriorly in the females, (2) the mesoscutum is posterolaterally evenly rounded in winged males, and (3) the fore wing crossvein 3r-m has a bulla. All these homoplasious synapomorphies are shared with *Dolichomutilla*. Considering the results of Brothers & Lelej (2017), as well as the results of the molecular analyses presented here, *Dolichomutilla* is transferred to Mutillini.

Mutillinae: *Pristomutillini* Waldren, trib.nov.

ZooBank registration: <https://zoobank.org/urn:lsid:zoobank.org:act:3B5B095A-9131-4935-AABB-CD824AD95F3C>

Clade 7A

A new tribe, *Pristomutillini* Waldren, **trib.nov.** (type genus: *Pristomutilla* Ashmead, 1903), is erected for three genera that were previously considered members of Myrmillinae and the mutilline tribe Ctenotillini: *Ceratotilla* Bischoff, 1920 (♂♀), *Pristomutilla* Ashmead, 1903 (♂♀), and *Viereckia* Ashmead, 1903 (♂♀). This new tribe is diagnosed by the following combination of characters in females: (1) the presence of a short, longitudinal carina on the postgenal bridge that is perpendicularly conjoined to the hypostomal carina at its postero-medial margin (although in *Viereckia* a longitudinal, transversely-striate sulcus is present on the post-genal bridge instead of a carina), (2) the posterodorsal margin of the propodeum is lined with spine-like processes or denticles (although in *Viereckia* they are reduced to distinct tubercles), (3) the second tergum has two whitish setal spots or yellowish integumental spots, (4) the pygidium is laterally bound by a carina and most of the surface of the plate is coarsely longitudinally striate (although in at least one *Pristomutilla* species it is transversely striate-rugose (Nonveiller, 1995)), (5) the mandible is apically bidentate with a small inner tooth, and (6) a scutellar scale is absent. In contrast to the other mutilline tribes, males have: (1) a convex, short, and weakly-ovate tegula and (2) the paramere in lateral view is apically straight.

Female-based characters that support these genera belonging to Mutillinae, rather than Myrmillinae, include: (1) the ventral mesopleural carina anterodorsad to the mesocoxa is reduced, (2) the lateral face of the pronotum has its posterior margin distinct throughout, (3) the meso-metapleural suture terminates at the posterior margin of the lateral face of the pronotum, (4) the pygidium is laterally bound by a carina and the surface of the plate is sculptured, and (5) the mandible is apically bidentate with a small inner tooth. Male-based characters that support these genera belonging to Mutillinae include: (1) the compound eye is distinctly emarginate internally, and (2) the stigma is unsclerotized, bound by veins, and is cell-like (at least for *Pristomutilla*; Brothers & Lelej (2017) coded *Viereckia* males as having a sclerotized stigma). *Pristomutillini* are Afrotropical and Oriental in distribution.

Mutillinae: *Psammothermini* Waldren, trib.nov.

ZooBank registration: <https://zoobank.org/urn:lsid:zoobank.org:act:171EF65F-FF6D-4370-9988-E1816014130C>

Clade 7D

A new tribe, *Psammothermini* Waldren, **trib.nov.** (type genus: *Psammotherma* Latreille, 1825), is erected for three genera that were previously considered members of the mutilline tribe Smicromyrmini: *Antennotilla* Bischoff, 1920 (♂), *Psammotherma* Latreille, 1825 (♂), and *Pseudocephalotilla* Bischoff, 1920 (♂♀). This new tribe is diagnosed by the following combination of characters in males: (1) the penial valves are apically enlarged, downcurved, and symmetrical in length, (2) the cuspis in lateral view is broad, concave, and internally covered with setae of varying density, (3) the basoventral margin of the volsella lacks a lobate expansion and associated long setae, (4) the paramere in lateral view is evenly arcuate and downcurved, (5) the third metasomal tergum is often mostly covered with appressed whitish setae, and (6) the second metasomal sternum often has a short felt line present. The previously unrecognized females of *Pseudocephalotilla* were transferred to this genus from *Smicromyrme* Thomson, 1870 by Brothers & Lelej (2017), and they were diagnosed at the genus level by Lelej & Williams (2023). A modified diagnosis of *Pseudocephalotilla* females is provided here and is based on the following combination of characters: (1) a genal carina is present, (2) the sculpture of the dorsum of the mesosoma and second metasomal tergum is longitudinally rugose and interspersed with punctures each bearing a seta, (3) a scutellar scale is present, (4) the ventral one-fourth to one-half of the mesopleuron and metapleuron are densely covered with appressed whitish setae, (5) the first metasomal tergum has a median whitish setal spot, (6) the second metasomal tergum has one median whitish setal spot or three median whitish setal spots arranged in a row, and (7) a pygidial plate is present and it is primarily longitudinally striate-rugose in sculpture. It is uncertain whether these characters are in part or entirely diagnostic for female psammothermines, as the

females of *Antennotilla* and *Psammotherma* are currently unknown (Lelej & Williams, 2023). Psammothermini are Afrotropical in distribution.

Two of the psammothermine genera, *Antennotilla* and *Psammotherma*, have males with pectinate antennae that are developed to varying degrees. The only other mutillid genus with pectinate antennae, the monotypic smicromyrmine genus *Ctenoceraea* Nonveiller, 1993 (♂), might also be a member of Psammothermini based on the original description and associated illustrations (Nonveiller, 1993). The form of the antennae and shape of the cuspis in lateral view support membership of *Ctenoceraea* to this tribe; however, the penial valves are not apically enlarged and downcurved as in *Antennotilla*, *Psammotherma*, and *Pseudocephalotilla*. Lelej & Williams (2023) suggested that *Ctenoceraea* may be congeneric with the female-based smicromyrmine genus *Guineomutilla* Suárez, 1977 due to both genera having the first metasomal segment with a distinct dorsal face that is anteriorly delimited by a transverse carina. They also suggested potential membership of *Ctenoceraea* to Trogaspidiini due to the slightly convex mesoscutellum and the presence of subbasal swellings on the eighth metasomal sternum. Due to its lack of apically enlarged and downcurved penial valves which are diagnostic for Psammothermini, *Ctenoceraea* is maintained in Smicromyrmini pending further study.

Mutillinae: Smicromyrmini Bischoff, 1920

Clade 7G (= Smicromyrmini sensu stricto), Clade 7D (= Psammothermini, **trib.nov.**)

The tribe Smicromyrmini was rendered nonmonophyletic by *Antennotilla* Bischoff, 1920, *Psammotherma* Latreille, 1825, and *Pseudocephalotilla* Bischoff, 1920 (clade 7D) being sister to clade 7E + Ctenotillini (clade 7F) + Smicromyrmini sensu stricto (clade 7G) (Figure S5). Brothers & Lelej (2017) noted the tribe was defined by a single unique synapomorphy: the volsella has a basal ventral lamellate expansion (although this character is not always present). Further, there are two homoplasious synapomorphies that define the group: (1) the pleurosomal carina is distinct, and together with hypostomal carina, forms a straight ridge that ends at the outer mandibular articulation, and (2) the second tergum has unpaired (i.e., odd-numbered) discal markings in the females. The genera in clade 7D are placed in a new tribe, Psammothermini, **trib.nov.**, and are discussed in the section dedicated to this tribe.

Mutillinae: Trogaspidiini Bischoff, 1920

Clade 7C (= Trogaspidiini sensu stricto), Clade 7B (= Mutillini)

The tribe Trogaspidiini (clade 7C; Figure 1d) was rendered polyphyletic by *Dolichomutilla* being recovered in Mutillini (clade 7B) (Figure S6). This genus lacks many of the diagnostic characters for

Trogaspidiini, and the general habitus is suggestive of Mutillini (Figure 1b, c). This genus is herein considered a member of Mutillini and is discussed more in detail in the Mutillinae: Mutillini section.

The tribe Peterseniini, which was found to be nonmonophyletic by Brothers & Lelej (2017) and was synonymized with Trogaspidiini, will be investigated in another UCE-based study dedicated to Trogaspidiini. The comparatively smaller number of trogaspidiines included in this study does not allow for an informative assessment here.

Brothers & Lelej (2017) noted that Trogaspidiini have a single unique synapomorphy: the first flagellomere is weakly flattened ventrally in the males (but is strongly flattened in a few). In addition, there are five homoplasious synapomorphies, including: (1) the propodeum has the dorsolateral margin carinate in the winged males, and (2) the first flagellomere is much longer than wide in the males.

Mutillinae: Zeugomutillini Waldren, trib.nov.

ZooBank registration: <https://zoobank.org/urn:lsid:zoobank.org:act:BD66A961-72C3-4817-BF8F-28CB611D7924>

Clade 7E

A new tribe, Zeugomutillini Waldren, **trib.nov.** (type genus: *Zeugomutilla* Chen, 1957), is erected for three genera that were previously considered members of the mutilline tribe Ctenotillini: *Montanomutilla* Nonveiller, 1979 (♀), *Strangulotilla* Nonveiller, 1979 (♂♀), and *Zeugomutilla* Chen, 1957 (♂♀). This new tribe is diagnosed by the following combination of characters in males: (1) the prementum is flat, (2) the mandible is apically bidentate with a large subapical inner tooth, (3) the ‘mesosternum’ is unarmed, (4) the seventh metasomal sternum is unarmed, (5) the paramere in lateral view is apically slightly upcurved, and (6) the parapenial lobes in dorsal view are relatively short and lamelliform with a shallow emargination separating them. These characters are in contrast with males of Ctenotillini which have the following combination of opposing characters: (1) the prementum is tuberculate, (2) the mandible is merely apically bidentate and lacks a subapical inner tooth, (3) the ‘mesosternum’ is laterally armed with a carina, process, or tubercle, (4) the seventh metasomal sternum is medially and/or laterally armed with a tubercle (except unarmed in *Ctenotilla caeca* (Radoszkowski, 1880) (Nonveiller, 1979)), (5) the paramere in lateral view is apically straight or slightly downcurved, and (6) the parapenial lobes in dorsal view are elongate with a deep emargination separating them. In their male diagnosis of *Zeugomutilla*, Lelej et al. (2017) state: “Sternum 7 with shining lateral tubercle.” In specimens of this genus examined by the first author, males do not have a tubercle on the seventh metasomal sternum (although the lateral margin of this sclerite is glabrous and slightly raised).

The females of Zeugomutillini and Ctenotillini are less clearly distinguished from one another. Both tribes have a transverse row of spines that line the posterodorsal margin of the propodeum (except for the ctenotilline genera *Arcuatotilla* and *Mimecomutilla* (Nonveiller, 1997,

1998)). Females of *Pristomutillini* also have propodeal spines (except for *Viereckia*), and can be distinguished from females of *Zeugomutillini* and *Ctenotillini* by the presence of a carina/sulcus on the postgenal bridge and in having a coarsely longitudinally-striate pygidial plate (with at least one species of *Pristomutilla* having it transversely striate-rugose (Nonveiller, 1995)). Further, *Pristomutillini* females have either two setal spots or two integumental spots on the second metasomal tergum, whereas *Zeugomutillini* and *Ctenotillini* females have zero spots or one median setal spot (except for the Oriental genus *Zeugomutilla* which can have zero spots or two setal spots (Lelej et al., 2017; Nonveiller, 1995)).

Nonveiller (1979) separated females of the primarily Afrotropical zeugomutilline genera—*Montanomutilla* and *Strangulotilla*—from several genera of the future tribe *Ctenotillini* Brothers & Lelej, 2017 (i.e., *Cephalotilla* Bischoff, 1920, *Chaetomutilla* Nonveiller, 1979, and *Ctenotilla* Bischoff, 1920) by the former two genera having a laterally-defined pygidial plate that is glabrous or weakly longitudinally striate in sculpture. The Oriental zeugomutillines (*Zeugomutilla* spp. and two *Strangulotilla* spp.) also have a glabrous or weakly longitudinally-striate pygidial plate in the known females (Lelej, 2005; Terine et al., 2021; Lelej et al., 2017). In contrast, *Ctenotillini* females have a granulate pygidial plate (although it is striate in three species of *Cephalotilla* (Nonveiller, 1979, 1995)). The sculpture of the pygidial plate is presently the most reliable character to separate females of these tribes and further study is necessary to better differentiate them. The presence/absence of a protarsal comb and the structure of the prementum may be additional characters to separate the females of these tribes. The female-based Afrotropical genus *Montanomutilla* is tentatively assigned to *Zeugomutillini* and discovery of the presently unknown males will facilitate a more accurate tribal placement for it. *Zeugomutillini* are Afrotropical and Oriental in distribution.

Sphaerophthalminae Schuster, 1949

Clade 8

The subfamily Sphaerophthalminae was recovered as monophyletic in both ML and MP analyses (Figure 3), whereas Brothers & Lelej (2017) did not recover Sphaerophthalminae as monophyletic due to *Euspinoliina* being variously placed throughout their trees depending on the analysis used. The only change to membership in Sphaerophthalminae is the unexpected addition of *Ephutini*, *stat.nov.*, from Mutillinae: Mutillini: *Ephutina* (Figure S4). The three sphaerophthalmine tribes recognized by Brothers & Lelej (2017)—*Dasymutillini*, *Pseudomethocini*, and *Sphaerophthalmini*—were all found to be nonmonophyletic, and a relatively major overhaul of the tribal composition of the subfamily is warranted given the results herein. Brothers & Lelej (2017) noted that Sphaerophthalminae have a single unique synapomorphy: the first tergum and/or propodeum has plumose pubescence in the females and the males. Further, the subfamily is supported by three homoplasious synapomorphies: (1) the mesopleural ridge is strong and is joined to the mesonotal tubercle, (2) the head has plumose pubescence in the

males, and (3) the male gonostylus is apically upcurved in lateral view. It is noteworthy that *Ephutini* and *Euspinoliina* both lack plumose setae, and with further study they may eventually be recognized as distinct from Sphaerophthalminae.

Several differences in tribal relationships among the Sphaerophthalminae were found between the ML and MP topologies (Figure 3). In the ML analysis, clade 8A (*Patquiata* *argentinensis* (André, 1907)) + (*Ephutini* + *Euspinoliini*) were sister to the remaining Sphaerophthalminae (Figure 3); in the MP analysis only clade 8A (*P. argentinensis*) and *Ephutini* were sister to the remaining Sphaerophthalminae (the latter including *Euspinoliini*) (Figure 3). The SH-aLRT/UFBoot values for the ML analysis were relatively low at 23.4 and 76, respectively. However, for the MP analysis, branch support for the clade 8A + *Ephutini* relationship was relatively high at 5,914. Another topological difference is that clade 8E (*Tallium* sp.) was sister to clade 8F (*Limaytilla* *pehuenche* Casal, 1964b + *Protophopsis* *venenaria* (Melander, 1903)) and the remaining Sphaerophthalminae in the ML analysis (Figure 3), while in the MP analysis clade 8E (*Tallium* sp.) and clade 8F (*L. pehuenche* + *P. venenaria*) were sister to each other (Figure 3). The former relationship had support values of 92.6 SH-aLRT and 97 UFBoot, while the latter had a branch support value of 3,742.

Sphaerophthalminae: *Dasymutillini* Brothers & Lelej, 2017

Clade 8H (= *Dasymutillini* sensu stricto), Clades 8F, 8I, 8J

The tribe *Dasymutillini* was rendered polyphyletic due to *Protophopsis* Schuster, 1947 being recovered as an unrelated lineage of Sphaerophthalminae (clade 8F) (Figure S7). Further, *Lomachaeta* Mickel, 1936b and two “*Ephutomorpha*” species were sister to *Neomutilla* Reed, 1898 (8J) + *Pseudomethocini* (8K) (Figure S7). The Australasian sphaerophthalmine fauna, with most species currently placed in the catch-all genus *Ephutomorpha* André, 1902 (Figure 1k), was under-sampled in this study; it appears that much of that fauna is closely related to the New World genus *Lomachaeta*. Brothers & Lelej (2017) recovered *Dasymutillini* as paraphyletic in most analyses, and only the male-based analysis resulted in a monophyletic *Dasymutillini*. Further, the tribe was not supported by any unique synapomorphies, but rather a single homoplasious synapomorphy: the eye is strongly convex in the females. The results herein support that the *Dasymutillini* sensu stricto are restricted to clade 8H.

Sphaerophthalminae: *Ephutini* Ashmead, 1903, *stat.nov.*

Clade 8C

This tribe is formally transferred to Sphaerophthalminae from Mutillinae: Mutillini: Mutillina. The position of this tribe in the results was unexpected given the morphology of the males, which have

emarginate compound eyes and elongate tegulae; these characters are diagnostic for male mutillines and are in contrast with the hemispherical eyes and rounded tegulae of most male Sphaerophthalminae. The strongly-petiolate first metasomal segment for both sexes, however, was unique among Mutillinae (Figure 1h) and is prevalent in Sphaerophthalminae (Figure 1i–k). A detailed morphological study is warranted in the context of the relationship of ephutines with other Sphaerophthalminae; seeing the taxon in a new light as a sphaerophthalmine will likely reveal noteworthy characters. Refer to the discussion for Mutillinae: Mutillini—the tribe Ephutini was transferred from—for further comments.

Sphaerophthalminae: Euspinoliini Brothers & Lelej, 2017, stat.nov.

Clade 8B

The pseudomethocine subtribe Euspinoliina was recovered as a non-pseudomethocine clade in both ML and MP analyses. This taxon is composed of the genera *Atillum* André, 1902, *Euspinolia* Ashmead, 1903, and *Hoplocrates* Mickel, 1937a, and these genera formed a clade in agreement with the results of Brothers & Lelej (2017). Brothers & Lelej (2017) noted the group is defined by a single unique synapomorphy: the fore tibia has an obliquely-elongate outer secretory pore in the males. In addition, there are 13 homoplasious synapomorphies. The group is here raised to the tribe level, Euspinoliini, stat. nov. For further discussion on this tribe, refer to the Sphaerophthalminae: Pseudomethocini section.

Sphaerophthalminae: Pseudomethocini Brothers, 1975

Clade 8K (= Pseudomethocini sensu stricto), Clade 8A, Clade 8B (= Euspinoliini, stat.nov.)

The tribe Pseudomethocini was rendered polyphyletic due the subtribe Euspinoliina and *Patquiatilla* Casal, 1962b forming a clade with Ephutini that is sister to the remaining Sphaerophthalminae (Figure S8). The genera comprising Euspinoliina were often recovered outside of Sphaerophthalminae in the results of Brothers & Lelej (2017) and were never recovered as being closely related to Pseudomethocina. Despite this, their preferred most-parsimonious tree was rearranged to retrofit the genera of Euspinoliina (*Atillum*, *Euspinolia*, and *Hoplocrates*) to reflect the older concepts of Pseudomethocini proposed by Brothers (1975) and Lelej & Nemkov (1997). The tribe Pseudomethocini sensu Brothers & Lelej (2017) was not supported by any unique synapomorphies, but rather by six homoplasious synapomorphies. The results of Brothers & Lelej (2017) and of the analyses herein reveal that the membership of Euspinoliina to Pseudomethocini is based on homoplasy and the former deserves tribal status: Euspinoliini, stat.nov. The Pseudomethocini sensu stricto are restricted herein to clade 8K.

Sphaerophthalminae: Sphaerophthalmini Schuster, 1949

Clade 8G (= Sphaerophthalmini sensu stricto), Clades 8D, 8E, 8F

The tribe Sphaerophthalmini was rendered nonmonophyletic due to component taxa being recovered in four separate sphaerophthalmine lineages (Figure S9). *Allotilla gibbosa* Schuster, 1949 and *Sphaerophthalma tenuiventris* (Spinola, 1851) were recovered as clade 8D, *Tallium* sp. was recovered as clade 8E, and *Limaytilla pehuenche* was recovered in clade 8F, whereas the remaining Sphaerophthalmini (sensu stricto) were recovered as clade 8G. As previously discussed in the Sphaerophthalminae section, the topologies of the ML and MP analyses differed regarding clade 8E (*Tallium* sp.) and clade 8F (*L. pehuenche* + *Protophopsis venenaria*) (Figure 3). The tribe Sphaerophthalmini sensu Brothers & Lelej (2017) was not known from any unique synapomorphies, but rather two homoplasious synapomorphies: (1) the hypostomal carina is simple in the males, and (2) the second sternum has a lateral felt line in males. In several of the Brothers & Lelej (2017) analyses, *Allotilla* Schuster, 1949 and *Tallium* André, 1902 were recovered outside of Sphaerophthalmini, which is supported by the results herein.

The Sphaerophthalmini sensu stricto are herein restricted to clade 8G, and this tribe is primarily composed of the species-rich Nearctic nocturnal fauna (Figure 1j). The Neotropical genus *Xystromutilla* André, 1905 is sister to this primarily Nearctic lineage. In addition, *Cystomutilla* André, 1896 was confirmed as a member of Sphaerophthalminae: Sphaerophthalmini, and along with *Hemutilla* Lelej, Tu & Chen (Tu et al., 2014), are the only representatives of this tribe in the Old World.

REVIEW OF THE BROTHERS & LELEJ (2017) STUDY

The valuable contribution of Brothers & Lelej (2017) towards better understanding the higher-level relationships within Mutillidae served as a reference for the present study. There are, however, a few problems with the approaches that were followed in their study which are now apparent in light of the results herein.

First, their taxa were treated at the genus level rather than the species level. Some mutillid genera are known to be heterogeneous assemblages that serve as placeholder taxa until their constituent species can be critically studied. The monophyly of these placeholder genera has yet to be investigated through phylogenetic inference and several genera in the Brothers & Lelej (2017) study fall into this category, including *Mutilla* Linnaeus, 1758, *Pseudomethoca* Ashmead, 1896, and *Timulla* Ashmead, 1899. Some placeholder genera, such as *Myrmilla* Wesmæl, 1851 and *Sphaerophthalma* Blake, 1871, were specified that they were considered in the strict sense; however, the former three placeholder genera were not. In the present study, both *Mutilla* and *Pseudomethoca* were recovered as nonmonophyletic (Figures 4, 5, respectively).

Second, the proposed higher-level taxa in Brothers & Lelej (2017) were neither described nor diagnosed, but were rather supported based on lists of primarily homoplasious synapomorphies. The synapomorphies provided for each higher taxon were based on the results of their maximum parsimony analyses or from post-analysis rearrangements that were made to their preferred most-parsimonious tree. There is a paucity of unique synapomorphies for many of the higher mutillid taxa, especially the tribes of Mutillinae and Sphaerophthalminae. Due to this, diagnoses using unique combinations of character states are likely necessary to delimit these higher taxa; this approach was followed for the three new tribes described in the present study.

Finally, some branches in the preferred most-parsimonious tree of Brothers & Lelej (2017) (Figures 5, 11, and 12 in their study) were rearranged post-analysis in order to retrofit their new results to match the higher classification hypotheses of Brothers (1975) and Lelej & Nemkov (1997) (Figures 1 and 13–16 in their study), and homoplasious synapomorphies were provided as support for these retrofitted higher taxa. As previously mentioned, there is a prevalence of morphological homoplasy in the family. The rationale for this decision is reflected in the first sentence for Figure 13 in their study: “Tree based on preferred tree (see Figure 5) but with branches re-arranged to make the potential recognizable groups (see Figure 12) monophyletic (length = 2858, ci = 0.19, ri = 0.60).” (Brothers & Lelej, 2017).

An example of this rearrangement involves the genera *Atillum*, *Euspinolia*, and *Hoplocrates*. These three genera have historically been considered members of the sphaerophthalmine tribe Pseudomethocini (Brothers, 1975; Lelej & Nemkov, 1997), which is broadly characterized by females having: (1) a large, quadrate head that is sometimes armed with processes, (2) a pear-shaped mesosoma that is more or less constricted at the propodeal spiracles in dorsal view, and (3) the first metasomal segment is sessile with the second segment. Males also have the first metasomal segment sessile with the second segment, and they often have a large head as well. In the preferred most-parsimonious tree and several other trees of Brothers & Lelej (2017), *Atillum*, *Euspinolia*, and *Hoplocrates* were not recovered as members of Sphaerophthalminae, but rather as sister to Sphaerophthalminae + (Dasylabrinae + (Myrmillinae + Mutillinae)). In addition, these three genera were not closely associated with Pseudomethocini sensu stricto in any of their results. Despite a lack of support for membership of *Atillum*, *Euspinolia*, and *Hoplocrates* to Pseudomethocini (and in many cases Sphaerophthalminae), these three genera were placed in Pseudomethocini as members of a new subtribe, Euspinoliina, with the following rationale:

“The two components [(Euspinoliina and Pseudomethocina)] of this grouping [(Pseudomethocini)] are not closely associated in any of the analyses, but they are placed together here on the basis of their consistent positions in the current classifications, and the fact that this arrangement adds only five steps when compared with that in the preferred tree (Figure 5; and see above). Because the two components are consistently shown as monophyletic in almost all of the

analyses, and acknowledging the uncertainties about their true relationships to each other, however, we propose that they be recognized as distinct subtribes.”

Our results reveal that Euspinoliina and Pseudomethocina are not closely related, and this supports the initial results of Brothers & Lelej (2017). Additional cases in which new results were retrofitted to older subfamilial and tribal concepts in Brothers & Lelej (2017) involve the following genera and groupings: *Allotilla* + *Photomorphus* Viereck, 1903 + *Tallium*, *Ancistrotilla* Brothers, 2012 + *Bothriomutilla* Ashmead, 1899 + *Eurymutilla* Ashmead, 1899 + *Odontomyrme* Lelej, 1983, *Apteromutilla*, *Brachymutilla*, *Dolichomutilla*, *Liotilla*, *Pristomutilla*, *Promecilla* André, 1902, *Protophopsis*, and *Pseudocephalotilla*. The situation involving the retrofitting of *Allotilla*, *Photomorphus*, and *Tallium* as members of Sphaerophthalmini is similar to that of Euspinoliina and Pseudomethocina. In Brothers & Lelej (2017), these three genera formed a clade and were not sister to the remaining Sphaerophthalmini, although both of these groups were recovered as early-branching clades in Sphaerophthalminae. The results of this study also revealed a polyphyletic Sphaerophthalmini, with *Allotilla*, *Photomorphus*, and *Tallium* each belonging to a separate early-branching clade in Sphaerophthalminae. Our results in part support the initial results of Brothers & Lelej (2017).

DIVERGENCE TIME ESTIMATES

The superfamily Pompiloidea, composed of the families Mutillidae, Myrmosidae, Pompilidae, and Sapygidae, was confirmed herein as monophyletic. The Pompiloidea were estimated to have emerged in the late Jurassic or in the Early Cretaceous at an inferred age of 154/144 Ma (Figure 6). This estimate is similar to that of Peters et al. (2017), who estimated Pompiloidea to have emerged 143 Ma. Other researchers inferred the age of Pompiloidea to be 116 Ma (Wilson et al. 2012) and 114 Ma (Branstetter, Danforth et al. 2017a). These latter two age estimates, however, are apparently too young for the superfamily due to the existence of the fossil sapygid species *Cretofedtschenkia santanensis* from the Crato Formation dated 122.46–112.6 Ma (median = 117.5 Ma). This species was placed by Osten (2007) in the enigmatic sapygid subfamily Fedtschenkiinae, and it was not used as a calibration reference in Wilson (2012) nor in Branstetter, Danforth et al. (2017a). A key synapomorphy for Sapygidae is the females having the sixth metasomal sternum forming a conical support for the sting. The placement of the female-based species *C. santanensis* in Sapygidae is herein supported as it has this character (Osten, 2007). Additional characters that support its classification in Fedtschenkiinae are discussed by Osten (2007). This fossil is the oldest known specimen among Pompiloidea.

The age of Pompilidae was inferred as 71/68 Ma. These dates are shortly before the Cretaceous-Palaeogene (K-Pg) boundary at 66 Ma. Other researchers inferred the age of Pompilidae to be 85 Ma (Wilson et al., 2012), 43.3 Ma (Waichert et al., 2015), 44 Ma (Branstetter, Danforth et al., 2017a), and 72 Ma (Peters et al., 2017). The oldest known pompilid fossil is the recently-described pepsine species

Cryptocheilus leleji (Waichert et al., 2019). This fossil originated from the Fur Formation dated 55.8–48.6 Ma (median = 52.2 Ma) and the median age of *C. leleji* was used to calibrate Pepsinae. As discussed in Waichert et al. (2019), *C. leleji* belongs to the subfamily Pepsinae due to the leg being smooth and lacking long, irregular spines. In addition, it lacks an inflection at the base of the Cu vein in the 2 M cell in the fore wing. Further, it was classified to the extant genus *Cryptocheilus* Panzer, 1806 based on several characters.

The age of Sapygidae was inferred as 113/118 Ma in the Early Cretaceous. UCE data for only two species of *Sapyga* Latreille, 1796 (Sapyginae) were available and none for Fedtschenkiinae, the subfamily from which the fossil calibration point was derived. Other age estimates for Sapygidae include 47 Ma (Wilson et al., 2012), 23 Ma (Branstetter, Danforth et al., 2017a), and 24 Ma (Peters et al., 2017), all of which are too young considering the discovery of *Cretofedtschenkia santanensis* from the Crato Formation dated 122.46–112.6 Ma (median = 117.5 Ma). Two other fossils are known for Sapygidae: a *Sapyga* sp. from Baltic amber (Brischke, 1886) and *Cretosapyga resinicola* Bennett & Engel, 2005. The latter species was described from a Burmese amber inclusion dated 99.7–94.3 Ma and was placed in its own subfamily, Cretosapyginae. It is doubtfully assigned to Sapygidae according to Osten (2007).

The age of Myrmosidae was inferred as 65/65 Ma, with the group arising shortly after the K-Pg boundary. Myrmosidae are represented in the fossil record by the kudakrumiine genus *Protomutilla*; a number of species are known from Baltic amber inclusions dated 37.2–33.9 Ma (median = 35.6 Ma) (Aleksandrova & Zaporozhets, 2008; Kaplan et al., 1977; Kosmowska-Ceranowicz, 2017). The median age was used to calibrate Kudakrumiinae. Other age estimates include 47 Ma (Wilson et al., 2012) and 97 Ma (Branstetter et al., 2017, albeit for the clade Myrmosidae + Mutillidae). Myrmosidae are primarily diagnosed by the dorsal carina on the hind coxa forming a lobate process in both sexes. Further, females of the subfamily Kudakrumiinae lack ocelli and also lack dense integumental punctuation; in contrast, both of these characters are present in Myrmosinae females. Last, Kudakrumiinae males have a simple, unmodified hypopygium, whereas Myrmosinae males have the hypopygium modified into various forms (e.g., trilobate, medially emarginate, etc.). The known specimens of *Protomutilla* have the aforementioned characters of Kudakrumiinae (Lelej, 1986).

The age of Mutillidae sensu stricto was inferred as 122/105 Ma in the Early Cretaceous. Other age estimates include 85 Ma (Wilson et al., 2012), 82 Ma (Branstetter, Danforth et al., 2017a), and 67 Ma (Peters et al., 2017). According to the age estimate results, Ticoplineae is the oldest extant mutillid subfamily and is dated at 74/71 Ma in the late Cretaceous (Figure 6). Two of the other early-branching subfamilies of Mutillidae, Pseudophotopsidinae and Rhopalomutillinae, were estimated to have arisen 13/15 Ma (Miocene) and 27/25 Ma (Oligocene), respectively. Several clades are broadly associated with the K-Pg boundary (66 Ma), with Dasylabrinae dated at 55/59 Ma and Sphaerophthalminae dated at 73/64 Ma. Further, the clade Dasylabrinae + (Odontomutillinae + (Myrmillinae + Mutillinae)) was dated at 68/66 Ma (Figure 6).

The fossil record for Mutillidae sensu stricto is relatively scant and is represented by two sphaerophthalmine tribes known from

Dominican amber: a single female specimen of Ephutini (*Ephuta clavigera* Brothers, 2003) and three male specimens of Dasymutillini (*Dasymutilla dominica* Manley & Poinar, 1991, *D. albifasciatus* Manley & Poinar, 1999, and an undescribed species of *Dasymutilla* (Manley & Poinar, 2003)). Dominican amber is dated from 20.4–13.7 Ma (median = 17.1 Ma) (Iturralde-Vinent & MacPhee, 1996). In addition, the extinct species *Cretavus sibiricus* Sharov, 1957 was originally placed in its own family, Cretavidae (Sharov, 1957), and it was later assigned to Mutillidae by Rasnitsyn (1975). This fossil specimen is only known from a right fore wing and it is from the Late Cretaceous in Russia dated from 99.7 to 94.3 Ma. *Cretavus sibiricus* was excluded from this study due to lack of additional characters which support its placement in Mutillidae. Regarding the fossil *Dasymutilla* species based on males, Williams et al. (2011) noted that they have the integument of the metasoma entirely black and the extruded genitalia of *D. albifasciatus* have a straight, setose, and relatively short cuspis. These characters are similar to males of the extant species *Dasymutilla militaris* (Smith, 1855) and are in contrast to members of the *Dasymutilla bioculata* species-group, which are the only other congeners that occur in the Greater Antilles. In addition, the fore wing is contrastingly banded in *D. albifasciatus* and the undescribed species (although in *D. dominica* the wings are damaged and their coloration is consequently not discernible). A banded fore wing is a rarely-observed character in Mutillidae, and the extant species *D. militaris* also shares this character with these extinct taxa (Manley & Pitts, 2007; Manley & Poinar, 1999, 2003). However, these extinct *Dasymutilla* species have a seta-filled pit on the second metasomal sternum, which *D. militaris* lacks. *Dasymutilla militaris* was inferred as sister to the majority of *Dasymutilla* and *Traumatomutilla* species by Williams (2012). The median Dominican amber age of 17.1 Ma was used to calibrate the clade *Dasymutilla* + *Traumatomutilla* using the Dasymutillini topology of Williams (2012) as a guide. The age of Dasymutillini was inferred herein as 38/35 Ma (Eocene).

With regard to Ephutini, *Onoretilla* Pagliano (Pagliano et al., 2017) was found to be sister to the remaining members of the tribe in the ML and MP analyses. The suspected females of this genus are unusual compared to other ephutines as they have an elongate, narrow mesosoma in dorsal view, relatively long legs, and a unique colour pattern on the mesosoma and metasoma (pers. obs.). The female-based fossil species *Ephuta clavigera* does not share these characters with the putative *Onoretilla* females, and it appears to be more closely related to the remaining taxa used in the analysis (i.e., *Ephuamelia*, *Ephuchaya*, and *Ephuta*). Consequently, the age of 17.1 Ma was assigned to the clade containing *Ephuamelia*, *Ephuchaya*, and *Ephuta*. The age of Ephutini was inferred herein as 33/32 Ma (Oligocene).

The tribes of the two most species-rich mutillid subfamilies, Mutillinae and Sphaerophthalminae, were estimated to have arisen during significantly different time periods (Figures S10 and S11). The primarily Afrotropical, Oriental, and Palearctic subfamily Mutillinae was dated at 40/35 Ma (Eocene), with its component tribes arising in the late Oligocene to Miocene (Figure S10). The primarily New World and Australasian subfamily Sphaerophthalminae was estimated to have emerged 73/64 Ma around the K-Pg boundary, with its component tribes primarily arising in the Eocene to Oligocene (Figure S11).

BIOGEOGRAPHY

Our results reveal several preliminary biogeographic patterns. One of these patterns involves Afrotropical mutillid taxa being recovered as sister to Oriental and/or Palaeartic mutillid taxa. The first example involves the subfamily Myrmillinae wherein the Afrotropical genera *Labidomilla* André, 1902 and *Odontotilla* Bischoff, 1920 are sister to the Palaeartic and Oriental myrmillines. Similarly, the Afrotropical and Oriental Odontomutillinae are sister taxa. The new mutilline tribe Pristomutillini, which is sister to the remaining Mutillinae, is primarily Afrotropical in distribution with a few Oriental members of *Pristomutilla* uncertainly assigned to this genus. In addition, the mutilline tribes Ctenotillini, Trogaspidiini, and Zeugomutillini all have Afrotropical genera sister to their remaining member genera. The new mutilline tribe Psammothermini is exclusively Afrotropical. The Rhopalomutillinae are primarily Afrotropical except for the genus *Pherotilla* Brothers, 2015, which is nested within the subfamily and is composed of Afrotropical and Oriental species (Brothers, 2015). The Ticopliinae have a less obvious dichotomy, although the subfamily is most species-rich in the Afrotropical region. The results regarding Dasylabrinae and the mutilline tribes Mutillini and Smicromyrmini differ from the previous examples, however, due to Palaeartic species recovered as sister to the remaining member taxa in this study. These latter three higher taxa are species-rich in the Afrotropical region and few representatives were included in this study. The Pseudophotopsidinae, which were recovered as sister to the remaining Mutillidae, are primarily Palaeartic in distribution with a few Afrotropical and Oriental species (Brothers & Lelej, 2017; Lelej & Brothers, 2008; Pagliano et al., 2020). Future studies that target these higher taxa using a more comprehensive coverage of constituent species will provide a clearer biogeographic picture.

With regard to Sphaerophthalminae—nearly all of which occur in the Nearctic, Neotropical, and Australasian regions—most of the early-branching clades are restricted to South America: clade 8A (*Patquiatilla argentinensis*, South America), clade 8B (Euspinolini, South America), clade 8C (Ephutini, North America + South America), clade 8D (*Allotilla gibbosa* + *Sphaerophthalma tenuiventris*, South America), clade 8E (*Tallium* sp., South America), and clade 8F (*Limaytilla pehuenche* + *Protophotopsis venenaria*, North America + South America). Further, most of the early-branching clades of Dasymutillini and Pseudomethocini are exclusively or partly South American, respectively. As for the Australasian sphaerophthalmines, only two representatives were included in this study from the catch-all genus *Ephutomorpha*. These specimens are nested within Sphaerophthalminae in clade 8I and are sister to the Nearctic species *Lomachaeta crocospinna* Pitts & Manley, 2004. Sphaerophthalminae may have had their origin in South America based on our results. A more comprehensive study of the Australasian sphaerophthalmine fauna in relation to the New World fauna is necessary to determine how many dispersal events occurred between these continents.

Last, a hypothesis regarding the biogeographic history of the Pompiloidea and Mutillidae is not attempted here as it is beyond the scope of the present study. The estimated ages inferred herein may serve as a reference for future biogeographic studies of these taxa.

CONCLUSION

The family Mutillidae has been redefined with Myrmosidae considered to be a separate family. Eight subfamilies are now recognized for Mutillidae, and the age of the family was inferred at 122/105 Ma in the Early Cretaceous. As revealed by the analysis of UCE data, most of the tribal concepts proposed by Brothers & Lelej (2017) are untenable and need reevaluation. Given the few unique synapomorphies for the subfamilies and tribes proposed by Brothers & Lelej (2017), a diagnostic approach using unique combinations of character states for each of the higher taxa is warranted. Homoplasy is widespread across the family and is particularly illustrated through the surprising addition of Ephutini to Sphaerophthalminae. The analyses herein have provided novel insights into the relationships between the higher taxa of Mutillidae that may not have been recognized through morphology alone. For example, considering Ephutini as a sphaerophthalmine rather than a mutilline may yield new, previously overlooked synapomorphies. It is hoped that these higher taxa can be approached with a new perspective, and this study will galvanize additional research on their delimitation.

AUTHOR CONTRIBUTIONS

GEORGE C. WALDREN: Conceptualization; methodology; software; data curation; investigation; validation; formal analysis; funding acquisition; visualization; project administration; resources; writing – original draft; writing – review and editing; supervision. **EMILY A. SADLER:** Software; formal analysis; writing – review and editing; data curation; methodology; validation; supervision; investigation. **ELIZABETH A. MURRAY:** Writing – review and editing; methodology; software; data curation. **SILAS BOSSERT:** Writing – review and editing; methodology; software; data curation. **BRYAN N. DANFORTH:** Funding acquisition. **JAMES P. PITTS:** Supervision; writing – review and editing; funding acquisition; resources; conceptualization; project administration.

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DATA AVAILABILITY STATEMENT

Raw sequence data are deposited at the NCBI SRA as BioProject PRJNA912170. All alignments, tree files, and the XML file used for (or produced by) the IQ-TREE, TNT, and BEAST analyses are deposited at Zenodo (<https://doi.org/10.5281/zenodo.7614399>). Contig assemblies (SPAdes and Trinity) and a table containing specimen data are also available at Zenodo. The Ant-Specific probe set sequence file (myBaits UCE Hymenoptera 2.5Kv2A) and the Principal Hymenoptera probe set sequence file (myBaits UCE Hymenoptera 2.5Kv2P) may be accessed at Figshare (<https://doi.org/10.6084/m9.figshare.4630375.v1>). The ZooBank registration for this contribution may be accessed at: <https://zoobank.org/urn:lsid:zoobank.org:pub:EECAAFEC-0908-4A78-BD36-B79579F98513>.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Paraphyly of Dasylabrinae: Dasylabrini (sensu Brothers and Lelej (2017)) (clade 4) due to inclusion of Dasylabrinae: Apteromutillini (represented by *Brachymutilla scabrosa* Bischoff, 1920). The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S2. Polyphyly of Myrmillinae (sensu Brothers and Lelej (2017)) (clade 6) due to *Ceratotilla septemmaculata* Bischoff, 1920 and *Vierckia acrisione* (Péringuey, 1898) being recovered in clade 7A. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S3. Polyphyly of Mutillinae: Ctenotillini (sensu Brothers and Lelej (2017)) (clade 7F) due to *Pristomutilla* sp. being recovered in clade 7A and *Strangulotilla* sp. and *Zeugomutilla pycnopyga* Chen, 1957 being recovered as clade 7E. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S4. Polyphyly of Mutillinae: Mutillini (sensu Brothers and Lelej (2017)) (clade 7B) due to the *Odontomutilla* genus-group being recovered as clade 5 and the *Ephuta* genus-group being recovered as clade 8C. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S5. Polyphyly of Mutillinae: Smicromyrmini (sensu Brothers and Lelej (2017)) (clade 7G) due to *Antennotilla phoebe* (Péringuey, 1899), *Psammotherma cyanochroa* (André, 1908), and *Pseudocephalotilla* sp. being recovered as clade 7D. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S6. Polyphyly of Mutillinae: Trogaspidiini (sensu Brothers and Lelej (2017)) (clade 7C) due to *Dolichomutilla* sp. being recovered in

Mutillinae: Mutillini (clade 7B). The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S7. Polyphyly of Sphaerophthalminae: Dasymutillini (sensu Brothers and Lelej (2017)) (clade 8H) due to *Protophopsis venenaria* (Melander, 1903) being recovered in clade 8F, *Lomachaeta crocopinna* Pitts & Manley, 2004 + two “*Ephutomorpha*” spp. being recovered as clade 8I, and *Neomutilla patagonica* (Fritz & Martínez, 1975) being recovered as clade 8J. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S8. Polyphyly of Sphaerophthalminae: Pseudomethocini (sensu Brothers and Lelej (2017)) (clade 8K) due to *Patquiaetilla argentinensis* (André, 1907) being recovered as clade 8A and Pseudomethocini: Euspinoliina being recovered as clade 8B. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S9. Polyphyly of Sphaerophthalminae: Sphaerophthalmini (sensu Brothers and Lelej (2017)) (clade 8G) due to *Allotilla gibbosa* Schuster, 1949 and *Sphaerophthalma tenuiventris* (Spinola, 1851) being recovered as clade 8D, *Tallium* sp. being recovered as clade 8E, and *Limaytilla pehuenche* Casal, 1964b being recovered in clade 8F. The asterisks indicate that SH-aLRT/UFBoot values both equal 100; if below 100, the numerical values are given in lieu of an asterisk. The scale bar represents the number of substitutions per site.

Figure S10. (A) Chronogram of Mutillinae tribes using BEAST. The purple bars represent 95% HPD intervals. (B) Chronogram of Mutillinae tribes using IQ-TREE. The purple bars represent confidence intervals.

Figure S11. (A) Chronogram of Sphaerophthalminae tribes using BEAST. The purple bars represent 95% HPD intervals. (B) Chronogram of Sphaerophthalminae tribes using IQ-TREE. The purple bars represent confidence intervals.

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