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Phylogenetics of Pteromalidae and Eulophidae (Hymenoptera: Chalcidoidea) With a Study of Cranial Bridges in Chalcidoidea

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ABSTRACT OF THE DISSERTATION

Phylogenetics of Pteromalidae and Eulophidae (Hymenoptera: Chalcidoidea) With a Study of Cranial Bridges in Chalcidoidea

by

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Phylogenetic studies on two different groups of Chalcidoidea were conducted. The subfamily Pteromalinae (Hymenoptera: Pteromalidae) was analyzed using the 28S D2-D5, Cytochrome b and Cytochrome Oxidase II gene regions, with focus on discovering the nearest relatives of the genus *Nasonia*. No support was found for monophyly of Pteromalinae, perhaps in part because of the low 28S molecular divergence within the group. Both maximum likelihood and Bayesian results indicate that *Nasonia* renders *Trichomalopsis* paraphyletic, and that *Trichomalopsis sarcophagae* is the sister group of the genus *Nasonia. Nasonia* and *T. sarcophagae* are both infected by *Wolbachia* bacteria, suggesting a history of *Wolbachia* infection that could be older than *Nasonia* itself. Other clades of Pteromalini that were 100% infected with *Wolbachia* among sampled species include the Australasian genus *Pseudanogmus* and *Coelopisthia* + *Diglochis*. Support was found for a monophyletic assemblage of taxa historically

placed in Miscogasterinae, and the subfamilies Cratominae, Miscogasterinae and Panstenoninae are synonymized under Pteromalinae **n. syn.** The genus *Diconocara* is transferred from Pteromalini to Miscogasterini **n. stat.** based on both molecular and morphological data.

A combined molecular and morphological phylogeny of the family Eulophidae is presented with focus on relationships within the subfamily Entedoninae. The 28S D2-D5 and CO1 gene regions were examined in partitioned maximum likelihood and Bayesian analyses, and an additional Bayes analysis was conducted to oberserve the effect of historically recognized morphological characters on the results. Eulophidae was strongly supported as monophyletic only when the genus *Trisecodes* was excluded. The subfamilies Eulophinae, Entiinae (=Euderinae) and Tetrastichinae were consistently monophyletic, but monophyly of Entedoninae was supported only in the combined morphological and molecular analysis. The lack of support from the molecular data was likely due to the form of the 3e´ subregion of the 28S D2 rDNA in the nominal subgenus of *Closterocerus*. The tribe Euderomphalini was excluded from a monophyletic Entiinae, suggesting that it should be retained in Entedoninae. Opheliminae **n. stat.** is raised from unplaced tribe to subfamily status, and a sister group relationship of Opheliminae + Entiinae was strongly supported. The genera *Neochrysocharis* **n. stat.** and *Asecodes* **n. stat.** were removed from synonymy with *Closterocerus* because molecular data corroborate their morphological differences. *Closterocerus* (*Achrysocharis*) *germanicus* was transferred to the genus *Chrysonotomyia* **n. comb.** based on molecular and morphological characters.

The posterior surface of the head was examined in several families of Chalcidoidea and interpreted according to theories of head capsule evolution as proposed by Snodgrass. Most chalcidoids have only a hypostomal bridge, but some species in the families Chalcididae, Eurytomidae, Pteromalidae, and Torymidae have postgenal bridges that may have been independently derived. Species of small-bodied parasitic wasps with a reduced head capsule, such as many Aphelinidae, Mymaridae, and Trichogrammatidae, lack important landmarks and cannot be easily interpreted without making inferences from related species. Several features provide potentially useful phylogenetic information, such as the presence of a postgenal bridge, extent of the hypostomal carina, extent of secondary posterior tentorial pits, and form of the mesal lamellae extending from the foramen magnum to the oral cavity. However, in many cases these characters present problems of homology that require a larger phylogenetic context to answer.

TABLE OF CONTENTS

CHAPTER 1

General Introduction.....1

References.....5

CHAPTER 2

Introduction.....9

Materials and Methods.....18

Results.....22

Discussion.....23

Conclusions.....39

References.....41

CHAPTER 3

Introduction.....66

Materials and Methods.....72

Results.....88

Discussion.....93

Conclusions.....106

References.....110

CHAPTER 4

Introduction.....146

Materials and Methods.....147

Results.....148

Conclusions.....154

References.....157

LIST OF TABLES

Table 1.....52
Table 2.....53
Table 3.....57
Table 4.....58
Table 5.....118
Table 6.....121
Table 7.....122
Table 8.....160

LIST OF FIGURES

Figure 1. *Nasonia vitripennis* posterior surface of head near foramen magnum.....59
Figure 2. RAxMLHPC BKL tree for Pteromalinae.....61
Figure 3. Bayes results for Pteromalinae.....63
Figure 4. Ventral surface of the petiole in selected Pteromalinae *sensu lato*.....64
Figure 5. Characters of the *Nasonia* Group.....65
Figure 6-9. Example habitus images of Eulophidae.....125
Figures 10-15. Characters of the antennal flagellum in Eulophidae.....126
Figures 16-21. Characters of antenna, head and pronotum in Eulophidae.....127
Figures 22-27. Characters of the head and pronotum in Eulophidae.....128
Figures 28-33. Characters of the mesosoma in Eulophidae.....129
Figures 34-39. Characters of the mesosoma in Eulophidae.....130
Figures 40-45. Characters of the legs, wings and metasoma in Eulophidae.....131
Figures 46-47. Characters of the male genitalia in Eulophidae.....132
Figure 48. RAxML molecular results for Eulophidae, 28S D2-D5 and CO1 regions.....134
Figure 49. Bayes molecular-only results for Eulophidae.....136
Figure 50. The 3e and 3e´ subregions for *Closterocerus trifasciatus* compared with that of other selected eulophids as aligned by the secondary structure model provided by Gillespie *et al.* (2005).....137
Figure 51. RAxML molecular results for Eulophidae with the 3e´subregion of the 28S D2 rDNA removed.....139
Figure 52. Bayes molecular-only results for Eulophidae with the 3e´ subregion of the 28S D2 rDNA removed.....141
Figure 53. Bayes combined morphological and molecular results for Eulophidae 143
Figure 54. Bayes combined morphological and molecular results for Eulophidae with selected state changes indicated.....145
Figure 55. Lack of cranial bridge in Xyelidae.....161
Figure 56. Hypostomal bridges and intermediate forms.....162
Figure 57. Hypostomal bridges and dubious cases.....163

Figure 58. Postgenal bridges and intermediate states.....164
Figure 59. Postgenal bridges and dubious cases.....165

CHAPTER 1

GENERAL INTRODUCTION

The parasitic wasp superfamily Chalcidoidea presents special problems for systematists because of the small size, generally less than 5 mm in length, coupled with no appreciable reduction in morphological complexity. However, the challenges of chalcidoid systematics are well worth surmounting because of the strong economic importance of the group. It constitutes one of the most important groups of animals for biological control of insects (Clausen 1978), and is one of the most diverse groups of Hymenoptera, with more than 20,000 described species (Noyes 2003) and possibly up to 400,000 species so far unknown to science (Noyes 2000).

The families Pteromalidae and Eulophidae are two of the most diverse and economically important families from among the 19 currently recognized in Chalcidoidea.

Pteromalidae is arguably the most morphologically and biologically diverse family of chalcidoids, with over 31 subfamilies containing over 3,500 species in nearly 600 genera (Noyes 2003). It is a notorious "dumping ground" of groups that do not fit in other chalcidoid families (Gibson *et al.* 1999). This may have been a historical accident due to the lack of distinctive characters used to define the core group of the family, currently contained in the subfamily Pteromalinae. Pteromalinae has never been precisely defined in a way that excludes other chalcidoid taxa, although most specialists considered it to be a recognizable group (Graham 1969, Bouček 1988). This diversity is contrasted

with a low degree of 28S D2 rDNA divergence (Heraty 2004), which was in that study less than the divergence for many morphologically uniform *genera* of Chalcidoidea. This raises the question of whether the diversity of the subfamily is largely a result of oversplitting by specialists, or whether the evolution of the 28S D2 molecule is unusual for the group.

The lack of a clear understanding of Pteromalinae and a concurrent lack of subgroups to classify the nearly 300 genera of the subfamily into informative units limits the context of evolutionary research of pteromaline genera. This is an especially visible problem with regards to the genus *Nasonia*, a model species for evolutionary and genetic research (Whiting 1967). All species of *Nasonia* harbor persistent infections of the reproductive manipulator bacteria *Wolbachia* (Breeuwer & Werren 1990, Werren 1997). The ease with which *Nasonia* can be reared in the laboratory, and the persistence of the infection within all species of the genus, has kept studies of this system at the forefront of *Wolbachia* research. The problems of pteromaline systematics has left one important question unanswered: does the shared history with *Wolbachia* extend further back than the origin of the currently recognized species of *Nasonia*? If so, it would be valuable to discover just how far back the relationship between the wasps and bacteria go. Furthermore, knowledge of additional related species of infected pteromalines should allow the selection of more meaningful species of Pteromalinae for comparisons when making evolutionary deductions. This study addersses these questions using molecular data with reference to morphological studies that are in progress.

The family Eulophidae is the most speciose family of Chalcidoidea, with over 4,500 currently recognized species (Noyes 2003). Recent phylogenies and anlayses of the group have created a controversy over the classification of some economically important eulophid parasitoids of leaf-mining flies and whiteflies (Gauthier *et al.* 2000, Gumovsky 2001, 2002). Gumovsky, using morphology (2001, 2002) or sometimes additional molecular data (2002) suggested that the genus *Closterocerus* was synonymous with other small-bodied genera of Entedoninae, such as *Neochrysocharis* and *Asecodes*, rather than being closely related to arguably more primitive genera such *Chrysonotomyia* and *Omphale* (Hansson 1990, 1994, 2002), and that the tribe Euderomphalini, consisting solely of whitefly parasitoids, be transferred to another subfamily, the Entiinae. These conclusions may have been based upon either oversimplification or mistaken interpretation of morphology (Burks 2003), suggesting that the proposed changes were misleading. This study uses molecular data to address this controversy by introducing new molecular data to test Gumovsky's conclusions.

Although it may seem that morphological data in Chalcidoidea are relatively well-understood, most character systems are in fact still being examined in order to establish a common ground for terminology and interpretation of structures across chalcidoid families. One system where this is necessary concerns interpretation of the cranial bridge on the posterior surface of the head. While some previous studies have addressed this structure in Pteromalidae and fig wasps (Bouček & Heydon 1997, Rasplus *et al.* 1998), a broader analysis is necessary to place these findings in a proper context. Most especially, determining how these structures fit into Snodgrass' (1960) ultimate interpretation of

cranial evolution was necessary. This study assesses variation in cranial bridges across Chalcidoidea and places obersved species within the standardized context of Snodgrass' previous study across insects.

The three studies presented in this dissertation comprise attempts to address questions of chalcidoid systematics and evolution by integrating morphological data with molecular data, and by moving discussion of chalcidoid variation into a more standardized framework. The common goals are to provide a more meaningful context for research into taxonomic problems within Chalcidoidea, and to move discussion of higher relationships of chalcidoid groups forward by using molecular data to provide more information for choosing between competing morphological hypotheses.

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CHAPTER 2

Molecular phylogeny of Pteromalinae with special reference to the placement of *Nasonia* (Hymenoptera: Chalcidoidea: Pteromalidae)

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Abstract. A molecular analysis of the subfamily Pteromalinae was conducted using the 28S D2-D5, Cytochrome b and Cytochrome Oxidase II gene regions, with the main goal of determining the nearest relatives of the genus *Nasonia*. Molecular divergence for 28S rDNA regions was very small compared to that for the mitochondrial regions, but greater than previously reported for the subfamily. Maximum likelihood and Bayesian results indicate that *Nasonia* renders *Trichomalopsis* paraphyletic, and that *Trichomalopsis sarcophagae* is likely the sister group of the genus *Nasonia. Trichomalopsis sarcophagae* differs from *N. vitripennis* by only one nucleotide base position for the 28S D2 regions, but differs from it in a number of bases in both mitochondrial regions. *Nasonia* and *T. sarcophagae* are both infected by *Wolbachia* bacteria. This suggests a history of *Wolbachia* infection that could be older than *Nasonia* itself. Additional clades of Pteromalini that were 100% infected with *Wolbachia* among sampled species were the Australasian genus *Pseudanogmus* and the clade of *Coelopisthia* + *Diglochis*. Our results provide some resolution for the phylogeny of Pteromalinae and related groups,

supporting a monophyletic assemblage of taxa historically placed in Miscogasterinae, but monophyly of Pteromalinae was not supported in any way. The subfamilies Cratominae, Miscogasterinae and Panstenoninae are synonymized under Pteromalinae n. syn. and Pteromalinae is broken up into the following tribes: Cratomini, Micradelini, Miscogasterini, Pachyneurini, Pteromalini, Rhaphitelini, Sphegigasterini, Termolampini and Trigonoderini, not all of which could be analyzed. *Diconocara* is transferred from Pteromalini to the paraphyletic tribe Miscogasterini because it formed a consistent outgroup to other Miscogasterini + Sphegigasterini.

Keywords. Molecular phylogeny, parasitoids, ribosomal, 28S, COII, Cytb, fig wasps.

Introduction

Chalcidoidea is one of the largest superfamilies in the family Hymenoptera, with over 20,000 described species (Noyes 2003), and probably with several times more undescribed species (Noyes 2000). It is also one of the most economically important groups of Hymenoptera, as almost all species are either parasitoids of other arthropods or phytophagous specialists (Noyes 2003). These life history traits make chalcidoids important for biological control of arthropod and weed pests, either as biological control agents or as complicating factors in biological control efforts (Noyes 1978, Greathead 1986, Heraty 2004). As well, it is likely the largest superfamily of Hymenoptera (Noyes 2002, 2007, Heraty & Gates 1993). Despite the significance of the group, it has a widely disputed family classification. Some chalcidoid families are generally considered

paraphyletic or polyphyletic (Noyes 1990, Quicke 1997, Gibson *et al.* 1999), and published phylogenies of Chalcidoidea reflect this opinion (Campbell *et al.* 2000). The rampant uncertainty, or even worse, certainty of error, in some aspects of chalcidoid classification is a major hurdle in reaching more meaningful conclusions involving the evolution of important chalcidoid species. Greater confidence in higher classification contributes to greater confidence in studying finer levels of classification by increasing the likelihood that the ingroup contains the appropriate selection of taxa, while facilitating selection of appropriate outgroup, and thus establishment of a proper context for evolutionary research.

Arguably the largest and most infamous problem in family classification of Chalcidoidea is the Pteromalidae. It contains by far the largest number of subfamilies in the Chalcidoidea, currently 31, and over 3,500 species contained in 590 genera (Noyes 2003). The large number of subfamilies reflects the generally accepted perception that Pteromalidae is a dumping ground for species not traditionally placed in other distinct families in modern treatments of Chalcidoidea (Gibson *et al.* 1999). Bouček (1988) is unusual in stating that Pteromalidae may be a natural group because most pteromalids can be derived from the subfamily Cleonyminae, and therefore the family may represent a monophyletic group with one or more paraphyletic subfamilies. This position has yet to be upheld by a phylogenetic analysis of Chalcidoidea (Campbell *et al.* 2000). The most likely scenario seems to be that most other chalcidoid families render Pteromalidae paraphyletic or polyphyletic through association with various different pteromalid subfamilies. This in itself illustrates the ironic phylogenetic importance of Pteromalidae

as a source of potential sister groups of currently accepted chalcidoid families. As Heraty & Darling (1984) were able to associate the former pteromalid subfamily Chrysolampinae with Perilampidae, similar studies may find that many current pteromalid subfamilies are better classified in other chalcidoid families or as separate families near the base of major lineages. The main source of inertia in recognizing pteromalid subfamilies as distinct families likely involves a resistance in erecting new families that have no clearly understood relationship to any other chalcidoid families. Furthermore, having a large number of minor and essentially undefined families would represent an obstacle to family identification of chalcidoids.

Many pteromalid subfamilies were intuitively placed in the family without any justification of why they would be more closely related to Pteromalinae than to other families of Chalcidoidea (Thomson 1876, 1878, Ashmead 1904, Graham 1969, Bouček 1988). A better understanding of which groups of Pteromalidae are actually closely related to Pteromalinae would allow for better investigations into subfamilies not closely related to Pteromalinae, with the aim of producing a more informative classification of Chalcidoidea. A proper analysis of Pteromalidae would require taking all of Chalcidoidea into account, a project currently underway (Munro *et al.* unpublished). However, a more focused analysis and redefinition of Pteromalinae would be a major step forward in chalcidoid phylogenetics.

Singling out a core group of pteromalids is not straightforward. Recognition that the subfamily Pteromalinae by default contains at least the “true” pteromalids can be deceptive. It contains a few other groups that may not rightly belong to the family at all,

such as the tribes Micradelini. Others, such as the tribe Termolampini, may be more closely related to other pteromalid subfamilies. Molecular evidence (Rasplus *et al.* 1998, Campbell *et al.* 2000) additionally suggests that some fig wasps not yet recognized as pteromalines may belong in that subfamily. Furthermore, a functional morphological definition of Pteromalinae and even of the tribe Pteromalini is completely absent in recent decades aside from a rough definition provided by identification keys that key them out in multiple terminal couplets (Graham 1969, Bouček 1988, Bouček & Rasplus 1991, Bouček & Heydon 1997). The last time either of these groups was defined morphologically was Ashmead's (1904) definition, which was based on the number of metatibial spurs and mandibular denticles, characters recognized for some time as being highly unreliable for deep classification (Graham 1969). Group membership of Pteromalidae has since expanded several times without a redefinition of the family, other than by ruling out all more easily defined groups of Chalcidoidea (Graham 1969, Bouček 1988). A more informative classification of Pteromalidae, using positive criteria instead of the current criteria based on absence of distinctive characters, is sorely needed.

The latest attempt to provide any sort of morphological character to help diagnostically define Pteromalidae, and potentially support its monophyly, used cranial

morphology (Bouček & Heydon 1997, Rasplus *et al.* 1998). They suggested that core pteromalids do not have a postoccipital bridge, but instead have a secondarily sclerotized gular area. This is more likely a reduced hypostomal bridge (Fig. 1), as the position of the posterior tentorial pits near the foramen magnum suggests that the neck membrane is not extensive enough to form a true gula (Snodgrass 1960, Burks & Heraty in prep.). Furthermore, a preliminary review in preparation of a morphological phylogeny of Chalcidoidea (Heraty *et al.* in prep.) has revealed that at least Eucharitidae and Perilampidae also share this character state. Molecular results (Munro *et al*. in prep.) do not link these two families to Pteromalinae, suggesting that the state has either evolved more than once among chalcidoids or is plesiomorphic for the group. Finally, Dzhanokmen's (1994) survey of pteromalid cranial morphology pointed out several pteromalids with a complete postgenal bridge, although the character apparently holds true across Pteromalinae.

Most previous phylogenetic analyses of Pteromalidae using morphology either focused on particular subfamilies (Gibson 2003, Desjardins 2007), or assumed *a priori* monophyly of all subfamilies (Dzhanokmen 2000), and did not make any final conclusions concerning membership of Pteromalidae or definition of Pteromalinae. Krogmann & Vilhelmsen (2006) included pteromalids in their analysis of Chalcidoidea using internal mesosomal morphology, finding that the subfamilies Asaphinae, Miscogasterinae, Panstenoninae and Pteromalinae formed a monophyletic unit while Cleonyminae and Spalangiinae consistently grouped with other chalcidoid families.

Other analyses of Pteromalidae have focused on the membership of Pteromalinae itself, and upon its relationship to the subfamily Miscogasterinae. Bouček & Heydon (1997) implied in their figure captions that Miscogasterinae should be a tribe of Pteromalinae, but it was not clear which taxa were to be included in the group. Likewise, the molecular analysis of Desjardins *et al*. (2007) concluded that Miscogasterinae *sensu* Bouček (1988) rendered Pteromalinae paraphyletic, but he stopped short of suggesting taxonomic changes. Campbell *et al.* (2000) agreed with Rasplus *et al.* (1998) that some non-pollinating fig wasps are apparently derived from Pteromalinae, further finding that among pteromalids only the subfamily Colotrechninae formed a monophyletic grouping with Pteromalinae. The only other molecular analysis of the family found that Panstenoninae rendered Pteromalinae paraphyletic, and that Spalangiinae was not closely related to core pteromalids (Krogmann & Abraham 2004). They suggested that Spalangiinae therefore may soon be returned to family rank.

The prospects for a molecular definition of Pteromalinae seemed promising when Heraty (2004) pointed out that the percentage sequence divergence for the 28S-D2 region for the subfamily Pteromalinae was only slightly over a tenth that of the genus *Aphelinus*. This is interesting given the wide range of morphological diversity in Pteromalinae (*sensu* Bouček 1988) compared to that of the genus *Aphelinus*. The causes of the discrepancy between 28S-D2 and morphological diversity are unknown, but the possibility of rapid speciation compared to that in other chalcidoid lineages cannot be ruled out.

The previously conducted molecular studies were only a beginning because none sampled more than 9 genera (< 3% of the total number) of Pteromalinae (Rasplus *et al.* 1998, Campbell *et al.* 2000, Desjardins *et al.* 2007, Krogmann & Abraham 2004), and several important morphologically defined units of the subfamily remain unsampled, as will be illustrated in a forthcoming key to genera of the subfamily (Burks in prep.). Additional data have not led to the discovery of morphological characters that support the monophyly of Pteromalinae or any group included within the subfamily. Additionally, low sample sizes of major pteromalid subgroups have contributed to a lack of perceived reliability of suggested taxonomic changes. For instance, while Spalangiinae may well belong to a different family from Pteromalinae, there are not enough data to deny that it could form a monophyletic unit with any other pteromalid groups, such as the morphologically similar pteromalid subfamily Cerocephalinae. Taxonomic changes before broad taxonomic sampling has been conducted could therefore lead to an unacceptable level of taxonomic instability, especially considering that out of the previously mentioned molecular analyses, only Desjardins *et al.* (2007) used more than one gene or provided any resampling support to gauge the strength of his results. In order to make more solid taxonomic statements, analyses should require targeted sampling of relevant taxa and a stronger focus upon using support measures.

Importance of *Nasonia* in pteromalid phylogenetics. Despite the poorly developed state of pteromaline phylogenetics, one of the most thoroughly studied insects is the pteromaline *Nasonia vitripennis* (Walker), a synanthropically distributed generalist parasitoid of muscoid flies. *Nasonia* has become a model organism for laboratory studies

because it is easy to rear and culture in large quantities and because its haplodiploid means of sex determination presents a valuable situation for genetic analysis (*e.g.* Whiting 1958; Velthius *et al.* 1965; Whiting 1967; Werren 1980, 1983; Drapeau & Werren 1999; Velthuis *et al.* 2004; van Opijnen *et al.* 2005), and analysis of extrachromosomal factors affecting its sex ratio, has led to many interesting discoveries (Werren *et al.* 1987, Breeuwer & Werren 1990, Gherna *et al.* 1991, Stouthamer *et al.* 1993). Our vast amount of knowledge of *Nasonia* presents a starting point for better understanding pteromaline phylogenetics.

Nasonia is a persistent host of A and B strains of the reproductive manipulator bacteria *Wolbachia* (Breeuwer & Werren 1990). This research led to the discovery of two additional Nearctic species of the genus (Darling & Werren 1990) that are ecological specialists, restricted mainly to muscoid flies present in bird nests. This is in contrast to the relative ecological generalist *N. vitripennis*. It was found that *Wolbachia* may have played a role in the speciation of the two Nearctic species of *Nasonia* (Breeuwer & Werren 1990, Coyne 1992, Werren 1997, Bordenstein *et al.* 2001, Zimmer 2001, Bordenstein 2003), because they are different in the different *Nasonia* species, render them reproductively incompatible, infect almost 100% of *Nasonia* individuals in the wild, and because establishment of different B strains in the populations apparently predated their genetic differences (Bordenstein *et al.* 2001). An ongoing project to sequence the genome of *Nasonia* species (Werren *et al.* unpublished) has revealed the presence of a fourth molecularly distinct species of the genus (Raychoudhury *et al.* in press), closely related to and sympatric with *Nasonia giraulti* Darling & Werren and *N. vitripennis*.

Despite our extensive knowledge of *Nasonia* genetics, the distinctness and placement of the genus within Pteromalinae is poorly understood. It does seem intuitively clear from morphology that *Nasonia* is near the type genus *Pteromalus* Swederus, but little is otherwise known. Morphological definitions of the genus provided by Graham (1969) and Wallace (1973) do not apply to the two more recently discovered species, which would instead key with difficulty to other genera in those references. Additionally, the definition provided by Darling & Werren (1991) applies more accurately to *N. vitripennis* than to the other two species, and does not distinguish it from other genera of Pteromalinae. Although Wallace (1973) attempted to characterize a "*Dibrachys* group" of genera that included *Nasonia* (Table 4) and thirteen other currently recognized genera, this group was ignored by most later authors or rejected as polyphyletic (Darling & Werren 1991). Finally, Graham (1969) mentioned an undescribed European species that he tenuously assigned to *Nasonia*, but this species was never mentioned in any literature again and the specimens could not be located in his collection.

An additional benefit to better understanding the placement of *Nasonia* within Pteromalinae stems from the strong probability that *Wolbachia* strains have coevolved with *Nasonia* for a unknown amount of time. The length of this association is not clear because it is essentially unknown if any near relatives of *Nasonia* possess related strains of *Wolbachia*, other than the knowledge that *Trichomalopsis dubia* (Ashmead) does not (Campbell *et al.* 1993). The relationship between *Nasonia* and *Wolbachia* is one of very few known cases where *Wolbachia* has had a known impact upon the speciation of insect hosts. Without better knowledge of which pteromalines are related to *Nasonia*, the length

of time that *Wolbachia* bacteria have maintained this important case of coevolution with the *Nasonia* lineage will remain poorly understood because it cannot be placed in any reasonable evolutionary context.

Materials and Methods

Taxa were chosen for analysis with the goal of maximizing taxonomic diversity, and eliminated if they could not be sequenced for an adequate number of genes, leading to a final dataset of 98 taxa (Table 1). Outgroup taxa were chosen according to their proximity to Pteromalinae in an overall molecular analysis of Chalcidoidea (Munro *et al.* unpublished), and according to their morphological similarity to Pteromalinae.

Most specimens were killed in 95% EtOH and stored at -80°F until extraction. The entire body was used for non-destructive extraction in most cases, but in some the metasoma was ground for extraction. The remainder of the body and whatever additional specimens remained from the same collection event were used as vouchers (Table 2), and deposited in the University of California, Riverside Entomology Research Museum (UCRC). Extractions were performed using the chelex method (Walsh *et al.* 1991), and kept at -80°F until needed. Table 2 lists the specimens used, their voucher numbers, and the Genbank accession numbers of the sequences.

Polymerase chain reactions were carried out in 20μl reactions using Promega Taq DNA polymerase (Madison, WI), Qiagen 10x PCR buffer (15 mM $MgCl_2$) and Qiagen 5x Q-solution (Valencia, CA). All genes were sequenced in the forward and reverse directions, and the resulting pair of chromatograms compared to find PCR or reading

errors. Primers were used as recommended from the following studies: 28S D2-Campbell *et al.* (1993, 2000); 28S D3 forward-Nunn *et al.* (1996); 28S D5 reverse-Schulmeister *et al.* (2003); COII-Villalba *et al.* (2002); Cytochrome b-Lopez-Vaamonde *et al.* (2001). Screening for presence of *Wolbachia* was conducted using general primers for the 16S ribosome of the bacteria (O'Neill *et al.* 1992). All PCR products were gene cleaned with the Bio 101 Geneclean Kit (Carlsbad, CA) using NaI and glassmilk. Cleaned samples were directly sequenced at either the San Diego State Microchemical Core Facility, The UC Riverside Genomics Center, or Genoscope (France).

Ribosomal sequences were aligned using the secondary structure model from Gillespie *et al*. (2005) with regions of ambiguous alignment aligned by eye. Mitochondrial sequences were verified to translate into valid amino acids, and did not have gaps. Molecular data were partitioned by gene region, with the protein-coding genes partitioned by codon position in both analyses.

Maximum likelihood bootstrap analysis was performed using RAxMLHPC 7.0.3 (Stamatakis 2006). The BKL (best-known likelihood) tree was chosen from a run of 200 inferences. One thousand bootstrap replicates were performed using the standard (slow) bootstrap method on a Intel Core 2 Duo Mac, each with a starting tree using the random number seed 21371, using the GTRMIX model and allowing RAxML to estimate model parameters. An initial rearrangement setting of 15 (i = 15), and the default number of categories (c = 25) were chosen for these analyses, determined using the iterative process recommended by Stamatakis (2006).

Bayesian analysis was performed using Mr. Bayes 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) on an AMD quad-core PC using a six parameter model for 33,330,000 generations with a burnin of 8,080,000 generations. A six parameter model with rates subject to a gamma distribution with a proportion of invariant sites (nst=6 rates=invgamma) was used for each separate partition as suggested by hierarchical likelihood ratio tests performed using MrModeltest (Nylander 2004). Repeated runs yielded the best results with 8 chains and 2 runs at a temperature of 20. The analysis did not reach convergence, but was stopped at an average standard deviation of split frequencies of 0.052 because of computational limitations. Results were summarized as a maximum credibility tree using TreeAnnotator 1.4.8 (Drummond & Rambaut 2007) using the same burnin as before, with posterior probability values plotted onto the maximum credibility tree (MCT). This method of displaying Bayes results was preferred since it provided better resolution than a 50% majority rule tree. While the importance of unsupported clades on this tree should not be overemphasized, the same caveat applies to a 50% majority rule tree. This method has the advantage of displaying the results on a resolved tree that has been selected because it is the tree with the maximum product of posterior clade credibilities. Advantages of this method include better tree resolution and the fact that the tree is an actual topology from among the posterior distribution of trees, chosen using an optimality criterion. The disadvantage of this method is that it may be misconstrued as indicating greater support for the resolved clades than is indicated by the data. Also, even though the MCT has the highest product

of posterior clade credibilities, the amount by which it exceeds other trees in this respect may be minimal.

Outgroup selection was a problem in both analyses. The tribe Systasini was selected as the outgroup because it was most distant from Pteromalinae both morphologically and molecularly. *Ormocerus* (Ormocerinae: Ormocerini), *Trigonoderopsis* (Pireninae), and non-pollinating fig wasps of the subfamilies Otitesellinae, Sycoecinae and Sycoryctinae were intended to be additional outgroups but were irretrievably classified within the ingroup. Other pteromalid taxa such as *Cleonymus* and Leptofoeninae were used as outgroups in some preliminary analyses but did not improve support or placement of the species from presumably non-pteromaline subfamilies. Cleonymine and leptofoenine taxa were removed because their 28S sequences were very different from the retained taxa, containing sequence arrays (blocks of nucleotide bases determined by alignment, usually regions of ambiguous alignment) absent from all included groups, suggesting a relationship too distant from Pteromalinae to be very helpful in the analysis.

Systasini was retained as the outgroup because of its general morphological and molecular similarity to Pteromalinae, and because it has a consistent morphological difference from Pteromalinae. This difference is that Systasini have thirteen flagellomeres instead of the fourteen found in Pteromalinae. One possible exception to this statement is the published description of the pteromaline species *Amphidocius schickae* as having the same number of flagellomeres as Systasini (Heydon & Bouček 1992). However, examination of a number of specimens reveals that this observation may

be in error, and that the first flagellomere in *A. schickae* is very tiny and usually hidden by the apex of the pedicel.

Results

The BKL tree in the RAxMLHPC analysis (Fig. 2) had a log likelihood score of -30,955.83. There was no bootstrap support for any previously recognized interpretation of Pteromalinae, and Sphegigasterini was the only higher level pteromaline taxon with strong support. There was bootstrap support (>75%) for monophyly of the pteromaline genera *Nasonia*, *Muscidifurax*, *Diglochis*, *Pseudanogmus* and *Pseudocatolaccus*, and for the sycoryctine genera *Philotrypesis* and *Sycoscapter*. Strongly supported multigeneric groupings included the clades of *Alticornis* + *Heteroschema*, *Arachnopteromalus* + *Grissellium*, *Coelopisthia* + *Diglochis*, *Conomorium* + *Dibrachoides*, *Dibrachys* + *Tritneptis*, *Neocatolaccus* + *Psilocera*, *Ogloblinisca* + *Miscogasteriella*, *Syntomopus* + *Thinodytes*, and the grouping of *Hemitrichus* (*Halticopterella* (*Pachycrepoideus* + *Toxeumorpha*). Although *Nasonia* and *Urolepis* were found to render *Trichomalopsis* paraphyletic, and *Trichomalopsis sarcophagae* placed as the sister group to *Nasonia*, there was no bootstrap support for these groupings.

In the Bayesian analysis, the maximum credibility tree found by TreeAnnotator (Fig. 3) had a log credibility value of -120.12. Almost all clades supported by the ML analysis were also supported by the Bayes results (Fig. 3) except that *Neocatolaccus* + *Psilocera* had no bootstrap support but were monophyletic. The Bayesian analysis did provide at least some support for groupings not well-supported by the ML analysis, such

as very mild support (74%) for a sister group relationship between *T. sarcophagae* and *Nasonia*, similarly weak support (71%) for monophyly of the genus *Pteromalus* Swederus, and for monophyly of Pachyneurini (78%). While these support values are hardly significant in Bayesian terms, they agree with morphological characters (Burks unpub.). There was strong support for an *Otitesella* Westwood (Otitesellinae) + *Trigonoderopsis* Girault (Pireninae) sister group relationship. This grouping was also monophyletic in the ML analysis, although without bootstrap support.

Discussion

Previous concepts of Pteromalinae and related groups. Given the state of pteromaline classification in previous literature, it is valuable here to clarify the usage of group names in this section. Bouček & Heydon (1997) most recently reclassified genera of Pteromalinae into tribes. They implied that the formerly separate subfamily Miscogasterinae should be a tribe within Pteromalinae. Unfortunately, they did not indicate what other tribes of Pteromalinae should be recognized, nor did they indicate which genera did not belong to Miscogasterini. This classification was not followed by Noyes (2003), who recognized Miscogasterinae as a separate subfamily with a classification roughly corresponding to that suggested by Bouček & Rasplus (1991) and Bouček (1988).

Pteromalinae *sensu lato* in the following sections is considered to be a group inclusive of *Cratomus*, Miscogasterinae, Pachyneurini Panstenoninae, and Sphegigasterini. It is not taken to include the non-pollinating fig wasp subfamilies

Otitesellinae, Sycoecinae and Sycoryctinae. Pteromalinae *sensu stricto* would include only Pachyneurini and Panstenoninae out of the aforementioned groups. Pteromalini is used in the following discussion to specifically rule out all previously mentioned groups except Panstenoninae, for reasons discussed below. While our analyses (Figs 2-3) indicate that Pteromalini has roughly the same scope as Pteromalinae *sensu stricto* itself, it is a useful name for unambiguously referring to a potential core group of Pteromalinae *s.s.* exclusive of Pachyneurini.

Miscogasterinae itself, historically considered near Pteromalinae (Graham 1969, Bouček 1988), has also been treated with varying limits. Herein, Miscogasterinae *sensu lato* would include Sphegigasterini and Trigonoderini as in most previous literature. It excludes Ormocerinae and Pireninae, which had been included by Graham (1969). Miscogasterini *sensu stricto* is used to unambiguously indicate the tribe (regardless of its placement in Miscogasterinae or Pteromalinae) as defined by Graham (1969) exclusive of all other groups discussed here.

Molecular divergence in Pteromalinae. Pteromalinae *sensu lato* (including all potential miscogasterine taxa) possess very little variation in the 28S D2-D5 regions. Heraty (2004) had found that the maximum sequence divergence was only 3.3% among 28S D2 sequences of 3 species, which was only slightly more than that for the genus *Aphelinus* Dalman. In this study, the maximum sequence divergence for Pteromalinae 28S D2 (88 included taxa) was 9.7% (Table 3) but for Pteromalini (74 included taxa) it was only 7.4%. Although much higher than previously reported, the divergence for Pteromalini was not much more than the 5.0% found for the genus *Orasema* Cameron

(Heraty 2004). For 28S D3-D5 the maximum sequence divergence is even less, at 3.4% for both Pteromalinae and Pteromalini. This is in strong contrast to the large values of sequence divergence in the two mitochondrial genes for Pteromalinae. The most conserved (2nd) codon positions, of COII and Cytochrome b varied by 16.8% and 22.8%, respectively. This raises a question: is 28S rDNA evolution is unusually slow in Pteromalinae, or is its mitochondrial evolution unusually fast?

Molecular divergence of COII and Cytochrome b has been examined for a few other Chalcidoidea. Auger-Rozenberg *et al.* (2006) found a divergence rate of up to 21.4% in Cyt-b sequences from *Megastigmus* Dalman (Torymidae) while finding 28S D2 divergences of only up to 2.2%. Divergence within the pollinator fig wasp genus *Ceratosolen* Mayr was up to 28% for COI and COII (Weiblen 2001), which was indicative of high rates of divergence within pollinator fig wasps in general in that study.

Divergence in COI has been examined in many Chalcidoidea, and this information can be applied to speculation on COII and Cytochrome b variation. Most notably, Opijnen *et al.* (2005) found a rate of synonymous divergence of up to 37.52% in *Nasonia*, which they found to be in agreement with reports of relatively rapid mitochondrial evolution among Apocrita in general (Crozier *et al.* 1989, Jermiin *et al.* 1994, Dowton & Austin 1995, Castro *et al.* 2002). Our results agree with these findings, although the fact remains that 28S divergence is relatively slow in Pteromalinae as compared to some other subfamilies of Chalcidoidea (Heraty 2004). This is in strong contrast to the very large number (nearly 300) of currently recognized genera of Pteromalinae (Noyes 2003), a rough indicator of high amounts of morphological

variation. This raises a possibility that there is taxon-specific constraint on 28S evolution in Pteromalinae as compared to other Chalcidoidea. While mechanisms for such a constraint are currently unknown, it bears further investigation.

This low divergence rate could be taken to imply that there should be some relatively distinctive and constant sections of the 28S rDNA that could be used as characters to define the group. Instead, such sections are lacking. Many other chalcidoid groups possess distinctive sequence motifs in the 28S D2-D5 regions that provide strong evidence for their monophyly (Munro *et al.* unpublished), but not Pteromalinae. Instead, pteromaline sequences can be quickly recognized through their lack of distinctive inserts or deletions relative to other chalcidoids. This general rule holds true for other subfamilies closely related to Pteromalinae as well (Figs 2-3), but not for more distantly related subfamilies such as Cleonyminae.

Relationships of Pteromalinae. No previous phylogenetic study involving pteromalines has shown significant statistical support for any grouping of pteromaline genera (Rasplus *et al.* 1998, Campbell *et al.* 2000, Krogmann & Abraham 2004, Desjardins 2007). Our data also provide no support for a monophyletic Pteromalinae *sensu lato* or *sensu stricto*, even though a number of other pteromalid subfamilies were included.. Rather, Pteromalinae formed an unresolved group mixed with Ormocerini, Otitesellinae, Pireninae, Sycoecinae and Sycoryctinae (Figs 2-3) without any clear indication of a core group that could be singled out as definitive Pteromalinae.

The non-pollinating fig wasp subfamilies Otitesellinae, Sycoecinae and Sycoryctinae could justifiably be placed into Pteromalinae based on previous studies

(Rasplus *et al.* 1998, Munro *et al.* unpub.), but *Ormocerus* (Ormocerinae: Ormocerini) and *Trigonoderopsis* (Pireninae) in particular do not fit any previously recognized morphological concept of Pteromalinae. However, inclusion of Ormocerinae and Pireninae *near* Pteromalinae is not unprecedented. These two current subfamilies were included in Miscogasterinae by Graham (1969), although that grouping has since been rejected based on morphological data (Bouček 1988). *Ormocerus* and *Trigonoderopsis* were intended to be outgroups, but their sequences are similar to those of other Pteromalinae both in base composition and in a lack of distinctive inserts or deletions.

The intrusion of other subfamilies into Pteromalinae in our results weakens any attempt at a molecular characterization of Pteromalinae. However, our results agree with the statement by Rasplus *et al.* (1998) that the non-pollinating fig wasps belong in Pteromalidae. They furthermore suggest that the subfamilies Otitesellinae, Sycoecinae, and Sycoryctinae are derived members of the subfamily Pteromalinae itself, as supported by the results of Munro *et al.* (unpub.). The current data are not strong enough to suggest taxonomic changes because of a lack of resampling support for those clades—it seems inadvisable to suggest taxonomic changes based on these data, because there is a strong possibility that the acquisition of new data could overturn these changes. Given the lack of certainty in placement of these taxa using current data, it seems best at this time to retain Ormocerinae, Otitesellinae, Pireninae, Sycoecinae and Sycoryctinae as distinct subfamilies of Pteromalidae with the understanding that additional data may indicate that they are better off as synonyms of Pteromalinae.

Despite the lack of molecular support for Pteromalinae *sensu stricto* or *sensu lato*, there is support for smaller groupings of pteromaline genera that will be discussed in the following sections. The previously described tribes of Pteromalinae *sensu stricto* have rarely been used (Ashmead 1904, Burks 1979), but there is a need to break down the nearly 300 genera of Pteromalini into more informative subgroupings. As we gain a better understanding of pteromaline relationships, those tribes will become more valuable as ways to refer to groups of pteromaline genera in more informative and less ponderous ways. The following sections provide recommendations on a tribe level classification of Pteromalinae *sensu lato* that seems appropriate until more information becomes available.

Cratomini, Miscogasterini, Sphegigasterini and Trigonoderini. The previously recognized subfamily Miscogasterinae has been the subject of controversy over the past few decades (Graham 1969, Bouček 1988, Heydon 1995, Bouček & Heydon 1997). Although Graham's (1969) classification included the current subfamily Ormocerinae and some Pireninae as miscogasterines, Bouček and other later authors used a different classification excluding those taxa, because of their lack of morphological and life history similarities with Miscogasterini.

Sphegigasterini and Trigonoderini have historically been retained in Miscogasterinae, although Bouček (1988) considered Trigonoderini as a tribe of Pteromalinae separate from Miscogasterinae. At times, the tribes Sphegigasterini (Bouček & Heydon 1997) and Trigonoderini (Bouček 1988) have been separated from core Miscogasterinae largely because Miscogasterinae *sensu lato* (inclusive of

Sphegigasterini and Trigonoderini) or Pteromalinae may not represent monophyletic groups with respect to one another based on speculation using morphology. A general trend in this controversy has been the lack of *explicit* character support for placing Sphegigasterini and Trigonoderini in Miscogasterinae, with only the occasional use of characters such as clypeal asymmetry or axillular size (Bouček 1988). However, even these characters are not constant within the defined groups.

Molecular data (Figs 2-3) support two alternative interpretations of relationships between the tribes formerly comprising Miscogasterinae *sensu lato*. Furthermore, they suggest a close relationship between these taxa and *Cratomus* and *Diconocara*, a relationship not previously suggested. ML results place a clade of Cratomini, Trigonoderini, Miscogasterini and Sphegigasterini within Pteromalini (Fig. 2). Bayes results place Cratomini + Trigonoderini at the base of Pteromalinae, with *Boharticus* Grissell interceding between them and the clade of *Pseudocatolaccus* + Miscogasterini + Sphegigasterini (Fig. 3). Neither of the two arrangements seems ideal, because Miscogasterinae *s.l.* has generally been considered primitive relative to Pteromalinae due to usually having complete notauli and a strongly indicated frenal groove (Graham 1969, Bouček 1988). The ML results do not agree with any previous concept of groupings in Pteromalidae. The concept of a primitive Miscogasterinae *s.l.* would be agreeable with the placement of Miscogasterinae *sensu lato* on the Bayes tree, but *Boharticus* and *Pseudocatolaccus* have no special characters in common with any miscogasterine taxa, and have incomplete notauli and a weakly indicated frenal groove as in most other Pteromalini. Another unexpected result is that Miscogasterini was rendered paraphyletic

by Sphegigasterini. The two tribes have always been considered to be closely related, but some authors differ on their memberships (Bouček 1988, Heydon 1995). No study has suggested a paraphyletic Miscogasterini, but none have addressed the question in a phylogenetic context.

Given that neither morphological nor our molecular data could clearly resolve Miscogasterinae from Pteromalinae, it seems best to follow the implied classification of Bouček & Heydon (1997) and recognize them as separate tribes within Pteromalinae as indicated in Table 2 and Figs 2-3. Miscogasterini, Sphegigasterini and Trigonoderini group together with the genera *Diconocara* and *Cratomus,* which had not previously been considered close to core miscogasterines.

Cratomus has usually been placed in its own subfamily, and *Diconocara* was described as a pteromaline. *Cratomus* was a supported (Bayes) or unsupported (ML) sister group to a monophyletic Trigonoderini in both analyses, but this grouping has never been suggested and currently has no known morphological support. Since it is not yet clear based on morphology where *Cratomus* should be placed, it seems best to retain it in the tribe Cratomini within Pteromalinae as recognized by Burks (1979).

Diconocara is morphologically distinct from Sphegigasterini and fits poorly in Pteromalini. However, there is strong molecular support for placing it as the sister group to the Miscogasterini/Sphegigasterini clade. Transferring *Diconocara* to Miscogasterini new placement seems best at this time in order to highlight the well-supported relationship found in this study (Figs 2-3). The paraphyly of Miscogasterini with respect to Sphegigasterini bears further investigation. It agrees with Heydon's (1995)

interpretation of a monophyletic Sphegigasterini based on the form of the ventral surface of the petiole (Fig. 4D). Because of the small number of genera sampled from these groups (Table 1), and because Sphegigasterini is strongly supported by molecular data, it seems best to retain Sphegigasterini as a distinct tribe until additional sequences can be obtained.

While these results are preliminary and largely unexpected aside from monophyly of Sphegigasterini, they do lend themselves towards an interesting conclusion. Sphegigasterini is defined by having a ventrally closed petiole (Fig. 4D) with a transverse carina along its antero-ventral edge (Heydon 1995). Most Pteromalini (notable exceptions mostly occurring in Pachyneurini) have a ventrally open and membranous petiole as in Fig. 4A, *Diconocara* has a partially closed petiole (Fig. 4B). *Sphaeripalpus* (Fig. 4C) and many other Miscogasterini have a closed petiole without a ventral carina, while all Sphegigasterini have a closed petiole with a ventral carina (Fig. 4D) except for a few known species in *Thinodytes* and *Tricyclomischus* Graham that have a strongly shortened petiole that is likely secondarily open ventrally. Both molecular analyses in this study suggest that ventral closure of the petiole is an end result of a gradual closure of the petiole along the lineage leading to Sphegigasterini. This suggests that the condition in Miscogasterini could be intermediate between the open condition in Pteromalini to the closed condition in Sphegigasterini. This illustrates some potential morphological plausibility for the molecular results showing Miscogasterini to be a paraphyletic grade.

Pachyneurini. Out of all existing pteromaline tribes, a modified version of Pachyneurini retains the best support in our analyses (Figs 2-3). Although ML results

place it as the sister group to all remaining Pteromalinae *sensu lato*, this placement is not statistically supported and may not be stable. Morphological support for this group includes the presence in all members of a short and thickened marginal vein of the forewing accompanied by a strong parastigmal break, the presence of a metasomal attachment complex involving a more strongly developed petiole anteriorly, and a more elongate first gastral sternite than found in other pteromalines. The anterior development of the petiole in Pachyneurini resembles that of Sphegigasterini, but has only lateral branches instead of the complete ventral carina found in the latter (Heydon 1995). Pachyneurini retains the genus *Coruna* as a basal member, which is interesting because it is one of the few pteromalids with complete notauli. *Grissellium* is the only analyzed member of Pteromalini which has complete notauli, but it was not near *Coruna* or any other taxon with complete notauli in the analyses (Figs 2-3). Instead, it grouped with other Pteromalini distant on the tree. This suggests that parallel reductions of the notauli has occurred within Pteromalinae. Having complete notauli therefore does not necessarily provide strong evidence of close relationship or even primitive placement within Pteromalini, despite the use of this character by previous authors (Graham 1969, Bouček 1988) as a means of supporting several different groupings.

Both maximum likelihood and Bayesian results (Figs 2-3) suggest the unprecedented inclusion of *Halticopterella*, *Hemitrichus*, *Rakosina* and *Toxeumorpha* in Pachyneurini. This placement is plausible using the morphological characters mentioned above, but we do not feel that official transfer of these taxa is currently warranted, since it is not yet clear how many additional genera should be transferred to Pachyneurini. Rather

than creating a new situation where some taxa are placed in Pachyneurini but morphologically similar taxa are left in Pteromalini, it seems better to wait for further revisionary work across all pteromaline genera to better define Pachyneurini in a broader context.

Panstenoninae. The single sampled species of *Panstenon* placed deep within the Pteromalini in both analyses, as an unsupported sister group to clades of various genera, including *Dibrachys*. These results agree with those of Krogmann & Abraham (2004). Panstenoninae and Pteromalini cannot be distinguished using molecular data and are so morphologically similar that *Panstenon* keys within the pteromaline section in recent identification keys (Bouček & Rasplus 1991, Bouček & Heydon 1997). Any recognition of Panstenoninae as a family-level group would inconveniently necessitate recognition of a large suite of other subfamilies, tribes, or subtribes from within Pteromalini, many of which would only contain a single genus. For these reasons we transfer all members of Panstenoninae to Pteromalini; new placement.

New tribe level classification of Pteromalinae. The previous sections propose changes that would divide the Pteromalinae analyzed in this study into the following tribes: Cratomini, Miscogasterini, Pachyneurini, Pteromalini, Sphegigasterini, and Trigonoderini. Panstenoninae is completely abolished as a family-level group. Some previously recognized tribes (Burks 1979) could not be analyzed, and should be retained as valid until they can be analyzed: Micradelini, Rhaphitelini, and Termolampini. This results in nine currently recognized tribes of Pteromalinae. Pteromalini itself should likely be broken up into several separate tribes in order to render it an informative group,

but such action requires a broader analysis of the group. The next few sections focus on Pteromalini itself, and upon some groups of interest within the tribe.

Pteromalini. The tribe Pteromalini is recognized here as a broad, undefined, potentially polyphyletic group containing almost all pteromaline genera. It is polyphyletic in both analyses, with only Pachyneurini and the designated outgroup Systasini and Pachyneurini placing outside of it in the ML results, and with only Systasini, Cratomini and Trigonoderini placing outside of it in the Bayes results. This means that the taxa from other subfamilies that place inside Pteromalinae in our analyses: Ormocerini, Otitesellinae, Pireninae, Sycoecinae and Sycoryctinae, also place inside Pteromalini. There is no support for monophyly of Pteromalini in our analyses but it is retained as a temporarily convenient grouping that allows recognition of the other, better supported, tribes of Pteromalinae without necessitating creation of a number of poorly supported tribes of pteromaline genera. At some point, it will likely be necessary to resurrect some long abandoned tribes from Ashmead's (1904) and other classifications to better characterize the variation within Pteromalini, as well as some newly established tribes. In the meantime, support for other, unofficial suprageneric groupings of pteromaline genera can be discussed. The advantage of such groups is that they can be established and altered without undue concern for monophyly or nomenclatural stability. They are therefore preferable in situations where no fully resolved and supported phylogeny is available.

Dibrachys Group. Our analysis found no support for any reasonably intact interpretation of the *Dibrachys* group (Table 4) as proposed by Wallace (1973) (boldface taxa; Figs 2-3). However, there is support for grouping certain pairs of genera. *Dibrachys*

grouped only with *Tritneptis*. *Conomorium* and *Dibrachoides* formed a well-supported clade in both analyses distant from other members of the *Dibrachys* group. *Coelopisthia* consistently grouped with *Diglochis* in a well-supported clade again isolated from other members of the *Dibrachys* group. The other included taxa from Wallace's *Dibrachys* Group: *Muscidifurax*, *Nasonia* and *Pseudocatolaccus*, were unrelated. A sister-group relationship of *Coelopisthia* + *Diglochis* had already been suggested based on morphology (Baur 2000) and is strongly supported by molecular data. Given that the *Dibrachys* group has been criticized in the literature (Darling & Werren 1990), it seems safe to conclude that such a grouping is not supported by molecular nor morphological data.

Muscidifurax. The muscoid fly parasitoid *Muscidifurax* Girault & Sanders consistently grouped with the non-pollinating fig wasp *Sycoscapter* of the subfamily Sycoryctinae, instead of with other pteromaline genera (Figs 2-3). There is no morphological evidence in favor of this grouping, and it lacked strong bootstrap or posterior probability support, and therefore may easily be overturned with the acquisition of new data. However, this grouping is interesting because of the taxa involved.

Muscidifurax is unusual among Pteromalinae in having seven funicular segments of the female flagellum (Bouček & Heydon 1997). It strongly resembles *Lariophagus* in overall morphologically, sharing similar sharp posterior propodeal corners, a strongly convex face, and similar shape of the antennal flagellomeres in males. Sycoryctinae is, like the subfamilies Otitesellinae, Sycoecinae and Sycophaginae, a separate subfamily mainly because its member inhabit figs and have a unifying diagnostic characteristic: the

last two metasomal tergites are elongate (Bouček 1988). These two traits do not indicate what relationship Sycoryctinae has with other chalcidoid groups, and therefore any suggested placement of the subfamily near another chalcidoid group should be taken seriously. These molecular results may prove valid, but it seems best at this time to avoid drawing final conclusions from data without strong support.

Nasonia Group. In all analyses *Nasonia* and *Urolepis* render *Trichomalopsis* paraphyletic, although resampling support for this clade is weak at best. The weak support is probably best attributed to the small amount of genetic divergence of pteromalines (Table 3) for the genes sampled. This grouping is congruent with one potentially valid interpretation of morphological data using a combination of characters, including presence of an occipital ridge (Fig. 5A, B: ocr), a dorsally convex propodeal nucha (Fig. 5A: nuc), and stronger sclerotization and sculpturing of the antecostal region of the first gastral sternite (Fig. 5C: gsf) than is usually found in Pteromalini (Fig. 4A). Admittedly, these characters are difficult to precisely define and interpret in some species, and none of them is unique within Pteromalini. There is extreme reduction or even sometimes absence of the occipital ridge in *Nasonia* (Fig. 5B: ocr), for instance, varying between individuals of the same species. It is an unfortunate reality in Pteromalinae that there appear to be no unique and unambiguous morphological characters available to define any suprageneric grouping at all, including this one. The only feasible common practice in defining pteromaline groups based on morphology requires the use of a combination of characters. The above combination of characters should function in separating the *Nasonia* Group from all other pteromalines.

We propose based on molecular and morphological evidence that these genera including *Usubaia* and *Gyrinophagus*, form a monophyletic *Nasonia* Group within Pteromalini. While the strongly sculptured first gastral sternite could suggest a close relationship with Pachyneurini, there is no other evidence to support this relationship.

There are no life history data, such as shared host relationships that support the *Nasonia* Group. However, it should be noted that different *Trichomalopsis* species parasitize a large suite of holometabolous insects. Several species parasitize muscoid flies, including *Trichomalopsis sarcophagae* (Gibson & Floate 2001, Noyes 2003), which was the sister group to *Nasonia* in all analyses. The consistent though weakly supported *T. sarcophagae + Nasonia* clade seems stable because *T. sarcophagae* differs from *Nasonia vitripennis* in only 1 base from over 1,100 bases of 28S D2-D5 rDNA, a finding confirmed by sequencing multiple specimens of both species. However, they differed in a number 76 amino acids in COII and 32 amino acids in Cytochrome b. Reference to *T. sarcophagae* as an outgroup to *Nasonia* supports a hypothesis of a shift from a more generalized habitat preference in *N. vitripennis* and *T. sarcophagae* to a more specific habitat preference in *N. giraulti* and *N. longicornis*—parasitizing muscoid flies in bird nests. It also suggests that there may be a longer than previously indicated history of *Wolbachia* association with the lineage leading up to *Nasonia*, although a more thorough analysis of *Wolbachia* phylogeny is required to fully elucidate the shared history of these organisms.

Occurrence of *Wolbachia* in Pteromalinae. There were three phylogenetic patterns found between *Wolbachia* and pteromalines: 1) the entire lineage of *Trichomalopsis*

sarcophagae (Gahan) and all *Nasonia* species is currently infected, 2) all three sampled species from the Holarctic *Coelopisthia* Förster/*Diglochis* Förster clade (*sensu* Baur 2000) are infected, 3) both sampled species of the Australian genus *Pseudanogmus* Dodd & Girault are infected, 4) *Neocatolaccus* (Ashmead) and *Psilocera* Walker formed an infected clade in the ML analysis.

Wolbachia infections were found in 26 (29.5%) of the 88 pteromaline species screened (Table 2). Infected taxa did not form a monophyletic grouping, as expected from previous surveys for *Wolbachia* across insects (Werren 1998). This indicates that *Wolbachia* has been introduced into Pteromalini from multiple different sources. Therefore, infection of pteromalines with *Wolbachia* is a phenomenon best investigated between very closely related pteromaline species and other insect species that they interact with. Investigation across the tribe Pteromalini for phylogenetic patterns seems uninformative at this time, but investigation within the clade of *Nasonia* + *Trichomalopsis* + *Urolepis* may prove more fruitful.

Recent discoveries have rendered phylogenetic research of *Wolbachia* strains problematic. Previously, infections by multiple strains of *Wolbachia* were only known involving strains from different supergroups (Werren 1997), but it has recently been discovered that multiple infections can involve *Wolbachia* from the same supergroup (Baldo *et al.* 2006, Raychoudhury *et al.* 2009). Because of this, it is not known how many multiple infections occur among the positive occurrences. An unfortunate implication of this discovery is that sequence data using multiple *Wolbachia* genes could be sampling different strains of *Wolbachia* instead of a single strain, providing misleading results.

Previous sequence data from these *Wolbachia* would also be potentially misleading if multiple strains from the same supergroup were found, because it would not be clear which strain the data were taken from.

Nasonia is important in *Wolbachia* research is because it is a rare situation where coevolution between an insect and *Wolbachia* has potentially occurred in insects, and in part because *Nasonia* may have a strong predilection to retain *Wolbachia* infections (Bordenstein *et al.* 2001). Part of the reason for this survey was to search for other clades of pteromalines in which similar evolutionary scenarios may have occurred, and to determine if pteromalines in general—instead of just *Nasonia*—have a higher than expected rate of *Wolbachia* infection. Of the different infected pteromaline clades discovered in this study, the *Coelopisthia*/*Diglochis* clade and the Australasian genus *Pseudanogmus* would seem to have the strongest possibility of exhibiting a system similar to that in *Nasonia*. Further research into the biology of these genera could be highly rewarding in gaining a greater understanding of any potential role of *Wolbachia* in insect evolution, with the caveat that these pteromalines are not as easily lab-reared as *Nasonia*.

Conclusions

While support for pteromalid relationships continues to be weak, our data provide a stronger context for defining the subfamily Pteromalinae and more reliably classifying the over 350 genera either included or related to the subfamily into more useful and more easily-handled subgroups. With reference to morphological data and additional molecular

data, it should be possible to achieve the goal of defining more solid tribes of Pteromalinae and making a stronger statement regarding their relationships.

While the D2-D5 subregions of 28S rDNA do not have an ideal signal to noise ratio for producing a well-resolved phylogeny of Pteromalinae when taken alone, they are helpful in constructing species-level phylogenies of some subgroups of Pteromalini and may be ideal for all levels of analysis of Miscogasterinae *sensu lato*. The best complement to 28S sequences for pteromalid phylogenetics would likely be rapidly evolving nuclear protein-coding genes. While ITS-2 and the nuclear protein-coding genes *Pten*, EF1-α and Long-wavelength Opsin, were attempted over the course of this project, they could not satisfy all necessary criteria, including the capability of being amplified and aligned across most pteromalines. *Pten* is useful for taxa in the *Nasonia* Section (Baudry *et al.* 2006), but it could rarely be amplified for taxa outside the immediate vicinity of *Nasonia*. While these genes have so far not proven ideal for analysis of pteromaline phylogenetics, the best option for improved results in the future likely includes the use of additional rapidly-evolving genes that can be aligned unambiguously.

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Table 1. Taxa analyzed by gene. Subfamilies indicated by gray shading. Classification reflects changes proposed in this study.

Higher taxon	species analyzed	28S D2	28S D3-D5	COII	Cytb
Ormocerinae	3	3	3	0	1
Ormocerini	1	1	1	0	0
Systasini	2	2	2	0	1
Otitesellinae	1	1	1	1	1
Pteromalinae	88	88	87	33	47
Cratomini	1	1	1	0	1
Miscogasterini	3	3	3	0	2
Pachyneurini	3	3	3	2	2
Pteromalini	74	74	70	29	40
Sphegigasterini	4	4	4	0	0
Trigonoderini	3	3	3	0	1
Pireninae	1	1	1	0	0
Sycoecinae	1	1	1	1	1
Sycoryctinae	4	4	4	0	1

Table 2. Specimens Used in This Study.

Taxon	Locality	Wolbachia infection	Museum No.	Genbank Accesion Numbers			
				28S D2	28S D3-D5	CO2	Cytb
Ablaxia n. sp.	Sweden: Öland	no	161089				
Alticornis sp.	MX: Quintana Roo	no	174895				
Amphidocius schickae	USA: CA	no	174903				
Anisopteromalus calandrae	India: Uttar Pradesh	no	174898				
Apsilocera sp.	Italy: Lazio	no	174871				
Arachnopteromalus dasys	USA: CA	no	174890				
Arthrolytus sp.	USA: NY	no	174877				
Boharticus n. sp.	USA: CA	yes	174905				
Bubekia sp.	Argen.: Missiones Prov.	no	161026				
Callitula bicolor	Italy: Lazio	no	174864				
Catolaccus sp.	Italy: Lazio	no	174867				
Cheiropachus quadrum	Sweden: Öland	no	161079				
Chlorocytus n. sp.	USA: CA	yes	161078				
Coelopisthia sp	Russia: Primorskiy kray	yes	174880				
Conomorium n. sp.	USA: CA	no	174849				
Coruna clavata	Russia: Primorskiy kray	no	161296				
Cratomus megacephalus	Canada: ON	no	00000807				
Diaziella sp.	Thailand: Surat Thani	yes	161300				
Dibrachoides dynastes	Italy: Lazio	yes	174863				
Dibrachys cavus	USA: CA	no	174904				
Diconocara petiolata	Russia: Primorskiy krai	no	00000820				
Diglochis sp. 1	Russia: Primorskiy krai	yes	174879				
Diglochis sp. 2	Russia: Primorskiy krai	yes	174881				

Euneura sp.	Chile: La Campana	yes	161102
Euteloida basalis	USA: CA	no	174845
Grissellium n. sp.	USA: CA	no	174893
Halticopterella sp.	Australia: QLD	yes	174906
Hemitrichus n. sp.	USA: CA	no	174861
Heteroschema sp.	MX: Quintana Roo	no	174896
Homoporus sp.	Russia: Primorskiy krai	no	174860
Homoporus mordellistenae	USA: CA	no	174846
Isoplatoides n. sp.	Australia: QLD	no	174855
Lamptrotatus n. sp.	USA: NM	yes	161023
Lariophagus distinguendus	USA: CA	no	174843
Lariophagus texanus	USA: NY	no	174878
Lyrcus n. sp.	USA: CA	no	174844
Lysirina polychroma	USA: CA	no	174847
Meraporus graminicola	Italy: Lazio	yes	174858
Merisus sp.	Italy: Lazio	no	174870
Mesopolobus sp.	Italy: Lazio	yes	174857
Miscogasteriella sp.	Papua New Guinea	no	174892
Monoksa dorsiplana	Argentina: La Rioja	no	174862
Muscidifurax raptor	USA†	no	174886
Muscidifurax raptorellus	USA†	no	174887
Muscidifurax uniraptor	USA†	yes	174852
Muscidifurax zaraptor	USA†	no	174894
Nasonia giraulti	USA†	yes	174889
Nasonia longicornis	USA†	yes	174888
Nasonia vitripennis	USA: NY	yes	000770
Neocatolaccus sp.	USA: GA	yes	161072
Norbanus sp.	Argen.: La Rioja	no	174899

Ogloblinisca americana	USA: LA	no	174891	
Ormocerus sp.	USA: CA	no	161348	
Otitesella sp.	Thailand: Surat Thani	no	161280	
Pachycrepoideus vindemmiae	USA†	no	174882	
Pachyneuron sp.	USA: CA	no	174853	
Panstenon sp.	Russia: Primorskiy krai	no	161293	
Philotrypesis sp 1	Thailand: Trang pr.	no	161272, 161273	
Philotrypesis sp. 2	unknown	no	J.Y. Rasplus	
Plutothrix sp.	USA: MI	no	161374	
Polstonia pelagocorphya	Colombia: Magdalena	no	91135	AY552173
Pseudanogmus sp. 1	Australia: QLD	yes	174848	
Pseudanogmus sp. 2	Australia: QLD	yes	174902	
Pseudocatolaccus guizoti	USA: CA	no	91131, 91132	AY552171
Pseudocatolaccus nitescens	Italy: Lazio	no	174869	
Psilocera sp.	USA: CA	yes	174850	
Pteromalus (Habrocytus) sp.	USA: CA	no	91131	AY552170
Pteromalus chrysos	Italy: Lazio	no	174909	
Pteromalus platyphilus	Italy: Lazio	no	174908	
Pteromalus puparum	unknown	no	*	AF379909
Rakosina n. sp.	Italy: Lazio	yes	174874	
Semiotellus sp.	Australia: QLD	no	161287	
Sphaeripalpus sp.	Italy: Lazio	no	161220	
Staurothyreus sp. 1	Italy: Lazio	no	174866	
Staurothyreus sp. 2	Italy: Lazio	no	174907	
Stenetra n. sp.	USA: CA	no	174851	
Stenoselma nigrum	Italy: Lazio	no	174875	
Sycoscapter sp.	Australia: QLD	no	161281	
Sycoscapter AJ00	unknown	no	J.Y. Rasplus	

Sycoscapter AJ02	unknown	no	J.Y. Rasplus	
Synedrus transiens sp.	Italy: Lazio	yes	174872	
Syntomopus sp.	USA: CA	no	174854	
Systasis sp.	Australia: SA	yes	161105	AY552172
Thinodytes sp.	USA: CA	no	91133	
Toxeumorpha sp.	Australia: QLD	yes	174856	
Trichomalopsis sp. 1	Italy: Lazio	no	174868	
Trichomalopsis sp. 2	Italy: Lazio	no	174873	
Trichomalopsis sp. 3	Italy: Lazio	no	174876	
Trichomalopsis sp. 4	India: Uttar Pradesh	no	174897	
Trichomalopsis apanteloctena	Japan (lab culture)	no	174900	
Trichomalopsis dubia	USA†	no	174885	
Trichomalopsis microptera	USA: OR	no	161027	
Trichomalopsis sarcophagae	USA†	yes	174884	
Trichomalus sp.	Italy: Lazio	no	174859	
Trigonoderopsis sp.	Thailand: Songkhla	no	174901	
Tritneptis sp.	Sweden: Vindelns kommun	yes	161206	
Urolepis maritima	USA: NV	no	161031	
Urolepis rufipes	USA†	no	174883	

* Sequence from Dowton & Austin (2001)
†From Werren Lab colonies

Table 3. Maximum % sequence divergence by gene, and maximum amino acid changes (Δaa) for mitochondrial proteins.

gene	Pteromalinae	Pteromalini
28S D2	9.7	7.4
28S D3-5	3.4	3.4
COII max Δaa	88	87
COII 1st	32.9	32.9
COII 2nd	18.0	16.8
COII 3rd	39.9	39.9
Cytb max Δaa	78	67
Cytb 1st	23.2	22.8
Cytb 2nd	9.2	8.6
Cytb 3rd	43	39.5

Table 4. Genera included in the *Dibrachys* group by Wallace (1973).

genus	region	species	usual host (B&R 1991, B&H 1997)
*Coelopisthia**	worldwide	12	Lepidoptera: Tortricidae
*Conomorium**	worldwide	5	unknown
Cyclogastrella	worldwide	9	Lepidoptera: Tortricidae
*Dibrachoides**	worldwide	3	Coleoptera: Curculionidae
*Dibrachys**	worldwide	18	Diptera: Tachinidae, Hymenopera
Helocasis	Holarctic	1	unknown
*Muscidifurax**	worldwide	5	muscoid Diptera
*Nasonia**	worldwide	3	muscoid Diptera
Platneptis	Czech, Hungary	1	Lepidoptera: Tortricidae
*Pseudocatolaccus**	Holarctic	13	Diptera: Cecidomyiidae
Schizonotus	worldwide	3	Coleoptera: Chrysomelidae
Stichocrepis	Palearctic	1	Lepidoptera: Geometridae
Systellogaster	Nearctic	2	Blattodea: Blattidae
*Tritneptis**	worldwide	11	Hymenoptera: Diprionidae, Lepidoptera

* = included in this study

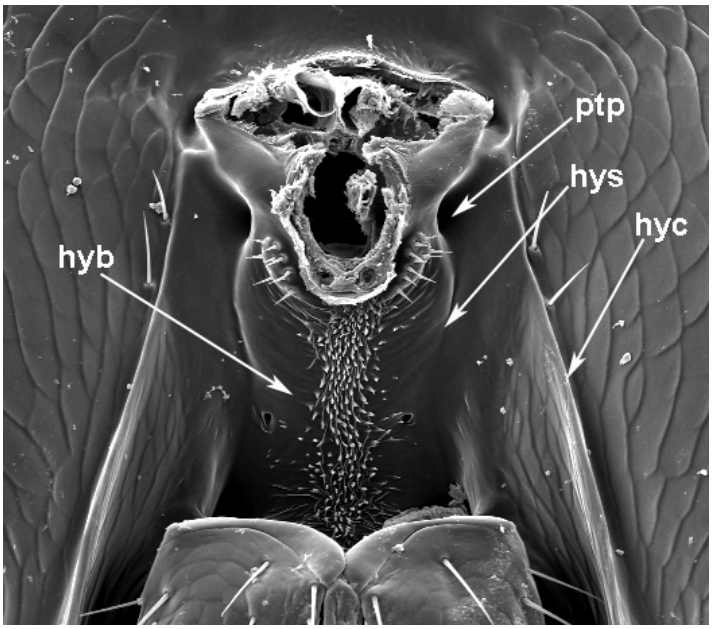


Figure 1. *Nasonia vitripennis* posterior surface of head near foramen magnum. Abbreviations: hyb = hypostomal bridge, hyc = hypostomal carina, hys = hypostomal sulcus, ptp = posterior tentorial pit.

Figure 2. RAxMLHPC BKL tree (-ln L 30,955.83). ML bootstrap values higher than 70% indicated on branches. Suprageneric taxa indicated by a vertical bar: black bar if monophyletic, gray bar if not monophyletic. *Dibrachys* group members indicated in bold. *Wolbachia* infected taxa indicated by an asterisk. Indicated taxonomic groupings reflect changes proposed in this article.

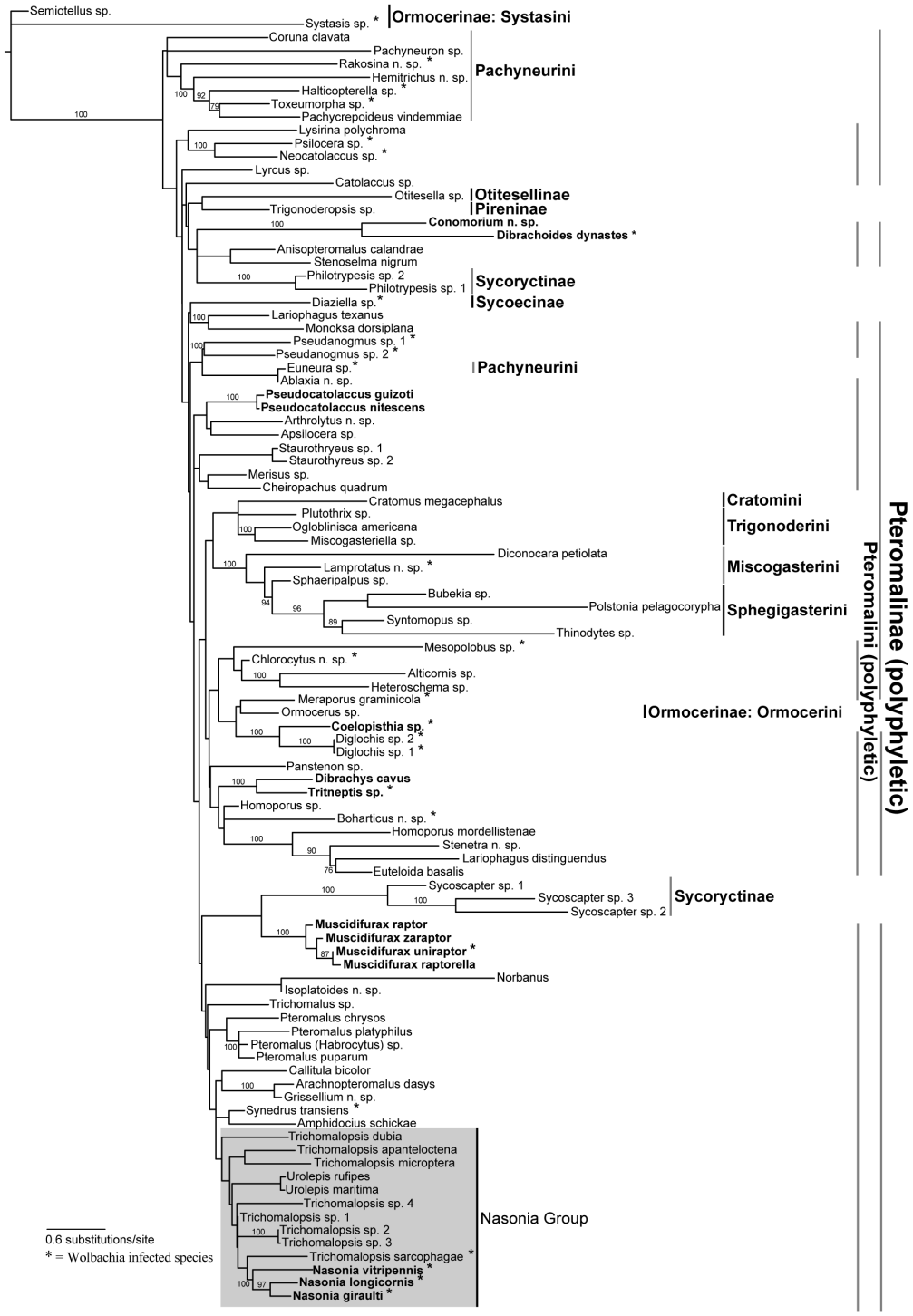
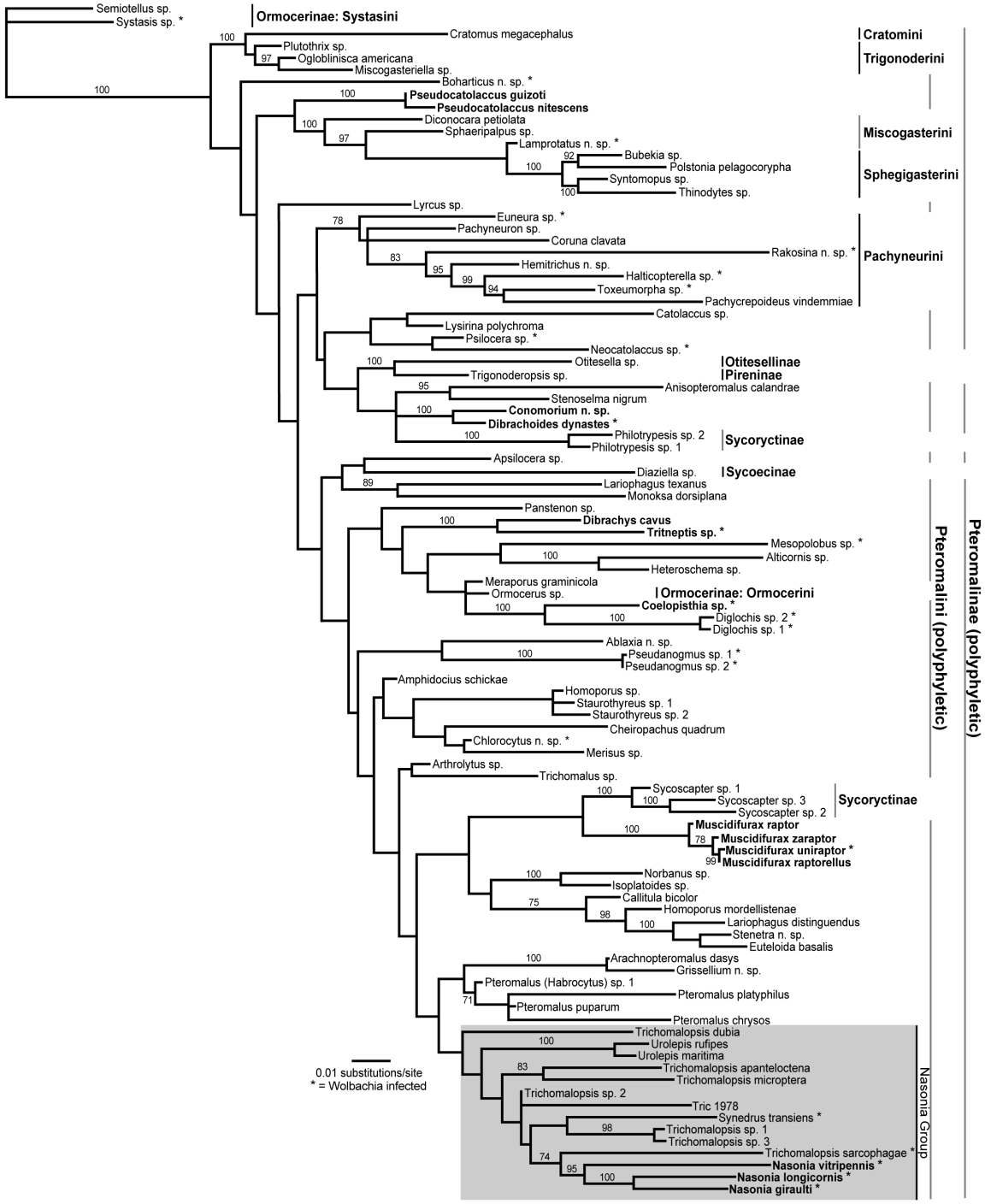

Semiotellus sp.
Systasis sp. *
Ormocerinae: Systasini
Coruna clavata
Pachyneuron sp.
Rakosina n. sp. *
Hemitrichus n. sp.
Halticopterella sp. *
Toxeumorpha sp. *
Pachycrepoideus vindemmiae
Pachyneurini
Lysirina polychroma
Psilocera sp. *
Neocatolaccus sp. *
Lyrcus sp.
Catolaccus sp.
Otitesella sp.
Otitesellinae
Trigonoderopsis sp.
Pireninae
Conomorium n. sp.
Dibrachoides dynastes *
Anisopteromalus calandrae
Stenoselma nigrum
Philotrypesis sp. 2
Philotrypesis sp. 1
Sycoryctinae
Diaziella sp.*
Sycoecinae
Lariophagus texanus
Monoksa dorsiplana
Pseudanogmus sp. 1 *
Pseudanogmus sp. 2 *
Euneura sp.*
Ablaxia n. sp.
Pachyneurini
Pseudocatolaccus guizoti
Pseudocatolaccus nitescens
Arthrolytus n. sp.
Apsilocera sp.
Staurothryeus sp. 1
Staurothyreus sp. 2
Merisus sp.
Cheiropachus quadrum
Cratomus megacephalus
Cratomini
Plutothrix sp.
Trigonoderini
Ogloblinisca americana
Miscogasteriella sp.
Diconocara petiolata
Lamprotatus n. sp. *
Miscogasterini
Sphaeripalpus sp.
Bubekia sp.
Polstonia pelagocorypha
Sphegigasterini
Syntomopus sp.
Thinodytes sp.
Mesopolobus sp. *
Chlorocytus n. sp. *
Alticornis sp.
Heteroschema sp.
Meraporus graminicola *
Ormocerus sp.
Ormocerinae: Ormocerini
Coelopisthia sp. *
Diglochis sp. 2 *
Diglochis sp. 1 *
Panstenon sp.
Dibrachys cavus
Tritneptis sp. *
Homoporus sp.
Boharticus n. sp. *
Homoporus mordellistenae
Stenetra n. sp.
Lariophagus distinguendus
Euteloida basalis
Sycoscapter sp. 1
Sycoscapter sp. 3
Sycoscapter sp. 2
Sycoryctinae
Muscidifurax raptor
Muscidifurax zaraptor
Muscidifurax uniraptor *
Muscidifurax raptorella
Norbanus
Isoplatoides n. sp.
Trichomalus sp.
Pteromalus chrysos
Pteromalus platyphilus
Pteromalus (Habrocytus) sp.
Pteromalus puparum
Callitula bicolor
Arachnopteromalus dasys
Grissellium n. sp.
Synedrus transiens *
Amphidocius schickae
Trichomalopsis dubia
Trichomalopsis apanteloctena
Trichomalopsis microptera
Urolepis rufipes
Urolepis maritima
Trichomalopsis sp. 4
Trichomalopsis sp. 1
Trichomalopsis sp. 2
Trichomalopsis sp. 3
Trichomalopsis sarcophagae *
Nasonia vitripennis *
Nasonia longicornis *
Nasonia girault *
Nasonia Group
0.6 substitutions/site
* = Wolbachia infected species
Pteromalinae (polyphyletic)
Pteromalini (polyphyletic)

Figure 3. Bayes results summarized as the maximum credibility tree (highest log clade credibility = -120.12), with posterior probability values higher than 70% indicated on branches. Suprageneric taxa indicated by a vertical bar: black bar if monophyletic, gray bar if not monophyletic. *Dibrachys* group members indicated in bold. *Wolbachia* infected taxa indicated by an asterisk. Indicated taxonomic groupings reflect changes proposed in this article.


Semiotellus sp.
Systasis sp. *
Ormocerinae: Systasini
Cratomus megacephalus
Plutothrix sp.
Ogloblinisca americana
Miscogasteriella sp.
Boharticus n. sp. *
Pseudocatolaccus guizoti
Pseudocatolaccus nitescens
Diconocara petiolata
Sphaeripalpus sp.
Lamprotatus n. sp. *
Bubekia sp.
Polstonia pelagocorypha
Syntomopus sp.
Thinodytes sp.
Lyrcus sp.
Euneura sp. *
Pachyneuron sp.
Coruna clavata
Rakosina n. sp. *
Hemitrichus n. sp.
Halticopterella sp. *
Toxeumorpha sp. *
Pachycrepoideus vindemmiae
Catolaccus sp.
Lysirina polychroma
Psilocera sp. *
Neocatolaccus sp. *
Otitesella sp.
Trigonoderopsis sp.
Anisopteromalus calandrae
Stenoselma nigrum
Conomorium n. sp.
Dibrachoides dynastes *
Philotrypesis sp. 2
Philotrypesis sp. 1
Apsilocera sp.
Diaziella sp.
Lariophagus texanus
Monoksa dorsiplana
Panstenon sp.
Dibrachys cavus
Tritneptis sp. *
Mesopolobus sp. *
Alticornis sp.
Heteroschema sp.
Meraporus graminicola
Ormocerus sp.
Coelopisthia sp. *
Diglochis sp. 2 *
Diglochis sp. 1 *
Ablaxia n. sp.
Pseudanogmus sp. 1 *
Pseudanogmus sp. 2 *
Amphidocius schickae
Homoporus sp.
Staurothyreus sp. 1
Staurothyreus sp. 2
Cheiropachus quadrum
Chlorocytus n. sp. *
Merisus sp.
Arthrolytus sp.
Trichomalus sp.
Sycoscapter sp. 1
Sycoscapter sp. 3
Sycoscapter sp. 2
Muscidifurax raptor
Muscidifurax zaraptor
Muscidifurax uniraptor *
Muscidifurax raptorellus
Norbanus sp.
Isoplatoides sp.
Callitula bicolor
Homoporus mordellistenae
Lariophagus distinguendus
Stenetra n. sp.
Euteloida basalis
Arachnopteromalus dasys
Grissellium n. sp.
Pteromalus (Habrocytus) sp. 1
Pteromalus platyphilus
Pteromalus puparum
Pteromalus chrysos
Trichomalopsis dubia
Urolepis rufipes
Urolepis maritima
Trichomalopsis apanteloctena
Trichomalopsis microptera
Trichomalopsis sp. 2
Tric 1978
Synedrus transiens *
Trichomalopsis sp. 1
Trichomalopsis sp. 3
Trichomalopsis sarcophagae *
Nasonia vitripennis *
Nasonia longicornis *
Nasonia giraulti *
0.01 substitutions/site
* = Wolbachia infected
Cratomini
Trigonoderini
Miscogasterini
Sphegigasterini
Pachyneurini
Otitesellinae
Pireninae
Sycoryctinae
Sycoecinae
Ormocerinae: Ormocerini
Sycoryctinae
Nasonia Group
Pteromalini (polyphyletic)
Pteromalinae (polyphyletic)

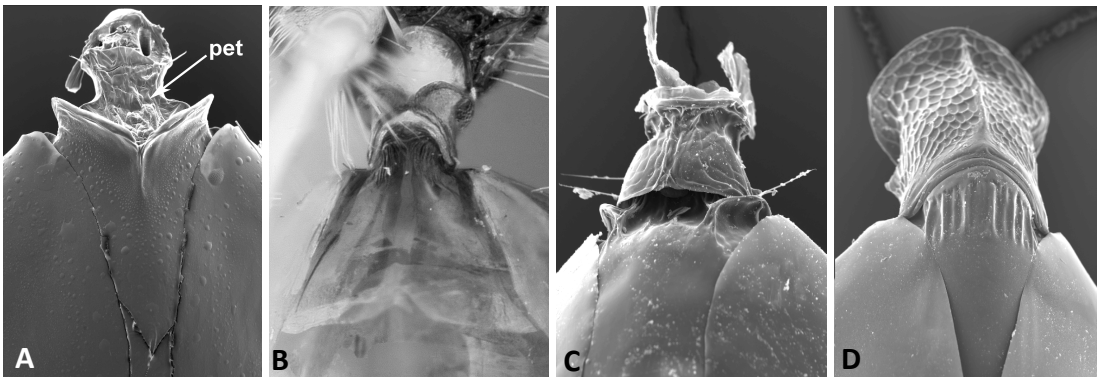


Fig. 4. Ventral surface of the petiole (pet) in selected Pteromalinae *sensu lato*. A. *Pteromalus* (Pteromalini). B. *Diconocara petiolata* (Miscogasterini). C. *Sphaeripalpus* sp. (Miscogasterini). D. *Halticoptera* sp. (Sphegigasterini).

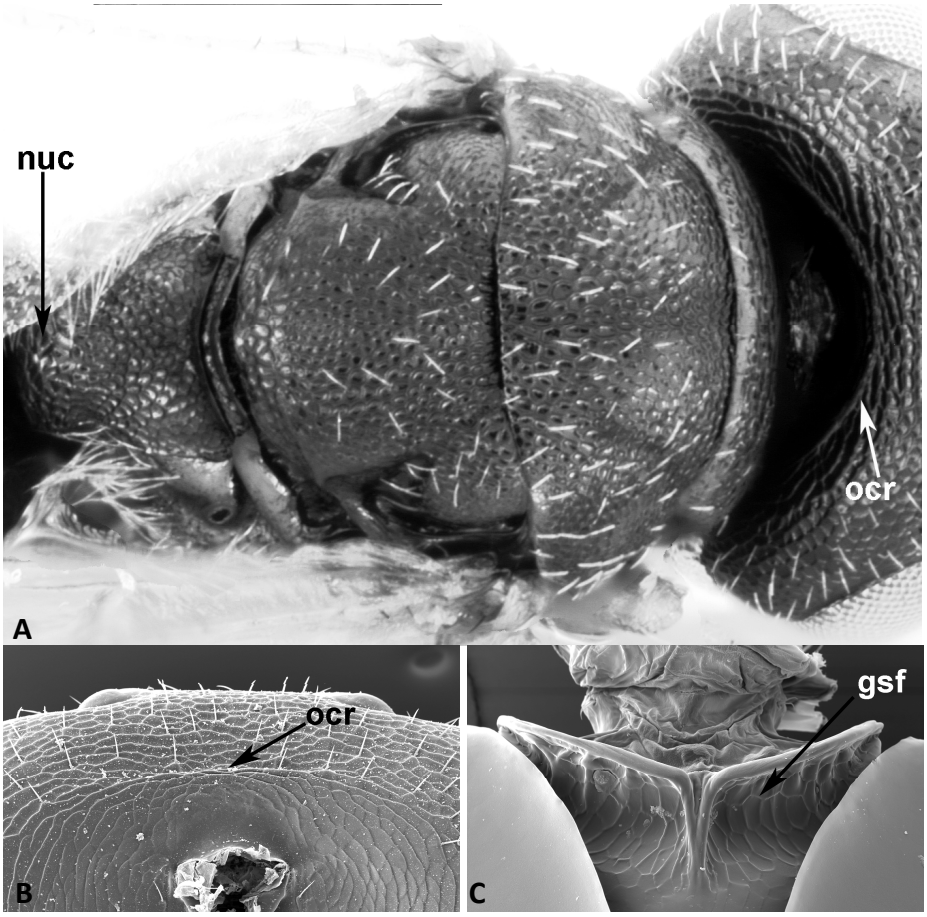


Fig. 5. Characters of the *Nasonia* Group. A. *Trichomalopsis tachinae* (Gahan), ocr = occipital ridge, nuc = propodeal nucha. B-C. *Nasonia girault*i, ocr = occipital ridge, gsf = ventral flange of first gastral sternite.

CHAPTER 3

Combined molecular and morphological phylogeny of Eulophidae (Hymenoptera: Chalcidoidea), with focus on the subfamily Entedoninae

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Abstract. A new combined molecular and morphological phylogeny of the Eulophidae is presented with special reference to the subfamily Entedoninae. We examined the 28S D2-D5 and CO1 gene regions in partitioned maximum likelihood and Bayesian analyses, and examined the effects of the addition of historically recognized morphological characters on the Bayesian analysis. Eulophidae was strongly supported as monophyletic only with exclusion of the enigmatic genus *Trisecodes*. The subfamilies Eulophinae, Entiinae (=Euderinae) and Tetrastichinae were consistently supported as monophyletic, but monophyly of Entedoninae was supported only in the combined analysis. The lack of support from the molecular data was due to the unusual 3e´ subregion of the 28S D2 rDNA in the nominal subgenus of *Closterocerus*. In all cases Euderomphalini was excluded from a monophyletic Entiinae, and we suggest that it be retained in Entedoninae. Opheliminae **n. stat.** is raised from tribe to subfamily status, and a sister

group relationship of Opheliminae + Entiinae is strongly supported. The genera *Neochrysocharis* **n. stat.** and *Asecodes* **n. stat.** are removed from synonymy with *Closterocerus* because strong molecular differences corroborate their morphological differences. *Closterocerus* (*Achrysocharis*) *germanicus* is transferred to the genus *Chrysonotomyia* **n. comb.** based on molecular and morphological characters.

Keywords. Molecular phylogenetics, parasitoids, ribosomal, COI.

Introduction

Eulophidae is one of the largest families of Chalcidoidea, with over 4,400 described species in 4 subfamilies (Noyes 2003). It is also one of the most diverse and economically important families in Chalcidoidea, with a large number of species important for biological control of agricultural pests, especially of leaf-mining Diptera (Clausen 1978). A number of other species are gall formers on a variety of plants, including *Eucalyptus* (Bouček 1988). However, their hosts are not limited to plants and insects. The diversity in life history strategies of eulophids is comparable to that of Chalcidoidea itself, with several unusual or unique examples. Some species are predators in spider egg sacs or in galls of mites or nematodes (LaSalle 1994). Although many eulophid genera are distributed worldwide, other potentially ancient subgroups of the family are Australasian (Bouček 1988).

Eulophidae and its major subgroups cannot be characterized succinctly in terms of natural history. The family is defined by a combination of characters that is not unique

within Chalcidoidea. All eulophids have twelve or fewer antennal segments, a small and straight protibial spur, and four or fewer tarsal segments, while most have a relatively long marginal vein (Gibson *et al.* 1999). The unplaced chalcidoid genus *Cales* Howard possesses all the above features, but like Trichogrammatidae is distinguished from Eulophidae in having a broad petiole allowing the mesophragma to extend into the metasoma (Burks 2003).

Although the defining characters of Eulophidae have come under suspicion because they are the apparent result of reduction from common groundplan chalcidoid states (LaSalle *et al.* 1997, Gauthier *et. al.* 2000), a core monophyletic group of eulophids is strongly supported in most molecular analyses (Campbell *et al.* 2000, Gauthier *et al.* 2000, Munro *et al.* unpublished). The more controversial issues remaining in eulophid taxonomy involve definition of its subfamilies and genera.

The subfamily Eulophinae has historically contained a diverse set of potentially ancient tribes in addition to the more characteristic genera near *Eulophus* Geoffroy (Bouček 1988). Gauthier *et al.* (2000) removed the primarily Australasian tribes Anselmellini, Keryini and Ophelimini from the subfamily based on 28S D2 molecular data and morphological differences. They transferred Keryini to Pteromalidae because of its gestalt morphological similarity to the mostly phytophagous subfamily Ormocerinae. Anselmellini and Ophelimini were left as *incertae sedis* in Eulophidae because no clear indication of their relationships was supported by the molecular data. They also transferred the genus *Elasmus* Westwood into Eulophinae as the sole member of the tribe Elasmini. Finally, Gauthier *et al* (2000) erected the tribe Cirrospilini for a set of

morphologically reduced genera usually placed within Eulophinae, but synonymized the previously recognized tribes Elachertini and Euplectrini under Eulophini.

Tetrastichinae is potentially the most diverse subfamily of Eulophidae in terms of species and life history traits (LaSalle 1994), but is notoriously not diverse morphologically and contain a large number of morphologically similar genera (Schauff *et al.* 1997). Ironically, the subfamily also lacks uniquely defining characters (LaSalle 1994), although it has been strongly supported molecularly (Gauthier *et al.* 2000, Munro *et al.* unpublished).

Entiinae (formerly Euderinae; Hansson 2009a) possesses a number of potentially plesiomorphic characters, such as a separate 9th metasomal tergite and complete notauli for most species (Coote 1994). Gumovsky (2002) transferred the tribe Euderomphalini into this subfamily based on a new interpretation of the morphology of its species. See below for a discussion of this interpretation.

Entedoninae contains two tribes, Entedonini and Euderomphalini. Entedonini was revised and redefined by Schauff (1991), while Euderomphalini was revised by LaSalle & Schauff (1994) and Hansson & LaSalle (2002). Although species of the tribe Entedonini are highly diverse in life histories, all host records of Euderomphalini indicate that they are parasitoids of whitefly. The tribe Platytetracampini was described in this subfamily by Bouček (1988) but was removed by Gauthier *et al.* (2000) because 28S D2 data placed the tribe near Anselmellini. While Entedonini is usually characterized as having only one pair of scutellar setae and a single dorsal submarginal vein seta (Schauff 1991, Schauff *et al.* 1997), this definition does not hold true across the tribe. Some

species in several different genera that clearly belong to Entedonini have several setae in one of these locations, and a few even have several setae in both locations. Additional characters provided by LaSalle & Schauff (1994) and further discussed by Gibson *et al.* (1999), such as pores on the male scape restricted to a ridge along the ventral edge, mesoscutal midlobe with two pairs of bristles, transverse facial sutures separated from the median ocellus, and tubercle present behind the propodeal spiracle, do not occur in all Entedonini either. Gumovsky (2002) proposed a new character for the definition of Entedonini, mentioning that the mesothoracic spiracle is hidden in all species of that tribe, but this feature is not found in the controversially placed Euderomphalini. Additionally, this character is present in various forms throughout a number of other chalcidoids, although it may be locally informative within Eulophidae.

The problem of imprecise morphological definitions applies to all four currently recognized subfamilies of Eulophidae and to most current tribes within these subfamilies (Burks 2003). It is therefore difficult to decide in which subfamily the more problematic groups such as the tribes Anselmellini, Euderomphalini, Ophelimini and Platytetracampini could belong. This suggests that molecular data could be helpful in determining the position of these groups, although for some of these groups the broader context of a phylogeny of Chalcidoidea will be needed (Munro *et al.* unpublished, Heraty *et al.* in prep.).

Further controversy exists in the generic classification within the tribe Entedonini. There is no agreement among specialists upon the generic classification of members of the tribe. This problem is most pronounced in the classification of small-bodied genera,

such as *Closterocerus* Westwood, *Neochrysocharis* Kurdjumov, and similar genera, with every expert in recent literature either using a different classification or expressing reservations about the one being used (Hansson 1990, 1994, 2002, Gumovsky 2001, 2002, Fisher & LaSalle 2005).

The disagreement over classification of entedonine genera focuses upon a debate over the reliability of certain morphological characters. Hansson (1990, 1994) discovered that the shape of the basiconic peg sensilla of the antennal flagellum differed among species that he reclassified accordingly from the genus *Chrysonotomyia* Ashmead to *Closterocerus* and *Neochrysocharis*. Most of the species transferred to *Closterocerus* were placed in the subgenus *Achrysocharis* Girault, while those with a carinate pedicel were retained in the nominal subgenus (discussed from here on as *Closterocerus sensu strictu*). Gumovsky (2001) suggested a different classification based upon delimitation of the clypeus and the presence of subtorular grooves, thus synonymizing *Neochrysocharis*, *Asecodes* Förster, and a number of other genera under *Closterocerus*. Gumovsky did not make reference to Hansson's antennal characters. Hansson (2004) later suggested a different definition of *Chrysonotomyia*, combining all species with a single set of volsellar spines on the male genitalia into that genus, but acknowledged subtorular grooves as a valid character. Some species of the subgenus *Closterocerus* (*Achrysocharis*) were reclassified into *Chrysonotomyia* based on the newly discovered genitalic character (Hansson 2004).

A common thread in this controversy is that the groups have been defined largely without a phylogenetic context, using only a small number of characters without

complete comparison across other potentially related genera. This approach especially ignores the concept of evolutionary polarity. While certain species may be similar in having a delimited clypeus for instance, it is quite possible that primitive entedonines also had a well-defined clypeus, such that its *lack*, not its presence, could instead be an informative character at some level. This approach also ignores the possibility of reversals or parallel evolution. There are no guarantees that every character has been derived only once within the lineage, and this possibility can adequately addressed only in a phylogenetic context.

The last published morphological phylogeny of Entedoninae was by Schauff (1991), but that study did not include the tribe Euderomphalini or several other genera of Entedonini from outside the Holarctic region. The molecular phylogenies of Eulophidae (Gauthier *et al.* 2000) and Entedoninae (Gumovsky 2002) did not, by the authors' own admission, make convincing statements on the classification of genera within Entedonini. The molecular analysis of Gumovsky (2002) focused more on placement of the tribe Euderomphalini. He concluded that euderomphalines should be transferred from Entedoninae to Entiinae based on 28S D2 ribosomal sequences, distinctness of the clypeus, and presence of apparently complete notauli. Again, this approach to morphological interpretation ignores the concept of character polarity. The presence of distinct notauli is certainly plesiomorphic throughout Chalcidoidea, again meaning that their loss, not their presence, could be informative at some level.

The taxonomic instability of Entedoninae has led to a problem in which it is not clear which classification to follow because there is no clear reason to prefer one

interpretation of generic characters over the other. Further, all previous phylogenies of Eulophidae and its subfamilies (Schauff 1991, Gauthier *et al.* 2000, Gumovsky 2002) used only parsimony as an optimality criterion. Gauthier *et al.* (2000) found strong bootstrap support for only Ophelimini, and only weak bootstrap support for Eulophinae and Tetrastichinae in their phylogeny of the family. Although they sampled a relatively large number of Entedoninae, and Entedonini + Euderomphalini formed a monophyletic group in their analysis, they found no bootstrap support for monophyly of the subfamily. There was also no strong bootstrap support for monophyly of any entedonine genus.

A more definitive classification of Eulophidae addressing available information would provide not only a more stable and informative classification, but a better framework for all aspects of research of family. The focus of this study is to provide additional molecular data using more recent analytical methods in an attempt to provide a more strongly supported phylogeny of Eulophidae, with the goal of providing a stronger, less equivocal, statement regarding the many controversies of eulophid classification.

Materials and Methods

A broad range of eulophids were chosen for this analysis based on morphological diversity and specimen availability (Table 1). Outgroups from three different families were chosen. Tetracampinae was chosen as an outgroup because of morphological (Bouček 1988) and molecular (Campbell *et al.* 2000, Munro *et al.* unpublished) proximity to Eulophidae. *Colotrechnus* Thomson and *Ceratogramma* De Santis were chosen as additional outgroups because of morphological similarity to Eulophidae. While

Rotoitidae, *Cales* Howard (Calesinae; family unplaced) and the pteromalid species *Idioporus affinis* LaSalle & Polaszek also possess four tarsomeres and a small protibial spur, they were excluded from this analysis because the 28S sequences for *Chiloe micropteron* Gibson & Huber, *Cales* and *I. affinis* were very different from those of eulophids (Munro *et al.* unpublished).

Most specimens were killed in 95% EtOH and stored at -80°F until extraction. The entire body was used for extraction using the chelex method (Walsh *et al.* 1991) in a non-destructive means in which the body was not macerated but removed from the Proteinase-K after a short time and cleaned for use as a primary voucher. DNA extracts were stored at -80°F until needed. All vouchers are stored at the University of California, Riverside Entomology Research Museum (UCRC). Table 1 lists the specimens used, their classification, general locality, UCRC voucher numbers, Genbank accession numbers of all sequences, and Morphbank image numbers for voucher specimens.

Polymerase chain reactions were carried out in 20µl reactions using Promega Taq DNA polymerase (Madison, WI), Qiagen 10x PCR buffer (15 mM $MgCl_2$) and Qiagen 5x Q-solution (Valencia, CA). All genes were sequenced in the forward and reverse directions, and the resulting pair of chromatograms compared to find PCR or reading errors. Primers and annealing temperatures are given in Table 2. PCR products were gene cleaned using the Bio 101 Geneclean Kit (Carlsbad, CA) with NaI and glassmilk. Cleaned samples were directly sequenced at either the San Diego State Microchemical Core Facility or the UC Riverside Genomics Center.

Ribosomal sequences were aligned using the secondary structure model from Gillespie *et al*. (2005) with regions of ambiguous alignment (RAA) aligned by eye. RAA's were retained in the analysis because they improved resolution of the ingroup. Mitochondrial sequences translated to valid amino acids and did not possess any gaps. Molecular data were partitioned by gene region, with 28S D2 and D3–D5 as separate partitions and CO1 partitioned by codon position. Maximum percent divergence values (uncorrected 'p') were calculated using PAUP* 4.0b10 (Swofford 2000).

Maximum likelihood bootstrap analysis was performed using RAxMLHPC 7.0.3 (Stamatakis 2006) using the GTRMIX model. An initial rearrangement setting of 10 (i = 10) and the default number of categories (c = 25) were used after completing the multistep process described in sections 5.2.1 and 5.2.2 of the program manual to determine the best values for those settings. The Best Known Likelihood (BKL) tree for each analysis was selected from a run of 200 inferences. One thousand bootstrap replicates were performed using the standard bootstrap method, each with a random starting tree using the random number seed 91583.

Bayesian analysis was performed using MrBayes 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). For each molecular partition a six parameter model with rates subject to a gamma distribution with a proportion of invariant sites (nst=6 rates=invgamma) was used as suggested by hierarchical likelihood ratio tests performed using MrModeltest (Nylander 2004). The morphological partition in the combined analysis was analyzed as unordered using the Mk model (Lewis 2001) with coding=variable and rates=gamma. In each analysis two independent simulations of four

simultaneous MCMC chains were ran, sampling every 1000 generations. Convergence was reached at 5 million generations in each analysis with <0.01 standard deviation of split frequencies. The burnin was 1.25 million generations for each analysis.

Morphological characters

The morphological component of this study includes 31 characters discussed in the text. It is meant to be a summary of the characters discussed here in a way that permits discussion in a defined context without providing undue bias in the combined analysis. This is in part because the molecular data are meant to be a test of the characters discussed here. A more definitive morphological analysis would require more characters and the inclusion of known genera and species groups, which is beyond the scope of this study. Terms follow those of Gibson (1997). Photographs were taken using either Auto-Montage software (Synoptics, Ltd., UK) or the EntoVision Mobile Imaging System (GT Vision LLC).

1. **Number of flagellomeres**: Coded using actual number or a letter substitute from **6** to **C** (=12).

The small terminal flagellomere found in some families of Chalcidoidea, including Pteromalidae, is here interpreted as a single segment as in Onagbola & Fadamiro (2008). The apparent maximum number of flagellomeres for Chalcidoidea is 14, based on the number found in Rotoitidae (Bouček & Noyes 1987) and in some other chalcidoid taxa, including *Colotrechnus* in this study. Eulophids have at most 10 flagellomeres, with a

variable number in all subfamilies except Entiinae, which have a constant number of 8. This character can become problematic when claval segments are fused or when there are several basal anelliform segments that are difficult to distinguish. In the case of the club, fused segments are not counted as if they were not fused. Anelliform segments were counted using slide-mounted specimens in species where the count could be problematic.

2. **Number of separate claval segments in females**: Coded using the actual number, from **1** to **4**.

The club is interpreted as the apical set of approximated flagellomeres, and therefore consists of at least one segment unless the flagellum is entirely absent. This character was chosen to best represent the difference in flagellar form between taxa such as *Closterocerus*, which have two funicular segments and three flagellomeres (Fig. 13), and from other Entedonini with three or more funicular segments and a correspondingly reduced number of claval segments (Fig. 5). Only females were used for this count because the number can vary between sexes in a pattern that is sometimes valuable for species distinction but is not informative across genera.

3. **Shape of flagellomeres in males**: **0** = cylindrical and without branches (Fig. 5: flg); **1** = nodose, with a rounded expanded section bearing elongate setae (Fig. 2); **2** = bearing two or three branches (Figs 6, 7); **3** = cylindrical apically but with a slight basal expansion (Fig. 8).

The form of the flagellum in males is variously modified in many groups of eulophids, although there are exceptional species with a cylindrical flagellum (3:0) in each of these groups (Bouček 1988, Burks 2003). Strongly nodose flagellomeres (3:1), with the expanded section bearing elongate setae, are found in males of most species of Entiinae (Fig. 2). Males of many species of Tetrastichinae have flagellomeres with a similar, always basal, expansion bearing elongate setae (3:2, Fig. 8). However, in these species the flagellomeres are more cylindrical, with a longer apical section and less distinct expansion. Rather than lump these two states occurring across subfamilies into the same state, it seems best to recognize the differences between them as potentially phylogenetically significant (3:1, 3:2).

Flagellar branches are found in males of *Elasmus* Westwood and in some genera of Eulophini. Three branches is the usual state in eulophines with branched flagellomeres (Fig. 6), but males of *Dicladocerus* Westwood have only two (Fig. 7). These conditions were lumped together as a single state (3.2) because separating *Dicladocerus* into a different state would needlessly create an autapomorphy in the analysis, and there appears to be no outstanding morphological difference in the morphology of the separate flagellomeres.

4. **Shape of basiconic peg sensilla of the flagellum**: **0** = symmetrical (Fig. 9); **1** = lightly asymmetrical, angular (Fig. 10); **2** = strongly asymmetrical, spear-shaped (Fig. 11). Hansson (1990, 1994, 1996) described variation in shape of the socketed, typically mushroom-shaped, basiconic peg sensilla between genera of Entedoninae and between

species groups of *Omphale* Haliday. Variation in this character is one principal reason for recognition of *Closterocerus* and *Neochrysocharis* as separate genera. It is possible that the spear-shaped form in state 2 (Fig. 11) could be derived exclusively from the slightly asymmetrical form in state 1 (Fig. 10), but there is no conclusive proof of this.

5. **Carinae of pedicel**: **0** = absent (Fig. 12: pdl); **1** = present (Fig. 13: carina).

The pedicel in all species of the nominal subgenus of *Closterocerus* is carinate (Fig. 13) along its dorsal and ventral edges (Hansson 1994). This state does not occur in the other subgenus, *Achrysocharis* Girault.

6. **Sulcus across vertex between median and lateral ocelli**: **0** = absent (Fig. 14); **1** = present (Fig. 15: sulcus).

Some genera of Euderomphalini possess a sulcus extending across the occipital triangle between the median and lateral ocelli (Hansson & LaSalle 2002). This sulcus is interpreted as different from the transverse facial sulci of most Entedonini, but may be more similar to the sulcus crossing the vertex behind the ocelli in *Ceranisus* Walker and other thrips parasitoids in Entedonini (Schauff 1991). The vertex sulcus in *Ceranisus* was coded as absent in this analysis because it would be autapomorphic among the included taxa. If additional species with this sulcus were included, that form of the sulcus would have been coded as a separate state in this character. It is not the same as the sulcus found in Euderomphalini (6:1) because it occurs posterior to all the ocelli.

7. **Vertex posterior carina**: **0** = absent (Fig. 14); **1** = present (Fig. 16: carina).

A sharply edged transition between the vertex and the occiput is found in many species across Eulophidae, but is important in separating the genus *Horismenus* Walker (absent) from *Pediobius* Walker (present) (Hansson 2002).

8. **Transverse facial sulcus**: **0** = absent; **1** = present and adjacent the median ocellus (Fig. 17: tfs); **2** = separated from the median ocellus by at least the diameter of the median ocellus (Fig. 18: tfs).

This character is a modified version of a previous interpretation of the transverse facial sulcus in Entedonini by LaSalle & Schauff (1994) that used the distance between the median ocellus and toruli as a point of comparison. It also incorporates, as state 1, a character introduced by Gauthier *et al.* (2000) as a potential synapomorphy of Entiinae (Fig. 17). The previous interpretation of the entedonine state is problematic because entedonine species in *Chrysonotomyia* and *Emersonella* Girault, among many others, have a transverse facial sulcus near the median ocellus that is apparently homologous to the more V-shaped sulcus found in most Entedonini (Fig. 18). The entedonine sulcus is separated from the median ocellus by a greater amount than found in most Entiinae, and therefore is interpreted as being different (8:2). Unfortunately, state 1 is not unique to Entiinae, as it is also found in some Euderomphalini. Probably the best interpretation of this variation is that while state 2 may be differently derived in different taxa, there are no known means of testing this possibility. Therefore, transverse facial sulci are probably most informative when compared between species of the same genus, and decrease in

value when compared at higher taxonomic levels unless some structural feature can be found to test the homology of sulci in different taxa.

9. **Subtorular grooves**: **0** = absent; **1** = present, extending from ventral edge of torulus (Fig. 18: stg); **2** = present, extending from lateral edge of torulus (Fig. 19: stg).

This character was described by Gumovsky (2001) as a reason for combining *Closterocerus*, *Neochrysocharis*, *Asecodes* and a number of other genera. These genera were then interpreted as different from *Chrysonotomyia* because the latter has a distinctly defined clypeus. It was later acknowledged by Hansson (2004) as a valid means of defining *Chrysonotomyia*, in combination with several other characters. The grooves found in Tetrastichinae (Fig. 19: stg) and Trichogrammatidae (9:2) are here interpreted as different because they contact the torulus near its lateral edge and expand dorsally instead of ending as simple grooves.

10. **Delimitation of clypeus**: **0** = delimited at least by lateral grooves (Figs 20, 21: cly); **1** = not delimited (Fig. 18).

Delimitation of the clypeus has been historically used to separate *Chrysonotomyia*, *Omphale*, *Parzaommomyia*, and some other genera of Entedonini from those without a delimited clypeus (Graham 1959, Bouček 1988, Hansson 1994, Hansson 1990, Gumovsky 2001, Hansson 2004). However, this character is problematic because the clypeus is distinct in some species not included in these genera and indistinct in some species found within these genera (Hansson 1996, Burks 2003). This discrepancy may be

due to its being interpreted inconsistently with respect to preconceived notions regarding each of these genera.

11. **Clypeus width**: **0** = not enlarged, width about equal to height (Fig. 21: cly); **1** enlarged, over 1.5 times as broad as high (Fig. 20: cly).

Most species of *Omphale* have a broadened clypeus (Fig. 21: cly), but in some species the clypeus is either not indicated or not broadened. While state 1 cannot serve as a synapomorphy for the entire genus (Hansson 1996), it does provide a link for several species groups. Variation in clypeal form is rare among other genera of Entedonini, but it does also occur in the genus *Clypecharis* Gumovsky and *Clypomphale* Bouček.

12. **Pronotal collar carina**: **0** = absent (Figs 23-27, 33); **1** = present (Fig. 16: prc).

An anterior carina extending transversely across the pronotal collar is present on the pronotal collar in a number of different eulophids. While this character may be homoplastic at the family level, it is locally informative for distinguishing some genera, such as *Pediobius* versus *Paracrias* Ashmead (Hansson 2002).

13. **Semicircular ridge of pronotum laterally**: **0** = absent; **1** = present (Fig. 22: carina).

State 1 (Fig. 22) was described by Gumovsky (2001) as a possible synapomorphy of *Achrysocharoides* Girault and *Entedon* Dalman. Although a similar ridge is found in some species of *Chrysocharis* (Burks 2003), this character is coded as specified by Gumovsky to use it in the conventional way.

14. **External completeness of notauli posteriorly**: **0** = reaching transscutal articulation (Figs 23, 26-27); **1** = not reaching transscutal articulation, essentially absent (Figs 24-25, 28, 33).

This character varies across all subfamilies of Eulophidae except for Entiinae and Tetrastichinae. While Krogmann & Vilhelmsen (2006) have shown that external incompleteness does not necessarily indicate internal incompleteness, the character is interpreted here in keeping with previous literature (Graham 1959, Bouček 1988). It can be problematic in cases where the external indication of the notauli ends as a gradually less defined groove. In those cases, if the groove could at all be traced to the transscutal articulation, it is interpreted as complete. Also, cases where the notauli end at a strongly advanced axilla instead of extending to the scutellar disc are not distinguished here because there is a smooth continuum between those two conditions among eulophids. The notauli are incomplete anteriorly (Fig. 23) in *Hubbardiella* Ashmead (Coote 1994), but this is an autapomorphy for the genus.

15. **Pairs of mesoscutal midlobe bristles**: Coded using the actual number, from **0** to **3** except that **3** includes counts of 3 or greater.

This character was used by Schauff (1991) as a potential means of defining Entedonini. However, it varies within Entedonini in a way that is often useful in distinguishing genera and species groups. The distinction between bristles and setae can sometimes be problematic, but in this analysis no distinction is made between them.

16. **Advancement of axillae**: **0** = not entirely advanced beyond anterior margin of scutellar disc (Figs 33: ax, 23-24, 26-28); **1 =** entirely advanced beyond anterior margin of scutellar disc (Fig. 25: ax).

The axillae are advanced entirely beyond the scutellar disc (Fig. 24) in some genera of Euderomphalini (LaSalle & Schauff 1994), and so strongly that they have been mistaken for the sidelobes of the mesoscutum (Gumovsky 2002). They are similarly advanced in the outgroup taxa *Ceratogramma* and *Colotrechnus*, but not in other eulophids.

17. **Pairs of scutellar disc setae**: Coded using the actual number, from **1** to **3** except that **3** includes counts of 3 or greater.

This presence of only one pair of setae on the scutellar disc has been used to help define Entedonini (Schauff 1991), although some species have additional setae. The character remains useful, however, because there are very few exceptions within each tribe of eulophids. As in character 15, no distinction is made between bristles and setae.

18. **Scutellar grooves**: **0** = absent (Figs 23-24, 28, 33); **1** = present as a U-shaped groove open anteriorly (Fig. 26: scg); **2** = present as parallel grooves open both anteriorly and posteriorly (Fig. 27: scg).

The scutellar grooves of all eulophids are here considered homologous. No eulophid subfamily or tribe is constant for either state 1 or 2, but Entiinae and

Euderomphalini all lack scutellar grooves. While some Eulophini characteristically have a U-shaped groove (Peck *et al.* 1964), this state also occurs in some Entedonini (Schauff 1991). Other Eulophini have parallel grooves instead. These are not the same as axillular grooves, which co-occur with the scutellar grooves in eulophids.

19. **Pit along scuto-scutellar sulcus between axilla and scutellar disc**: **0** = absent (Figs 24, 26, 33); **1** = present (Fig. 28: pit).

This characteristic pit is found in *Horismenus* (Hansson 2002) and *Podkova* Gumovsky. It is apparently unique to these genera among eulophids.

20. **Propleura**: **0** = posterior margins diverging angularly along prosternum (Fig. 29: ppl); **1** = posterior margins transverse, diverging at right angles at prosternum (Figs 30: ppl, 31).

State 1 was used by Gauthier *et al.* (2000) as a potential synapomorphy of Eulophini minus *Dicladocerus*. It also occurs in some species of *Elasmus*, which has a continuous grade of variation between the two states. State 1 is not found in any other eulophids but does occur in other families of Chalcidoidea such as Pteromalidae.

21. **Mesepisternal projection over posterior margin of prepectus**: **0** = absent; **1** = present (Fig. 31: mep).

A narrow lobe-like projection from the mesepisternum extending anteriorly to slightly overlap the posterior margin of the prepectus was described by Schauff (1991) as

a synapomorphy of *Horismenus* (Fig. 31). It has since been found in some other entedonine genera, including *Pediobius* (Hansson 2002).

22. **Expansion of mesepimeron over metapleuron**: **0** = mesepimeron not expanded over metapleuron (Fig. 31); **1** = mesepimeron expanded, overlapping metapleuron (Fig. 32: mse).

The mesepimeron is strongly expanded in some genera of Entiinae, becoming convex laterally and overlapping the metapleuron, hiding most of its surface from view (Fig. 32). In other eulophids the mesepimeron is flat and either abuts or only slightly overlaps the edge of the metapleuron (Fig. 31).

23. **Median carina of propodeum**: **0** = not flattened dorsally (Fig. 26); **1** = flattened dorsally (Figs 33: mc, 28).

The median propodeal carina of most species of *Horismenus* (Fig. 28) and *Paracrias* (Fig. 33: mc) is broadly flattened along its length and may also project to the metapleuron (Schauff 1991). This character also occurs in some species of *Pediobius* (Hansson 2002).

24. **Setae of propodeal disc**: **0** = not curving mesad; **1 =** curving mesad (Fig. 34).

The curvature of the setae along the lateral surface of the propodeal disc, not including the propodeal callus setae, curve mesad in all Tetracampinae (Peck *et al.* 1964, Bouček 1988) and is a likely synapomorphy of the subfamily. This character does not

occur in Eulophidae, where most species have an entirely bare propodeal disc (Figs 26, 28, 33). Most Chalcidoidea lack setae in this area, and absence or a few straight setae is plesiomorphic.

25. **Number of tarsomeres in fore leg**: Coded using the actual number, from **3** to **5**.

Rotoitidae, almost all Eulophidae, and a few species of Aphelinidae, Pteromalidae and male Agaonidae have four tarsomeres for all legs. *Trisecodes agromyzae* Delvare & LaSalle is the only exceptional eulophid, having only three. Among the outgroup taxa, *Colotrechnus* has five and *Ceratogramma* has three.

26. **Protibial spur**: **0** = stout and curved (Fig. 35: pts); **1** = slender and straight (Fig. 36: pts).

The presence of a reduced protibial spur has historically been used to help define Eulophidae (Peck *et al.* 1964, Bouček 1988), but also occurs in Tetracampidae, Trichogrammatidae, and arguably in some other families of Chalcidoidea (LaSalle *et al.* 1997). Although variation exists in this character within Eulophidae and other families, it does not vary among the taxa included in this analysis and is here interpreted in its more conventional sense.

27. **Number of tarsomeres in mid leg compared to fore leg**: **0** = same; **1** = one less.

Male Tetracampinae have four tarsomeres for the mid leg in males, but five for the other legs (Peck *et al.* 1964, Bouček 1988). This may be a synapomorphy for the subfamily.

28. **Submarginal vein setae**: Coded using the actual number, from **1** to **3** except that **3** includes counts of 3 or greater.

Most Entedonini have only two submarginal vein setae, and this character has been used as a potential synapomorphy of Entedonini (Schauff 1991). Although it varies in other eulophids, most notably in Tetrastichinae, it remains a useful character.

29. **Postmarginal vein length**: **0** = more than half stigmal vein length (Fig. 37: pmv); **1** = half or less stigmal vein length (Fig. 38: pmv).

The postmarginal vein in most species of Tetrastichinae is extremely short or absent (LaSalle 1994). While this character also occurs in some species of Entedoninae and varies within Tetrastichinae, it remains a convenient character for Tetrastichinae in the absence of any known universal diagnostic characters for the subfamily (Gibson *et al*. 1999).

30. **Epipygium (Mt9) in females**: **0** = separate from Mt8 (Fig. 39: Mt9); **1** = fused with Mt8, forming a syntergum (Fig. 40: Mt8+9).

All Entiinae except *Beornia* Hedqvist and *Hubbardiella* have a separate Mt9 in females (Coote 1994). This character does not occur in any other eulophids, although it is present in some other Chalcidoidea.

31. **Number of volsellar digital spines**: Coded using the actual number, from **1** to **2** (Figs 41, 42: vds).

Most eulophids have a pair of spines (Fig. 41: vds) as in the outgroup taxa. A single spine is present on each volsellar digitus (Fig. 42: vds) in *Chrysonotomyia* Hansson (2004).

Results

Initial molecular analyses. The most optimum tree from the RAxML analysis had a log likelihood score of -17,392.54 (Fig. 43). There was 100% bootstrap support for Eulophidae minus *Trisecodes agromyzae*, the only eulophid with three tarsomeres. Although *T. agromyzae* placed as the sister group to the rest of Eulophidae, there was no bootstrap support for this placement. Among sampled subfamilies, there was 100% bootstrap support for monophyly of the eulophid subfamily Tetrastichinae and the outgroup subfamily Tetracampinae (Tetracampidae). Eulophinae was weakly (85%) supported as monophyletic, but there was 100% support for monophyly of its tribes Cirrospilini and Eulophini. The clade of Tetrastichinae + Eulophinae was strongly (96%) supported as sister group to remaining Eulophidae. With *Colotrechnus ignotus* as the

designated outgroup, Tetracampinae + *Ceratogramma* formed the sister group to Eulophidae including *T. agromyzae*.

The clade containing Entedoninae, Entiinae, and *Ophelimus maskelli* received 100% bootstrap support. However, Entedoninae was not monophyletic because *Closterocerus sensu strictu* placed as sister group to all other members of this clade. Entiinae was monophyletic, but without bootstrap support. There was 100% bootstrap support for the clade of *Ophelimus maskelli* + Entiinae. *Bellerus* was placed between the strongly supported clades of *Astichus* and *Beornia* + *Euderus* + *Hubbardiella*.

Within Entedoninae, Euderomphalini was paraphyletic with respect to all remaining Entedonini because *Neopomphale* was placed as sister group to the remaining Euderomphalini + Entedonini. There was little bootstrap support for relationships within Entedoninae, but the clades of *Chrysonotomyia* + *Closterocerus germanicus* and *Omphale* + *Parzaommomyia* each received 100% bootstrap support. There was a 100% supported clade of Entedonini minus *Chrysonotomyia*, *Closterocerus sensu strictu*, *Omphale*, *Parzaommomyia* and *Tropicharis*. Within this clade there was strong support for the clades *Pediobomyia* + *Rhynchentedon* (96%) and *Horismenus floridensis* + *Horismenus longicornis* (90%) and weak support (82%) for the clade of *Achrysocharoides* + *Entedon*. Among entedonines with more than one species sampled, only *Omphale* was monophyletic.

The Bayes analysis (Fig. 44) usually indicated stronger support for all clades shared with the ML analysis, but provided no convincing support (< 70% posterior probability) for monophyly of Eulophidae when *Trisecodes agromyzae* was included.

The two analyses agreed in most other respects, except that Bayes results placed *Neopomphale* within a monophyletic but poorly supported (63%) Euderomphalini, and in placement of different species within the same "core" Entedonini clade supported by the ML analysis. Some clades in Entedonini were supported by posterior probability values of above 80% that were not supported in the ML analysis, including a 99% supported clade of *Asecodes* + *Neochrysocharis* + *Pediobomyia* + *Pediobius* + *Rhynchentedon*, an 89% supported clade of *Horismenus* + *Paracrias*, and an 83% supported clade of *Astichomyiia* + *Ceranisus* + *Emersonella*.

Molecular analysis without the 28S D2 3e´ subregion. In an attempt to explain why the placement of *Closterocerus sensu strictu* differs so strongly between the molecular and morphological analyses, the sequence alignment was investigated. It was discovered in both *Closterocerus tau* and *C. trifasciatus* that the 3e´ subregion, consisting of six bases, was very different from that of other Entedonini. It also did not canonically pair with the 3e subregion (Fig. 45), which should be its reverse complement according to the secondary structure model. The 3e and 3e´ subregions do not canonically pair in several other eulophines, but with less extreme disparity as in *Closterocerus*. The 3e subregion of *Closterocerus s.s.* was identical to some Entedoninae and even Entiinae (Fig. 45). Most members of the tribe Entedonini are fixed for both subregions, but the 3e´ sequence for both species of *Closterocerus s.s.* more strongly resembles that of a euderomphaline or entiine by having a cytosine in the 5th position, by having only two adenines instead of three, and by having a thymine at the third position. Separate

molecular-only analyses were performed with the 3e´ subregion deleted from all taxa (Figs 46-47) to observe what affect this subregion had on the analysis.

The revised ML analysis (Fig. 46) differed from the previous one (Fig. 43) only in the placement of species within Entedoninae. *Closterocerus s.s.* and *Chrysonotomyia* formed a clade, as in the subsequent morphological analysis. *Neopomphale* is placed as sister group to the clade of remaining Euderomphalini plus *Tropicharis cecivora* from Entedonini. Entedonini and Euderomphalini were therefore still not monophyletic with respect to one another despite the more conventional placement of *Closterocerus* and *Neopomphale*. One unanticipated effect was that the clade of *Achrysocharoides* + *Entedon* moved to a shallower node to become the sister group of *Pleurotroppopsis*. *Neochrysocharis* and *Pediobius* continued to be associated in a paraphyletic assemblage with *Asecodes*, *Pediobomyia*, and *Rhynchentedon*, but the two species of *Neochrysocharis* moved closer together and closer to *Asecodes* while still rendering *Pediobius* paraphyletic.

The Bayes results without the 3e´subregion (Fig. 47) were very different, although they again placed *Closterocerus* s.s. with *Chrysonotomyia*. *Neopomphale* placed strangely, as the sister group to *Ophelimus* + Entiinae. The remainder of Euderomphalini rendered Entedonini paraphyletic, being placed as sister group to *Omphale* + *Parzaommomyia*. The clade of *Achrysocharoides* + *Entedon* remained at a deeper node in these results, as sister group to the other Entedonini that lack a well-defined clypeus (10:0). Despite the inconsistent placement of euderomphaline taxa in this analysis, most nodes had very strong support. The placement of euderomphalines in this analysis

implies that the 3e´ subregion contains important phylogenetic information for that group; that information appears to have been lost by deleting the subregion from the analysis.

Combined molecular and morphological analysis. A combined Bayes analysis (Figs 48-49) was performed including the 28S D2 3e´ subregion and 31 morphological characters (Table 3). The 3e´ subregion was retained despite the findings of the previous analysis because there is no reason to conclude that the subregion is a result of mistakes in sequencing, because it is found in both included species of *Closterocerus s.s.*. As well, the region was included as a test to determine the effect of morphological characters upon the entire molecular dataset, and especially to determine if morphological characters would move *Closterocerus s.s.* into Entedonini, while retaining *Tropicharis cecivora* in Entedonini and *Neopomphale* in Euderomphalini.

The combined results showed strong support for all subfamilies and tribes. Most significantly, there was 100% posterior probability support for a monophyletic Entedoninae, 99% for Entedonini, 94% for Euderomphalini and 98% for Entiinae. Monophyly of Eulophidae continued to be unsupported when *Trisecodes agromyzae* was included but strongly supported (100%) without it. The tree topology outside of Entedoninae was the same as in the molecular analysis except for two clades. In Cirrospilini, *Cirrospilus* formed a weakly supported (78%) clade with *Zagrammosoma* instead of with *Aulogymnus*. In Entiinae, *Bellerus* formed an unsupported clade with *Astichus* instead of placing between it and the remaining entiines.

All relationships within Euderomphalini received between 87-89% posterior probability support, with *Neopomphale* forming a clade with *Cabeza* and *Euderomphale* as sister group to a *Dasyomphale* + *Entedononecremnus* clade.

Tropicharis cecivora placed as sister group to all remaining Entedonini with *Closterocerus s.s* as sister group to the rest. *Chrysonotomyia* + *Omphale* + *Parzaommomyia* formed a poorly supported clade of taxa that all share a completely defined clypeus (character 10:0). However, this character remains homoplastic within Entedonini because of the placement of *T. cecivora*, which also possesses this state. The clade of *Achrysocharoides* + *Entedon* + *Pleurotroppopsis* is retained in this analysis as in the morphological analysis (Fig. 42), but was poorly supported (58%). *Asecodes* and *Neochrysocharis* formed a well-supported (93%) clade as sister group to an even better supported (100%) clade of *Pediobomyia* + *Pediobius* + *Rhynchentedon*. There was also strong (97%) support for monophyly of *Horismenus*. The clade of *Astichomyiia* + *Ceranisus* + *Emersonella* also receives stronger support here (91%) than in any previous analysis.

Discussion

Support for monophyly of Eulophidae. Maximum likelihood (ML) and Bayesian analyses agree on the higher classification of Eulophidae in all respects except for the classification of Entedoninae (Figs 43-44, 46-49). Monophyly of Eulophidae, excluding *Trisecodes agromyzae*, is strongly supported in all analyses. *Trisecodes* has only three tarsomeres instead of four, and while described as an entedonine, it was placed there with

some doubt because it bears no strong similarity to any other known entedonines (Delvare & LaSalle 2000). *Trisecodes* is unusual among entedonines in having three pairs of mesoscutal midlobe setae (15:3), three pairs of scutellar setae (17:3), and in having only one submarginal vein seta instead of two (28:1). It shares a V-shaped transverse facial sulcus (8:2) with other entedonines (as in Fig. 18). Results from an analysis across all chalcidoid families using 28S D2-D5 and 18S rDNA place *T. agromyzae* far outside of an otherwise monophyletic Eulophidae (Munro *et al.* unpublished), but do not consistently associate it with any other family. These results put family placement of *T. agromyzae* in doubt, but do not indicate a better placement for this monotypic genus. Regardless of family classification, there seems to be no justification for placing *T. agromyzae* in any current subfamily of Eulophidae, and it seems best to consider it as *incertae sedis* within Eulophidae, awaiting further information to better elucidate its placement.

***Ophelimus* and monophyly of Entiinae.** All molecular and combined analyses indicate a monophyletic Entiinae, but with support only in the Bayesian analyses. However, the clade of *Ophelimus* + Entiinae, a group not recognized by any previous author, was strongly supported in all molecular and combined analyses.

Ophelimus had previously been placed in Eulophinae along with a number of other genera in the tribe Ophelimini (Bouček 1988). Gauthier *et al.* (2000) removed most of the other genera to form the tribe Cirrospilini. This left only *Ophelimus* and *Australsecodes* Girault in a reduced Ophelimini that was then placed as *incertae sedis* within Eulophidae because it strongly differed from Eulophinae for 28S D2 data.

While it is tempting to transfer ophelimines to Entiinae to render it into a named subfamily that is strongly supported in all molecular analyses, this ignores the lack of known morphological similarity between entiines and ophelimines. Most importantly, ophelimines lack all three of the characters specified by Coote (1994) as helpful in recognizing Entiinae: a bare area under the fore wing marginal vein exposing ventral admarginal setae to view, scutellum overhanging the reduced and concave axillula, and the separated Mt9 (character 30: 0, Fig. 39). These characterize all Entiinae except *Beornia* and *Hubbardiella* (Fig. 40). While these three characters are neither unique to nor universally found in Entiinae, there have been no similarities found between ophelimines and entiines that are not also found in all other eulophid subfamilies. Combining the two would therefore result in a group that is more difficult to characterize morphologically than either of the currently separate groups. The only benefit of combining ophelimines and entiines into a single subfamily would be the ability to refer to the strongly supported clade of *Ophelimus* + Entiinae as a subfamily in the current molecular analyses. This seems to be a very minor gain compared to the drawbacks of producing a newly delimited subfamily that cannot currently be defined morphologically. For these reasons, it seems best to acknowledge the molecularly supported sister group relationship between ophelimines and entiines by recognizing them as equal in taxonomic rank—elevating Opheliminae to subfamily rank **new status** and retaining *Australsecodes* in Opheliminae until it can also be analyzed molecularly.

In the analyses by Gauthier *et al.* (2000) the Neotropical entiine genus *Bellerus* Walker placed outside Eulophidae, with either *Idioporus affinis* or *Kerya* Bouček. This

raised doubts as to its family and subfamily affinities. All molecular and combined analyses in the current study place it solidly within Entiinae, however. The only consistently supported clade within Entiinae was the grouping of *Beornia* + *Euderus* + *Hubbardiella*. Although *Beornia* and *Hubbardiella* are the only entiines with a fused Mt8+9 (character 30: 1, Fig. 40), *Hubbardiella* consistently placed with weak support as sister group to *Euderus* in all molecular and combined analyses.

Monophyly of Eulophinae. The reduced version of Eulophinae as defined by Gauthier *et al.* (2000) and the two sampled tribes Cirrospilini and Eulophini were at least weakly supported as monophyletic in all molecular and combined analyses. Gauthier *et al.* (2000) pointed out that all in Eulophini, except for the genera *Colpoclypeus* Lucchese and *Dicladocerus*, the propleura diverge at right angles upon reaching the prosternum (character 20: 1, Fig. 30). This raised some doubts concerning placement of the two exceptions. *Colpoclypeus* was not available for sequencing, but *Dicladocerus westwoodi* consistently placed within Eulophini in all molecular and combined analyses, between the clades *Eulophus* + *Pnigalio* and *Elachertus* + *Euplectrus*. This suggests that a reversal in propleural form has occurred at least once within Eulophini.

As an additional investigation, 28S D2-D5 sequence and morphological data for *Elasmus polistis* Burks were added to the dataset and the analyses redone to evaluate their placement within Eulophinae, as proposed by Gauthier *et al.* (2000). The results of these analyses (not shown) agreed with the analyses that did not include *E. polistis*, and placed *E. polistis* in Eulophinae in a weakly supported groupings with *Elachertus* and *Euplectrus.* However, boostrap support was poorer in ML analyses throughout the tree.

The *E. polistis* sequence is highly divergent, and forms a long branch that we reason causes a greater amount of disagreement between bootstrap replicates. Because *E. polistis* is not contained in either of the two subfamilies that were the primary focus of this study (Entedoninae and Entiinae), and because its inclusion reduced the quality of the overall results, *E. polistis* was not included in the final results. We suspect that an analysis containing a much larger and more diverse set of Eulophinae would resolve this problem.

Monophyly of Entedoninae. The only eulophid subfamily that was paraphyletic in any molecular analysis was Entedoninae, likewise its two tribes Entedonini and Euderomphalini were paraphyletic (Figs 43-44, 46-49) in all but the combined morphological and molecular analysis (Fig. 49). While the placement of *Trisecodes agromyzae* had been admittedly controversial (Delvare & LaSalle 2000), there had never been any doubt concerning the subfamily placement of *Closterocerus* Westwood. Our initial ML and Bayes analyses (Figs 43-44) placed *Closterocerus sensu strictu* as the sister group to other Entiinae + Entedoninae. This is in agreement with the previous analysis by Gauthier *et al*. (2000), suggesting that these findings are unlikely to be due to sequencing error. However, removal of the 3e´ subregion in the 28S D2 rDNA from all sequences in the analysis resulted in the placement of *Closterocerus* s.s. as the sister group to *Chrysonotomyia* (Figs 46-47), which is a more acceptable hypothesis. The 3e´ subregion in both sampled species of *Closterocerus* s.s. is very different from that of other entedonines (Fig. 45) and could be both the defining trait of the subgenus and the cause of its misplacement.

Subfamily placement of Euderomphalini. Gumovsky (2002) transferred Euderomphalini to Entiinae based on 28S D2 data, the apparently posteriorly complete notauli in all members of each group (character 14: 0, Fig. 23), and the distinctness of the clypeus (character 10: 0) in at least some members. Each of these criteria appears to be problematic.

The interpretation of the notauli as complete in euderomphalines was a novel interpretation based on the state in *Euderomphale* Girault (Fig. 25), where LaSalle & Schauff (1994) had previously considered the notauli to not be indicated externally. If the notauli were complete, this state would be shared with Entiinae. The disagreement over the extent of the notauli is based on differing interpretations of a pair of dorsal thoracic sulci in *Euderomphale* (Fig. 25: sulcus). Positional homology suggests that these sulci are part of the transscutal articulation, which separates the axillae and scutellum from the mesoscutum (Gibson 1997), meaning that they cannot be the notaular grooves. More specifically, the tegula and the posterior notal wing process are landmarks that can be used to recognize the lateral surfaces of the mesoscutum and axilla. The tegula (Fig. 25: tgl) abuts the lateral aspect of the mesoscutum mesally. The posterior notal wing process (Fig. 25: pnwp) extends between the fore wing and hind wing bases, connecting with the dorsal sclerites of the mesosoma with two arm-like processes. The anterior arm ends at the anterior edge of the lateral surface of the axilla at the fore wing base. The posterior arm reaches the scutellum behind the axilla, separating the axilla from the metanotum. Because the posterior notal wing process occurs alongside the axilla for its entire length, it and the wing bases themselves are reliable indicators of the location of the axilla. In

Euderomphale (Fig. 25: ax) the axilla is advanced almost entirely anterior to the scutellum, and the mesoscutum is left with only a small sidelobe that is not delimited by a notaular groove. The axilla is almost entirely expressed as a flat dorsal surface, with only a very short and steep posterior slope. Because the notauli are best interpreted as incomplete in *Euderomphale*, this condition cannot be validly used as a similarity with Entiinae.

Even if one is not convinced by the condition in *Euderomphale*, it is even more clear that *Entedononecremnus* (Fig. 24), another euderomphaline genus, has no externally indicated notauli. Its more typically shaped axillae are only weakly advanced anteriorly and extend posteriorly as a long slope towards the metanotum as in most other chalcidoids. Gumovsky (2002) acknowledged this, but maintained that the state in *Euderomphale* was different. Our interpretation is that the notauli are externally incomplete for all Euderomphalini.

The change in interpretation of this character leaves only clypeal form and 28S D2 data supporting a grouping of Euderomphalini + Entiinae. With the addition of 28S D3-D5 and CO1 data, this grouping does not occur, and instead Euderomphalini groups with Entedonini (Figs. 43-44, 46-49). While clypeal form in the Entiinae and Euderomphalini may be similar in some taxa, the clypeus is not indicated in some species of both groups. This leaves no unambiguous support for Euderomphalini + Entiinae, and therefore it seems best to retain Euderomphalini in Entedoninae.

Monophyly of Entedonini and Euderomphalini. Euderomphalini was not monophyletic in the initial ML analysis (Fig. 43) because *Neopomphale* placed as the

sister group to a clade of remaining Euderomphalini + all Entedonini aside from *Closterocerus sensu strictu*. The initial Bayes analysis (Fig. 44) indicated a poorly supported but monophyletic Euderomphalini. ML analyses with the 3e´ subregion removed (Figs 46-47) indicated a paraphyletic Euderomphalini with respect to *Tropicharis cecivora*. Bayes analysis with the 3e´ subregion removed caused Euderomphalini to render Entedonini paraphyletic but with *Neopomphale* placing with Opheliminae and Entiinae.

The combined Bayes analysis (Fig. 49) indicated a well-supported monophyletic Euderomphalini. It also indicated strong support for a monophyletic grouping of *Cabeza, Euderomphale* and *Neopomphale*, all of which have a transverse sulcus or sharp carina extending across the vertex between the median and lateral ocelli (character 6:1, Fig. 15).

Monophyly of Euderomphalini in the molecular-only analyses seems to be strongly impacted by a tendency for *Neopomphale* and *T. cecivora* to approximate each other or possibly one another's respective tribe. No sequence block or gap could be found to explain this pattern.

Generic relationships within Entedonini. The combined Bayes analysis (Figs 48-49) is the preferred phylogeny because it provides strongly supported results that are relatively easily explained morphologically. However, some potentially valid alternative relationships occur in some of the other analyses, most importantly the *Chrysonotomyia* + *Closterocerus* s.s. clade (Figs 46-47) found when the 3e´ subregion is removed. This allows some interpretation of the results in light of morphological variation. All genera except *Chrysocharis* Förster formed either strongly supported groupings or fit into

weakly supported groupings corresponding to previously published morphological hypotheses.

***Closterocerus sensu lato*.** The unexpected placement of *Closterocerus s.s.* outside Entedoninae in 28S D2 results by Gauthier *et al.* (2000) or as the sister group to all remaining Entedonini Gumovsky (2002) cast strong doubt upon Gumovsky's (2001) synonymy of *Asecodes* and *Neochrysocharis* under *Closterocerus*. These results are here confirmed by independent sequencing of species in the affected taxa and the addition of 28S D3-D5 and CO1 data. However, the anomalous sequence of the 3e´ subregion in *Closterocerus* s.s. appears to be the cause of this placement, even though it is only six bases long. Removal of this subregion from the analysis "rescues" *Closterocerus* s.s. into Entedonini, but as the sister group to *Chrysonotomyia* (Figs 46-47). This placement is supported morphologically by the shared presence of slightly asymmetrical basiconic peg sensilla (character 4: 1, Fig. 10) and subtorular grooves meeting the torulus at its ventral edge (character 9: 1, Fig. 18). Inclusion of these and other characters into the combined molecular and morphological analysis (Figs 48-49) brings *Closterocerus* s.s. back into Entedonini but as the sister group to all members of the tribe aside from *Tropicharis cecivora*. No analysis supports inclusion of *Asecodes* and *Neochrysocharis* with *Closterocerus*. Instead, combined results (Figs 48-49) suggest that they are closely related to a clade of *Pediobomyia* + *Pediobius* + *Rhynchentedon*. A similar relationship between *Asecodes*, *Neochrysocharis*, and *Pediobius* was found independently by Gumovsky (2002) using 28S D2 data. While these results could easily be dismissed as morphologically implausible, there is no known restriction on eulophid evolution that

could support such a dismissal. If valid, these results suggest that the form of the basiconic peg sensilla (character 4, Figs 9-10) in this particular case may be a more reliable indicator of phylogenetic relationship than the presence of subtorular grooves (character 9, Fig 18). In summary, there is no molecular evidence supporting the synonymy of *Asecodes* and *Neochrysocharis* under *Closterocerus*. In light of the conflict from both molecular and morphological data, we propose that *Asecodes* and *Neochrysocharis* be reinstated as valid genera **new status**.

While *Neochrysocharis* is paraphyletic with respect to *Asecodes* and some species of *Pediobius* in the molecular-only analyses (Figs 43-44, 46-47), it is monophyletic in the combined analysis (Figs 48-49). There is no known morphological reason to expect *Neochrysocharis* to be paraphyletic with respect to *Pediobius*. A proper investigation of the monophyly of *Neochrysocharis* will require investigation of additional species from these taxa.

***Closterocerus* and *Chrysonotomyia*.** Hansson (2004) suggested a novel set of characters defining *Chrysonotomyia*, most importantly the presence of a single spine on the volsellar digitus (character 31: 1, Fig. 42) and an at least partially delimited clypeus (character 10: 0, Fig. 20). He reclassified some Neotropical and Nearctic species from the subgenus *Closterocerus* (*Achrysocharis*) into *Chrysonotomyia* based on this character, but other members of the subgenus were not discussed. The European species *Closterocerus* (*Achrysocharis*) *germanicus* (Erdös) included in our analysis renders an otherwise monophyletic *Chrysonotomyia* paraphyletic in all analyses and corresponds to the new definition of the genus. We therefore transfer it to *Chrysonotomyia* as

Chrysonotomyia germanica (Erdös) **new placement**. These results suggest that all other members of *Closterocerus* (*Achrysocharis*) should be examined as potential members of the genus *Chrysonotomyia*.

While the two genera placed together only in molecular analyses with the 3e´ subregion removed (Figs 46-47), a close relationship between *Chrysonotomyia* and *Closterocerus* is supported by the shared presence of slightly asymmetrical basiconic peg sensilla (character 4: 1, Fig. 10) and subtorular grooves extending from the ventral edge of the torulus (character 9: 1, Fig. 18). This relationship is presented as an alternative to placement of *Chrysonotomyia* near *Omphale*, but there is currently not enough data to decide between these alternatives.

***Omphale* and other Entedonini with delimited clypeus.** Gumovsky & Ubaidillah (2002) and Hansson (2004) listed a number of genera that are similar to *Omphale* in possessing a delimited clypeus (character 10:0, Figs 20-21). The genera from these two lists included in this study are *Astichomyiia*, *Chrysonotomyia*, *Parzaommomyia* and *Tropicharis*. None of the analyses in the current study produced a monophyletic assemblage of these genera, but the combined Bayes analysis and initial ML analysis (Figs 43, 48-49) both present an unsupported clade of *Chrysonotomyia* + *Omphale* + *Parzaommomyia*. The only supported monophyletic relationship between any genera with a delimited clypeus was *Omphale* + *Parzaommomyia*, which was supported in all analyses (Figs 43-44, 46-49).

Astichomyiia consistently placed near the genus *Emersonella*, forming a supported monophyletic clade with it and *Ceranisus* in the combined analysis (Figs 48-

49), and does not appear to actually have a delimited clypeus (Hansson 2002: Fig. 404). Given that *Astichomyiia* also possesses a pronotal collar carina (character 12: 1), which is absent in *Omphale*, we suggest that there is no evidence of any close relationship between it and *Omphale*.

Chrysonotomyia placed as sister group to *Closterocerus* s.s. in the analyses with the 3e´ subregion removed (Figs 46-47), or in other analyses as sister group to a strongly supported clade (Figs 43-49) of entedonines usually interpreted as lacking a delimited clypeus. The potential relationship between *Chrysonotomyia* and *Closterocerus* s.s. is discussed in the above section.

Tropicharis placed either as sister group to most other Entedonini (Figs 43-44, 48-49), or within Euderomphalini (Figs 46-47) in the molecular analyses. The combined analysis (Figs 48-49) places it at the base of a grade including *Chrysonotomyia*, *Closterocerus* s.s., and *Omphale* + *Parzaommomyia*. This grade leads to a strongly and consistently supported clade of entedonines usually interpreted as lacking a delimited clypeus. This arrangement seems plausible if one concludes that a delimited clypeus in the groundplan state for Entedonini, the character being lost multiple times in entedonine evolution. This scenario is supported by the relatively weakly, only laterally indicated clypeus in some species of *Chrysonotomyia* (Hansson 2004) and the loss of clypeus delimitation in some species of *Omphale* itself (Hansson 1996). Given that delimitation of the clypeus is therefore an apparently vaguely determined and easily lost character, it seems plausible that the it has been lost multiple times independently over the course of entedonine evolution, as suggested by the combined analysis (Figs 48-49).

***Horismenus* and similar genera.** Hansson (2002) suggested a close relationship between the genera *Alachua* Schauff & Bouček, *Edovum* Grissell, *Horismenus* and *Paracrias* based on propodeal sculpture and the form of the median carina (character 23:1, Figs 28, 33). Later, Hansson (2009b) synonymized *Edovum* and *Alachua* under *Horismenus*. No species from the former genus *Edovum* were included in this analysis, but the others formed a monophyletic and supported group in all molecular-only analyses (Figs 43-44, 46-47). They did not form a monophyletic group in the combined analysis (Figs 48-49), in which *Paracrias* was placed in an unresolved grouping with the *Horismenus* clade, *Chrysocharis*, and the clade of *Astichomyiia* + *Ceranisus* + *Emersonella*. *Horismenus floridensis*, the only species from the former genus *Alachua*, placed with other *Horismenus* species in all analyses.

Paracrias differs from *Horismenus* in a number of morphological characters (Hansson 2004), most importantly in lacking the scuto-scutellar pit. This may explain why it renders *Horismenus* paraphyletic in all the combined analysis. Because of these differences, it seems best to retain *Paracrias* as a separate genus.

***Pediobius* and similar genera.** Morphological similarity between *Pediobius* and the genera *Pediobomyia* and *Rhynchentedon* was recognized by Bouček (1988). In all analyses *Pediobomyia* and *Rhynchentedon* were sister groups forming a clade with at least one species of *Pediobius*. The three genera formed a strongly supported monophyletic clade in the combined analysis (Figs 48-49), but not in the molecular analyses (Figs 43-44, 46-47). Given the lack of molecular support for *Pediobius*, it seems unwise to synonymize any genera with it at this time.

***Entedon* and similar genera.** Gumovsky (2007) listed a set of genera possessing a longitudinal carina on the lateral surface of the pronotum (character 13:1, Fig. 22). Three of these genera were included in this analysis: *Achrysocharoides, Entedon* and *Pleurotroppopsis*. These genera formed a monophyletic unit in two analyses, the combined analysis (Figs 48-49) and the ML analysis with the 3e´ subregion removed (Figs 46-47). This clade was not strongly supported in any analyses, and *Pleurotroppopsis* placed in poorly supported groupings among the other entedonines lacking a defined clypeus in the other analyses. *Achrysocharoides* and *Entedon* formed a strongly supported clade in all analyses except the ML analysis with 3e´ removed.

***Astichomyiia, Ceranisus* and *Emersonella*.** In all analyses, these three genera formed a moderately supported clade. Hansson (2002) recognized the morphological similarity between *Astichomyiia* and *Emersonella* but also listed some similarities between *Astichomyiia* and *Closterocerus*, a grouping that is not supported by molecular data. *Ceranisus* is part of an assemblage of entedonine parasitoids of thrips united by the presence of a transverse groove across the vertex (Triapitsyn & Morse 2005). No morphological data have suggested a relationship between this group and either *Astichomyiia* or *Emersonella*, but *Ceranisus* and *Thripobius* Ferrière formed an unsupported monophyletic group with *Emersonella* in the analysis by Gauthier *et al*. (2000).

Conclusions

Our results present the first published phylogenetic analysis of Eulophidae where the subfamily Entedoninae has been supported as monophyletic. The phylogenetic hypothesis

presented in the combined Bayes analysis (Figs. 48-49) presents strongly supported nodes providing answers to some controversies concerning eulophid morphology.

The initial impetus for this study was to determine if new molecular data could be used to address conflicting hypotheses concerning placement of Euderomphalini, *Asecodes*, and *Neochrysocharis* based on morphology—and in the case of Euderomphalini 28S D2 data. The addition of 28S D3-D5 and CO1 data provided clarity in that they indicated a lack of justification for both the transfer of Euderomphalini to Entiinae, and the synonymy of *Asecodes* and *Neochrysocharis* under *Closterocerus*. The addition of morphological characters led to much stronger answers that provided well-supported nodes with alternative placements for the taxa involved in both controversies.

Investigation of sequences alignments revealed that the unexpected placement of *Closterocerus* s.s. in previous molecular analyses (Gauthier *et al.* 2000, Gumovsky 2002) could be explained by an unusual sequence for a block of six bases in 28S D2 rDNA, the 3e´ block. It seems plausible that this block no longer pairs with the 3e block, and that at least some of it should be treated as an RAA in *Closterocerus*. This would mean that the placement for *Closterocerus* outside Entedonini in previous molecular studies was due to a violation of assumptions of the analysis—specifically the assumption that the entire 3e´ block is comparable between *Closterocerus* and other Eulophidae. This provides a clearly justifiable reason for potentially misleading results in molecular analyses that do not acknowledge this change in *Closterocerus*. Morphological data were also useful in overriding this aberrant information, resulting in a more traditional placement of this genus. This finding raises a new question concerning the placement of *Closterocerus s.s.*

with respect to *Chrysonotomyia* and other taxa morphologically close to *Omphale.* Further investigation should focus on increased taxon sampling of these genera to determine if it is a paraphyletic grade at the base of Entedonini, or whether it forms a clade. This finding also suggests that a general approach of investigating sequence alignment of anomalously placed species can be fruitful in molecular analyses. It is not recommended that wholesale deletion of inconvenient chunks of sequence be performed. Instead, changes to the alignment should be made when taxa are found that violate assumptions used in constructing the alignment.

The preferred hypothesis (Figs 48-49) of eulophid relationships based on molecular and morphological data makes strong statements concerning entedonine phylogenetics. A core group of entedonines was supported in all analyses, excluding *Closterocerus* s.s. and almost all analyzed genera previously considered close to *Omphale* by Hansson (2004) and Gumovsky & Ubaidillah (2002). While this clade could be characterized by the lack of a delimited clypeus, clypeal distinctness varies in the excluded taxa as well and some disagreement exists over interpretation of the character itself (such as in the case of *Astichomyiia*). The strongly supported placements for most eulophid genera should provide a strong context for future analyses of eulophid phylogenetics at subfamily, genus and species levels. It seems likely that the addition of more species to the analysis will provide more clarity for those genera without a strongly supported placement.

Our results put family placement of *Trisecodes agromyzae* into question. This species differs from all other Eulophidae in having three tarsomeres instead of four. An

analysis across chalcidoid families (Munro *et al.* unpublished) indicates that this species does not belong in Eulophidae. However, there is no clear indication of its family placement using either molecular or morphological data.

Disagreement between the combined analysis (Figs 48-49) and the molecular-only analyses (Figs 43-44, 45-46) indicates that some controversy yet remains in eulophid phylogenetics. The addition of more gene regions to the molecular analyses should provide greater clarity in future analyses. Morphological analyses should be improved through more thorough investigation of variation between the species and species groups within the involved genera. While it is possible to succinctly characterize many eulophid genera morphologically, such characterizations often fall apart when all known species are examined (Burks 2003). Rather than providing confusion, such variation could provide greater clarity in morphological hypotheses if the variation is analyzed in a phylogenetic context.

Finally, we recommend that unsupported nodes not be taken very seriously in any analysis, including this one. The best reason to avoid making taxonomic changes to recognize clades that are not well-supported by bootstrap or posterior probability values is that these changes could very easily be overturned by the addition of only a few characters to the analysis. It is important in the interest of both taxonomic stability and the potential informative value of classifications that changes not be made lightly.

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506–513.

Table 1. Specimens Used in This Study

Taxon	Classification	Locality	voucher #	Genbank Accession Numbers		
				28S D2	28S D3-D5	CO1
Achrysocharoides sp.	Eulophidae: Entedoninae: Entedonini	Italy	161085			
Aprostocetus sp.	Eulophidae: Tetrastichinae: Tetrastichini	CA	49012	AY599265		
Asecodes sp.	Eulophidae: Entedoninae: Entedonini	Italy	161135-40			
Astichomyiia latiscapus	Eulophidae: Entedoninae: Entedonini	Costa R.	161116, 161197			
Astichus mirrisimis	Eulophidae: Entiinae	Austral.	92142	AY599261		
Astichus n. sp.	Eulophidae: Entiinae	Austral.	92141	AY599260		
Aulogymnus n. sp.	Eulophidae: Eulophinae: Cirrospilini	CA	161048			
Bellerus sp.	Eulophidae: Entiinae	Chile	161250-1			
Beornia n. sp.	Eulophidae: Entiinae	Austral.	161042, 161044			
Cabeza n. sp.	Eulophidae: Entedoninae: Euderomphalini	Argent.	161082			
Ceranisus menes	Eulophidae: Entedoninae: Entedonini	India	161120-1			
Ceratogramma sp.	Trichogrammatidae: Trichogrammatinae	Owen/Jer				
Chrysocharis sp.	Eulophidae: Entedoninae: Entedonini	CA	161050, 161192-3			
Chrysonotomyia sp.	Eulophidae: Entedoninae: Entedonini	Thai.	161076, 161097			
Chrysonotomyia maculata	Eulophidae: Entedoninae: Entedonini	CA	161130-161134			
Cirrospilus coachellae	Eulophidae: Eulophinae: Cirrospilini	CA	776	AY599268		
Closterocerus germanicus	Eulophidae: Entedoninae: Entedonini	Ukraine	161156-60			
Closterocerus tau	Eulophidae: Entedoninae: Entedonini	CA	161070, 161107			
Closterocerus trifasciatus	Eulophidae: Entedoninae: Entedonini	Germany	161090			
Colotrechnus ignotus	Pteromalidae: Colotrechninae	CA	161379			
Crataepus marbis	Eulophidae: Tetrastichinae: Tetrastichini	France	175179	AY599262		
Dasyomphale chilensis	Eulophidae: Entedoninae: Euderomphalini	Chile	161065-8			
Dicladocerus westwoodi	Eulophidae: Eulophinae: Eulophini	Italy	174915			
Elachertus sp.	Eulophidae: Eulophinae: Eulophini	Italy	161043, 161115			

Emersonella planiceps	Eulophidae: Entedoninae: Entedonini	Costa R.	161149-55	
Entedon diotimus	Eulophidae: Entedoninae: Entedonini	Sweden	161141-8	
Entedononecremnus sp.	Eulophidae: Entedoninae: Euderomphalini	CA	175196	
Epiclerus sp. 1	Tetracampidae: Tetracampinae	Italy	161340	
Epiclerus sp. 2	Tetracampidae: Tetracampinae	CA	174775	
Euderomphale sp.	Eulophidae: Entedoninae: Euderomphalini	CA	161523	
Euderus sp.	Eulophidae: Entiinae	Austral.	174911	AY599259
Eulophus sp.	Eulophidae: Eulophinae: Eulophini	Russia	174914	
Euplectrus sp.	Eulophidae: Eulophinae: Eulophini	Italy	161110	
Foersterella reptans	Tetracampidae: Tetracampinae	Italy	174913	
Hadrotrichodes waukheon	Eulophidae: Tetrastichinae: Tetrastichini	CA	161071	
Horismenus floridensis	Eulophidae: Entedoninae: Entedonini	Costa R.	161101	
Horismenus longicornis	Eulophidae: Entedoninae: Entedonini	Costa R.	161096	
Horismenus n. sp.	Eulophidae: Entedoninae: Entedonini	Costa R.	161122-5	
Horismenus petiolatus	Eulophidae: Entedoninae: Entedonini	Costa R.	161169-77	
Hubbardiella n. sp.	Eulophidae: Entiinae	Hondur.	174912	AY599258
Neochrysocharis sp.	Eulophidae: Entedoninae: Entedonini	Italy	161075	
Neochrysocharis clinias	Eulophidae: Entedoninae: Entedonini	Italy	161184-5	
Neopomphale sp.	Eulophidae: Entedoninae: Euderomphalini	Chile	161381	
Omphale chryseis	Eulophidae: Entedoninae: Entedonini	Sweden	161161-8	
Omphale radialis	Eulophidae: Entedoninae: Entedonini	Italy	161095	
Ophelimus maskelli	Eulophidae: Opheliminae	Italy	161366	
Paracrias pubicornis	Eulophidae: Entedoninae: Entedonini	Costa R.	161187-91	
Parzaommomyia sp.	Eulophidae: Entedoninae: Entedonini	Austral.	161113	
Pediobomyia canaliculata	Eulophidae: Entedoninae: Entedonini	Costa R.	161073	
Pediobius sp.	Eulophidae: Entedoninae: Entedonini	Kenya	161212-6	
Pediobius alaspharus	Eulophidae: Entedoninae: Entedonini	Sweden	161117-9	
Pediobius pullipes	Eulophidae: Entedoninae: Entedonini	Costa R.	161126-9, 161186	

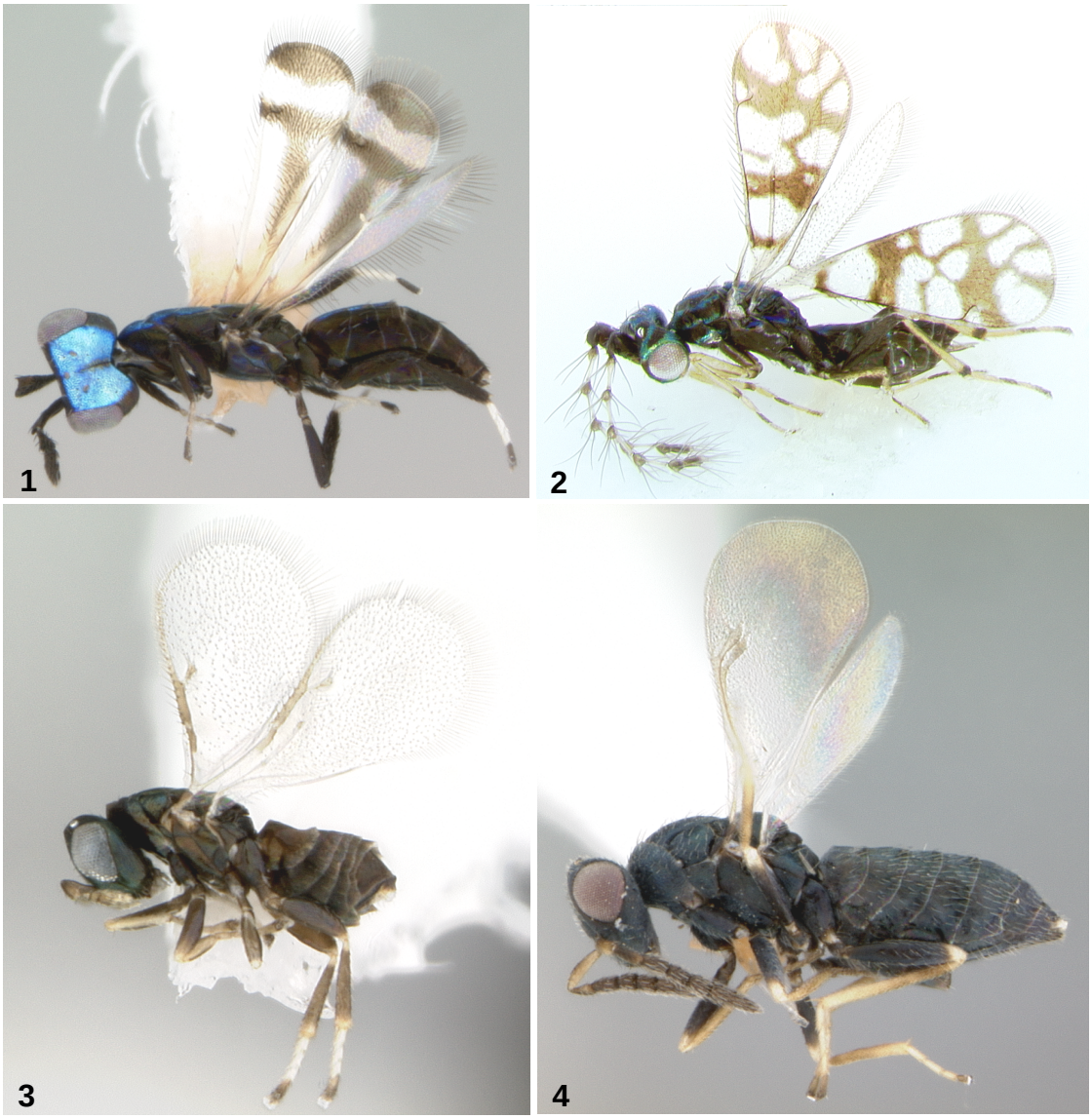
Platyplectrus sp.	Eulophidae: Eulophinae: Eulophini	Thai.	161036, 161093	
Pleurotroppopsis sp.	Eulophidae: Entedoninae: Entedonini	Thai.	161038	
Pnigalio sp.	Eulophidae: Eulophinae: Eulophini	CA	49088	AY599279
Rhynchentedon maximus	Eulophidae: Entedoninae: Entedonini	Thai.	161178-83	
Tetracampe sp.	Tetracampidae: Tetracampinae	Russia	174910	
Trisecodes agromyzae	Eulophidae: *incertae sedis*	Hondur.	161204	
Tropicharis cecivora	Eulophidae: Entedoninae: Entedonini	Costa R.	161194-6	
Zagrammosoma sp.	Eulophidae: Eulophinae: Cirrospilini	CA	49013	AY599263

Table 2. Coding of morphological characters for combined analysis.

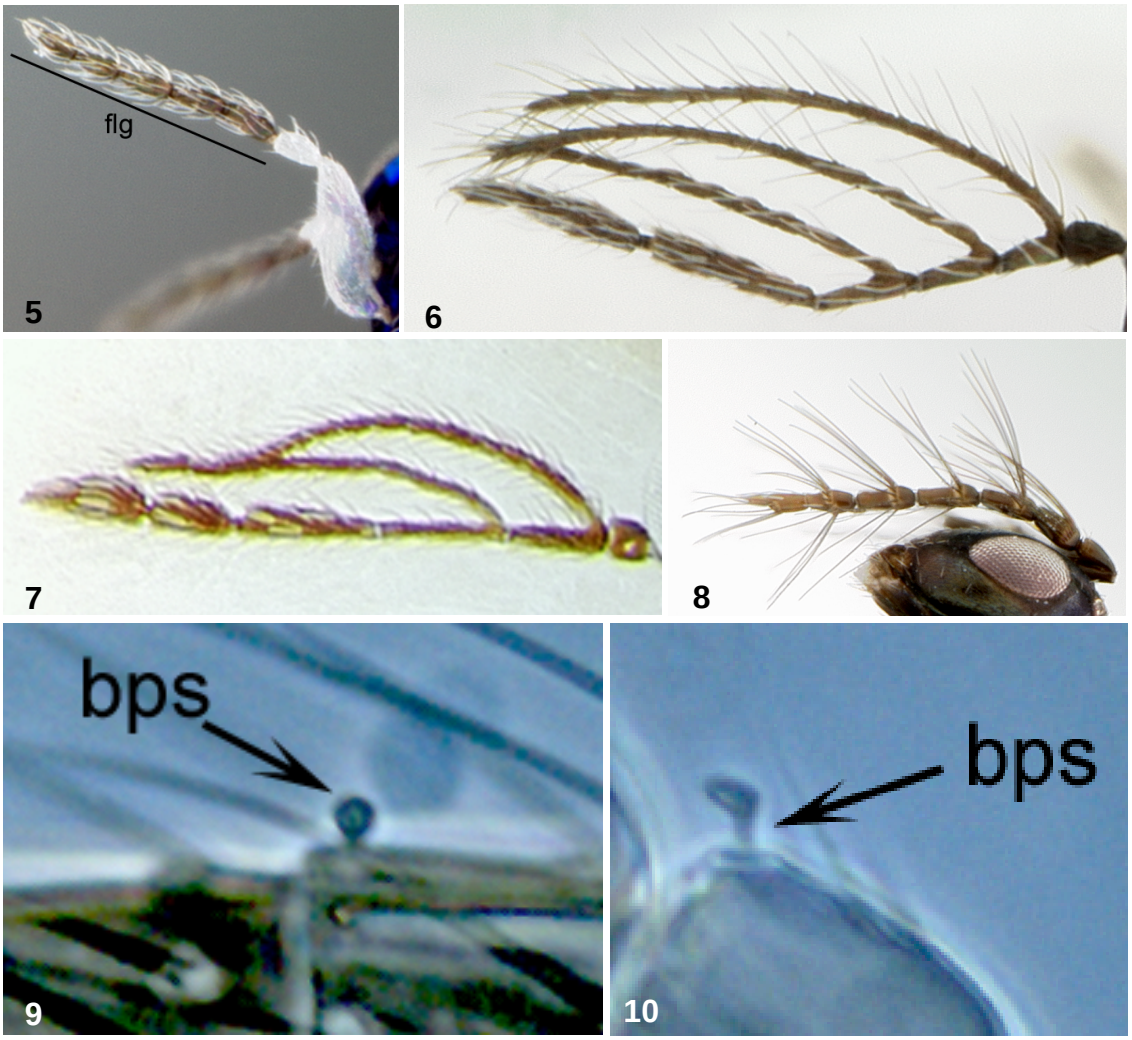
Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Pteromalidae: Colotrechninae																															
Colotrechnus ignotus	C	4	0	0	0	0	0	0	0	1	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	5	0	3	0	1	2
Trichogrammatidae																															
Ceratogramma sp.	8	3	0	0	0	0	0	1	2	1	0	0	0	0	2	1	2	0	0	0	0	0	0	0	1	3	0	2	0	1	2
Tetracampidae: Tetracampinae																															
Epiclerus sp. 1	A	3	0	0	0	0	0	1	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	1	1	5	1	3	0	1	2
Epiclerus sp. 2	A	3	0	0	0	0	0	1	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	1	1	5	1	3	0	1	2
Foersterella reptans	8	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	1	1	5	1	3	0	1	2
*Tetracampe*sp.	8	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	2	0	0	0	0	0	0	1	1	5	1	3	0	1	2
Eulophidae																															
Entedoninae: Entedonini																															
Achrysocharoides sp.	6	1	0	0	0	0	0	1	0	1	0	0	1	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Asecodes sp.	6	1	0	0	0	0	0	1	1	1	0	0	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Astichomyiia latiscapus	6	3	0	0	0	0	0	1	0	1	0	1	0	1	1	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Ceranisus menes	6	3	0	0	0	0	0	1	0	1	0	0	0	1	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Chrysocharis sp.	8	2	0	1	0	0	0	1	0	1	0	0	0	1	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Chrysonotomyia sp.	6	3	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	1
Chrysonotomyia maculata	6	3	0	1	0	0	0	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Closterocerus germanicus	6	3	0	1	0	0	0	1	1	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	1
Closterocerus tau	6	3	0	1	1	0	0	1	1	1	0	0	0	1	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Closterocerus trifasciatus	6	3	0	1	1	0	0	1	1	1	0	1	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Emersonella planiceps	6	1	0	0	0	0	0	1	0	1	0	1	0	1	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Entedon ?*diotimus*	6	2	0	0	0	0	0	1	0	1	0	1	1	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Horismenus floridensis	6	2	0	0	0	0	0	1	0	1	0	1	0	1	2	0	1	0	1	0	1	0	1	0	1	4	0	2	0	1	2
Horismenus longicornis	6	2	0	0	0	0	0	1	0	1	0	1	0	0	2	0	1	0	1	0	1	0	1	0	1	4	0	2	0	1	2
Horismenus n. sp.	6	2	0	0	0	0	0	1	0	1	0	1	0	0	2	0	1	2	1	0	1	0	1	0	1	4	0	2	0	1	2
Horismenus petiolatus	6	1	0	0	0	0	0	1	0	1	0	1	0	0	2	0	1	0	1	0	1	0	1	0	1	4	0	2	0	1	2
Neochrysocharis clinias	6	3	0	0	0	0	0	1	1	1	0	0	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Neochrysocharis formosa	6	3	0	0	0	0	0	1	1	1	0	0	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2

Omphale chryseis	6	1	0	2	0	0	0	1	0	0	1	0	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Omphale radialis	6	1	0	2	0	0	0	1	0	1	1	0	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Paracrias pubicornis	6	3	0	0	0	0	1	1	0	1	0	0	0	1	2	0	1	0	0	0	0	0	1	0	1	4	0	2	0	1	2
Parzaommomyia sp.	6	2	0	2	0	0	0	1	1	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Pediobomyia canaliculata	6	2	0	0	0	0	1	1	0	1	0	0	0	0	2	0	1	0	0	0	1	0	0	0	1	4	0	2	0	1	2
Pediobius alaspharus	6	2	0	0	0	0	1	1	0	1	0	1	0	1	2	0	1	0	0	0	1	0	0	0	1	4	0	2	0	1	2
Pediobius pullipes	6	2	0	0	0	0	1	1	0	1	0	1	0	0	2	0	1	0	0	0	1	0	0	0	1	4	0	2	0	1	2
Pediobius sp.	6	2	0	0	0	0	1	1	0	1	0	1	0	1	2	0	1	0	0	0	1	0	0	0	1	4	0	2	0	1	2
Pleurotroppopsis sp.	6	2	0	0	0	0	1	1	0	1	0	1	1	0	2	0	1	2	0	0	0	0	0	0	1	4	0	2	0	1	2
Rhynchentedon maximus	6	2	0	0	0	0	1	1	0	1	0	1	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Trisecodes agromyzae	7	3	0	0	0	0	0	1	0	1	0	0	0	0	3	0	3	0	0	0	0	0	0	0	1	3	0	1	1	1	2
Tropicharis cecivora	6	3	0	2	0	0	1	1	0	0	0	0	0	0	2	0	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Entedoninae: Euderomphalini																															
Cabeza n sp.	6	3	0	0	0	1	0	1	0	0	0	0	0	1	0	1	2	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Dasyomphale chilensis	6	3	0	0	0	0	0	2	0	0	0	0	0	1	3	0	2	0	0	0	0	0	0	0	1	4	0	3	0	1	2
Entedononecremnus sp.	6	3	0	0	0	0	0	0	0	0	0	0	0	1	3	0	2	0	0	0	0	0	0	0	1	4	0	3	0	1	2
Euderomphale sp.	6	3	0	0	0	1	0	0	0	0	0	0	0	1	2	1	2	0	0	0	0	0	0	0	1	4	0	3	0	1	2
Neopomphale sp.	6	3	0	0	0	1	0	2	0	1	0	0	0	1	1	1	1	0	0	0	0	0	0	0	1	4	0	2	0	1	2
Entiinae																															
Astichus mirissimis	8	3	2	0	0	0	0	2	0	1	0	0	0	0	3	0	2	0	0	0	0	1	0	0	1	4	0	3	0	0	2
Astichus n. sp.	8	3	2	0	0	0	0	2	0	1	0	0	0	0	2	0	2	0	0	0	0	1	0	0	1	4	0	3	0	0	2
Bellerus sp.	8	3	2	0	0	0	0	0	0	1	0	0	0	0	1	0	2	0	0	0	0	0	0	0	1	4	0	3	0	0	2
Beornia sp.	8	2	0	0	0	0	0	0	0	0	0	0	0	0	3	0	2	0	0	0	0	0	0	0	1	4	0	3	0	1	2
Euderus sp.	8	3	2	0	0	0	0	2	0	0	0	0	0	0	3	0	2	0	0	0	0	0	0	0	1	4	0	3	0	0	2
Hubbardiella sp.	8	3	0	0	0	0	0	0	0	0	0	0	0	0	3	0	3	0	0	0	0	0	0	0	1	4	0	3	0	1	2
Eulophinae: Cirrospilini																															
Aulogymnus sp.	7	3	0	0	0	0	0	1	0	1	0	0	0	0	3	0	2	1	0	0	0	0	0	0	1	4	0	3	0	1	2
Cirrospilus sp.	6	3	0	0	0	0	0	1	0	1	0	0	0	0	3	0	2	1	0	0	0	0	0	0	1	4	0	3	0	1	2
Zagrammosoma sp.	6	3	0	0	0	0	0	1	0	1	0	0	0	0	3	0	2	1	0	0	0	0	0	0	1	4	0	3	0	1	2
Eulophinae: Eulophini																															
Dicladocerus westwoodi	8	3	1	0	0	0	0	0	0	1	0	0	0	1	3	0	2	1	0	0	0	0	0	0	1	4	0	3	0	1	2
Elachertus sp. 1	7	2	0	0	0	0	0	0	0	1	0	0	0	0	3	0	2	2	0	1	0	0	0	0	1	4	0	3	0	1	2
Elachertus sp. 2	7	2	0	0	0	0	0	0	0	1	0	0	0	0	3	0	2	2	0	1	0	0	0	0	1	4	0	3	0	1	2

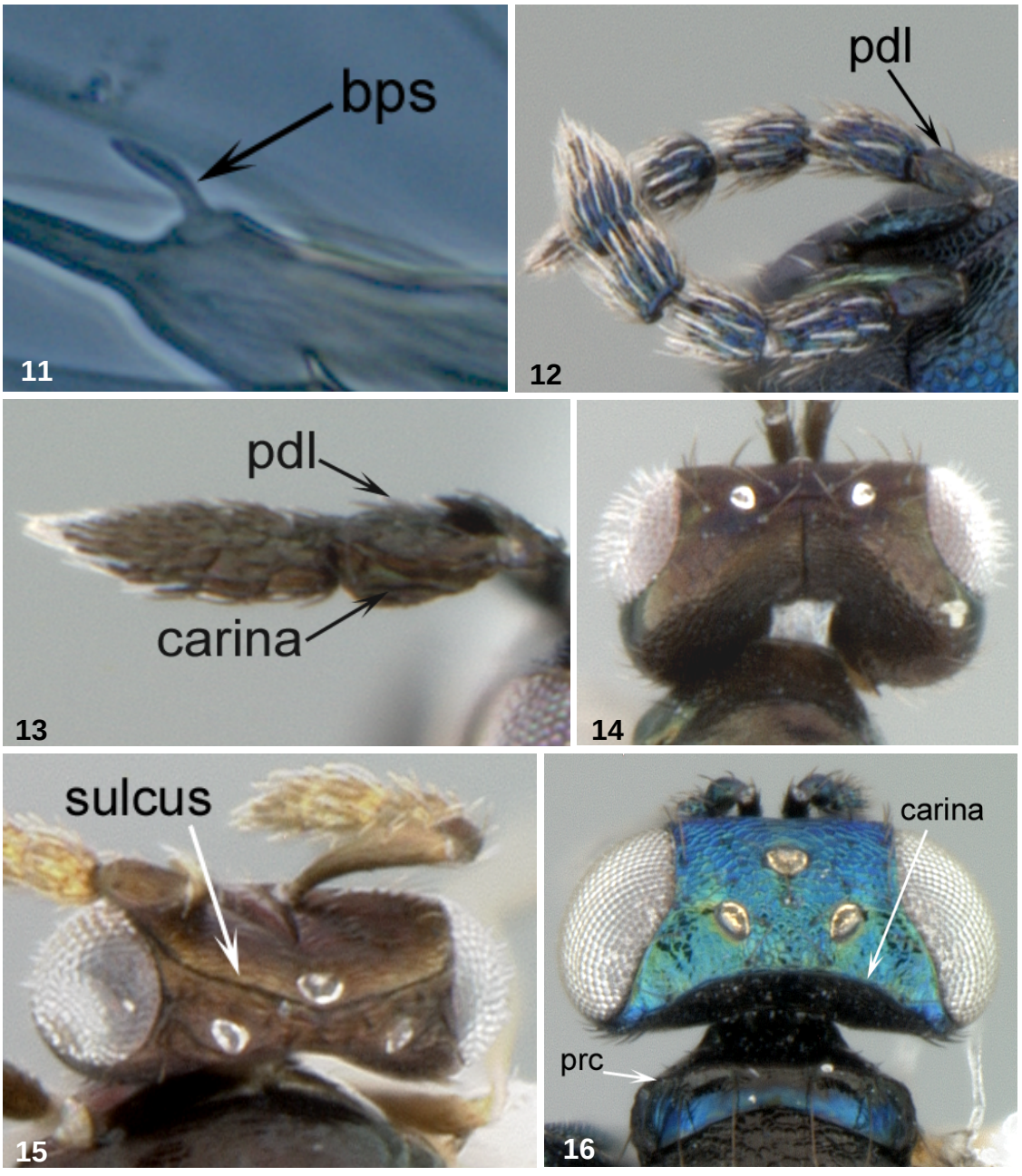
Eulophus sp.	7	3	1	0	0	0	0	0	0	1	0	0	0	1	3	0	2	0	0	1	0	0	0	0	1	4	0	3	0	1	2
Euplectrus sp.	7	2	0	0	0	0	0	0	0	1	0	1	0	0	3	0	2	0	0	1	0	0	0	0	1	4	0	3	0	1	2
Pnigalio sp.	7	2	1	0	0	0	0	0	0	1	0	0	0	1	3	0	2	0	0	1	0	0	0	0	1	4	0	3	0	1	2
Ophelimini																															
Ophelimus maskelli	8	3	0	0	0	0	0	0	0	1	0	0	0	0	1	0	2	0	0	0	0	0	0	0	1	4	0	1	0	1	2
Tetrastichinae: Tetrastichini																															
Aprostocetus sp. 1	8	3	3	0	0	0	0	0	2	1	0	0	0	0	3	0	2	1	0	0	0	0	0	0	1	4	0	3	1	1	2
Aprostocetus sp. 2	8	3	3	0	0	0	0	1	2	1	0	0	0	0	3	0	2	1	0	0	0	0	0	0	1	4	0	3	1	1	2
Crataepus marbis	8	3	0	0	0	0	0	1	0	1	0	0	0	0	3	0	2	1	0	0	0	0	0	0	1	4	0	3	1	1	2
Hadrotrichodes waukheon	8	3	0	0	0	0	0	0	2	1	0	0	0	0	3	0	2	1	0	0	0	0	0	0	1	4	0	3	1	1	2



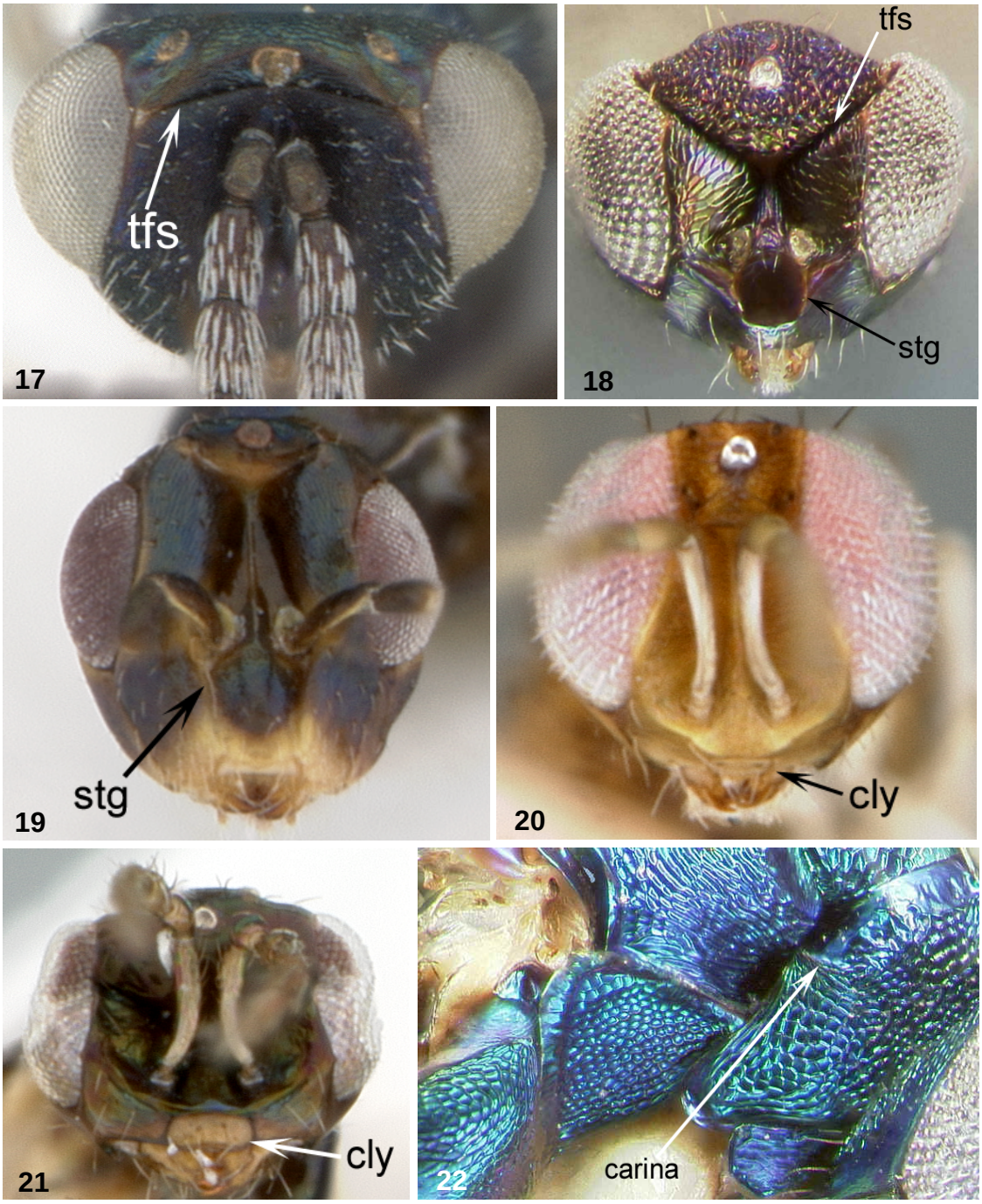
Figures 1-4. Habitus of selected eulophids. **1.** *Closterocerus tau* (Entedoninae: Entedonini). **2.** *Astichus* sp. (Entiinae). **3.** *Ophelimus maskelli* (Opheliminae). **4.** *Aprostocetus* sp. (Tetrastichinae).



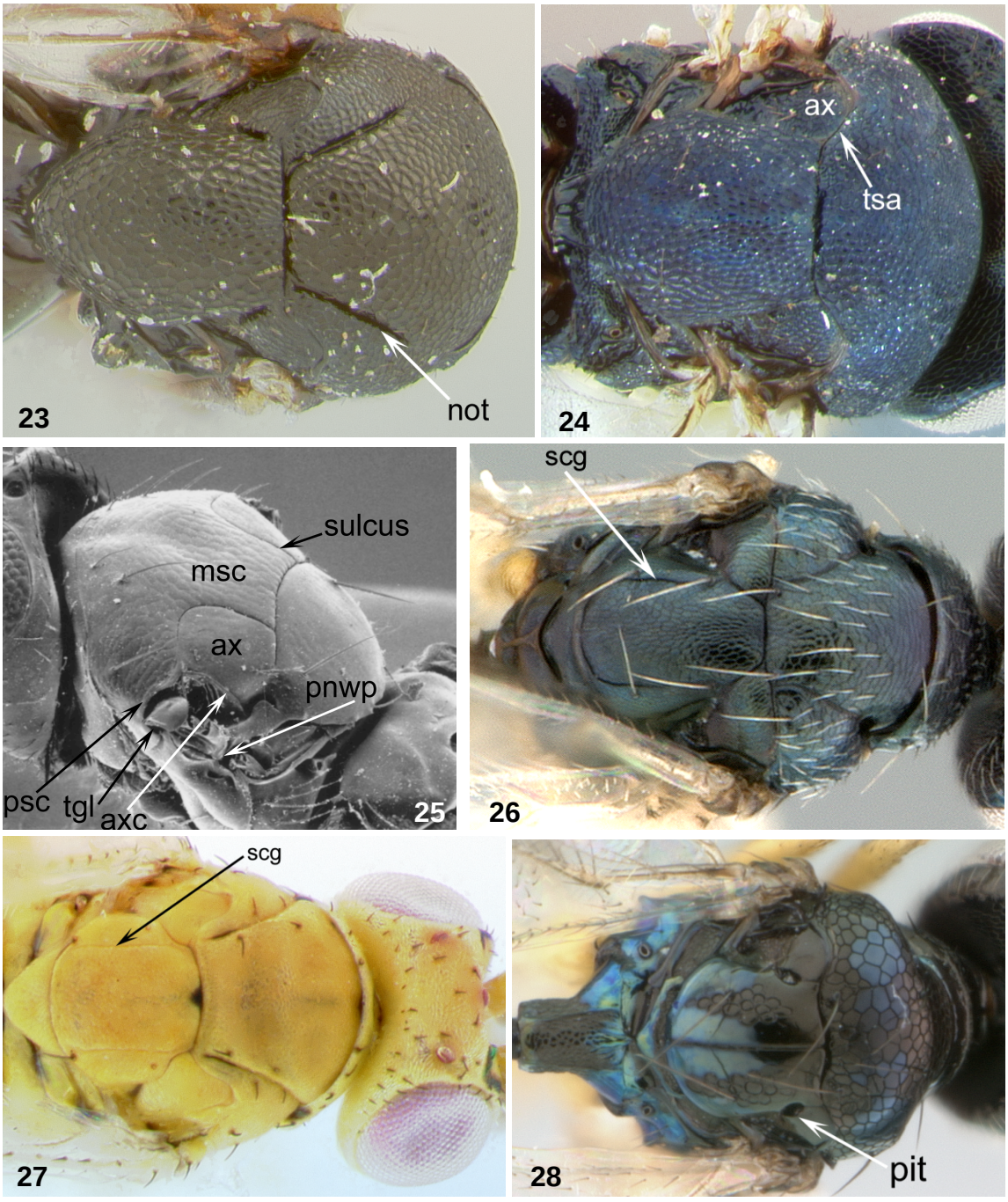
Figures 5-10. Characters of the antennal flagellum. **5-8.** Antennae of male Eulophidae. **5.** *Achrysocharoides* sp. **6.** *Pnigalio* sp. **7.** *Dicladocerus westwoodi*. **8.** *Aprostocetus* sp. **9-10.** Basiconic peg sensilla variation. **9.** *Neochrysocharis* sp. **10.** *Closterocerus* sp.



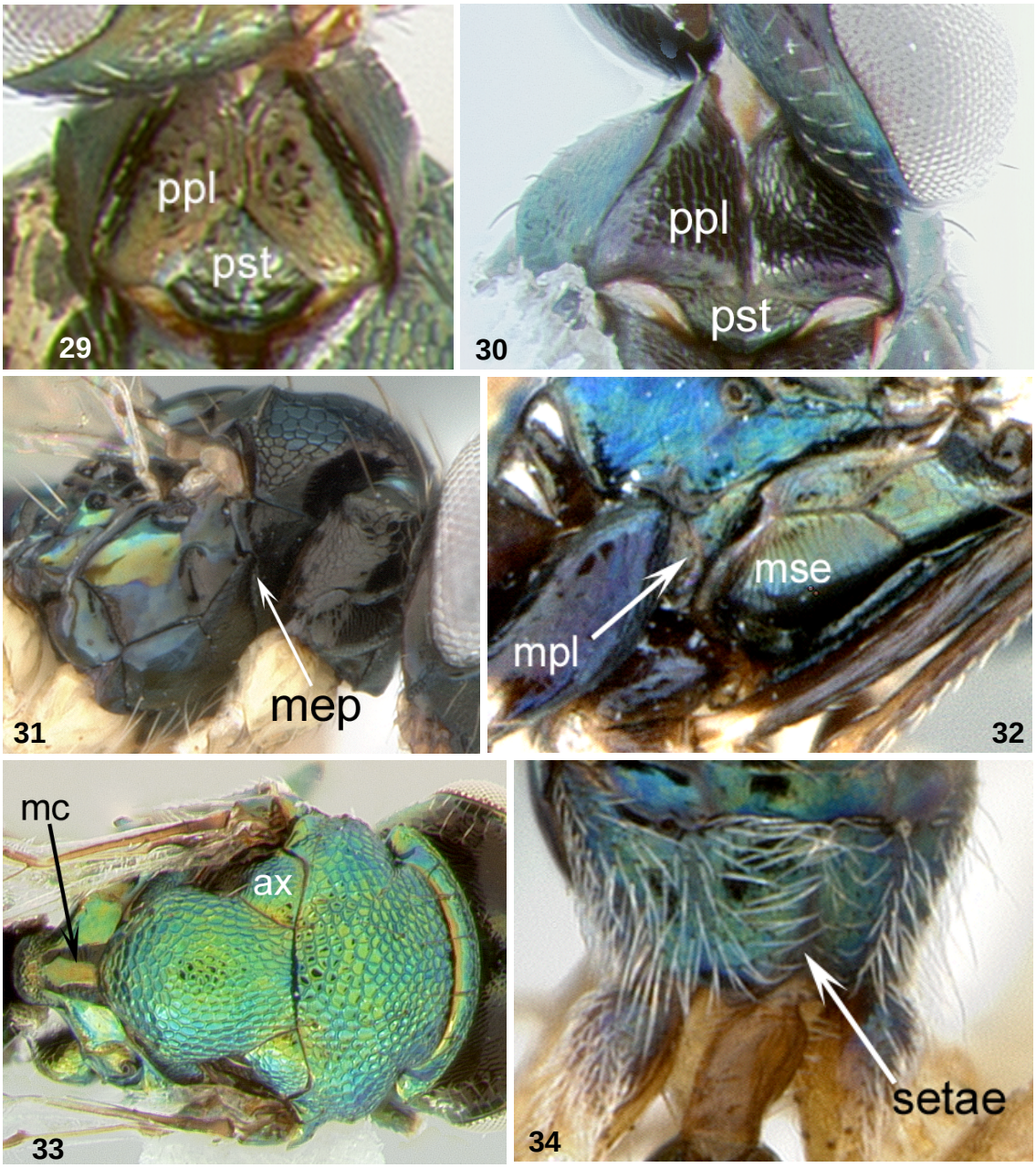
Figures 11-16. Characters of antenna, head and pronotum. **11.** *Omphale* sp. basiconic peg sensilla. **12.** *Pediobius pullipes* antenna, pdl = pedicel. **13.** *Closterocerus tau* antenna: pdl = pedicel. **14.** *Asecodes* sp. vertex. **15.** *Neopomphale* sp. vertex. **16.** *Pediobius pullipes* vertex and pronotum, prc = pronotal collar carina.



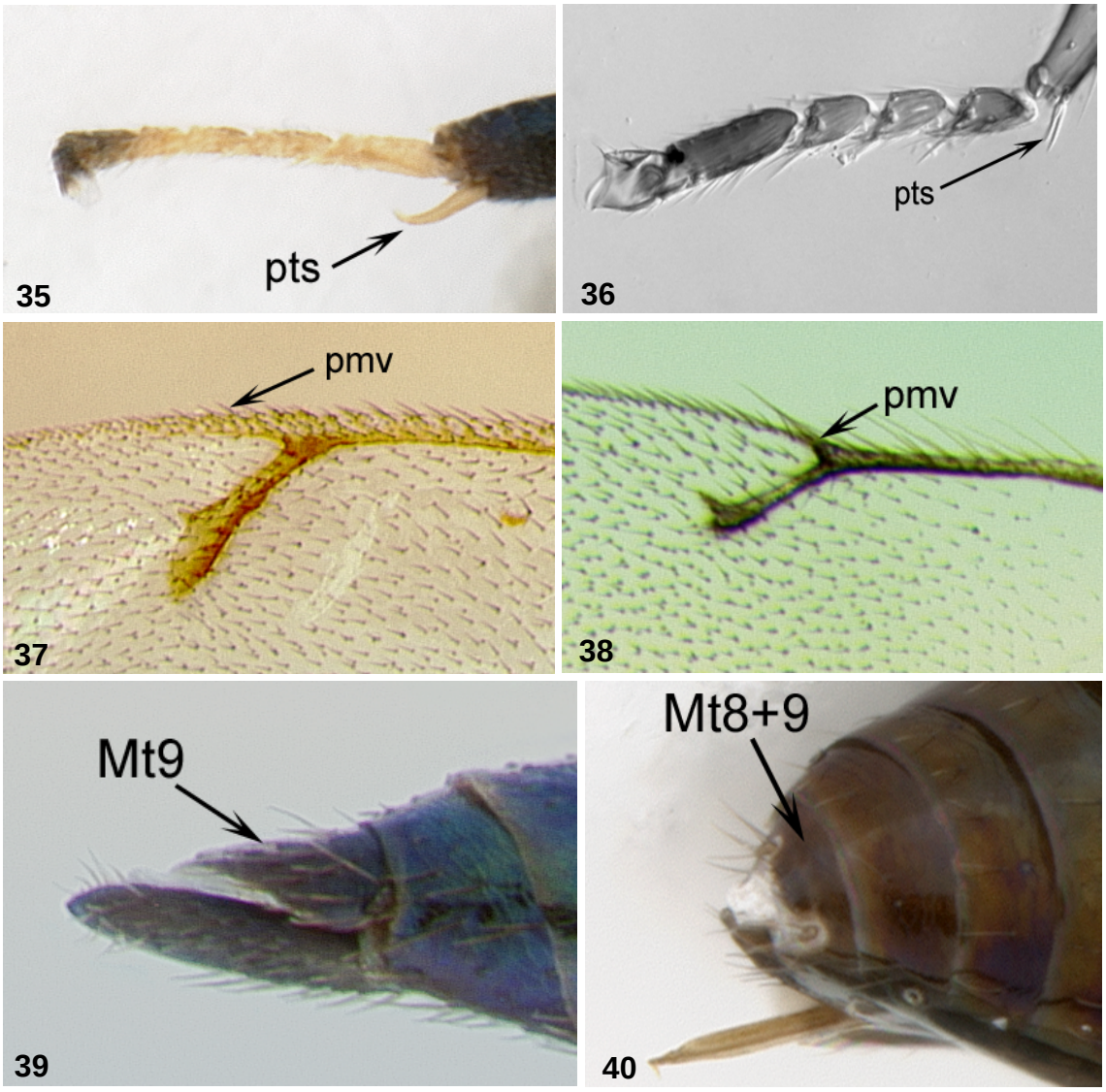
Figures 17-22. Characters of the head and pronotum. **17.** *Euderus* sp., tfs = transverse facial suture. **18.** *Closterocerus trifasciatus*, stg = subtorular groove. **19.** *Aprostocetus* sp. **20.** *Chrysonotomyia germanica* **n. comb.**, cly = clypeus. **21.** *Omphale* sp. **22.** *Achrysocharoides* sp.



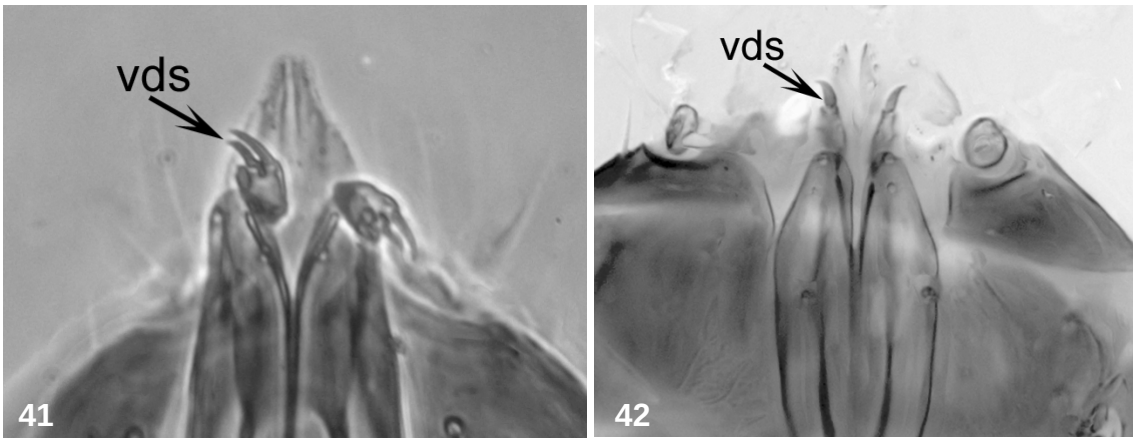
Figures 23-28. Characters of the mesosoma. **23.** *Hubbardiella* n. sp., not = notaulus. **24.** *Entedononecremnus* sp., ax = axilla, tsa = trans-scutal articulation. **25.** *Euderomphale* sp., axc = axillar carina, msc = mesoscutum, pnwp = posterior notal wing process, psc = parascutal carina, tgl = tegula. **26.** *Elachertus* sp., scg = scutellar groove. **27.** *Cirrospilus* sp. **28.** *Horismenus petiolatus*.



Figures 29-34. Characters of the mesosoma. **29.** *Dicladocerus* sp. prothorax, ventral. **30.** *Necremnus* sp. prothorax, ventral. **31.** *Horismenus petiolatus*, mep = mesepisternal projection. **32.** *Astichus* sp., mse = mesepimeron, mpl = metapleuron. **33.** *Paracrias arizonensis*, ax = axilla, mc = median carina. **34.** *Epiclerus* sp. propodeum.



Figures 35-40. Characters of the legs, wings and metasoma. **35.** *Colotrechnus ignotus* fore tarsus (pts = protibial spur). **36.** *Euderomphale* sp. fore tarsus. **37.** *Aulogymnus* sp. forewing venation (pmv = postmarginal vein). **38.** *Aprostocetus* sp. forewing venation. **39.** *Euderus* sp. gastral apex. **40.** *Beornia* sp. gastral apex.



Figures 41-42. Characters of the male genitalia. **41.** *Closterocerus* sp., vds = vosellar digitus spine. **42.** *Chrysonotomyia* sp.

Figure 43. RAxML molecular results, 28S D2-D5 and CO1 regions. Log likelihood score -17,392.54. Bootstrap values above 70% indicated on branches. Suprageneric taxa indicated by a vertical bar. Black bars indicate monophyletic groups, gray bars indicate non-monophyletic groups. *Closterocerus* s.s. indicated in bold.

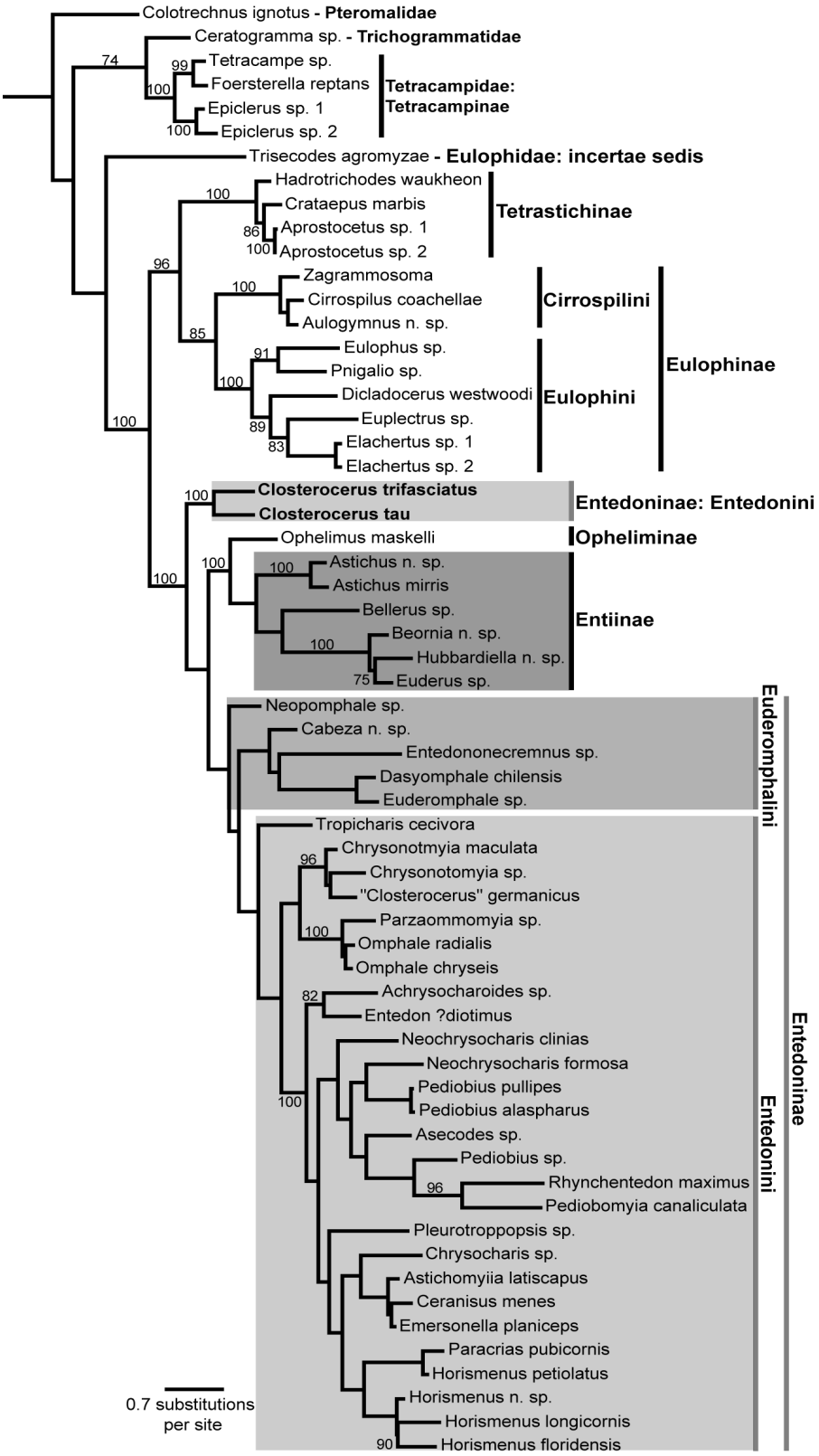
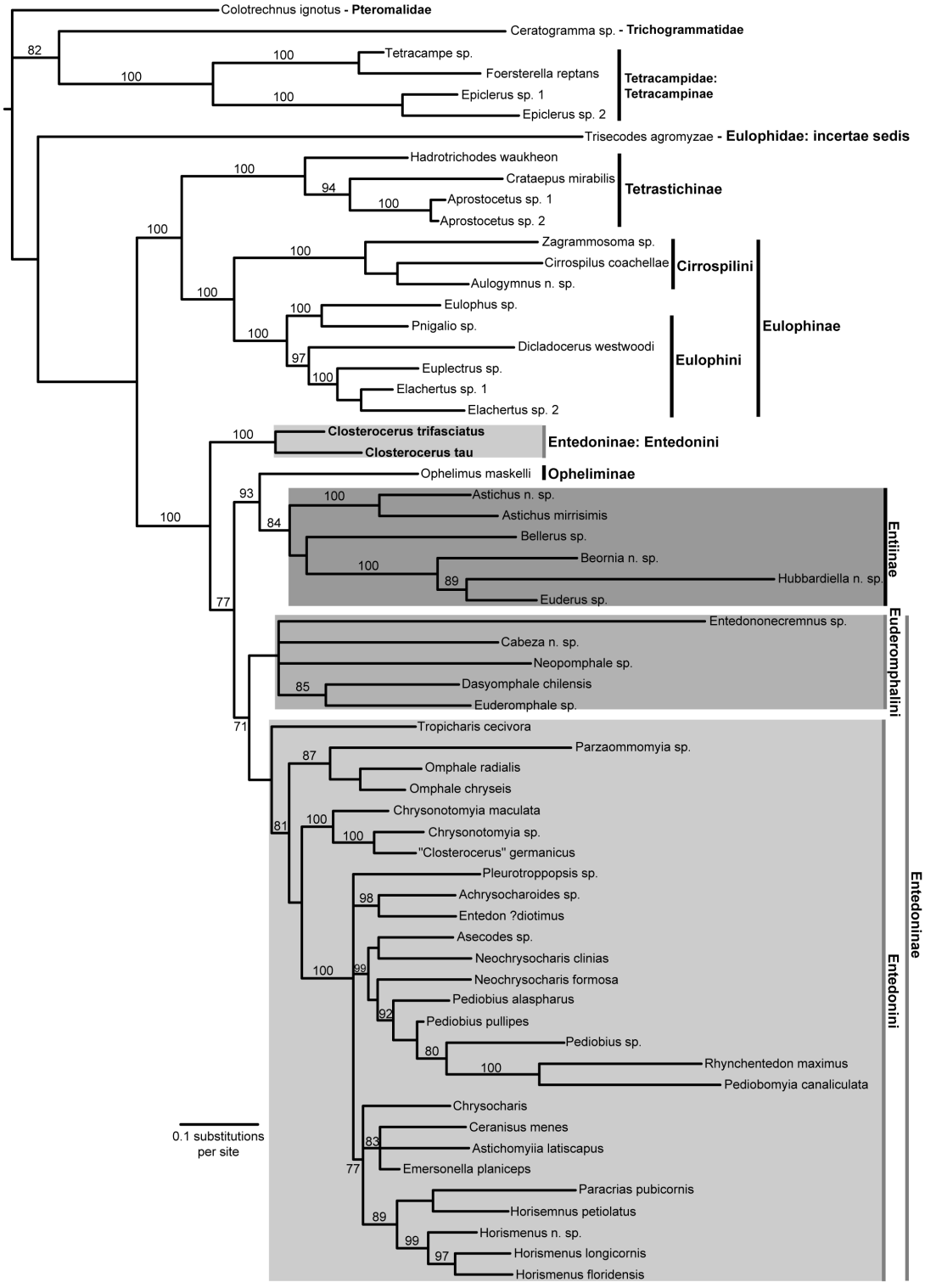

Colotrechnus ignotus - Pteromalidae
Ceratogramma sp. - Trichogrammatidae
Tetracampe sp.
Foersterella reptans
Epiclerus sp. 1
Epiclerus sp. 2
Tetracampidae: Tetracampinae
Trisecodes agromyzae - Eulophidae: incertae sedis
Hadrotrichodes waukheon
Crataepus marbis
Aprostocetus sp. 1
Aprostocetus sp. 2
Tetrastichinae
Zagrammosoma
Cirrospilus coachellae
Aulogymnus n. sp.
Cirrospilini
Eulophus sp.
Pnigalio sp.
Dicladocerus westwoodi
Euplectrus sp.
Elachertus sp. 1
Elachertus sp. 2
Eulophini
Eulophinae
Closterocerus trifasciatus
Closterocerus tau
Entedoninae: Entedonini
Ophelimus maskelli
Opheliminae
Astichus n. sp.
Astichus mirris
Bellerus sp.
Beornia n. sp.
Hubbardiella n. sp.
Euderus sp.
Entiinae
Neopomphale sp.
Cabeza n. sp.
Entedononecremnus sp.
Dasyomphale chilensis
Euderomphale sp.
Euderomphalini
Tropicharis cecivora
Chrysonotmyia maculata
Chrysonotomyia sp.
"Closterocerus" germanicus
Parzaommomyia sp.
Omphale radialis
Omphale chryseis
Achrysocharoides sp.
Entedon ?diotimus
Neochrysocharis clinias
Neochrysocharis formosa
Pediobius pullipes
Pediobius alaspharus
Asecodes sp.
Pediobius sp.
Rhynchentedon maximus
Pediobomyia canaliculata
Pleurotroppopsis sp.
Chrysocharis sp.
Astichomyiia latiscapus
Ceranisus menes
Emersonella planiceps
Paracrias pubicornis
Horismenus petiolatus
Horismenus n. sp.
Horismenus longicornis
Horismenus floridensis
Entedonini
Entedoninae
0.7 substitutions per site

Figure 44. Bayes molecular-only results summarized on a 50% majority rule tree with branch lengths included. Posterior probability values higher than 70% indicated on branches. Suprageneric taxa indicated by a vertical bar. Black bars indicate monophyletic groups, gray bars indicate non-monophyletic groups. *Closterocerus* s.s. indicated in bold.


Colotrechnus ignotus - Pteromalidae
Ceratogramma sp. - Trichogrammatidae
Tetracampe sp.
Foersterella reptans
Epiclerus sp. 1
Epiclerus sp. 2
Tetracampidae: Tetracampinae
Trisecodes agromyzae - Eulophidae: incertae sedis
Hadrotrichodes waukheon
Crataepus mirabilis
Aprostocetus sp. 1
Aprostocetus sp. 2
Tetrastichinae
Zagrammosoma sp.
Cirrospilus coachellae
Aulogymnus n. sp.
Cirrospilini
Eulophus sp.
Pnigalio sp.
Dicladocerus westwoodi
Euplectrus sp.
Elachertus sp. 1
Elachertus sp. 2
Eulophini
Eulophinae
Closterocerus trifasciatus
Closterocerus tau
Entedoninae: Entedonini
Ophelimus maskelli
Opheliminae
Astichus n. sp.
Astichus mirrisimis
Bellerus sp.
Beornia n. sp.
Hubbardiella n. sp.
Euderus sp.
Entiinae
Entedononecremnus sp.
Cabeza n. sp.
Neopomphale sp.
Dasyomphale chilensis
Euderomphale sp.
Euderomphalini
Tropicharis cecivora
Parzaommomyia sp.
Omphale radialis
Omphale chryseis
Chrysonotomyia maculata
Chrysonotomyia sp.
"Closterocerus" germanicus
Pleurotroppopsis sp.
Achrysocharoides sp.
Entedon ?diotimus
Asecodes sp.
Neochrysocharis clinias
Neochrysocharis formosa
Pediobius alaspharus
Pediobius pullipes
Pediobius sp.
Rhynchentedon maximus
Pediobomyia canaliculata
Chrysocharis
Ceranisus menes
Astichomyiia latiscapus
Emersonella planiceps
Paracrias pubicornis
Horisemnus petiolatus
Horismenus n. sp.
Horismenus longicornis
Horismenus floridensis
Entedoninae
Entedonini
0.1 substitutions per site

	3e		3e'
Closterocerus trifasciatus	CCGT-T	…	AATGCG
Chrysocharis sp.	CCGT-T	…	AAATGG
Entedononecremnus sp.	TCGT-T	…	AATCGA
Hubbardiella n.sp	CCGT-T	…	AAGAGG
Euderus sp.	CTGT-T	…	ATGCGG
Astichus n. sp.	CCTG-T	…	ATCTGG
Astichus mirissimis	CCGT-T	…	ATCTGG
Zagrammasoma sp.	CCGGTC	…	GTCCGG
Aprostocetus sp. 1	CCGA-T	…	A-TTGG
Aprostocetus sp. 2	CCGG-T	…	G-CCGG
Cirrospilus sp.	CCGG-T	…	G-CCGG

Figure 45. The 3e and 3e´ subregions for *Closterocerus trifasciatus* compared with that of other selected eulophids as aligned by the secondary structure model provided by Gillespie *et al.* (2005). Species of the tribe Entedonini are highlighted. Other Entedonini have the same sequence as *Chrysocharis* sp. in these subregions. *Closterocerus tau* has the same sequence as *C. trifasciatus* in these subregions. Intervening bases between the two subregions omitted.

Figure 46. RAxML molecular results with the 3e´subregion of the 28S D2 rDNA removed. Log likelihood score -14399.405046. Bootstrap values above 70% indicated on branches. Suprageneric taxa indicated by a vertical bar. Black bars indicate monophyletic groups, gray bars indicate non-monophyletic groups. *Closterocerus* s.s. indicated in bold.

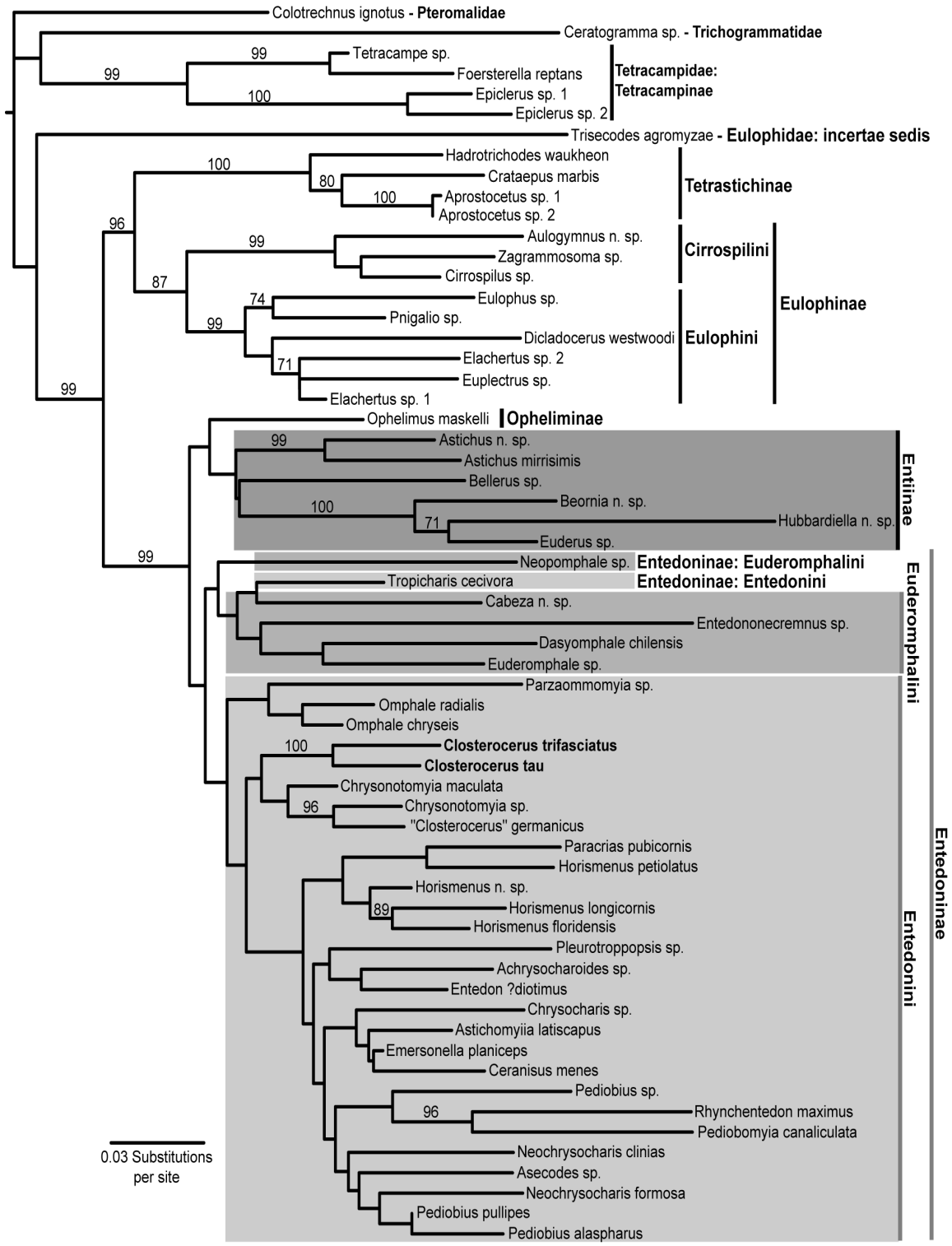

Colotrechnus ignotus - Pteromalidae
Ceratogramma sp. - Trichogrammatidae
Tetracampe sp.
Foersterella reptans
Epiclerus sp. 1
Epiclerus sp. 2
Tetracampidae: Tetracampinae
Trisecodes agromyzae - Eulophidae: incertae sedis
Hadrotrichodes waukheon
Crataepus marbis
Aprostocetus sp. 1
Aprostocetus sp. 2
Tetrastichinae
Aulogymnus n. sp.
Zagrammosoma sp.
Cirrospilus sp.
Cirrospilini
Eulophus sp.
Pnigalio sp.
Dicladocerus westwoodi
Elachertus sp. 2
Euplectrus sp.
Elachertus sp. 1
Eulophini
Eulophinae
Ophelimus maskelli
Opheliminae
Astichus n. sp.
Astichus mirrisimis
Bellerus sp.
Beornia n. sp.
Hubbardiella n. sp.
Euderus sp.
Entiinae
Neopomphale sp.
Entedoninae: Euderomphalini
Tropicharis cecivora
Entedoninae: Entedonini
Cabeza n. sp.
Entedononecremnus sp.
Dasyomphale chilensis
Euderomphale sp.
Euderomphalini
Parzaommomyia sp.
Omphale radialis
Omphale chryseis
Closterocerus trifasciatus
Closterocerus tau
Chrysonotomyia maculata
Chrysonotomyia sp.
"Closterocerus" germanicus
Paracrias pubicornis
Horismenus petiolatus
Horismenus n. sp.
Horismenus longicornis
Horismenus floridensis
Pleurotroppopsis sp.
Achrysocharoides sp.
Entedon ?diotimus
Chrysocharis sp.
Astichomyiia latiscapus
Emersonella planiceps
Ceranisus menes
Pediobius sp.
Rhynchentedon maximus
Pediobomyia canaliculata
Neochrysocharis clinias
Asecodes sp.
Neochrysocharis formosa
Pediobius pullipes
Pediobius alaspharus
Entedonini
Entedoninae
0.03 Substitutions per site

Figure 47. Bayes molecular-only results with the 3e´ subregion of the 28S D2 rDNA removed, summarized on a 50% majority rule tree with branch lengths included. Posterior probability values higher than 70% indicated on branches. Suprageneric taxa indicated by a vertical bar. Black bars indicate monophyletic groups, gray bars indicate non-monophyletic groups. *Closterocerus* s.s. indicated in bold.

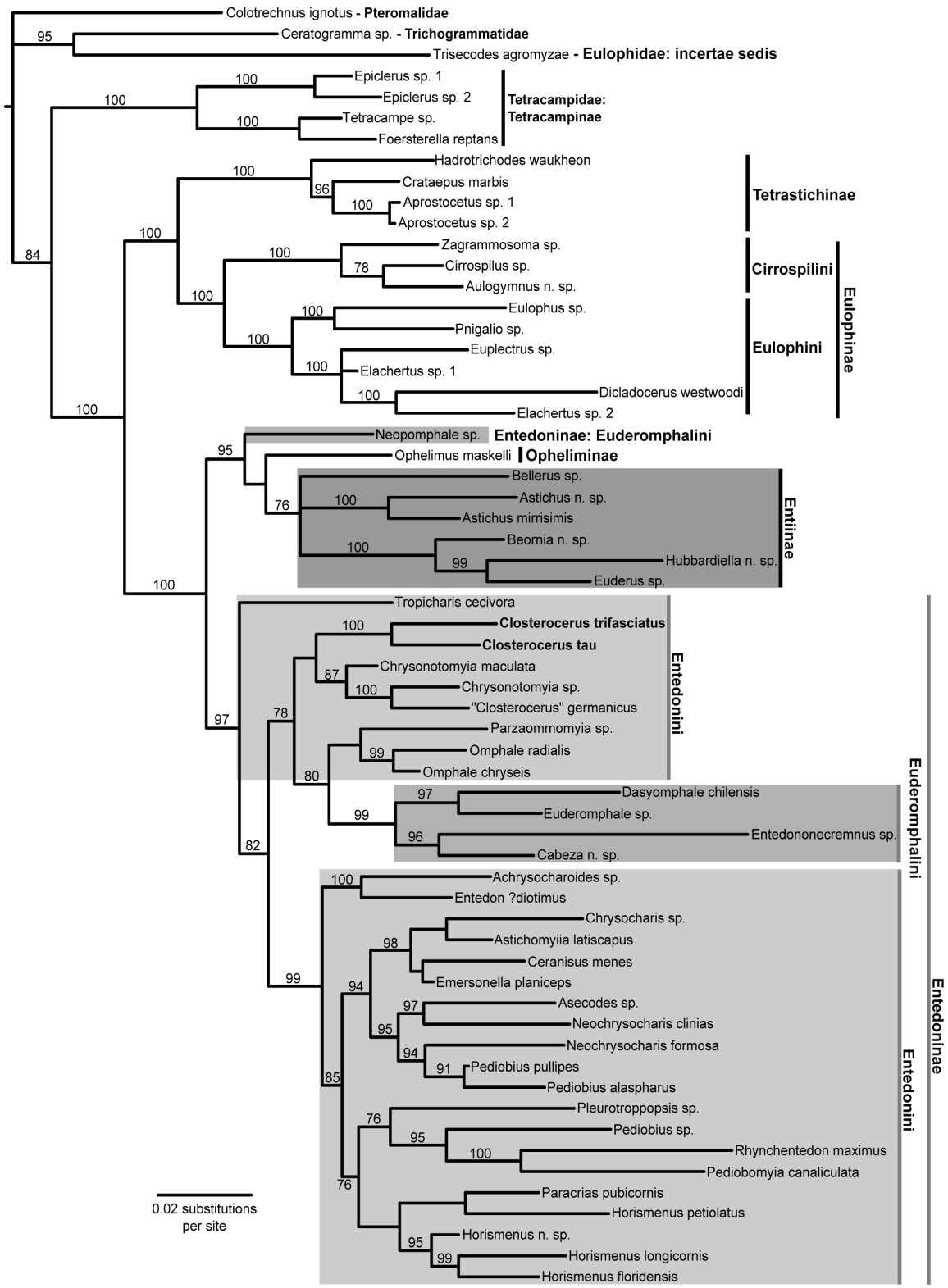

Colotrechnus ignotus - Pteromalidae
Ceratogramma sp. - Trichogrammatidae
Trisecodes agromyzae - Eulophidae: incertae sedis
Epiclerus sp. 1
Epiclerus sp. 2
Tetracampe sp.
Foersterella reptans
Tetracampidae: Tetracampinae
Hadrotrichodes waukheon
Crataepus marbis
Aprostocetus sp. 1
Aprostocetus sp. 2
Tetrastichinae
Zagrammosoma sp.
Cirrospilus sp.
Aulogymnus n. sp.
Cirrospilini
Eulophus sp.
Pnigalio sp.
Euplectrus sp.
Elachertus sp. 1
Dicladocerus westwoodi
Elachertus sp. 2
Eulophini
Eulophinae
Neopomphale sp.
Entedoninae: Euderomphalini
Ophelimus maskelli
Opheliminae
Bellerus sp.
Astichus n. sp.
Astichus mirrisimis
Beornia n. sp.
Hubbardiella n. sp.
Euderus sp.
Entiinae
Tropicharis cecivora
Closterocerus trifasciatus
Closterocerus tau
Chrysonotomyia maculata
Chrysonotomyia sp.
"Closterocerus" germanicus
Parzaommomyia sp.
Omphale radialis
Omphale chryseis
Entedonini
Dasyomphale chilensis
Euderomphale sp.
Entedononecremnus sp.
Cabeza n. sp.
Euderomphalini
Achrysocharoides sp.
Entedon ?diotimus
Chrysocharis sp.
Astichomyiia latiscapus
Ceranisus menes
Emersonella planiceps
Asecodes sp.
Neochrysocharis clinias
Neochrysocharis formosa
Pediobius pullipes
Pediobius alaspharus
Pleurotroppopsis sp.
Pediobius sp.
Rhynchentedon maximus
Pediobomyia canaliculata
Paracrias pubicornis
Horismenus petiolatus
Horismenus n. sp.
Horismenus longicornis
Horismenus floridensis
Entedonini
Entedoninae
0.02 substitutions per site

Figure 48. Bayes combined morphological and molecular results summarized on a 50% majority rule tree with branch lengths included. Posterior probability values higher than 70% indicated on branches. Suprageneric taxa indicated by a vertical bar. *Closterocerus* s.s. indicated in bold.

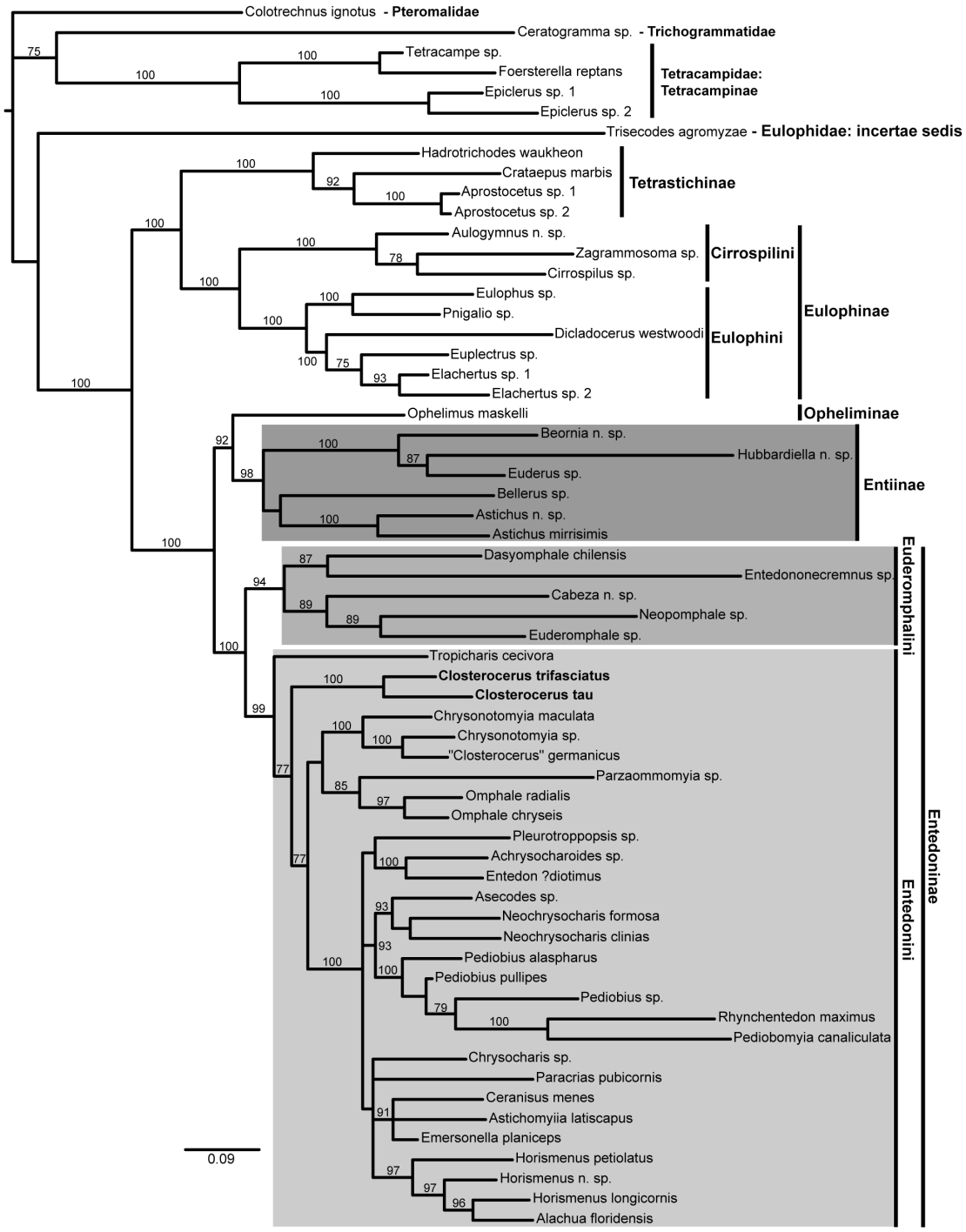
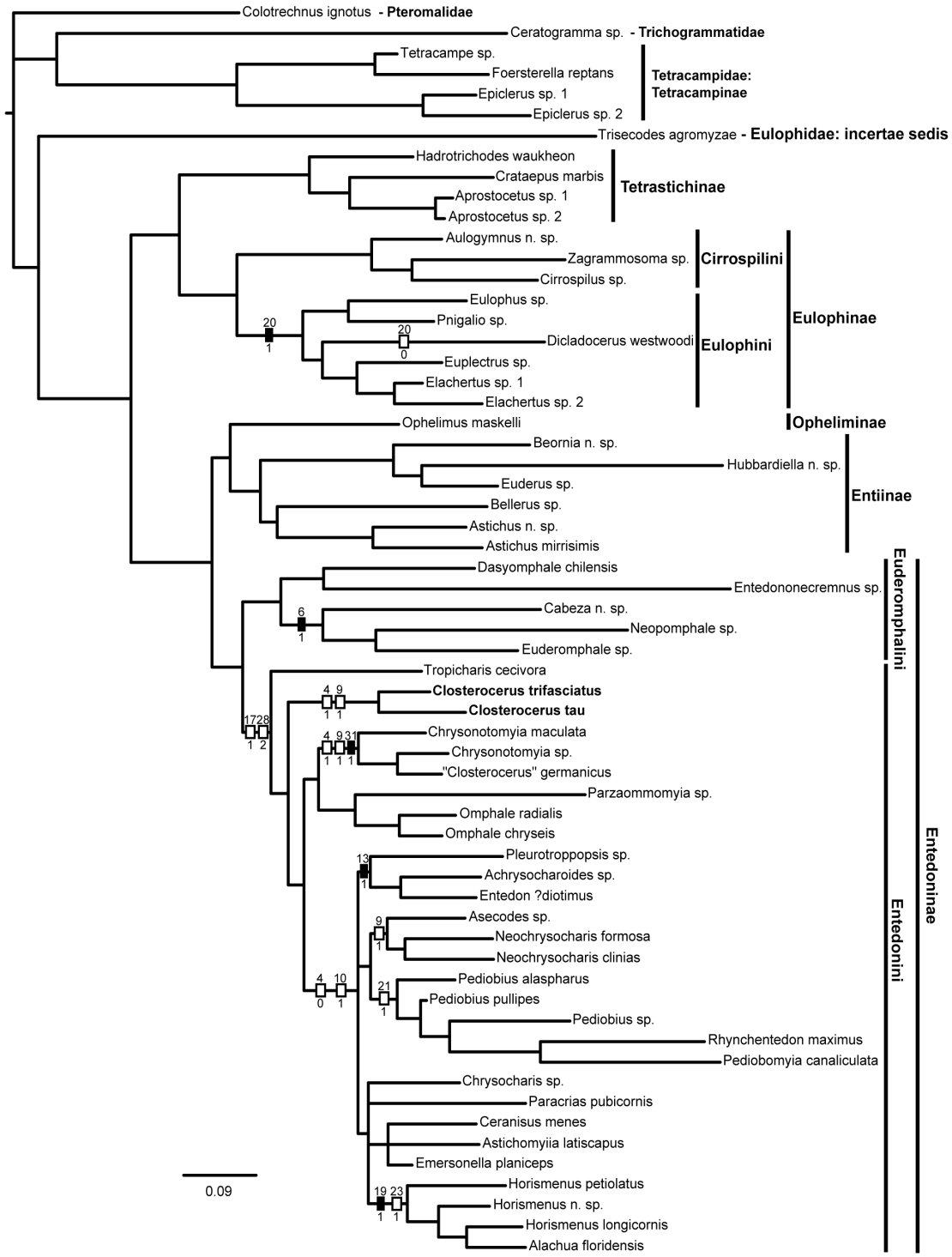

Colotrechnus ignotus - Pteromalidae
Ceratogramma sp. - Trichogrammatidae
Tetracampe sp.
Foersterella reptans
Epiclerus sp. 1
Epiclerus sp. 2
Tetracampidae: Tetracampinae
Trisecodes agromyzae - Eulophidae: incertae sedis
Hadrotrichodes waukheon
Crataepus marbis
Aprostocetus sp. 1
Aprostocetus sp. 2
Tetrastichinae
Aulogymnus n. sp.
Zagrammosoma sp.
Cirrospilus sp.
Cirrospilini
Eulophus sp.
Pnigalio sp.
Dicladocerus westwoodi
Euplectrus sp.
Elachertus sp. 1
Elachertus sp. 2
Eulophini
Eulophinae
Ophelimus maskelli
Opheliminae
Beornia n. sp.
Hubbardiella n. sp.
Euderus sp.
Bellerus sp.
Astichus n. sp.
Astichus mirrisimis
Entiinae
Dasyomphale chilensis
Entedononecremnus sp.
Cabeza n. sp.
Neopomphale sp.
Euderomphale sp.
Euderomphalini
Tropicharis cecivora
Closterocerus trifasciatus
Closterocerus tau
Chrysonotomyia maculata
Chrysonotomyia sp.
"Closterocerus" germanicus
Parzaommomyia sp.
Omphale radialis
Omphale chryseis
Pleurotroppopsis sp.
Achrysocharoides sp.
Entedon ?diotimus
Asecodes sp.
Neochrysocharis formosa
Neochrysocharis clinias
Pediobius alaspharus
Pediobius pullipes
Pediobius sp.
Rhynchentedon maximus
Pediobomyia canaliculata
Chrysocharis sp.
Paracrias pubicornis
Ceranisus menes
Astichomyiia latiscapus
Emersonella planiceps
Horismenus petiolatus
Horismenus n. sp.
Horismenus longicornis
Alachua floridensis
Entedonini
Entedoninae
0.09

Figure 49. Bayes combined morphological and molecular results with selected state changes indicated. Numbers above rectangles indicate character number; those below the rectangles indicate character state number. Filled rectangles indicate unambiguous changes, while unfilled rectangles indicate changes that are homoplastic on the tree. Suprageneric taxa indicated by a vertical bar. *Closterocerus* s.s. indicated in bold.


Colotrechnus ignotus - Pteromalidae
Ceratogramma sp. - Trichogrammatidae
Tetracampe sp.
Foersterella reptans
Epiclerus sp. 1
Epiclerus sp. 2
Tetracampidae: Tetracampinae
Trisecodes agromyzae - Eulophidae: incertae sedis
Hadrotrichodes waukheon
Crataepus marbis
Aprostocetus sp. 1
Aprostocetus sp. 2
Tetrastichinae
Aulogymnus n. sp.
Zagrammosoma sp.
Cirrospilus sp.
Cirrospilini
Eulophus sp.
Pnigalio sp.
Dicladocerus westwoodi
Euplectrus sp.
Elachertus sp. 1
Elachertus sp. 2
Eulophini
Eulophinae
Ophelimus maskelli
Opheliminae
Beornia n. sp.
Hubbardiella n. sp.
Euderus sp.
Bellerus sp.
Astichus n. sp.
Astichus mirrisimis
Entiinae
Dasyomphale chilensis
Entedononecremnus sp.
Cabeza n. sp.
Neopomphale sp.
Euderomphale sp.
Euderomphalini
Tropicharis cecivora
Closterocerus trifasciatus
Closterocerus tau
Chrysonotomyia maculata
Chrysonotomyia sp.
"Closterocerus" germanicus
Parzaommomyia sp.
Omphale radialis
Omphale chryseis
Pleurotroppopsis sp.
Achrysocharoides sp.
Entedon ?diotimus
Asecodes sp.
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Neochrysocharis clinias
Pediobius alaspharus
Pediobius pullipes
Pediobius sp.
Rhynchentedon maximus
Pediobomyia canaliculata
Chrysocharis sp.
Paracrias pubicornis
Ceranisus menes
Astichomyiia latiscapus
Emersonella planiceps
Horismenus petiolatus
Horismenus n. sp.
Horismenus longicornis
Alachua floridensis
Entedonini
Entedoninae
0.09

CHAPTER 3

Understanding the Posterior Surface of the Head in Chalcidoidea (Hymenoptera)

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Abstract. Variation in structures of the posterior surface of the head in Chalcidoidea is compared and interpreted according to theories of head capsule evolution within Hymenoptera as proposed by Snodgrass. Most chalcidoids have only a hypostomal bridge, but some species in the families Chalcididae, Eurytomidae, Pteromalidae, and Torymidae have varying forms of postgenal bridges. Species with a reduced head capsule, such as many Aphelinidae, Mymaridae, and Trichogrammatidae, lack important landmarks that cannot be easily interpreted without making inferences from related species. Several features provide potentially useful phylogenetic information, such as the presence of a postgenal bridge, extent of the hypostomal carina, extent of secondary posterior tentorial pits, and form of the mesal lamellae extending from the foramen magnum to the oral cavity. However, in many cases these characters present problems of homology that may not be answerable at this time.

Keywords. Tentorial bridge, hypostomal bridge, postgenal bridge, parasitoid evolution.

Introduction

The superfamily Chalcidoidea is a diverse and apparently monophyletic grouping of notoriously small-bodied wasps, with most species parasitic or developing in plant galls (Gibson *et al.* 1999). There are at least 20,000 described species of chalcidoids (Noyes 2003), but possibly up to 400,000 species total (Noyes 2000). While most chalcidoids are from 3-5 mm in length (Gibson 1993), the males of the mymarid species *Dicopomorpha echmepterygis* Mockford can be as small as 0.139 mm (Mockford 1997). One may expect such small-bodied insects to have relatively uniform reduction in cranial morphology, but instead chalcidoids exhibit almost every known type of cranial bridge and intermediate state, lacking only a gular bridge. The great diversity of cranial bridges in chalcidoids could provide a valuable suite of phylogenetic characters, but there are many difficulties in the interpretation of homology. There remains some disturbing intermediacy and some potentially highly reduced states that complicate comparisons of different chalcidoid families. This summary begins with taxa having a reduced or absent cranial bridge, and ends with taxa having a postgenal bridge. Our intent is to provide a starting point for comparative discussion of cranial morphology in chalcidoids, based upon a relatively broad selection of taxa, and as a beginning for assessment of these features for their phylogenetic value.

There has been little published on cranial bridge morphology in Chalcidoidea, with the exception of reviews of Torymidae (Grissell 1995), Agaonidae *sensu lato* (Rasplus 1998), Pteromalidae (Dzhanokmen 1994, Bouček & Heydon 1997), and Eurytomidae (Lotfalizadeh *et al.* 2007). The relative lack of material published on this

region of the body is most probably due to the difficulty in interpreting exterior features in a meaningful way. For example, there is more than just a simple pair of tentorial pits and a pair of sulci along the cranial bridge in many chalcidoids, which may represent internal modifications not be easily interpreted without dissection of the head to determine the location of the posterior tentorial pits as a primary landmark. Even after dissection, some unresolved questions may remain, especially concerning the presence of postgenal lobes or the ultimate occurrence of a postgenal bridge in cases where the head capsule becomes an essentially fused unit posteriorly without landmarks.

This study aims to investigate a broad range of families to discover and assess possible head characters of value in chalcidoid phylogenetics. We seek to establish a standardized set of terms and interpretations to better facilitate comparisons of species from different families, with a foundation built on the comprehensive study by Snodgrass (1960). Finally, the broad phylogenetic context of our study should provide a more meaningful phylogenetic context for examination of cranial variation across and within families, subfamilies, and tribes of Chalcidoidea.

Materials and Methods

Terms and positional interpretations are based on those of Snodgrass (1960). Dissections of the head capsule were conducted on specimens mounted on SEM stubs with conductive silver paint, with the head attached face-up. Optical photographs were taken using the EntoVision Mobile Imaging System (GT Vision LLC).

Taxa illustrated are listed in Table 1. Some figures were taken from referenced publications. Others are original photos from the University of California, Riverside Entomology Research Museum, with voucher numbers listed.

Results

Absence of a cranial bridge (Fig. 1): The bridge is assumed to be primitively absent in taxa where the labial ***postmentum*** (Fig. 1A: **Pmt**, 1B: **psmt**) extends dorsally to connect near the posterior tentorial pits at the occipital foramen. In these taxa, there is no intervening sclerite between the labial connection and the occipital foramen. The hypostomae extend separately to the postocciput and merge with it. This condition has not been found in Chalcidoidea nor for any other Apocrita. It is discussed here in order to provide greater context for the following discussions. It is known from the sawfly groups Xyelidae and Tenthredinoidea (Beutel & Vilhelmsen 2007), and therefore may be the primitive state for Hymenoptera.

Hypostomal bridge (Figs 2-3): One kind of cranial bridge commonly found in Chalcidoidea is the ***hypostomal bridge*** (hyb). It is formed by a fusion of postoccipital and hypostomal structures between and along the postgenae. Within the fused hypostomal/postoccipital structure, hypostomal structures theoretically exist below the first pair of posterior tentorial pits and postoccipital structures exist above the pits. The ***hypostomal sulci*** (hys) extend continuously towards the occipital foramen, meeting the

posterior ***tentorial pits*** (ptp) and there merge with the postoccipital suture unless obliterated.

In a hypostomal bridge, the hypostomal sulci are by definition not interrupted by postgenal structures. Extra pits may occur on the hypostomal bridge (Fig. 2E), but are usually not associated with the tentorium. If they are associated with extra posterior tentorial arms, they are designated as secondary posterior tentorial pits (Fig. 2B). Although the hypostoma and other subgenal structures are not perfectly separate from postgenal structures in Hymenoptera, there are some useful landmarks for recognizing them. The maxillo-labial complex attaches to a special projection from the hypostoma (Fig. 2D), and the oral cavity is surrounded by hypostomal structures.

Formation of a hypostomal bridge occurs by encroachment of hypostomal lobes (HL) across the area between the occipital foramen and the oral cavity. When united mesally they establish the hypostomal bridge (hyb), a sclerotized structure that separates the occipital foramen from the oral cavity (Fig. 2D). Inference of a hypostomal bridge (hyb) can become problematic in two ways. First, if the hypostomal sulci (hys) are obliterated as in Fig. 3D, there can appear to be a postgenal bridge instead; in which case, there will likely not be any internal structures that could be used to determine with certainty what kind of bridge it is. Second, if all posterior cranial structures are reduced, the head may appear to have the primitive condition of no posterior cranial bridge at all. Some taxa with elongate heads have a very narrow hypostomal bridge interposed between the barely separated postgenae (Fig. 3A, hyb). As long as the hypostomal bridge

is continuously interposed between the postgenae, however narrowly, no postgenal bridge is present.

Examination with the mouthparts extended can reveal a sclerotized transverse bar at the oral cavity (Fig. 2D: bar) bearing the attachment of the maxillo-labial complex and serving as the ventral termination point for the hypostomal sulcus.

In Pteromalinae (Fig. 2E), Perilampidae, and Eucharitidae, the hypostomal bridge becomes sunken below the posterior surface of the head. The bridge loses the transvese bar along the oral margin, and appears to be less sclerotized. We reject the possibility that the bridge could have been formed through novel sclerotization of neck membrane, because the ***hypostomal sulci*** (hys) meet the ***posterior tentorial pits*** (ptp), and are apparently present alongside the entire length of the bridge.

Some taxa, such as a few species of *Asaphes* (Fig. 2F) and *Ablerus*, have one or more (solid or split) independent plates or flaps extending from the postocciput that covers the hypostomal bridge. The presence of this extension is not always easy to discern externally, but can be inferred if the **mesal lamellae** (= ***ornamentation of median stripe*** in Lotfalizadeh *et al.* 2007) of the hypostomal bridge are discovered under the extension after it has been removed.

Some taxa have a very narrow hypostomal bridge that is often difficult to interpret. In many pteromalids such as Spalangiinae and Leptofoeninae, there is a narrow hypostomal bridge (Fig. 3A: hyb) intervening between the postgenae along the entire distance between the occipital foramen and the oral cavity. It is possible that the postoccipital bridge of Agaonidae (Rasplus *et al.* 1998) is a raised narrow hypostomal

bridge as well, but confirmation of this awaits dissection to determine the position of the posterior tentorial pits and to see if the structure is part of the cranial bridge or if it is a different structure originating independently.

In *Megastigmus* (Fig. 3B), the apparent hypostomal sulci (hys) are marked externally along the cranial bridge, but they do not represent any clear internal structure. Instead, there is an inflected internal ridge of hypostomal structure that extends to the postocciput and is continuous with the tentorium. Internally, a hyaline lamina connects the anterior tentorial arm with the ridge along much of its length. In taxa with a complete postgenal bridge, such as Toryminae, the posterior tentorial arms are enlarged, and the hyaline lamina from the tentorium extends along the lateral edges of the fused postoccipital/hypostomal areas dorsal to the postgenal bridge. It is not clear if this kind of cranial bridge is homologous with the other types found so far. One important difference from Pteromalinae is that in Megastigminae the hypostomal carina (hyc) does not extend dorsally along the bridge, but instead ends or becomes inflected at the cranial midline; in other examples of hypostomal bridges, there is a clear line of separation between lateral and mesal hypostomal structures. The shape of the hypostomal carina in Megastigminae is similar to that of Toryminae, but in Toryminae the hypostomal sulci do not extend along the cranial bridge. The states in *Megastigmus,* and more clearly in *Ormyrus,* are probably intermediates, in which the postgenal lobes are encroaching across the hypostomal bridge.

Inference of a hypostomal bridge becomes difficult when the hypostomal carinae are strongly reduced (Figs 3C-D). It then relies upon finding the hypostomal sulci (Fig.

3C: hys), or through finding a consistently sunken sclerotized bridge that seems separate from the postgenae. A postgenal bridge remains absent because the cranial bridge includes hypostomal/postoccipital structures along its entire length. The cranial bridge of *Coccophagus* (Fig. 3D) and most other small-bodied Chalcidoidea is therefore inferred to be a hypostomal bridge even though there is no definite sign of a hypostomal sulcus or carina. In *Cales* (Fig. 3E), there are postgenal lobes, which could be an intermediate state in the formation of a postgenal bridge.

A few chalcidoids, such as *Chiloe micropteron*, apparently have a fully separated sclerite composed of a fusion of hypostomal and postoccipital structures (Fig. 3F: hyb). This is not interpreted as a gula because the gula is characterized by sclerotization of neck membrane and ventral migration of the posterior tentorial pits. There is no indication that the conditions characterizing a gula are satisfied in *C. micropteron*, and therefore it seems best interpreted as a hypostomal bridge with deep hypstomal sulci. It is probably not a postoccipital plate, because there is no sign of any bridge or mesal lamellae under this structure.

Postgenal bridge (Figs 4-5): This is a cranial bridge consisting of a mesad extension of the postgenae across the area below the occipital foramen. This bridge extends over the remaining hypostomal structures, including the hypostomal bridge (Figs 4A-C). It does not primarily include any postoccipital structures, but can merge with all surrounding parts of the head capsule. The hypostomal carina does not extend dorsally along the cranial bridge in these taxa, but instead is restricted to enclosing the area along the oral

cavity (Figs 4C-4F). However, something approaching this condition also occurs in many taxa with potentially only a hypostomal bridge, such as *Megastigmus* (Fig. 3B). The mesal lamellae extend over the postgenal bridge, and therefore are rarely useful for establishing any basis of positional homology. They are useful when they are covered by postgenal lobes that do not form a complete bridge (Fig. 5A).

The postgenal bridge may become more visibly fused with surrounding cranial structures, making its interpretation more problematic. Dissection of the head reveals that the pit dorsad of the bridge is connected to the posterior tentorial arm, and therefore is a secondary posterior tentorial pit (Fig. 4E: ptp). In some taxa such as many Toryminae (Figs 5B-5C), the tentorial arm itself is expanded and its connection forms one long sulcus exteriorly. In species with extreme fusion of cranial structures, a postgenal bridge is inferred if the hypostomal carinae (Figs 4C-4F: hyc) approximate or meet immediately above the oral cavity ***and*** the hypostomal sulci do not extend dorsally along the length of the bridge. In *Chromeurytoma* (Fig. 4F), the postgenal bridge (pgb) is hardly elevated above the hypostomal bridge (poc), but is essentially the same state that is found in *Eurytoma* (Fig. 4E) and in some Chalcididae, including *Acanthochalcis nigricans* Cameron (not shown).

Some taxa have a discernable gradual acquisition of a postgenal bridge in different ways, most commonly with the postgenae overlapping hypostomal areas, but not forming a complete fusion across the head. This kind of “postgenal bridge” (Fig. 5A: “pgb”) is for the purposes of this discussion not considered to be an actual postgenal bridge. In this case the postgenae likely cover a hypostomal bridge, and only partially

cover the mesal lamellae along the midline of the hypostomal bridge. Families containing species with “postgenal bridges” like this usually contain other species with a variably exposed hypostomal bridge. It should be possible in these cases to construct a transformation series leading from a completely exposed hypostomal bridge as in Fig. 2F to a completely hidden one like that in Fig. 5A.

Outgroup taxa, such as the superfamilies Mymarommatoidea, Platygasteroidea, and Proctotrupoidea, exhibit a similar range of variation to that of Chalcidoidea. While a thorough examination of these states is beyond the scope of this study, many species in these taxa have ambiguous cranial bridges that are also very difficult to interpret. A broader context, examining many species in each superfamily, would be necessary to fully understand this variation.

Conclusions

Phylogenetic utility of cranial bridge characters. While some posterior cranial characters can be coded with little ambiguity in some chalcidoids, there is a high degree of uncertainty concerning many structures, sometimes with no ready solution. This uncertainty mainly involves classification of some cranial bridges as postgenal or hypostomal bridges, especially in cases where the hypostomal sulci are obliterated. It seems best to completely avoid using categorizations that could lead to misleading results. Therefore, the best solution for coding posterior cranial characters of chalcidoids is to define characters using only consistently identifiable structures, such as the hypostomal carina and posterior tentorial pits.

The dichotomy between having a hypostomal bridge and a postgenal bridge is theoretically false, considering that having a hypostomal bridge is just one necessary step towards having a postgenal bridge. This should mean that presence of a postgenal bridge is a meaningful character, but this does not mean that character coding is simple. While both Toryminae and Cleonyminae possess species with a postgenal bridge, for instance, the bridges do not look quite the same in the two subfamilies (Figs 4C-E, 5B). The "postgenal bridge" of *Acmopolynema* is certainly not of the same type at all (Fig. 5A), and should not be coded the same. It is possible that a postgenal bridge has been derived many separate times in Chalcidoidea, but that it is locally informative within groups where it is derived. That said, possession of a postgenal bridge is likely the most valuable character found in this study. It must be coded carefully to prevent rampant homoplasy, probably in such a way that it will support monophyly of Toryminae, for instance, but not necessary support grouping it with Cleonyminae or Eurytominae, since the form of the bridge is different in each of these three subfamilies. The best approach could be to code the finer details of the structures, rather than forcing this purely descriptive category (postgenal bridge) to represent deeper evolutionary significance than it can easily support.

Most other potentially useful characters at the family and subfamily levels are continuous and vague in nature, such as the level of the hypostomal bridge relative to the postgena in Pteromalinae, Eucharitidae, and Perilampidae (Fig. 2E). The extent and shape of the hypostomal carina may be a useful character, but the homology of this potentially composite structure is dubious. It is possible that the parallel carinae extending alongside

the hypostomal bridge in many taxa (Figs 2C-F), may be a different structure from the one that extends between the postgenal bridge and the oral cavity in Eurytominae and Chromeurytominae (Figs 4D-F). The interruption of this carina on the cranial bridge of *Cleonymus* (Fig. 4C) and Toryminae (Figs 4D, 5B) is another potentially useful character. The presence or absence of postgenal lobes is likely usually not a valuable character, because fusion with the hypostomal bridge may be highly variable. This fusion could lead to situations where the lobes cannot be evaluated with any confidence, or even to a dubious determination that they are present based on surface sculpturing patterns.

Placement of the primary posterior tentorial pits apparently does not vary in Chalcidoidea, but the presence of secondary pits or an elongation of the primary pits may be valuable characters (Figs 4D-F, 5B-C), as long as their association with the tentorial bridge is confirmed. The presence of extra, non-tentorial, pits and postoccipital plates are characters that deserve further evaluation to determine what function these structures may have. They may vary even within species, but not enough data exist to confirm this. Finally, the mesal lamellae may be valuable at many levels of taxonomy, especially considering how much they can vary within genera (Lotfalizadeh *et al.* 2007).

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Table 1. Taxa illustrated in this review, with voucher numbers and references indicated where applicable.

Taxon	Superfamily	Family	Subfamily	Voucher number
Apis mellifera Linnaeus	Apoidea	Apidae	Apinae	--
Coccophagus rusti Compere	Chalcidoidea	Aphelinidae	Coccophaginae	
Eretmocerus eremicus Rose & Zolnerowich	Chalcidoidea	Aphelinidae	Eretmocerinae	
Eurytoma sp.	Chalcidoidea	Eurytomidae	Eurytominae	--
Acmopolynema varium	Chalcidoidea	Mymaridae	--	--
Asaphes sp.	Chalcidoidea	Pteromalidae	Asaphinae	
Chromeurytoma sp.	Chalcidoidea	Pteromalidae	Chromeurytominae	
Cleonymus sp.	Chalcidoidea	Pteromalidae	Cleonyminae	
Eunotus sp.	Chalcidoidea	Pteromalidae	Eunotinae	
Scutellista caerulea (Fonscolombe)	Chalcidoidea	Pteromalidae	Eunotinae	
Doddifoenus wallacei Burks & Krogmann	Chalcidoidea	Pteromalidae	Leptofoeninae	
Nasonia vitripennis (Walker)	Chalcidoidea	Pteromalidae	Pteromalinae	
Chiloe micropteron Gibson & Huber	Chalcidoidea	Rotoitidae	--	
Megastigmus transvaalensis (Hussey)	Chalcidoidea	Torymidae	Megastigminae	
Glyphomerus stigma (Fabricius)	Chalcidoidea	Torymidae	Toryminae	--
Torymus sp.	Chalcidoidea	Torymidae	Toryminae	--
Cales noacki Howard	Chalcidoidea	*incertae sedis*	--	
Pelecinus polyturator (Drury)	Proctotrupoidea	Pelecinidae	--	--
Nematus ribesii (Scopoli)	Tenthredinoidea	Tenthredinidae	Nematinae	--
Dolichovespula maculata (Linnaeus)	Vespoidea	Vespidae	Vespinae	--
Xyela julii (Brébisson)	Xyeloidea	Xyelidae	Xyelinae	--
Xyela minor Norton	Xyeloidea	Xyelidae	Xyelinae	--

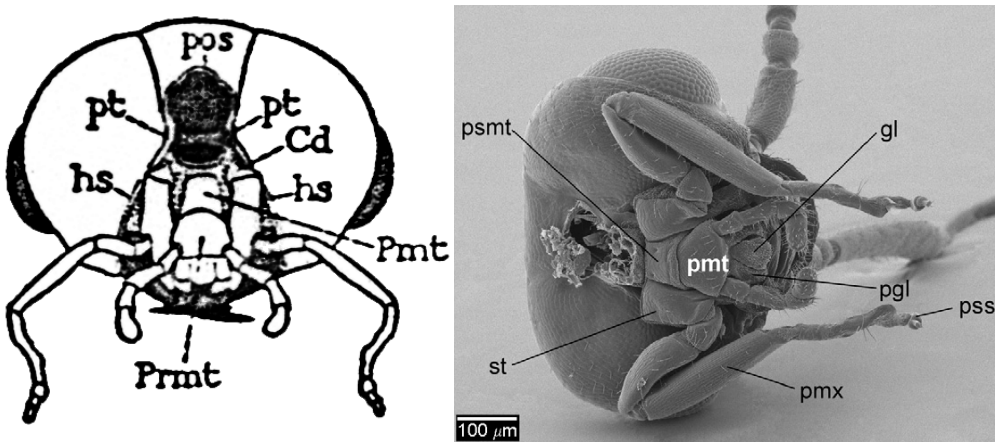

Figure 1: Lack of a cranial bridge in Xyelidae. A. *Xyela minor*, hs = hypostomal sulcus, Pmt = postmentum, pt = posterior tentorial pit (Snodgrass 1960). B. *Xyela julii* head, gl = glossa, pgl = paraglossa, pmt = prementum, pmx = maxillary palp, psmt = postmentum, pss = pseudosegments of palp (Beutel & Vilhelmsen 2007).

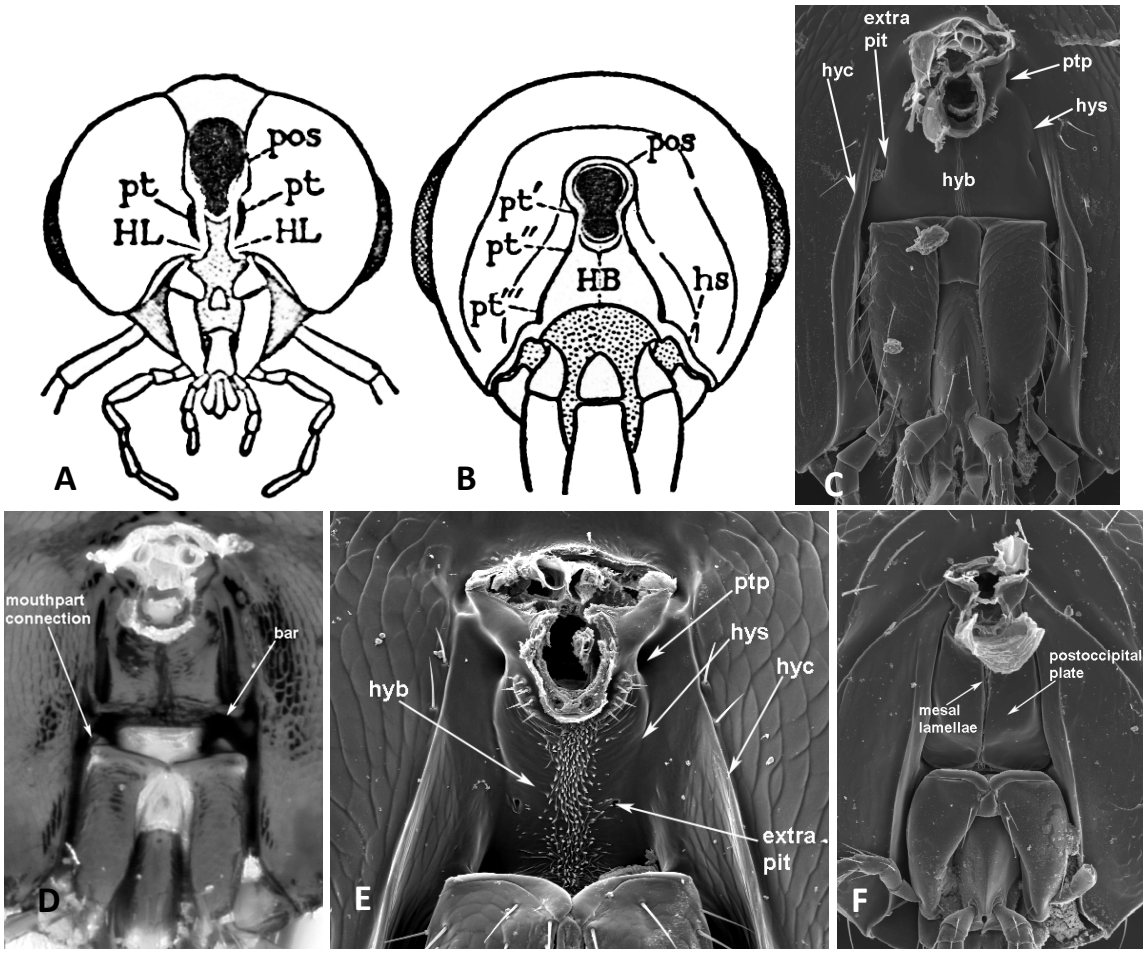

Figure 2: Hypostomal bridges and intermediate forms. A. *Nematus ribesii* with hypostomal lobes but no bridge (Tenthredinoidea: Tenthredinidae), HL = hypostomal lobe, pos = postoccipital sulcus, pt = posterior tentorial pit (Snodgrass 1960). B. *Pelecinus polyturator* (Proctotrupoidea: Pelecinidae), HB = hypostomal bridge, hs = hypostomal sulcus, pt´-pt´´´ = posterior tentorial pits. C. *Eunotus* sp. (Pteromalidae: Eunotinae), hyb = hypostomal bridge, hyc = hypostomal carina, hys = hypostomal sulcus, ptp = posterior tentorial pit. D. *Scutellista caerulea* (Pteromalidae: Eunotinae). E. *Nasonia vitripennis* (Pteromalidae: Pteromalinae). F. *Asaphes* sp. (Pteromalidae: Asaphinae).

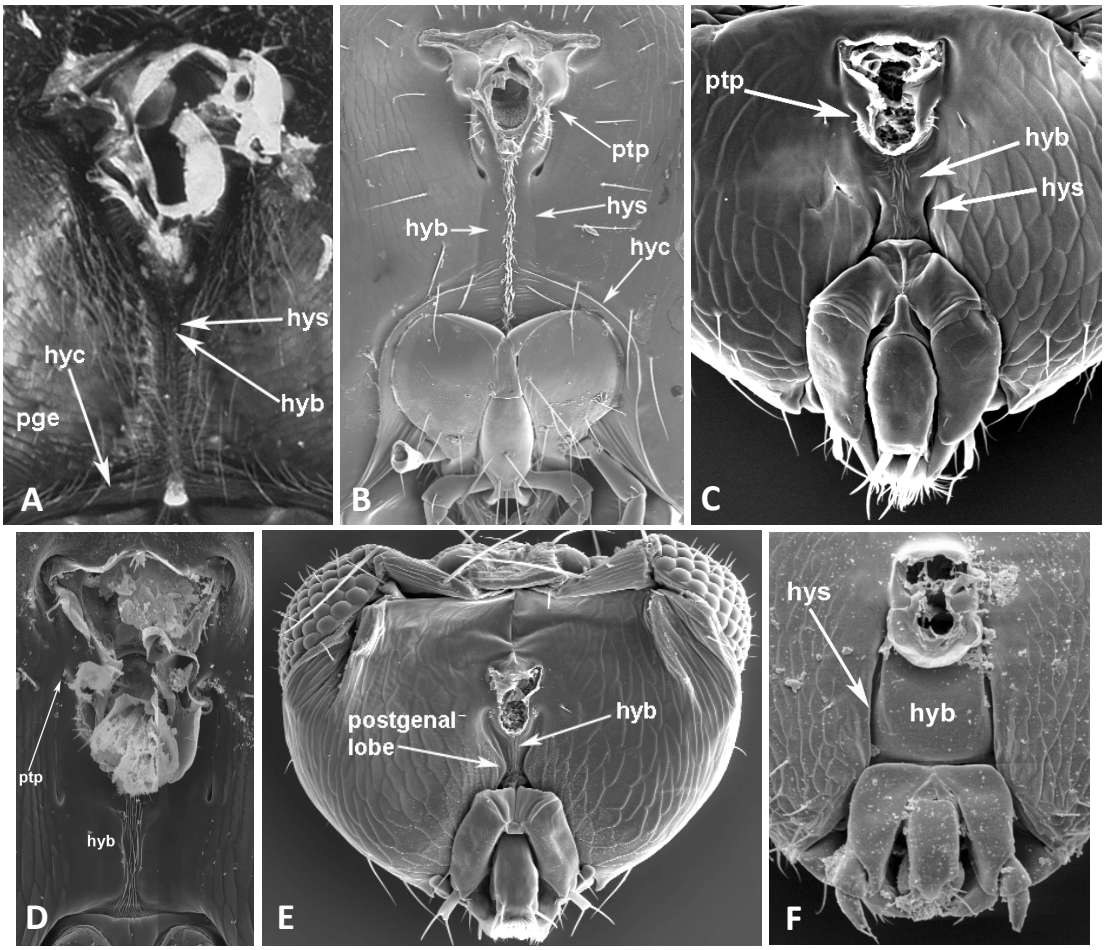

Figure 3. Hypostomal bridges and dubious cases, hyb = hypostomal bridge, hyc = hypostomal carina, hys = hypostomal sulcus, ptp = posterior tentorial pit. A. *Doddifoenus wallacei* (Pteromalidae: Leptofoeninae). B. *Megastigmus transvaalensis* (Torymidae: Megastigminae). C. *Eretmocerus eremicus* (Aphelinidae: Eretmocerinae). D. *Coccophagus rusti* (Aphelindae: Coccophaginae). E. *Cales noacki* (Chalcidoidea: *incertae sedis*). F. *Chiloe micropteron* (Rotoitidae).

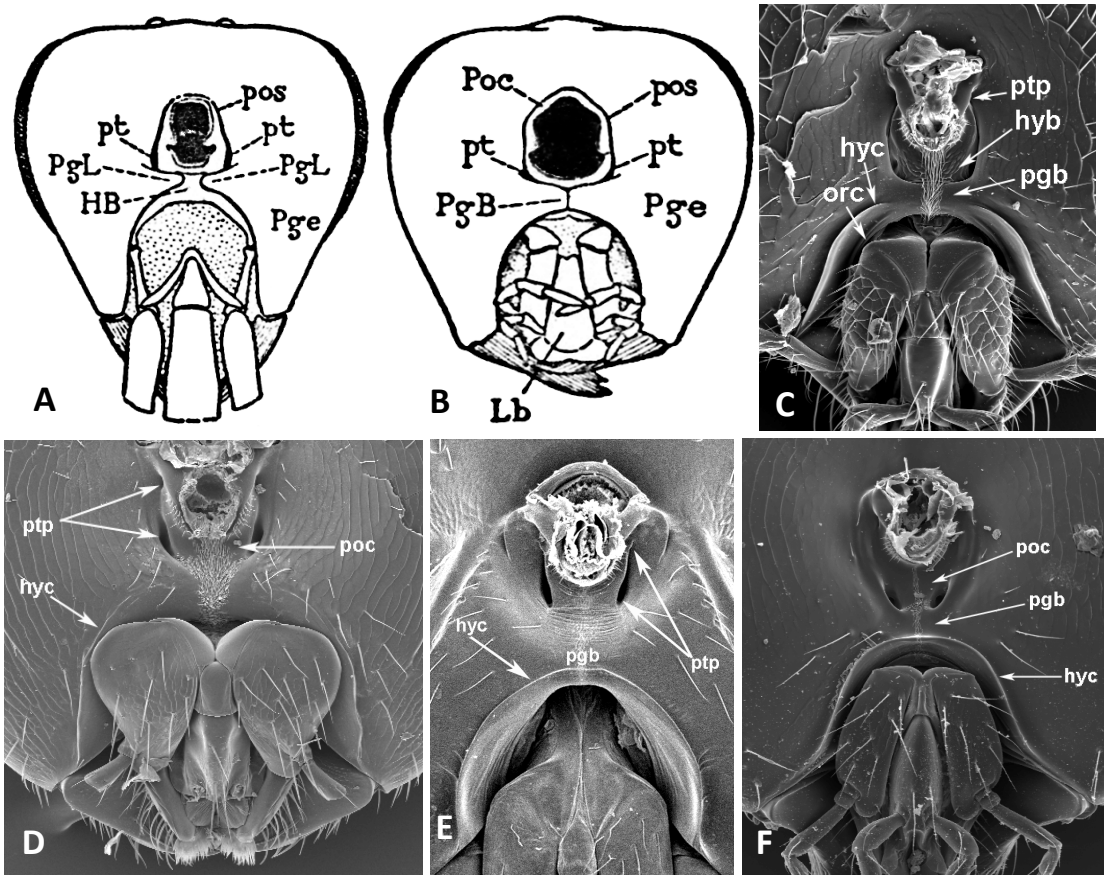

Figure 4. Postgenal bridges and intermediate states. A. *Apis mellifera* (Apoidea: Apidae) with postgenal lobes partially encroaching over a hypostomal bridge, HB = hypostomal bridge, Pge = postgena, PgL = postgenal lobe, pt = posterior tentorial pit (Snodgrass 1960). B. *Dolichovespula maculata* (Vespoidea: Vespidae) with a complete postgenal bridge (Snodgrass 1960). C. *Cleonymus* sp. (Pteromalidae: Cleonyminae), hyb = hypostomal bridge, hyc = hypostomal carina, orc = oral carina, pgb = postgenal bridge, ptp = posterior tentorial pit. D. *Torymus* sp. (Torymidae: Toryminae). E. *Eurytoma* sp. (Eurytomidae: Eurytominae). F. *Chromeurytoma* sp. (Pteromalidae: Chromeurytominae).

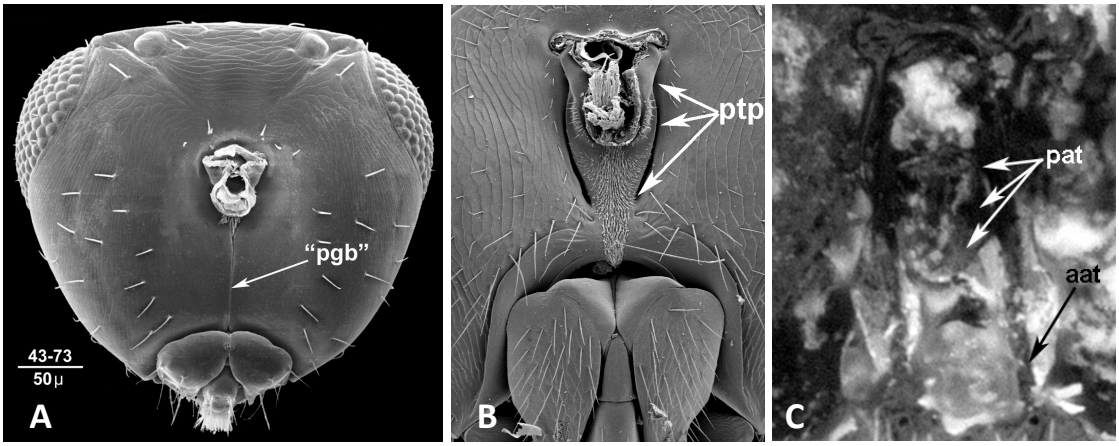

Figure 5: Postgenal bridges and dubious cases. A. *Acmopolynema varium* (Mymaridae), "pgb" = near postgenal bridge covering mesal lamellae. B-C. *Glyphomerus stigma* (Torymidae: Toryminae). B. External surface, ptp = posterior tentorial pit forming an extended sulcus. C. Internal surface, aat = anterior tentorial arm, pat = posterior tentorial arm as it attaches to the external surface of the head as an elongate structure.