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Multi-gene phylogeny and divergence estimations for Evaniidae (Hymenoptera)

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Ensign wasps (Hymenoptera: Evaniidae) develop as predators of cockroach eggs (Blattodea), have a wide distribution and exhibit numerous interesting biological phenomena. The taxonomy of this lineage has been the subject of several recent, intensive efforts, but the lineage lacked a robust phylogeny. In this paper we present a new phylogeny, based on increased taxonomic sampling and data from six molecular markers (mitochondrial 16S and COI, and nuclear markers 28S, RPS23, CAD, and AM2), the latter used for the first time in phylogenetic reconstruction. Our intent is to provide a robust phylogeny that will stabilize and facilitate revision of the higher-level classification. We also show the continued utility of molecular motifs, especially the presence of an intron in the RPS23 fragments of certain taxa, to diagnose evaniid clades and assist with taxonomic classification. Furthermore, we estimate divergence times among evaniid lineages for the first time, using multiple fossil calibrations. Evaniidae radiated primarily in the Early Cretaceous (134.1-141.1 Mya), with and most extant genera diverging near the K-T boundary. The estimated phylogeny reveals a more robust topology than previous efforts, with the recovery of more monophyletic taxa and better higher-level resolution. The results facilitate a change in ensign wasp taxonomy, with Parevania, syn. nov., and Papatuka, syn. nov. becoming junior synonyms of Zeuxevania, and Acanthinevania, syn. **nov.** being designated as junior synonym of *Szepligetella*. We transfer 30 species to Zeuxevania, either reestablishing past combinations or as new combinations. We also transfer 20 species from Acanthinevania to Szepligetella as new combinations.

Multi-gene phylogeny and divergence estimations for Evaniidae (Hymenoptera)

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15 ABSTRACT

Ensign wasps (Hymenoptera: Evaniidae) develop as predators of cockroach eggs (Blattodea), have a 16 wide distribution and exhibit numerous interesting biological phenomena. The taxonomy of this lineage 17 has been the subject of several recent, intensive efforts, but the lineage lacked a robust phylogeny. In this 18 paper we present a new phylogeny, based on increased taxonomic sampling and data from six molecular 19 markers (mitochondrial 16S and COI, and nuclear markers 28S, RPS23, CAD, and AM2), the latter 20 used for the first time in phylogenetic reconstruction. Our intent is to provide a robust phylogeny that 21 will stabilize and facilitate revision of the higher-level classification. We also show the continued utility 22 of molecular motifs, especially the presence of an intron in the RPS23 fragments of certain taxa, to 23 diagnose evaniid clades and assist with taxonomic classification. Furthermore, we estimate divergence 24 times among evaniid lineages for the first time, using multiple fossil calibrations. Evaniidae radiated 25 primarily in the Early Cretaceous (134.1-141.1 Mya), with and most extant genera diverging near the 26 K-T boundary. The estimated phylogeny reveals a more robust topology than previous efforts, with the 27 recovery of more monophyletic taxa and better higher-level resolution. The results facilitate a change in 28 ensign wasp taxonomy, with Parevania, syn. nov., and Papatuka, syn. nov. becoming junior synonyms 29 30 of Zeuxevania, and Acanthinevania, syn. nov. being designated as junior synonym of Szepligetella. We transfer 30 species to Zeuxevania, either reestablishing past combinations or as new combinations. We 31 also transfer 20 species from Acanthinevania to Szepligetella as new combinations. 32

INTRODUCTION

Ensign wasps (Hymenoptera: Evaniidae) are common, nearly cosmopolitan, and include approximately 34 500 extant species in 21 genera, although many species remain to be described (Deans, 2005). Their 35 biology lies at the precipice between wasps that provision their young with prey and parasitic wasps that 36 deposit their offspring to feed on one host. A female evaniid wasp lays a single egg within a cockroach 37 egg case and their offspring feeds on the unhatched cockroach eggs. Because their larvae feed on multiple 38 hosts, ensign wasps are regarded as predators as opposed to parasitoids (Huben, 1995). However, the 39 intimate association that larval evaniids have with their prey is much more reminiscent of parasitoid 40 behavior. Despite these interesting biological features, there is scant research aimed at understanding 41 their evolution and natural history. This predicament remains, in part, due to ongoing instability in their 42 classification and the lack of robust diagnostic tools and inadequate taxon descriptions. Taxonomic work 43 over the last 20 years, however, including a key to genera (Deans and Huben, 2003), a comprehensive 44 species catalog (Deans, 2005, treating all ca. 500 species), descriptions of fossils (Deans et al., 2004; 45 Jennings et al., 2013, 2012, 2004), and updated (Deans and Kawada, 2008) and semantically-enhanced

species-level revisions (Balhoff et al., 2013; Mikó et al., 2014) have substantially increased the potential 47 for research on these insects. 48

Deans et al. (2006) also published the first phylogeny of the family, which was an attempt to test the 49 historic generic and tribal classifications. Of the 17 included genera, four were represented by single 50 specimens: Papatuka Deans, Rothevania Huben (monotypic), Thaumatevania Ceballos (monotypic),

51 and Trissevania Kieffer. Six genera were found to be monophyletic in both a parsimony and Bayesian 52

analysis, including: Acanthinevania Bradley, Decevania Huben, Evania Fabricius, Evaniscus Szépligeti, 53

Micrevania Benoit, and Semaeomyia Bradley. Although Prosevania Kieffer was always recovered, 54

with one possibly misplaced specimen of Szepligetella Bradley, it is likely that Prosevania may also 55

be monophyletic. Several other genera were consistently recovered as paraphyletic or in unresolved 56

polytomies, including Brachygaster Leach, Evaniella Bradley, Hyptia Illiger, Szepligetella Bradley, 57 Parevania Kieffer, and Zeuxevania Kieffer. The latter two genera were consistently recovered in a clade 58

with Papatuka Deans, and Deans et al. (2006) suggested that these taxa may be congeneric based on 59

the molecular results and inconsistencies in the morphological character that separates these two genera 60 (presence of fore wing 1RS in *Parevania*). They also suggested *Evaniella* may be monophyletic as it 61

was consistently recovered with the exception of one aberrant taxon, since described as its own genus 62 63

(Alobevania Deans and Kawada, 2008)).

The only tribal classification put forth for Evaniidae was by Bradley (1908), who suggested two 64 tribes for the ten genera described at the time: Hyptiini (including Evaniella, Evaniscus, Hyptia, Pare-65 vania, Semaeomyia, and Zeuxevania) and Evaniini (including Acanthinevania, Evania, Prosevania, and 66 Szepligetella). This tribal classification was not supported by Deans et al. (2006). There was not enough 67 resolution to confidently resolve relationships among evaniid genera to develop a better tribal classification. 68 Deans et al. (2006) did suggest that the New World taxa with reduced wing venation (including *Evaniscus*, 69

Decevania, Hyptia, Rothevania, and Semaeomyia) were monophyletic and could represent a tribe. 70

The poorly resolved phylogenies published by Deans et al. (2006) may be attributed to low taxonomic 71 sampling, as only 54 ingroup taxa were included, or, more likely, a lack of informative sites in the sequence 72 data. The resulting "backbone polytomy", where higher-level classifications remain elusive, is common in 73 other phylogenies of Hymenoptera that use the same or similar sets of genes (Dowton and Austin, 2001; 74

Mardulyn and Whitfield, 1999; Pitz et al., 2007). Divergence times for members of Evaniidae have not 75

been estimated before. Several recent studies on Hymenoptera have estimated stem-age divergences for 76

Evanioidea ranging from 175 Ma to 221 Ma (Ronquist et al., 2012a; Zhang et al., 2015; Peters et al., 77 2017; Branstetter et al., 2017). Unfortunately, the small sample size for Evanioidea in all of these studies

78 (1–3 exemplars) and uncertainty in phylogenetic relationships of Evanioidea within Hymenoptera resulted 79

in wide confidence intervals around the estimates. Based on all fossils placed within Evanioidea, it is 80

likely that the superfamily diversified in the Middle Jurassic but may have originated as early as the late 81 Triassic (Li et al., 2018). 82

83 Here we attempt to gain a better understanding of higher-level relationships among genera and better test the monophyly of genera, using an increased taxonomic and genetic sampling dataset, including 84 a handful of new protein-coding genes. Our intent is to provide a robust phylogeny that will stabilize 85 and facilitate revision of the higher-level classification. We also show the continued utility of molecular 86 motifs, first described for Evaniidae by Deans et al. (2006), to diagnose clades and assist with taxonomic 87 classification. Furthermore, we estimate divergence times among evaniid lineages for the first time, using 88 multiple fossil calibrations to understand of the timing of diversification in Evaniidae. 89

MATERIALS AND METHODS 90

Taxon sampling 91

A list of taxa and sequences utilized in this study is presented in Table 1 (more details in Table S1). 92

Exemplars were obtained for 89 evaniid specimens, across 17 genera, and five outgroup taxa, including 93

two species of Gasteruption (Gasteruptiidae) and three species of Pristaulacus (Aulacidae), for a total 94

of 94 taxa. All evaniid genera were represented except four rare genera: Afrevania, Brachevania, 95

Thaumatevania, and Vernevania. We were only able to include one representative of Alobevania and 96

Rothevania (monotypic), and *Papatuka*. Where possible, sampling was increased for genera that were 97

previously recovered as paraphyletic by Deans et al. (2006). 98

taxon	Ext.	DV#	28S	AM2	CAD1	CAD2	RPS23	COI	16S
Gasteruption 300	300		Х		Х	X	Х	Х	D
Gasteruption 244	244		Х		Х		Х	Х	
Pristaulacus strangaliae	176			X			Х	Х	
Pristaulacus fasciatus	299							Х	
Pristaulacus 21	306	21	D				Х	D	D
Acanthinevania 240	240		Х	X	X	X	Х	Х	
Acanthinevania 242	242		Х	X	X	X	Х		
Acanthinevania princeps	246		Х		Х	X	Х	Х	
Acanthinevania 001	271	001	D	X	Х	X	Х	D	D
Acanthinevania 033	289	033	D	X	Х	X	Х	D	D
Acanthinevania 049	292	049	D	X	Х	X	Х	D	D
Alobevania gattiae	200	039	D	X	Х	X		Х	D
Brachygaster minutus	273	030	Х		Х	X	Х	D	D
Brachygaster minutus	512				X	X		Х	
Brachygaster 037	286		D		X		Х		D
Brachygaster 050	290		D				Х		D
Decevania 502	502				X	X			
Decevania 513	513			X				Х	
Decevania 004	274	004	D	X		X	Х	D	D
Decevania 005	301	005	D				Х		D
Decevania 063	296	063	D	X	X	X	Х	D	D
Evania 175	175		Х				Х	Х	
Evania albofacialis	275	020	D		X	X	Х	D	D
Evania appendigaster	207	046	D		X	X	Х	D	D
Evania 496	496		Х			X	Х	Х	
Evania 002	189	002	D		X	X		D	D
Evaniella 230	230		Х	X	X	X	Х	Х	
Evaniella 234	234		Х	X		X	Х	Х	
Evaniella 237	237			X	X			Х	
Evaniella 485	485			X	X	X	Х		
Evaniella 486	486			X	X		Х		
Evaniella 493	493				X		X	Х	
Evaniella semaeoda	220	058	D				X	D	D
Evaniella 019	192	019	D	X	X		X	D	
Evaniella 025	307	025	D		X		X	D	D
Evaniella 045	206	045	D	X	X		X		D
Evaniscus marginatus	213	052	D				X		D
Evaniscus rufithorax	206		D			X	X	Х	D
Hyptia 232	232			X	X	X	X	Х	
Hyptia 487	487			X				Х	
Hyptia 501	501			X	X			X	
Hyptia 511	511			X	X			X	
Hyptia amazonica	235		_		X	X		Х	
Hyptia floridana	291	009	D	X	X	X		D	D
Hyptia 007	302	007	D		X		X	D	D
Hyptia 008	303	008	D				X	D	D
Micrevania difficilis	283	006	D		X			X	D
Micrevania 061	288	061	D	X				D	D
Micrevania 066	298	066	D			X		D	D
Micrevania 026	308	026	D			D		D	D
Papatuka capensis	227	065	D			X	X	Х	D
Parevania 172	172		X	X	X		X		
Parevania 174	174		Х	X			X	Х	

Continued on next page

NOT PEER-REVIEWED

taxon	Ext.	DV#	28S	AM2	CAD1	CAD2	RPS23	COI	16S
Parevania 041	295	041	D	Х	Х	Х	Х	D	D
Parevania 057	219	057	D	Х	Х	Х	Х		D
Parevania 064	276	064	D		Х	Х	Х	D	D
Prosevania fuscipes	224	062	D			Х		X	D
Prosevania 497	497		Х	Х	Х	Х		Х	
Prosevania 498	498				Х			X	
Prosevania 508	508							Х	
Prosevania 027	309	027	D	Х		Х		X	D
Prosevania 034	277	034	D					D	D
Prosevania 036	284	036	D		Х	Х		Х	D
Prosevania 044	205	044	D	Х	Х	Х	Х	D	D
Rothevania valdivianus	239	048	D	Х	Х	Х		D	D
Semaeomyia 489	489				Х	Х	X	X	
Semaeomyia 509	509		Х			Х	X	X	
Semaeomyia 510	510		Х			Х	X	X	
Semaeomyia leucomelas	305	016	D			Х	X	D	D
Semaeomyia 012	197	012	D		Х	Х		D	D
Semaeomyia 051	279	051	D			Х	Х	D	D
Semaeomvia 059	293	059	D	Х		Х	X	D	D
Szepligetella 170	170				Х		Х	Х	
Szepligetella 231	231		Х		Х	Х	Х	X	
Szepligetella 233	233		Х	Х	Х	Х	Х	Х	
Szepligetella 236	236		Х	Х	Х	Х		X	
Szepligetella 238	238		Х	Х	Х	Х	X	Х	
Szepligetella 241	241				Х	Х	Х	Х	
Szepligetella 243	243		Х	Х	Х		Х	Х	
Szepligetella 247	247				Х		Х	X	
Szepligetella 248	248		Х		Х		Х	X	
Szepligetella sericea	297					Х	Х	X	
Szepligetella 047	208	047	D	Х	Х		Х	D	D
Szepligetella 055	280	055		Х	Х		X	D	D
Szepligetella 056	294	056	D	Х	Х	Х	X	X	D
Szepligetella 285	285			Х	Х	Х	X	X	
Trissevania anemotis	282	038	D	Х		Х	Х	D	D
Trissevania 507	507					Х			
Zeuxevania 499	499				Х	Х			
Zeuxevania 500	500				Х	Х		X	
Zeuxevania 503	503					Х			
Zeuxevania 505	505		Х		Х	Х	X	X	
Zeuxevania 015	191	015	D		Х			D	D
Zeuxevania splendidula	312	031	D		X	X	X	Х	D
% amplified			71	44	66	64	67	86	50
% parsimony-informative			40	44	49	53	35	60	40

Table 1 – Continued from previous page

Table 1. Taxonomic and genetic sampling. Exemplars used by Deans et al. (2006) are listed with the reference from that paper (DV#) beside the internal voucher number (Ext.) Genes for each taxon are marked with an **X** if amplified in this study and **D** if amplified by Deans et al. (2006). Gene codes: 28S = 28S rDNA; AM2 = alpha-mannosidase II; CAD1 and CAD2 = carbamoyl-phosphate sythetase-asparate transcarbamoylase-dihydroorotase (CAD) (for amplicon regions for each segment, see Figure 1); RPS23 = Ribosomal Protein S23; COI = cytochrome oxidase I; 16S = 16S rDNA.

99

Each exemplar not identified to species represents a putative morphospecies, as many species remain

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¹⁰¹ undescribed. Several DNA extracts and some sequences were used from Deans et al. (2006), as indicated ¹⁰² in Table 1. Vouchers were deposited at the Frost Entomological Museum, at The Pennsylvania State

¹⁰³ University, or in repositories stipulated by collecting permits and/or loan agreements. See supplementary

¹⁰⁴ CSV file (EvaniidPhylogenyVouchers.csv).

105 Gene selection

We utilized DNA from six different genes, including two mitochondrial (mt) genes (16S ribosomal DNA 106 (16S) and cytochrome c oxidase I (COI)) and four nuclear genes (28S ribosomal DNA (28S), ribosomal 107 protein S23 (RPS23), carbamoyl-phosphate synthetase-aspartate transcarbamoylase-dihydroorotase (CAD) 108 and alpha-mannosidase II (AM2)). Diagrams of the gene structures of CAD, RPS23, and AM2 are 109 presented in Figure 1. The diagrams were produced based on annotations of the genomic reference 110 sequences from Apis mellifera Linnaeus, 1758 (NCBI RefSeq ID: GCF_000002195.4) and Nasonia 111 vitripennis (Ashmead, 1904) (NCBI RefSeq ID: GCF_000002325.3), visualized in NCBI's Sequence 112 Viewer (http://www.ncbi.nlm.nih.gov/tools/sviewer) and Geneious v.6.0.6 (Biomatters 113 Ltd.) The annotations include information on the introns, exons, organization of coding regions and 114 protein product features. Conserved domains in the protein products were also identified via a BLASTx 115 116 search (Altschul et al., 1990) against NCBI's Conserved Domains Database (CDD) (Marchler-Bauer et al., 2015). The genetic regions corresponding to the identified domains are included for reference in 117 the diagrams as well as the primers used in this study (primer sequences are listed in Table S2). Further 118 background about the three protein coding genes is provided below since the amplified regions or genes 119 utilized are novel for phylogenetic studies. All sequences are available in NCBI's Genbank (https: 120 //www.ncbi.nlm.nih.gov/genbank/) under accession numbers KY082187-KY082565. 121

122 **CAD**

CAD is a long and complex gene which codes a "fusion" protein, that is, a protein with multiple 123 enzymatic activities: glutamine-amidotransferase (GATase), carbamoylphosphate synthetase (CPSase), 124 dihydroorotase (DHOase) and aspartate/ornithine transcarbamoylase (ATCase/OTC). There are 26 exons 125 and 25 introns in both Apis and Nasonia, although intron loss has been reported in the CPS as small chain 126 region in some Braconidae (Sharanowski et al., 2011). CPSase is divided in two domains: one for a short 127 chain, which includes GATase, and one for a long chain. The long chain is also subdivided, consisting 128 of two subunits (N-terminal + ATP-binding region), one oligomerization domain, and one MGS-like 129 (methylglyoxal synthetase-like) domain. These two CPSase chains are coded by 14 exons. Various 130 segments of this gene have been used in other phylogenetic studies of insects, particularly for lineages 131 diversifying within the last 150 million years (Danforth et al., 2006; Moulton and Wiegmann, 2004; 132 Winterton and De Freitas, 2006). The regions we analyzed are within the CPSase domains, extending 133 between exons 3 to 5 (Figure 1A). 134

135 **RPS23**

Ribosomal protein S23 (Figure 1B) is part of the small ribosomal subunit (Wool, 1979). It has a binding 136 site for mRNA and is associated with the eukaryotic initiation factor of the translation process (NCBI-137 CDD:cd03367). This gene has been previously used in macro-evolutionary phylogenetic studies on 138 Hymenoptera (Sharanowski et al., 2010) and Arthropoda (Aleshin et al., 2009; Timmermans et al., 2008) 139 and as an EPIC (exon-primed, intron-crossing) marker for population-level studies (Lohse et al., 2011, 140 2010). RPS23 is well conserved in sequence and structure across Hymenoptera, with the variation 141 concentrated in the introns. In both Apis and Nasonia, there are three exons (3bp, 159bp, and 270bp 142 in length) and 2 introns (339bp and 84bp in Apis; 353bp and 79bp in Nasonia). The amplified region 143 covers the downstream region of exon 2, full intron 2, and about half of exon 3, which contains the 144 aminoacyl-tRNA interaction site and therefore is expected to be conserved. 145

146 **AM2**

We performed sequence similarity searches with tBLASTx (Altschul et al., 1990), using Hymenopteran
expressed sequence tags (ESTs) from Sharanowski et al. (2010) against proteins of *Apis mellifera* and *Nasonia vitripennis*. Our search focused on genes with regions of variability (for putative phylogenetic
signal), limited introns and relatively long exons, and regions of sequence conservation (for priming sites).
Alpha-mannosidase II is a glycoside hydrolase involved in the catabolism of carbohydrates (Gonzalez and
Jordan, 2000) and has not been explored for phylogenetic studies. There has been a shift in the placement

¹⁵³ of the second intron between *Nasonia* and *Apis* (Figure 1C), and thus we labeled the exons 2a and 2b

to demonstrate the homology with labeled exon 3 in both taxa. Three main protein domain regions are identifiable in the reference sequences: (1) an N-terminal catalytic domain of Golgi alpha-mannosidase II, which is entirely in exon 2a in *Apis*, but overlaps the second intron in *Nasonia*, and therefore also lies in exon 2b; (2) a middle domain, which is located in exon 3; and (3) and a C-terminal, which is located in exon 4 (Figure 1C). The amplified area is contained in the region that corresponds to the N-terminal in *Apis*, ending before the second intron (Figure 1C). No intron was amplified in the evanioid taxa used in this study, and thus the gene structure is more similar to *Apis* in the amplified region.

161 Extraction and Sequencing

Extraction of genomic DNA was performed following the manufacturer's protocols using the DNeasyTM 162 Tissue Kit (Qiagen, USA). Exemplars were either whole body extracted or only the separated thorax 163 and metasoma were used as the use of the head often resulted in low DNA concentrations in Evaniids. 164 COI was amplified using the protocols outlined in Schulmeister et al. (2002), with the primers developed 165 for that study or using the universal primers developed by Folmer et al. (1994) and following protocols 166 outlined in Namin et al. (2014) (Table S2). Sequences for 16S mitochondrial rDNA were used from Deans 167 et al. (2006), which were based on primers and protocols developed in previous studies (Dowton and 168 Austin, 1994; Whitfield, 1997). Amplification of the D2-D3 region of 28S was performed using either 169 primers developed byDowton and Austin (2001) or primers newly developed for this study (Table S2), 170 due to difficulty with amplification of some taxa. CAD sequences were amplified in two discontinuous 171 fragments using newly developed primers (Figure 1; Table S2: CAD1, CAD2). For CAD1, three reverse 172 primers were developed to either reduce degeneracy or due to amplification difficulties in some taxa, and 173 a touchdown protocol was also used to increase specificity of the reaction (Table S2). For CAD2, two sets 174 of primers were developed, the second set (CAD-Amel379F/CAD-Amel479R) slightly internal to the 175 first (CAD-Amel368F/CAD-Amel482R). If no amplification product was achieved with the first set of 176 primers, the second set was used alone or as a nested re-amplification of the product obtained with the 177 first set. RPS23 was amplified using primers developed by Lohse et al. (2011) and in conjunction with 178 a second newly developed reverse primer and amplified with a touchdown protocol (Figure 1B; Table 179 S2). Primers were also designed to amplify AM2, with an internal forward primer (AM2-Amel356F) 180 amplifying a much shorter fragment (Figure 1C, Table S2), which increased the number of taxa for which 181 we achieved amplification success. 182

All PCR amplifications were carried out using $0.2-1 \ \mu g$ DNA extract, $1 \times$ Standard Taq Buffer (New 183 England Biolabs, USA) (10 mm Tris-HCl, 50 mm KCl, 1.5 mm MgCl2), 200 µm dNTP, 4 mm MgSO4, 184 400 nm of each primer, 1 unit of Taq DNA polymerase (New England Biolabs, USA) and purified water to 185 a final volume of 25 μ L. PCR products were visualized on a 1% agarose gel. Occasionally 5% Dimethyl 186 sulfoxide (DMSO, Sigma-Aldrich, USA) was added as a PCR additive when non-specific bands occurred. 187 This additive has been shown to increase PCR yield with GC-rich templates (Farell and Alexandre, 2012). 188 Nested re-amplifications were performed using $0.5 \,\mu$ L of PCR product as DNA template (concentrations 189 varied depending on first PCR reaction success). PCR purification was performed using ExoSAP-IT 190 (Affymetrix, USA) following the manufacturer's instructions, except using 25% of the suggested reagent 191 amount. If double bands were visualized on the gel following PCR, a subsequent 50 μ L reaction was run 192 on gel cut bands, the product ran on a 2.5% agarose gel, and purified using the QIAquick Gel Extraction 193 Kit (Qiagen, USA) following the manufacturer's protocols. Sequencing was carried out using the BigDye 194 Terminatory 3.1 Cycle Sequencing Kit (Applied Biosystems, U.S.A.), with reaction products sequenced 195 on an Applied Biosystems 3730xl DNA Analyzer at the Genomic Sciences Laboratory, North Carolina 196 State University. Contigs were assembled and trimmed for quality using Geneious. 197

198 Sequence alignment

The protein-coding genes were aligned by translating the sequences and setting the correct reading frame 199 in BioEdit (Hall, 1999). Sequences were then aligned as proteins using MAFFT (Katoh and Standley, 200 2013) on the EMBL-EBI webserver (Li et al., 2015) under default settings and then back translated 201 to nucleotides. Introns present in CAD1 and RPS23 were excluded from the dataset prior to multiple 202 sequence alignment. Ribosomal DNA sequences were aligned following secondary structure models 203 204 developed by Gillespie et al. (2005a,b) and modified by Deans et al. (2006) for Evaniidae. Regions of ambiguous alignment (RAA), expansion and contraction (REC), and slipped-strand compensation (RSC) 205 were excluded from the analysis, following Deans et al. (2006). For analysis of sequence motifs, intron 206

were aligned using MAFFT with a gap opening penalty of 2 and gap extension penalty of 0.5 to limit excessive gaps in the alignment.

209 Phylogenetic analyses

The optimal partitioning scheme and models of evolution for the concatenated analysis were determined 210 using PartitionFinder v.1.1.1 (Lanfear et al., 2012). Character sets were predefined by gene, and by codon 211 position for the 5 protein-coding genes for a total of 17 partitions (CAD1 and CAD2 were partitioned 212 separately). The Bayesian information criterion was used to select among models implemented in 213 MrBayes version 3.2 Ronquist et al. (2012b), with the greedy search algorithm and branch lengths 214 unlinked. The optimal scheme included two partitions. The first partition included the 3rd codon positions 215 for CAD1, CAD2, AM2, and RPS23 under the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). 216 The remaining 13 predefined partitions were included together under the general time reversible model 217 (GTR). Both partitions included a parameter for invariant sites and rate heterogeneity modeled under a 218 gamma distribution. We observed notable differences in nucleotide composition across taxa for some 219 genes (calculated in MEGA v.6 Tamura et al., 2013), and thus, tested for base composition homogeneity 220 using chi-square tests in PAUP* (Swofford, 2002) (Supplementary Material Table S3). For CAD1 and 221 222 *RPS23* the intron was removed.

Phylogenies were estimated using MrBayes 3.2, either on the CIPRES Science Gateway (Miller et al., 223 2010) or the ComputeCanada WestGrid computational facility. Parameters were unlinked and site specific 224 rates were allowed to vary across partitions. Analyses were performed with two independent searches 225 and four chains. All concatenated analyses were run for 10 million generations, sampling every 2000th 226 generation. Individual gene trees were analyzed with 5 million generations, sampling every 1000th. 227 Convergence diagnostics, stationarity, and appropriate mixing were assessed with Tracer v1.6 (Rambaut 228 and Drummond, 2009), and a suitable burn-in was chosen based on the parameter values. Trees from 229 the posterior distribution were summarized post burn-in with a majority rule consensus and manipulated 230 for better visualization using FigTree v.1.3.1 (Rambaut, 2012) and modified for publication using Adobe 231 Illustrator (Adobe Systems, Inc. San Jose, CA). The final nexus file is available through Penn State's 232 ScholarSphere repository (DOI: 10.18113/S1D06H). 233

234 Divergence time estimations

An uncorrelated log-normal relaxed clock as implemented in the program BEAUTi and BEAST v.1.8.2 (Drummond et al., 2002, 2012) was used to estimate divergence times. The same partitions and models of molecular evolution were applied to each partition as in the phylogenetic analysis. We utilized the Birth-Death process for incomplete sampling (Stadler, 2009) and started with a random tree. Only the calibration for the entire ingroup (Evaniidae) was constrained to be monophyletic which was well supported from the Bayesian analysis.

241 We utilized six fossil calibration points with each fossil assigned to the crown group for which they belonged (see Fossil Calibrations in Supplementary Material) (Brues, 1933; Nel et al., 2002; Peñalver et al., 242 2010; Jennings et al., 2004, 2013; Jennings and Krogmann, 2009; Rasnitsyn et al., 1998; Sawoniewicz 243 and Kupryjanowicz, 2003). We performed two separate analyses to examine uncertainty with respect to 244 maximum bounds for clade ages. For the first analysis we used log-normal distributions. The age of the 245 fossil determined the hard minimum bound, as the clade to which it belongs must be at least that old. We 246 then chose a mean and standard deviation so that the 95% highest priority density interval (95% HDP) for 247 the divergence estimation of the clade was from 2 to 25 million years prior to the age of the fossil. The 25 248 million year demarcation is arbitrary, but it seems reasonable and follows Cardinal and Danforth (2013). 249 For the second analysis we chose hard maximum bounds based on previous knowledge of the fossil record 250 and the evolutionary relationships among the included taxa, which are justified (Supplementary Material 251 - Fossil Calibrations) for each calibration. Generally, we chose the mean as the average between the 252 hard minimum and maximum bounds and then set the standard deviation so that the 95% HDP spanned 253 the range from the minimum to the maximum bound. For both analyses, initial values were set to the 254 mean and the ucld.mean prior was set to exponential with a mean of 0.05. Although these values are 255 somewhat arbitrary, according to the authors of the program, they are unlikely to have an effect on 256 the analysis (Drummond and Rambaut, 2007; Drummond et al., 2012). All other parameters and the 257 258 Markov-chain Monte Carlo settings were left at the default settings. The xml input files for both the lognormal and normal distribution analyses are available through Penn State's ScholarSphere repository 259 (DOI: 10.18113/S1D06H). 260

261 RESULTS AND DISCUSSION

262 Phylogenetic analyses

The final concatenated data set consisted of 3097 characters total: *COI* (681bp), *16S* (371bp, excluding RAAs), *28S* (428bp, excluding RAAs), *AM2* (672bp), *CAD1* (417bp, excluding the intron), *CAD2* (321bp), and *RPS23* (207bp, excluding the intron). Individual gene trees are depicted in Supplementary Figures S1-S7. The null hypothesis for base composition homogeneity was rejected for *AM2* ($\chi 2 = 368.819$, df =120; P = 0.000000000) and *COI* ($\chi 2 = 562.535$, P = 0.0000000) (Table S3). Average nucleotide composition across all genes and gene regions analyzed are depicted in Table S3.

The Bayesian analysis of the concatenated dataset recovered a well resolved tree with most clades 269 well supported (pp >0.95) (Figure 2). Clades recovered across the individual gene trees and for the 270 concatenated analysis are summarized in Table S4 and gene trees are depicted in Figures S1-S7. We 271 also performed a Maximum Likelihood analysis with RaxML v8.2.4 (Stamatakis, 2014, 2006) (Figure 272 S15) under the GTR+CAT model and auto-determination of bootstrap replicates. The phylogenies 273 obtained from BEAST (Figure 3), Mr.Bayes (Figure 2), and RaxML (Figure S15) were very similar except 274 relationships among species varied within genera and Micrevania was not monophyletic in the Bayesian 275 analysis (Figure 2). The placement of *Rothevania* also varied across analyses. 276

In the concatenated analysis (Figure 2), Evaniidae was recovered as monophyletic with high support (pp = 1.0). Of the 15 genera included in the analysis with more than one representative, nine were recovered as monophyletic, including *Evaniscus*, *Decevania*, *Semaeomyia*, *Evania*, *Hyptia*, *Brachygaster*, *Prosevania*, *Trissevania*, and *Evaniella*. All clades representing monophyletic genera had posterior probabilities of 1.0. Although *Micrevania* was recovered as paraphyletic, it was recovered as monophyletic in other analyses, as mentioned above, the divergence analysis (Figure 3), ML analaysis (Fig S.15) and the *16S* and *COI* individual gene analyses (Table S3) and previously by Deans et al. (2006).

Similar to the previous study (Deans et al., 2006), Parevania and Zeuxevania were recovered as 284 285 paraphyletic with respect to each other, but in a well-supported clade (pp = 1.0) with *Papatuka*, in the concatenated analysis as well as five of the seven gene trees (Table S4). Interestingly, all of these taxa 286 have a distinct sequence motif at the 3' end of the RPS23 intron: GTTTGTTTTGYAG (Fig. S9). No other 287 evaniid taxa have a similar motif at the 3' end (Fig. S8), and thus the motif is diagnostic for this clade. 288 *Trissevania* and *Evania* were recovered as sister taxa with high support (pp = 1.0) in the concatenated 289 analysis and these two taxa were recovered as sister to Zeuxevania + Parevania + Papatuka (pp = 0.98) 290 (Figure 2). But there was little support for these higher level relationships among in the individual gene 291 trees (Table S4). Brachygaster was recovered as sister to Prosevania with strong support (pp = 1.0) but 292 was only recovered in the CAD2 gene tree (Fig. S4). Micrevania was also recovered as the sister to 293 all remaining evaniids, followed by *Brachygaster + Prosevania* in the concatenated analysis. Yet, the 294 position of these taxa fluctuated widely among the individual gene trees, likely due to inconsistent taxon 295 sampling across the gene trees. 296

Acanthinevania and Szepligetella were consistently recovered together (pp = 1.0 in the concatenated 297 analysis (Figure 2) and all gene trees except 16S (Table S4), but were paraphyletic with respect to 298 each other. Interestingly, all members of Acanthinevania and Szepligetella have a GATCTAAC motif 299 (Fig. S10) in the *RPS23* intron that is not shared with any other evaniid taxa (Fig. S8), highlighting 300 their close evolutionary relationship. There are also two diagnostic motifs within regions of ambiguous 301 alignment (RAAs) that were excluded from the phylogenetic analyses. All members of Acanthinevania and 302 Szepligetella have the motif TAAAAT in RAA8 (Fig. S11) and the motif TGCAYT within RAA12 (Fig. 303 S12). Evaniella was recovered as the sister group to Acanthinevania + Szepligetella in the concatenated 304 analysis and in three genes trees (Table S4). Members of all three genera share a 9bp diagnostic motif in 305 RAA10 in 28S: YTCGAWAAA (Fig. S12). Most other evaniid taxa do not have this many base pairs in 306 307 this position (usually 2–4bp); the ones that do have longer motifs are radically different in sequence (the full alignment is available in Scholarsphere, DOI:10.18113/S1D06H). Alobevania was recovered as sister 308 to Evaniella + (Acanthinevania + Szepligetella), with strong support in the concatenated analysis, and 309 with moderate support (pp = 0.88) in the 28S gene tree. This result is unsurprising given that these taxa 310 were once treated as *Evaniella* (Deans and Huben, 2003). 311

New world taxa with reduced wing venation (*Evaniscus*, *Decevania*, *Hyptia*, *Rothevania*, and *Semaeomyia*) are recovered together in a well-supported clade (pp = 1.0), in the concatenated analysis (Figure 2). This clade is only recovered in the *CAD1* gene tree (Figure S4), possibly due to lower taxonomic sampling in some individual gene trees due to failed amplification. However, these taxa are present

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in various combinations throughout the individual gene trees, but the relationships among taxa fluctuate

317 widely, which is also reflected in the lower support values in the concatenated tree for relationships among

these genera (Figure 2).

319 Divergence time analyses

The phylogenies obtained from the two Bayesian uncorrelated relaxed clock analyses using BEAST were 320 both identical (Figure 3 (simplified chronogram from the log-normal distribution) and Fig S14 (normal 321 distribution)). Other than slight differences among species within genera, and the recovery of Micrevania 322 as monophyletic, the trees were very similar to the tree obtained from the analysis with MrBayes (Figure 323 2). Estimates of divergence time from both analyses, using either a log-normal and normal distribution 324 are listed in Table 2. The log-normal analysis estimated younger divergence times for all clades (Table 325 2). This was expected as the calibration bounds were constrained within 25 million years of the fossil's 326 age in the log-normal analysis, but were allowed to vary across a larger span of time in the normal 327 distribution analysis based on interpretations of the fossil record. It is likely that the normal analysis 328 uses too broad a range, with the maximum bound being set too far away from the oldest known fossil 329 for the crown lineage, and thus we depict the log-normal analysis (Figure 3) and use these dates to draw 330 inferences about evaniid clade divergence. Evaniidae was estimated to diverge around 137 million years 331 ago (Mya) (134.1-141.1). Although the superfamily was not the focus of this study, Evanioidea had 332 an estimated mean age of 168 Mya (135.9–199.0), consistent with other previous estimates suggesting 333 Evanioidea diverged in the mid-late Jurassic (Peters et al., 2017; Branstetter et al., 2017). Branches 334 leading to *Micrevania*, *Prosevania*, and *Brachygaster* split sometime around the end of the Cretaceous, 335 with means ranging between 60-73 Mya (Table 2). Other extant genera likely diverged sometime in the 336 early Cenozoic and these lineages were likely all present before the start of the Neogene (Figure 3, Table 337 2). 338

	Log-normal - Age (My) mean (95% HDP)	Normal - Age (My) mean (95% HDP)
Gasteruption (Gasteruptiidae)	38.6 (18.5–59.3)	46.3 (27.2–69.4)
Pristaulacus (Aulacidae)	49.1 (45.4–54.7)	48.9 (23.3-73.6)
Evaniidae	136.8 (134.1–141.4)	151.5 (135.9-166.7)
Brachygaster	60.7 (40.5-86.4)	72.1 (49.5–96.3)
Decevania	37.6 (25.2–51.1)	47.8 (31.6-64.0)
Evania	45.3 (33.3–58.6)	55.4 (40.5-70.7)
Evaniella	69.3 (55.5-84.0)	88.6 (73.5–104.3)
Evaniscus	66.0 (40.8-89.5)	80.4 (50.1–110.3)
Hyptia	50.7 (45.7–57.8)	65.4 (50.7-81.2)
Micrevania	67.8 (38.4–94.8)	80.1 (52.5–111.8)
Prosevania	72.1 (58.6–86.4)	85.7 (67.6–103.8)
Semaeomyia	59.0 (46.6–72.5)	76.9 (61.1–94.0)
Szepligetella s.l.	49.0 (38.3-60.1)	60.6 (48.6-72.1)
Trissevania	32.0 (17.5–50.4)	38.5 (20.5–57.3)
Zeuxevania s.l.	55.6 (45.8–66.3)	76.0 (59.6–92.2)

Table 2. Estimates of divergence times for Evaniidae (bolded) and outgroups based on an uncorrelated log-normal relaxed clock analyses. Six fossil calibrations were used (see Supplementary Material) with maximum bounds for clade ages set using a log-normal (Analysis 1) and normal distribution (Analysis 2). For each analysis the mean age in millions of years (My) and the 95% highest posterior density interval (HDP, equivalent to a confidence interval) is provided.

Novel genes and molecular signatures

Alpha-mannosidase 2 (AM2) has never been used before in phylogenetic studies. This gene has a mix of

- conserved and variable sites (44% parsimony-informative sites), but it failed the test for base composition
- homogeneity, which can cause systematic bases in phylogenetic analyses (Phillips et al., 2004; Rodríguez-
- Ezpeleta et al., 2007; Sharanowski et al., 2011). RY-coding this gene did not change the results obtained

from the concatenated analysis. Unfortunately amplification of AM2 was difficult, even with the addition 344 of PCR additives such as DMSO, causing a high amount of missing data. Gel cuts were often necessary 345 to achieve clean sequences for several genes, but particularly AM2. RPS23 was highly conserved in the 346 exonic regions, and thus may be better suited for deeper level studies across families. There were distinct 347 molecular signatures within the intronic region that would be very useful for lower level studies, such as 348 across species, or population-level studies (see Lohse et al., 2010). The molecular motifs in the RPS23 349 intron were useful for delimiting genera and diagnosing congenerics (see taxonomic implications, below). 350 The individual gene trees for both regions of CAD were relatively well resolved (Figures S4-5) and similar 351 to other studies (Desjardins et al., 2007; Sharanowski et al., 2011), which demonstrates good utility for 352 353 resolving phylogenetic relationships in Hymenoptera.

Alignments based on secondary structure for rDNA have been very useful for delimiting highly variable regions to exclude from analyses to achieve better phylogenetic results (Gillespie et al., 2005b; Pitz et al., 2007). However, variable regions have useful information with phylogenetic and taxonomic utility, as demonstrated by Sharanowski et al. (2011), who included variable regions (RECs, RAAs, and RSCs) if the variation in sequence length had a standard deviation less than one. Here we demonstrate the utility of some of these regions for diagnosing genera (Figures S11-12) and use these data to improve taxonomic classifications (see Taxonomic implications below).

361 Taxonomic implications

Relative to the Deans et al. (2006) study, the addition of several more genes and taxa clearly led to increased resolution. For example, an additional four genera were recovered as monophyletic, and higher level relationships were more resolved and better supported. Our understanding of evaniid relationships remains incomplete, but, based on mounting evidence here and through our observations of morphology, we feel comfortable proposing the following classificatory changes.

New synonyms of Zeuxevania and new combinations Parevania, syn. nov., and Papatuka, syn. 367 nov., are congeneric with and junior synonyms of Zeuxevania. Bradley (1908) also suspected that these 368 two taxa were congeneric and treated *Parevania* as a subgenus of *Zeuxevania*. These taxa are consistently 369 recovered together in well-supported clades across individual gene trees and within the concatenated 370 analyses, but are polyphyletic with respect to each other (Table S4). Additionally, there are molecular 371 signatures within the RPS23 intron that support their shared evolutionary history (Fig. S9). ARD has 372 observed thousands of specimens of these taxa and can find no consistency in the presence or absence of 373 the fore wing vein 1RS, which was the only character purported to separate Parevania and Zeuxevania 374 (Deans and Huben, 2003). 375

Following the taxonomy of Hedicke (1939), we hereby transfer the following species back to *Zeuxevania: albitarsus* (Cameron, 1899); *annulicornis* (Turner, 1927); *atra* (Kieffer, 1916); bisulcata (Kieffer, 1911); *curvicarinata* (Cameron, 1899); *kriegeriana* (Enderlein, 1905); *leucostoma* (Kieffer, 1910); *longicalcar* (Kieffer, 1911); *punctatissima* (Kieffer, 1911); *rubra* (Cameron, 1905); *sanguineiceps* (Turner, 1927); schlettereri Bradley 1908; *schoenlandi* (Cameron, 1905); *semirufa* (Kieffer, 1907).

We also transfer the following species to *Zeuxevania* for the first time: *aurata* (Benoit, 1950), **comb. nov.**; *brevis* (Brues, 1933), **comb. nov.**; *broomi* (Cameron, 1906), **comb. nov.**; *emarginata* (Kieffer, 1911), **comb. nov.**; *kasauliensis* (Muzaffer, 1943), **comb. nov.**; *laeviceps* (Enderlein, 1913), **comb. nov.**; *madegassa* (Benoit, 1952), **comb. nov.**; *meridionalis* (Cameron, 1906), **comb. nov.**; *micholitzi* (Enderlein, 1905), **comb. nov.**; *ortegae* (Ceballos, 1966), **comb. nov.**; *plana* (Benoit, 1952), **comb. nov.**; *producta* (Brues, 1933), **comb. nov.**; *remanea* (Brues, 1933), **comb. nov.**.

Papatuka was originally described from a single, apterous specimen (Deans, 2002) and was since
 expanded to include other, winged species (Deans, 2005). The morphology of these species, which is
 also reflected in the molecular data, is not substantially different from *Zeuxevania*, and we transfer those
 species to *Zeuxevania: alamunyiga* (Deans, 2002), comb. nov.; *capensis* (Schletterer, 1886), comb. nov.;
 longitarsis (Kieffer, 1904), comb. nov.

New synonym of *Szepligetella* and new combinations There is also abundant evidence to support *Acanthinevania* as congeneric with *Szepligetella*. They are consistently recovered together in a clade but neither appears to be monophyletic by itself (Table S4). The primary diagnostic characters that separated these two primarily Australian genera include: *Szepligetella* with the third labial palpomere swollen; *Acanthinevania* with an elongated head relative to *Szepligetella*; and *Acanthinevania* with labium folded strongly anteriorly and thus appearing long and narrow, not broad and flat as in most *Szepligetella* (Deans
and Huben, 2003). Our observations of more than 1,000 specimens reveal that these character states (e.g.,
face long vs. face short) fall along phenotypic gradients, with no discrete sets of states. Several molecular
characters link (but do not separate) these genera, including motifs present in the *RPS23* intron and at
least two regions of 28S (Figs S10-12).
We treat *Acanthinevania*, **syn. nov.**, as *Szepligetella* and transfer the following species to *Szepligetella*:

australis (Schletterer, 1886), comb. nov.; braunsi (Kieffer, 1911), comb. nov.; braunsiana (Kieffer, 403 1911), comb. nov.; clavaticornis (Kieffer, 1911), comb. nov.; erythrogaster (Kieffer, 1904), comb. nov.; 404 eximia (Schletterer, 1886), comb. nov.; genalis (Schletterer, 1886), comb. nov.; humerata (Schletterer, 405 406 1889), comb. nov.; leucocras (Kieffer, 1911), comb. nov.; longigena (Schletterer, 1889), comb. nov.; lucida (Schletterer, 1889), comb. nov.; mediana (Schletterer, 1889), comb. nov.; princeps (Westwood, 407 1841), comb. nov.; quinquelineata (Kieffer, 1904), comb. nov.; rufiventris (Kieffer, 1911), comb. nov.; 408 scabra (Schletterer, 1889), comb. nov.; sericans (Westwood, 1851), comb. nov.; striatifrons (Kieffer, 409 1904), comb. nov.; szepligeti (Bradley, 1908), comb. nov.; versicolor (Kieffer, 1904), comb. nov.; 410 villosicrus (Kieffer, 1904), comb. nov. 411

Emerging tribal classification A new tribal classification for Evaniidae is warranted, given the lack of 412 support for Bradley's (1908) original (>100 year-old) tribal concepts (Deans, 2005; Deans et al., 2006; 413 Deans and Huben, 2003). Mikó et al. (2014) recently described Trissevaniini, to include Trissevania and 414 Afrevania, and, based on our results here, molecular work by (Deans et al., 2006), and prior morphological 415 work by us and our colleagues (Deans and Huben, 2003; Deans and Kawada, 2008; Kawada and Azevedo, 416 2007; Kawada, 2011) we have an opportunity to revise Hyptiini to include those New World genera 417 with reduced wing venation: Evaniscus, Hyptia, Rothevania, Semaeomyia, and Decevania. We remove 418 Brachygaster, Evaniella, and Zeuxevania from Hyptiini (see Bradley, 1908). This updated concept of 419 Hyptiini can be separated from other evaniid taxa by the absence of at least the fore wing RS+M, and 420 usually many other apical veins (see Figs. 1, 9, 11, 16, 17 in Deans and Huben, 2003), and its origin in 421 the New World. 422

423 Evaniid divergence and evolution

Evaniids diverged in the Early Cretaceous (ca. 134.1–141.1 Mya), when numerous modern cockroach 424 fossils have been found (Grimaldi and Engel, 2005), although cockroaches with oothecae are thought 425 to have much earlier origins in the Late Carboniferous (Legendre et al., 2015). Most of the extant 426 evaniid genera diverged sometime near the K-T boundary, which may indicate that the mass extinction 427 played a role in the divergence of multiple new lineages of ensign wasps. Whether or not there has been 428 co-cladogenesis with modern cockroach lineages remains to be tested but would be hampered by the lack 429 of known host relationships for most evaniids (Deans, 2005). For evaniids, as for most Hymenoptera, 430 basic natural history research is needed to understand the trophic relationships among wasps and their 431 hosts. 432

433 CONCLUSION

We provide here a more robust and well-resolved phylogeny for Evaniidae than previous studies, which 434 will facilitate ongoing evolutionary and taxonomic work. Indeed, the new synonyms and combinations 435 proposed above help us progress towards a stable classification that reflects evolutionary relationships. 436 Building on prior results (Deans et al., 2006), our data also reveal new, useful markers for Hymenoptera 437 (AM2 and RPS23) and continue to support the utility of shared molecular motifs in defining major clades 438 in Evaniidae. Our results indicate that Evaniidae diverged in the early Cretaceous with most genera 439 diversifying in the late Cretaceous or early Tertiary. The results also highlight important targets for future 440 data collection, especially near the base of the tree (*Micrevania*) and the relationships within each genus. 441 More intensive sampling, especially with the addition of morphological data and fossils (e.g., Ronquist 442 et al., 2012a), is the logical next step in providing a tribal classification and more refined estimates for 443 divergence times. 444

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450 **REFERENCES**

- Aleshin, V., Mikhailov, K., Konstantinova, A., Nikitin, M., Rusin, L. Y., Buinova, D., Kedrova, O., and
- Petrov, N. (2009). On the phylogenetic position of insects in the Pancrustacea clade. *Molecular Biology*,
 43(5):804–818.
- ⁴⁵⁴ Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment ⁴⁵⁵ search tool. *Journal of molecular biology*, 215(3):403–410.
- Balhoff, J. P., Mikó, I., Yoder, M. J., Mullins, P. L., and Deans, A. R. (2013). A semantic model for species
 description applied to the ensign wasps (Hymenoptera: Evaniidae) of New Caledonia. *Systematic* Distance (205), (200, (50)
- 458 Biology, 62(5):639–659.
- Bradley, J. C. (1908). The Evaniidæ, ensign-flies, an archiac family of Hymenoptera. *Transactions of the American Entomological Society*, 34(2):101–194.
- Branstetter, M. G., Danforth, B. N., Pitts, J. P., Faircloth, B. C., Ward, P. S., Buffington, M. L., Gates,
 M. W., Kula, R. R., and Brady, S. G. (2017). Phylogenomic insights into the evolution of stinging

wasps and the origins of ants and bees. *Current Biology*, 27(7):1019–1025.

- Brues, C. T. (1933). The parasitic Hymenoptera of the Baltic amber. part 1. *Bernstein-Forschungen*, 3:4–172.
- Cardinal, S. and Danforth, B. N. (2013). Bees diversified in the age of eudicots. *Proceedings of the Royal* Society of London B: Biological Sciences, 280(1755).
- ⁴⁶⁸ Danforth, B. N., Fang, J., and Sipes, S. (2006). Analysis of family-level relationships in bees (Hy-⁴⁶⁹ menoptera: Apiformes) using 28S and two previously unexplored nuclear genes: CAD and RNA
- polymerase II. *Molecular Phylogenetics and Evolution*, 39(2):358–372.
- 471 Deans, A. and Huben, M. (2003). Annotated key to the ensign wasp (Hymenoptera: Evaniidae) genera
- of the world, with descriptions of three new genera. *Proceedings of the Entomological Society of*
- 473 *Washington*, 105:859–875.
- ⁴⁷⁴ Deans, A. R. (2002). *Papatuka alamunyiga* Deans, a new genus and species of apterous ensign wasp
 ⁴⁷⁵ (Hymenoptera: Evaniidae) from Kenya. *Zootaxa*, 95(1):1–8.
- ⁴⁷⁶ Deans, A. R. (2005). Annotated catalog of the world's ensign wasp species (Hymenoptera: Evaniidae),
 ⁴⁷⁷ volume 34. American Entomological Institute.
- ⁴⁷⁸ Deans, A. R., Basibuyuk, H. H., Azar, D., and Nel, A. (2004). Descriptions of two new Early Cretaceous
- (Hauterivian) ensign wasp genera (Hymenoptera: Evaniidae) from lebanese amber. *Cretaceous Research*, 25(4):509–516.
- 481 Deans, A. R., Gillespie, J. J., and Yoder, M. J. (2006). An evaluation of ensign wasp classification
- (Hymenoptera: Evaniidae) based on molecular data and insights from ribosomal RNA secondary
 structure. *Systematic Entomology*, 31(3):517–528.
- ⁴⁸⁴ Deans, A. R. and Kawada, R. (2008). *Alobevania*, a new genus of neotropical ensign wasps (Hymenoptera:
- Evaniidae), with three new species: integrating taxonomy with the World Wide Web. *Zootaxa*, 1787:28– 486 44.
- ⁴⁸⁷ Desjardins, C. A., Regier, J. C., and Mitter, C. (2007). Phylogeny of pteromalid parasitic wasps (Hy-⁴⁸⁸ menoptera: Pteromalidae): initial evidence from four protein-coding nuclear genes. *Molecular*
- *Phylogenetics and Evolution*, 45(2):454–469.
- Dowton, M. and Austin, A. D. (1994). Molecular phylogeny of the insect order Hymenoptera: Apocritan
 relationships. *Proceedings of the National Academy of Sciences USA*, 91(21):9911–9915.
- ⁴⁹² Dowton, M. and Austin, A. D. (2001). Simultaneous analysis of 16S, 28S, COI and morphology in the
- ⁴⁹³ Hymenoptera: Apocrita—evolutionary transitions among parasitic wasps. *Biological Journal of the* ⁴⁹⁴ *Linnean Society*, 74(1):87–111.
- ⁴⁹⁵ Drummond, A. J., Nicholls, G. K., Rodrigo, A. G., and Solomon, W. (2002). Estimating mutation
- ⁴⁹⁶ parameters, population history and genealogy simultaneously from temporally spaced sequence data.
- ⁴⁹⁷ *Genetics*, 161(3):1307–1320.
- ⁴⁹⁸ Drummond, A. J. and Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees.
- *BMC evolutionary biology*, 7(1):214.
- ⁵⁰⁰ Drummond, A. J., Suchard, M. A., Xie, D., and Rambaut, A. (2012). Bayesian phylogenetics with
- ⁵⁰¹ BEAUti and the BEAST 1.7. *Molecular biology and evolution*, 29(8):1969–1973.

- Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of
 mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular marine*
- ⁵⁰⁴ *biology and biotechnology*, 3(5):294–299.
- ⁵⁰⁵ Gillespie, J. J., Munro, J. B., Heraty, J. M., Yoder, M. J., Owen, A. K., and Carmichael, A. E. (2005a).
- A secondary structural model of the 28S rRNA expansion segments D2 and D3 for chalcidoid wasps
- ⁵⁰⁷ (Hymenoptera: Chalcidoidea). *Molecular Biology and Evolution*, 22(7):1593–1608.
- ⁵⁰⁸ Gillespie, J. J., Yoder, M. J., and Wharton, R. A. (2005b). Predicted secondary structure for 28S and 18S ⁵⁰⁹ rRNA from Ichneumonoidea (Insecta: Hymenoptera: Apocrita): impact on sequence alignment and
- ⁵¹⁰ phylogeny estimation. *Journal of Molecular Evolution*, 61(1):114–137.
- Gonzalez, D. S. and Jordan, I. K. (2000). The α-mannosidases: phylogeny and adaptive diversification.
 Molecular Biology and Evolution, 17(2):292–300.
- 513 Grimaldi, D. and Engel, M. S. (2005). Evolution of the Insects. Cambridge University Press.
- ⁵¹⁴ Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program
- for Windows 95/98/NT. In *Nucleic acids symposium series*, volume 41, pages 95–98. [London]: Information Retrieval Ltd., c1979-c2000.
- Hasegawa, M., Kishino, H., and Yano, T.-a. (1985). Dating of the human-ape splitting by a molecular
 clock of mitochondrial dna. *Journal of molecular evolution*, 22(2):160–174.
- Hedicke, H. (1939). Evaniidae. In Hedicke, H., editor, *Hymenoptorum Catalogus Pars 9*. Dr. W. Junk,
 Gravenhage.
- Huben, M. (1995). Evaniidae. In Hanson, P. and Gauld, I., editors, *The Hymenoptera of Costa Rica*, pages 195–199. Oxford University Press.
- Jennings, J. T., Austin, A. D., and Stevens, N. B. (2004). Hyptiogastrites electrinus Cockerell, 1917,
- from Myanmar (Burmese) amber: redescription and its placement within the Evanioidea (Insecta: Hymenoptera). *Journal of Systematic Palaeontology*, 2(2):127–132.
- Jennings, J. T. and Krogmann, L. (2009). A new species of *Pristaulacus* kieffer (Hymenoptera: Aulacidae) from Baltic amber. *Insect Systematics & Evolution*, 40(2):201–207.
- Jennings, J. T., Krogmann, L., and Mew, S. L. (2012). *Hyptia deansi* sp. nov., the first record of Evaniidae (Hymenoptera) from Mexican amber. *Zootaxa*, 3349(1):63–68.
- Jennings, J. T., Krogmann, L., and Priya, P. (2013). Happy birthday Willi Hennig!—*Hyptia hennigi* sp. nov. (Hymenoptera: Evaniidae), a fossil ensign wasp from Eocene Baltic amber. *Zootaxa*, 3731:395–398.
- Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7:
 improvements in performance and usability. *Molecular biology and evolution*, 30(4):772–780.
- Kawada, R. (2011). Pictorial key for females of *Decevania* Huben (Hymenoptera, Evaniidae) and
 description of a new species. *ZooKeys*, (116):59–84.
- Kawada, R. and Azevedo, C. (2007). Taxonomic revision of the neotropical ensign wasp genus *Decevania* (Hymenoptera: Evaniidae). *Zootaxa*, 1496(1):1–30.
- Lanfear, R., Calcott, B., Ho, S. Y., and Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular biology and*
- *evolution*, 29(6):1695–1701.
- Legendre, F., Nel, A., Svenson, G. J., Robillard, T., Pellens, R., and Grandcolas, P. (2015). Phylogeny of
- ⁵⁴² Dictyoptera: dating the origin of cockroaches, praying mantises and termites with molecular data and ⁵⁴³ controlled fossil evidence. *Plos ONE*, 10(7):e0130127.
- Li, L., Rasnitsyn, A. P., Shih, C., Labandeira, C. C., Buffington, M., Li, D., and Ren, D. (2018). Phylogeny
- of Evanioidea (Hymenoptera, Apocrita), with descriptions of new Mesozoic species from China and Myanmar. *Systematic Entomology*, 43(4):810–842.
- Li, W., Cowley, A., Uludag, M., Gur, T., McWilliam, H., Squizzato, S., Park, Y. M., Buso, N., and
- Lopez, R. (2015). The embl-ebi bioinformatics web and programmatic tools framework. *Nucleic acids research*, 43(W1):W580–W584.
- Lohse, K., Sharanowski, B., Blaxter, M., Nicholls, J. A., and Stone, G. N. (2011). Developing EPIC markers for chalcidoid hymenoptera from EST and genomic data. *Molecular ecology resources*,
- 552 11(3):521-529.
- Lohse, K., Sharanowski, B., and Stone, G. N. (2010). Quantifying the Pleistocene history of the oak gall parasitoid *Cecidostiba fungosa* using twenty intron loci. *Evolution*, 64(9):2664–2681.
- Marchler-Bauer, A., Derbyshire, M. K., Gonzales, N. R., Lu, S., Chitsaz, F., Geer, L. Y., Geer, R. C., He,
- J., Gwadz, M., Hurwitz, D. I., Lanczycki, C. J., Lu, F., Marchler, G. H., Song, J. S., Thanki, N., Wang,

- Z., Yamashita, R. A., Zhang, D., Zheng, C., and Bryant, S. H. (2015). CDD: NCBI's conserved domain
 database. *Nucleic Acids Research*, 43(D1):D222–D226.
- Mardulyn, P. and Whitfield, J. B. (1999). Phylogenetic signal in the coi, 16s, and 28s genes for inferring relationships among genera of Microgastrinae (Hymenoptera; Braconidae): evidence of a high
- diversification rate in this group of parasitoids. *Molecular Phylogenetics and Evolution*, 12(3):282–294.
- ⁵⁶² Mikó, I., Copeland, R. S., Balhoff, J. P., Yoder, M. J., and Deans, A. R. (2014). Folding wings like a
- cockroach: a review of transverse wing folding ensign wasps (Hymenoptera: Evaniidae: *Afrevania* and *Trissevania*) *PLoS ONE* 9(5):e94056
- ⁵⁶⁴ *Trissevania*). *PLoS ONE*, 9(5):e94056.
- ⁵⁶⁵ Miller, M. A., Pfeiffer, W., and Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference
- of large phylogenetic trees. In *Gateway Computing Environments Workshop (GCE)*, 2010, pages 1–8.
 IEEE.
- Moulton, J. K. and Wiegmann, B. M. (2004). Evolution and phylogenetic utility of CAD (rudimen-
- tary) among mesozoic-aged eremoneuran Diptera (Insecta). *Molecular phylogenetics and evolution*, 31(1):363–378.
- ⁵⁷¹ Namin, H. H., Iranpour, M., and Sharanowski, B. J. (2014). Phylogenetics and molecular identification of ⁵⁷² the *Ochlerotatus communis* complex (Diptera: Culicidae) using DNA barcoding and polymerase chain
- reaction-restriction fragment length polymorphism. *The Canadian Entomologist*, 146(1):26–35.
- ⁵⁷³ Nel, A., Martínez-Delclòs, X., and Azar, D. (2002). A new ensign-fly from the Lower-Middle Miocene Do-
- ⁵⁷⁵ minican amber (Hymenoptera, Evaniidae). Bulletin de la Société entomologique de France, 107(3):217–
 ⁵⁷⁶ 221.
- Peters, R. S., Krogmann, L., Mayer, C., Donath, A., Gunkel, S., Meusemann, K., Kozlov, A., Podsiad-
- lowski, L., Petersen, M., Lanfear, R., Diez, P. A., Heraty, J., Kjer, K. M., Klopfstein, S., Meier, R.,
- Polidori, C., Schmitt, T., Liu, S., Zhou, X., Wappler, T., Rust, J., Misof, B., and Niehuis, O. (2017).
- Evolutionary history of the Hymenoptera. *Current Biology*, 27(7):1013–1018.
- Peñalver, E., Ortega-Blanco, J., Nel, A., and Delclòs, X. (2010). Mesozoic Evaniidae (Insecta: Hymenoptera) in Spanish amber: Reanalysis of the phylogeny of the Evanioidea. *Acta Geologica Sinica (Englich Edition)* 84(4):800–827
- ⁵⁸³ (English Edition), 84(4):809–827.

Phillips, M. J., Delsuc, F., and Penny, D. (2004). Genome-scale phylogeny and the detection of systematic
 biases. *Molecular biology and evolution*, 21(7):1455–1458.

- Pitz, K. M., Dowling, A. P., Sharanowski, B. J., Boring, C. A., Seltmann, K. C., and Sharkey, M. J. (2007).
 Phylogenetic relationships among the Braconidae (Hymenoptera: Ichneumonoidea): A reassessment of
- ⁵⁸⁸ Shi, Chen, and van Achterberg (2005). *Molecular Phylogenetics and Evolution*, 43(1):338–343.
- Rambaut, A. (2012). FigTree v. 1. 4. Molecular evolution, phylogenetics and epidemiology. Edinburgh,
 UK: University of Edinburgh, Institute of Evolutionary Biology.
- Rambaut, A. and Drummond, A. (2009). Tracer v1.5. Available at http://tree.bio.ed.ac.uk/
 software/tracer/ Accessed 19 February 2018.
- Rasnitsyn, A. P., Jarzembowski, E. A., and Ross, A. J. (1998). Wasps (Insecta: Vespida = Hymenoptera)
 from the Purbeck and Wealden (Lower Cretaceous) of southern England and their biostratigraphical
- and palaeoenvironmental significance. *Cretaceous Research*, 19(3-4):329–391.
- Rodríguez-Ezpeleta, N., Brinkmann, H., Roure, B., Lartillot, N., Lang, B. F., and Philippe, H. (2007). Detecting and overcoming systematic errors in genome-scale phylogenies. *Systematic Biology*, 56(3):389–
- ⁵⁹⁸ 399.
- ⁵⁹⁹ Ronquist, F., Klopfstein, S., Vilhelmsen, L., Schulmeister, S., Murray, D. L., and Rasnitsyn, A. P. (2012a).
- A total-evidence approach to dating with fossils, applied to the early radiation of the Hymenoptera. *Systematic Biology*, 61(6):973–999.
- Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu,
- L., Suchard, M. A., and Huelsenbeck, J. P. (2012b). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61(3):539–542.
- ⁶⁰⁵ Sawoniewicz, J. and Kupryjanowicz, J. (2003). *Evaniella eocenica* sp. nov. from the Baltic amber ⁶⁰⁶ (Hymenoptera: Evaniidae). *Acta Zoologica Cracoviensia*, 46(Suppl. - Fossil Insects):267–270.
- ⁶⁰⁷ Schulmeister, S., Wheeler, W. C., and Carpenter, J. M. (2002). Simultaneous analysis of the basal lineages
- of Hymenoptera (Insecta) using sensitivity analysis. *Cladistics*, 18(5):455–484.
- ⁶⁰⁹ Sharanowski, B. J., Dowling, A. P., and Sharkey, M. J. (2011). Molecular phylogenetics of Braconidae
- (Hymenoptera: Ichneumonoidea), based on multiple nuclear genes, and implications for classification.
- ⁶¹¹ *Systematic Entomology*, 36(3):549–572.

Peer Preprints

- Sharanowski, B. J., Robbertse, B., Walker, J., Voss, S. R., Yoder, R., Spatafora, J., and Sharkey, M. J.
 (2010). Expressed sequence tags reveal Proctotrupomorpha (minus Chalcidoidea) as sister to Aculeata
- ⁶¹⁴ (Hymenoptera: Insecta). *Molecular phylogenetics and evolution*, 57(1):101–112.
- 615 Stadler, T. (2009). On incomplete sampling under birth-death models and connections to the sampling-
- based coalescent. *Journal of theoretical biology*, 261(1):58–66.
- Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thou sands of taxa and mixed models. *Bioinformatics*, 22(21):2688–2690.
- Stamatakis, A. (2014). Raxml version 8: a tool for phylogenetic analysis and post-analysis of large
 phylogenies. *Bioinformatics*, 30(9):1312–1313.
- Swofford, D. L. (2002). PAUP* Phylogenetic analysis using parsimony (*and other methods). Version 4
 beta. Sunderland, MA: Sinauer Associates Inc, software.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, 30(12):2725–2729.
- Timmermans, M., Roelofs, D., Mariën, J., and Van Straalen, N. (2008). Revealing pancrustacean
- relationships: Phylogenetic analysis of ribosomal protein genes places Collembola (springtails) in
- a monophyletic Hexapoda and reinforces the discrepancy between mitochondrial and nuclear DNA
- markers. *BMC Evolutionary Biology*, 8(1):83.
- ⁶²⁹ Whitfield, J. B. (1997). Molecular and morphological data suggest a single origin of the polydnaviruses
- among braconid wasps. *Naturwissenschaften*, 84(11):502–507.
- Winterton, S. and De Freitas, S. (2006). Molecular phylogeny of the green lacewings (Neuroptera:
 Chrysopidae). *Austral Entomology*, 45(3):235–243.
- Wool, I. G. (1979). The structure and function of eukaryotic ribosomes. Annual review of biochemistry,
- 634 48(1):719–754.
- Zhang, C., Stadler, T., Klopfstein, S., Heath, T., and Ronquist, F. (2015). Total-evidence dating under the
- fossilized birth-death process. *Systematic Biology*, 65:228–249.



Figure 1. Diagrammatic gene maps for: (**A**) carbamoyl-phosphate sythetase-asparate transcarbamoylase-dihydroorotase (*CAD*); (**B**) ribosomal protein S23 (*RPS23*); and (**C**) alpha-mannosidase II (*AM2*). Dotted lines mark protein domains and features. For *CAD* and *AM2*, *Apis* and *Nasonia* gene diagrams are shown individually as references due to substantial differences in exon locations. The bottom diagram in each gene map depicts the regions amplified in this study. In *CAD*, intron 13 in *Nasonia* has been scaled down due to an incomplete sequence in the GenBank entry. Primers are named according to the amino acid position in the *Apis mellifera* protein. Forward primers are in dark green and reverse primers in light green. See Table S2 for primer combinations. Abbreviations: *CPS*, carbamoyl-phosphate synthase; *GAT*, glutamine aminotransferase; *DHO*, dihydroorotase; *MGS*, methylglyoxal-like; *OTC*, ornithine carbamoyltransferase; *SN1*, N-terminal of subunit 1 in CPS large chain; *N2*, N-terminal of subunit 2 in CPS large chain; *N2*, N-terminal of subunit 2 in CPS large chain; olig., oligomerization domain.



Figure 2. Bayesian analysis of phylogenetic relationships among Evaniidae. The outgroups were removed and placed above the ingroup tree for better visualization (the scale has been retained). Posterior probabilities are listed beside each clade.



Figure 3. Simplified chronogram showing estimated divergence times for Evaniidae with six fossil calibrations and maximum clade ages under a lognormal distribution. Monophyletic genera have been collapsed for better visualization of the divergence estimations of the major clades. The blue bars indicate the 95% highest posterior density interval (HDP, also listed in Table 1). The scale is in millions of years. Mean age is listed above each clade and posterior probabilities are listed below .

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