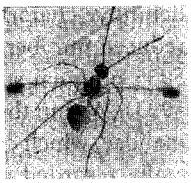
Molecular Systematics of the Chalcidoidea Using 28S-D2 rDNA



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Introduction

Chalcidoidea are recognised to contain somewhere on the order of 18,500 described species distributed in 20 families and 89 subfamilies (Noyes 1990; Gibson *et al.* 1999). Estimates of the number of species range somewhere between 60 000 and 100 000 (Noyes 1978, 1990; Gordh 1979). Ecologically and economically, they are one of the most important groups for control of other insect populations (Noyes 1978; LaSalle 1993). However, after more than 200 years of descriptive work, the taxonomy and classification of Chalcidoidea is still unresolved, frequently revised, and largely lacking a consensus in understanding of monophyly at higher taxonomic levels (Gibson *et al.* 1999). Monophyly of many higher taxonomic groups, including larger family groups such as Eulophidae, Aphelinidae, Pteromalidae and Eupelmidae, has not been determined (Gibson 1989, 1990, 1995; Noyes 1990; Heraty *et al.* 1997). As the taxon of focus becomes narrowed to either subfamilies or families with few included genera, the problem is not one of defining monophyly but of positing relationships to other groups of Chalcidoidea. New characters for the analysis of relationships are necessary to solve these problems but only after thorough surveys are undertaken to more accurately estimate the real distribution, homology and transformation of each feature (Heraty *et al.* 1994, 1997).

Until recently, classification of Chalcidoidea has been based on morphological similarities and differences rather than on shared apomorphies. As a result, some families are generally regarded as paraphyletic, if not polyphyletic (Woolley 1988; Gibson 1989, 1990; Noyes 1990). Above the family level, inclusion of Mymarommatidae or Mymaridae in Chalcidoidea has been debated (Kozlov & Rasnitsyn 1979; Rasnitsyn 1980; Gibson 1986), with Mymarommatidae currently excluded as a separate superfamily (Goulet & Huber 1993). The number of chalcidoid families has stabilised at 20 (Goulet & Huber 1993), but disagreement still persists over placement of several subfamilies (e.g. Akapalinae, Calesinae, Chrysolampinae, Eriaporinae, Eunotinae, Philomidinae and Sycoryctinae). The inability to resolve the placement of these taxa into family-level groups using available morphological criteria clearly indicates that new additional character systems are needed.

Three major groups of taxa are currently considered in the Chalcidoidea. These groups are usually referred to as the mymarid, eulophid and pteromalid lineages, with the pteromalid lineage often subdivided into chalcidid, torymid and encyrtid sub-lineages. Trichogrammatidae and Aphelinidae are usually placed in the 'eulophid lineage' along with Elasmidae, Eulophidae and Signiphoridae. This eulophid group has not been characterised as monophyletic based on

definitive characters but rather on a preponderance of reductions (e.g. fewer antennal articles, reduced number of tarsomeres). Combined, the eulophid lineage accounts for 675 genera and 5955 nominal species of Chalcidoidea (Noyes 1978; LaSalle & Gauld 1991; Goulet & Huber 1993). Within the 'pteromalid lineage', problems centre around lack of resolution within Pteromalidae, which contains between 32 and 42 subfamilies (Gibson *et al.* 1999). This group is generally regarded as being paraphyletic or polyphyletic and the typical 'dumping ground' for unplaced taxa within the Chalcidoidea. Embedded within this lineage are the Eupelmidae and Encyrtidae. The three subfamilies of Eupelmidae are likely a grade taxon closely related to the pteromalid subfamily Cleonyminae according to Gibson (1990). Monophyly of Encyrtidae is demonstrated by several external (Noyes & Hayat 1994) and internal structures (Heraty *et al.* 1997). Most genera of Encyrtidae can be assigned to one of the two recognised subfamilies; however, the relationships among tribes within Encyrtidae are unclear (Trjapitzin & Gordh 1978; Noyes & Hayat 1994). Similar problems of classification exist for almost all higher level taxa within the Chalcidoidea.

Convergence and extreme divergence of morphological traits lead to much of the taxonomic confusion. In the Chalcidoidea, a large number of similar characters are considered non-homologous, even though they have similar structure; for example, presence of antennal rami, reduced number of tarsomeres, loss of the mesofurcal bridge, presence of a linea calva on the fore wing, and enlargement of the acropleuron (Gibson 1986, 1989; Heraty *et al.* 1997). Some features, such as the expanded mesopleuron of the Eupelmidae and Encyrtidae, can be dismissed as being convergent on the basis of detailed morphological studies (e.g. Gibson 1989), but others such as the linea calva of Aphelinidae and Encyrtidae are structurally almost identical, causing problems with their classification. In some Aphelinidae and Encyrtidae, a peculiar socketed tooth on the mandible is extremely similar in both structure and function, but must be interpreted as being convergent within these two divergent lineages (Heraty & Schauff 1998). Each of the above features is 'locally' important for identification of monophyletic lineages, but at the superfamily level, or 'globally', they are presumed convergent.

Considerable discussion exists on the classification and placement of taxa within the Chalcidoidea (cf. Bouček 1988; Hayat 1994; Gibson *et al.* 1999). However, character-based phylogenetic studies have usually been confined to assessment of relationships within families, often with some reference made to characteristics of other closely related families. A few papers focus on relationships at the family level, but again these discuss relationships or characteristics of only a limited number of families (LaSalle & Noyes 1985; Bouček & Noyes 1987). At the superfamily level, only one study has focused on a character system across a large representation of taxa (Heraty *et al.* 1997). Noyes (1990) presented the only 'dendrogram' of relationships so far (cf. Heraty *et al.* 1997), but this was admittedly an intuitive hypothesis and not based on synapomorphies. Some major problems facing a morphologically based phylogenetic analysis of the Chalcidoidea are simply the sheer number of taxa, the extreme diversity of form, and the tendency towards reduction of similar characters in unrelated taxa. Even complex morphological features, such as an enlarged mesopleuron and jumping mechanisms in subfamilies of Eupelmidae, some Aphelinidae, Tanaostigmatidae and Encyrtidae, may be convergent (Gibson 1989).

Molecular systematics offers a different set of characteristics that may be used to assess hypotheses of monophyly. Major lineages of Hymenoptera have been surveyed using mitochondrial 16S rDNA sequences (Derr *et al.* 1992a, 1992b; Dowton & Austin 1994, 1995) to assess earlier hypotheses of relationships based on morphological characters and to evaluate hypotheses of

single or multiple origins of parasitism within Hymenoptera. Inferences of evolutionary affiliations of different studies using nucleotide sequences have been inconclusive in demonstrating relationships among sawflies but appear to be relatively congruent for relationships among Apocrita (Carmean *et al.* 1992; Dowton & Austin 1994, 1995). Within these analyses Chalcidoidea have been shown to be monophyletic, but this inference was based upon at most three species (Derr *et al.* 1992a, 1992b; Dowton & Austin 1994, 1995).

Within Chalcidoidea, several studies have begun to address higher relationships but usually with only a few taxa. Different species and populations of *Nasonia* Ashmead and *Trichomalopsis* Crawford (Pteromalidae) were analysed using nucleotide sequences of the ITS2 and 28S-D2 regions of the rRNA transcript (Campbell *et al.* 1993). Using either *Trichomalopsis* as an outgroup for the ITS2 data or *Melittobia* Westwood (Eulophidae) as an outgroup for the more conserved 28S data, the relationships among species of *Nasonia* were the same (*N. vitripennis* (Walker) + (*N. giraulti* Darling + *N. longicornis* Darling)). These results were concordant with phylogenetic trees obtained for their cytoplasmic incompatability bacteria of the genus *Wolbachia* (Breeuwer *et al.* 1992). Relationships among several species of *Trichogramma* Westwood were analysed using ITS2 (Frenk *et al.* 1996). Machado *et al.* (1996) used mitochondrial 12S to analyse relationships of the subfamilies Agaoninae, Otitisellinae, Sycoryctinae and Sycophaginae (Agaonidae) with Doryctinae (Braconidae) as an outgroup. Similar results were obtained from analyses of the 28S-D2 region using more reasonable outgroups (Eurytomidae, Figitidae and Ichneumonidae) and other representatives of Agaonidae *sensu* Bouček (1988) (at least two each of Epichrysomallinae, Otitisellinae, Sycophaginae, Sycoryctinae and Sycoecinae) (Rasplus *et al.* 1998). The results suggest that Agaonidae is not monophyletic with Agaoninae having a very distant relationship with other subfamilies currently included in Agaonidae.

The D2 expansion region of 28S rDNA was shown previously to have one to six substitutional differences between species of *Nasonia*, 10–11 between *Nasonia* and *Trichomalopsis* (both Pteromalinae) and 42–46 between these Pteromalidae and *Melittobia* (Eulophidae) (Campbell *et al.* 1993). This degree of genetic variation was considered to provide an appropriate phylogenetic signal at the generic and family levels, and was chosen for a broader molecular phylogenetic analysis of the Chalcidoidea reported here. N.B. The authors for genera and species are given in Appendix 1.

Materials and Methods

Samples

Voucher specimens of almost all taxa sampled (see Appendix 1) to date are deposited in the Entomology Research Museum at the University of California, Riverside or the collection at INRA, Montpellier, France. A few specimens of species not commonly collected but easily identified do not have vouchers. Sequences for several eulophid and encyrtid taxa were provided by Donald Quicke (Imperial College, London; identified by a 'q' following the generic name in Fig. 1), and sequences for *Uscana* and *Trichogramma fuentesi* Torre were provided by Richard Stouthammer (Wageningen Agricultural University, The Netherlands). A goal of the taxon sampling was to obtain sequences for two or more representatives of each higher taxonomic group (tribe or subfamily). Of the 109 species analysed to date, six belong to the outgroups Cynipoidea and Scelionidae, and one species, *N. vitripennis*, was duplicated in the analysis from different populations (United States and France), and sequenced independently in the Campbell and Rasplus

laboratories. Eighteen of the 20 families of Chalcidoidea are represented, but only 39 (43.8%) of the 89 subfamilies.

Methods

Specimens were killed and stored by freezing at –85°C or were collected into 80–100% EtOH. DNA extractions followed the methods outlined in Campbell *et al.* (1993). PCR was performed in 25µl reactions using a GeneAmp® Kit (Perkin Elmer Cetus, Norwalk, Conn.). The majority of samples were cloned using a plasmid vector (Invitrogen TA Cloning™ System), but 28 taxa were sequenced directly. Primer sequences for PCR amplification of the D2 expansion region of 28S rDNA and direct sequencing are: forward primer 5'-CGT GTT GCT TGA TAG TGC AGC-3', and reverse primer 5'-TTG GTC CGT GTT TCA AGA CGG-3'. Sequencing was done initially using ^{33}P-dATP based autorads on a Genomyx thermal sequencer and later using an infrared dye system on a LI-COR 4200 automated sequencer. The universal Sp6 and T7 primers were used to sequence plasmid 28S-D2 inserts. Both top and bottom strands were completely sequenced.

Nucleotide sequences were aligned initially using the ClustalW subprogram on MacVector v 6.5 (Oxford Molecular) with the majority of remaining sequences aligned manually. Gaps were treated as missing values. Phylogenetic analyses were performed on PAUP 4.0b2a (Sinauer Associates, Inc.) using the random addition sequence search algorithm with 25 replicates through three iterations beyond the point where there was no change in tree length. Each iteration was started using the seed number from the shortest tree of the previous iteration. Successive approximations character weighting (Carpenter 1988) was performed on the shortest tree using the maximum value of the rescaled consistency index and a base weight of 1000; hereafter referred to as the reweighted analyses. Successive iterations did not produce a stable result (increasing length with each iteration), and the final tree was selected after four iterations, the last three of which produced a tree of the same length (the same tree) when all characters were reweighted to unity.

Results

The aligned 28S-D2 data matrix consisted of 863 bases, of which 299 were constant and 158 autapomorphic. A total of 406 informative sites were found among chalcidoids, which is more than found in the mitochondrial 16S region (217 across Apocrita; Dowton & Austin 1995). One tree was recovered from the parsimony analysis with a length of 3437 steps, consistency index of 0.274 and a retention index of 0.529 (Fig. 1). After successive weighting, a single tree of 3457 steps with a slightly lower consistency index (0.272) and retention index (0.525) was obtained. The reweighted tree was different from the most parsimonious tree, however, many of the relationships were recovered in both trees (bold lines, Fig. 1). The two major apical clades indicated in Figure 1 were recovered in both analyses but three taxonomic groups (Cleonyminae + Chalcidinae, Eucharitidae, and *Eusandalum*) shifted to the other of these two clades in the reweighted tree (marked by an asterisk). In all analyses (including our studies with fewer taxa or different alignments), Mymaridae are the sister group to remaining Chalcidoidea and *Cales* (Calesinae; currently unplaced at family level) is positioned basally.

Of the genera represented by more than one species, none of the included species were misplaced into non-congeneric taxa. Forty-four species representing 18 genera were, respectively, placed as monophyletic in both unweighted and reweighted parsimony analyses. Species of six genera (*Brachymeria*, *Eurytoma*, *Megastigmus*, *Nasonia*, *Podagrion* and *Spalangia*) were sequenced

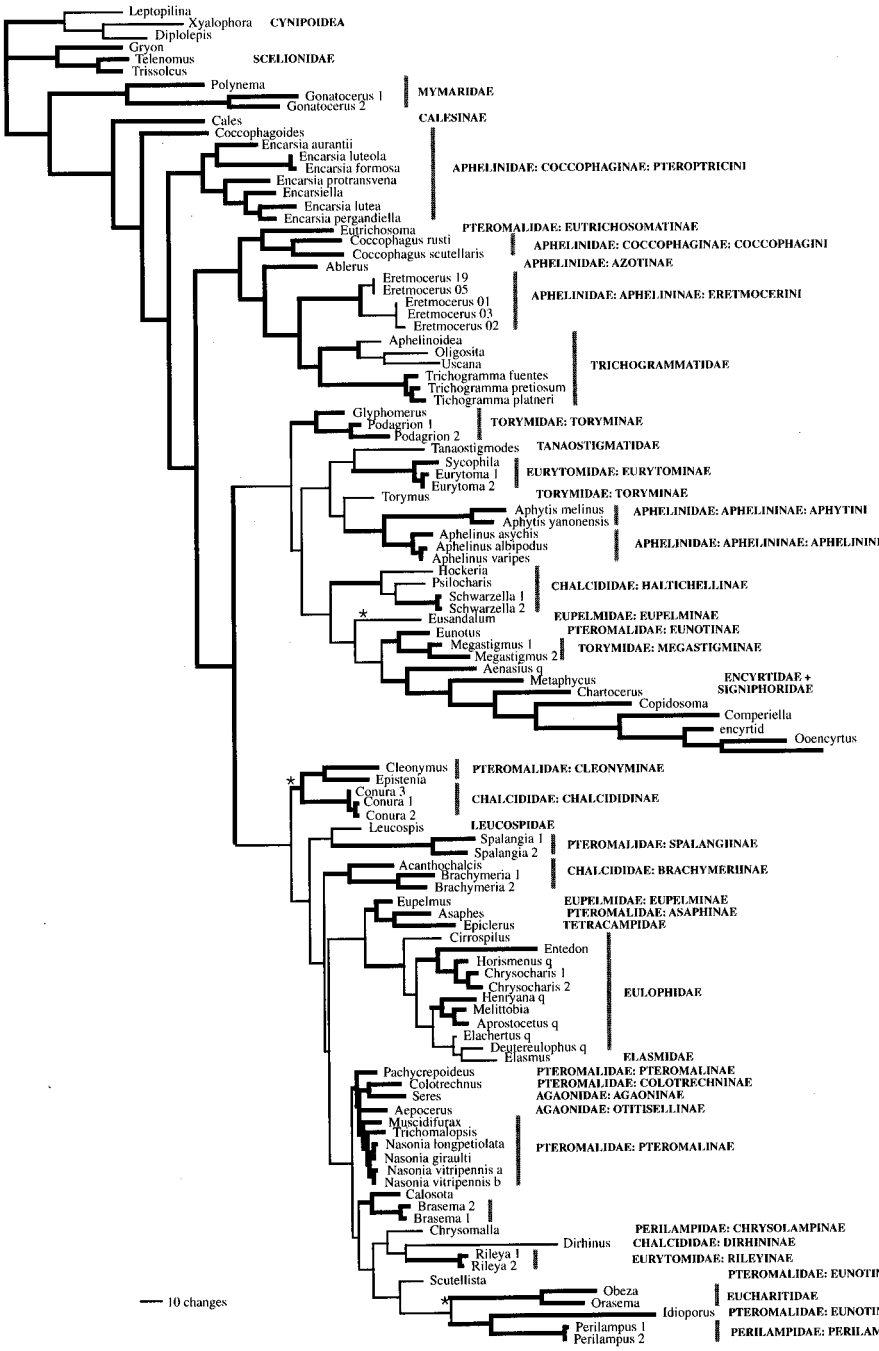

Figure 1 Single most parsimonious tree (phylogram) using the 28S-D2 region (Length 3437 steps, consistency index 0.27, retention index 0.53). Bold lines represent branches supported by both parsimony and successive approximations weighting analyses. Thin lines were supported only in the parsimony analysis. Taxonomic groups indicated by shaded bars. Asterisk indicates clades or taxa that join a different major clade in the reweighted tree.

separately as blind tests to check for sequence fidelity in the Campbell and Rasplus laboratories. The molecular data set also placed the six species representing *Encarsia* as monophyletic, but this clade also included *Encarsiella*, a relationship which is supported by another study incorporating more species of *Encarsia* (Babcock & Heraty unpublished).

Of 12 families represented by more than one genus, three were monophyletic: Mymaridae, Trichogrammatidae and Eucharitidae. Eulophidae was monophyletic but included *Elasmus* (Elasmidae) grouped within the Eulophinae. The placement of *Elasmus* within Eulophinae rather than as a sister group of Eulophidae was proposed initially by John Noyes (pers. comm.) based on similar host characteristics. The inclusion of this and additional species of *Elasmus* within Eulophinae has been supported in a more complete analysis of eulophid genera using the same genetic region (Gauthier *et al.* 2000). Encyrtidae was also monophyletic, but included *Chartocerus* (Signiphoridae). We have attempted to verify the sequence of *Chartocerus* with that of two other species of *Signiphora* but have had difficulties with amplifying the region. Until the sequence is verified this placement is tentative.

Of subfamilies represented by multiple genera, seven were monophyletic: Aphelininae (based only on Aphytini and Aphelinini), Haltichellinae, Cleonyminae, Brachymeriinae (*sensu lato*), Tetrastichinae and Entedoninae, and Pteromalinae (excluding *Pachycrepoideus*). The Eulophinae (*Cirrospilus, Deuteroeulophus* and *Elachertus*) was polyphyletic in the unweighted analysis but monophyletic (including *Elasmus*) in the reweighted analysis.

Not all higher taxa were coherently resolved. None of the three genera of Eunotinae (*Scutellista, Eunotus* and *Idioporus*) grouped together. Toryminae were separated into two groups (*Glyphomerus* + *Podagrion* and *Torymus*) in both the unweighted and reweighted analyses. Within Eupelmidae, neither Eupelminae (*Eupelmus* and *Brasema*) or Calosotinae (*Calosota* and *Eusandalum*) formed a subfamily grouping, although *Brasema* formed a group with *Calosota*. The Coccophaginae (Aphelinidae) were split into a paraphyletic Pteroptricini (*Coccophagoides, Encarsia* and *Encarsiella*) at the base of the tree, and Coccophagini (*Coccophagus*) which was grouped with *Eutrichosoma* (Pteromalidae: Eutrichosomatinae) on both trees; the latter grouping being an unlikely hypothesis. Of three families expected to be monophyletic based on a consensus of findings from morphological studies, Torymidae (Toryminae and Megastigminae), Chalcididae (Chalcidinae, Brachymeriinae, Haltichellinae and Dirhininae) and Eurytomidae (Rileyinae and Eurytominae) were not. Also, Chrysolampinae, which are generally assigned to either Pteromalidae or Perilampidae, were not affiliated with either group. Eupelmidae were scattered across the tree and showed no affinities with Cleonyminae, Tanaostigmatidae or Encyrtidae. The various tribes of Aphelinidae also were scattered across the tree, as were the Pteromalidae. However, as might be expected for such a diverse assemblage, the latter result was not unexpected. Surprisingly, the subfamilies of Chalcididae showed no immediate common affiliation with each other, although genera within each subfamily grouped together.

The higher level relationships, basically the backbone of the cladogram, are generally unstable and can change if fewer taxa or different alignments are considered. Different rearrangements usually correspond with relationships supported only in the parsimony tree (thin branches, Fig. 1), although even some of the well-supported relationships (bold branches) can change. Some relationships are very stable even with different taxa or alignments. Across different analyses, the relationship of *Ablerus* (Azotinae) and *Eretmocerus* + Trichogrammatidae remain unchanged. Also, *Cales* (Calesinae) and the Pteroptricini are usually placed basally, although in some analyses these are replaced as basal by *Perilampus* and *Idioporus*. Some relationships

change depending on the taxa being included. For example, a sister group relationship between Tanaostigmatidae and Encyrtidae (LaSalle & Noyes 1985) was not recovered in this analysis, but did occur in some of the earlier analyses with fewer taxa. Adding more taxa may recover this relationship. Notably, across the Chalcidoidea, the number of base changes does not always correlate with consistency of relationships. For example, the clade formed by Pteromalinae, Colotrechninae, Agaoninae and Otitisellinae has relatively few substitutional differences but the relationships remain consistent in all analyses, especially in the clade that includes *Aepocerus* and *Nasonia*.

Discussion

An important goal of this study is to represent all of the terminal taxa of interest (families, subfamilies or tribes) by two or more divergent taxa, as proposed by Wheeler *et al.* (1993). Where we have a high degree of confidence in the relationships of closely related taxa based on morphological evidence, for example two unequivocally placed genera of the same subfamily, they should group together on trees produced by molecular evidence (cf. Patterson *et al.* 1993). If not, then we need to either re-evaluate our initial assumptions of relationship or quality of the molecular data. If we can accept our initial assumptions of relationships, then informative nucleotide changes are best if they are shared by all included members of a taxon (synapomorphic) and, potentially, worst if they are shared by few members of that same taxon and a distantly related taxon (homoplastic). Presumably, derived character states shared by divergent groups within a taxon are more likely to be indicative of synapomorphic changes rather than autapomorphic changes.

Results from the 28S-D2 data set are encouraging and indicate that the region is appropriate for analysis of chalcidoid taxa, which may have diverged in the Upper Cretaceous or early Tertiary. Including outgroup taxa, 80% (88/110) of the species are placed into some form of realistic grouping (generic or family group taxon) based on morphological evidence. From our analysis, there was no support for the so-called eulophid or pteromalid lineages. Fourteen subfamilies are represented by only a single species and essentially remain unverified in the data set. The 28S-D2 dataset shows greatest support at the subfamily and generic levels, although five families, Mymaridae, Encyrtidae (+ *Chartocerus*), Eulophidae (+ *Elasmus*), Eucharitidae, and Trichogrammatidae are placed as monophyletic or paraphyletic with one other taxon. Based on morphological evidence, four families expected to be monophyletic (Chalcididae, Eurytomidae, Perilampidae and Torymidae) were not. Eunotinae was the only subfamily not having any of the species grouped together. In part this lack of a coherent relationship may be justified based on recent discussions on the placement of *Idioporus*, in which they were put into Eunotinae as a distinct tribe, but only after consideration of shared characteristics with Eriaporinae (unplaced at the family level), Aphelinidae and Eulophidae (LaSalle *et al.* 1997).

The genera of Eulophidae were all placed into a monophyletic group with monophyly of the Tetrastichinae and Entedoninae supported, while Eulophinae were supported only in the reweighted analysis. Elasmidae are usually placed as the sister group of Eulophidae based on a similar reduction of antennal segments and a simple calcar. The association of Elasmidae with Eulophinae has not been proposed on morphological characters, but instead due to a similar habit of attacking leaf-mining Lepidoptera (Noyes pers. comm.). The results using molecular data support use of character reductions for defining Eulophidae.

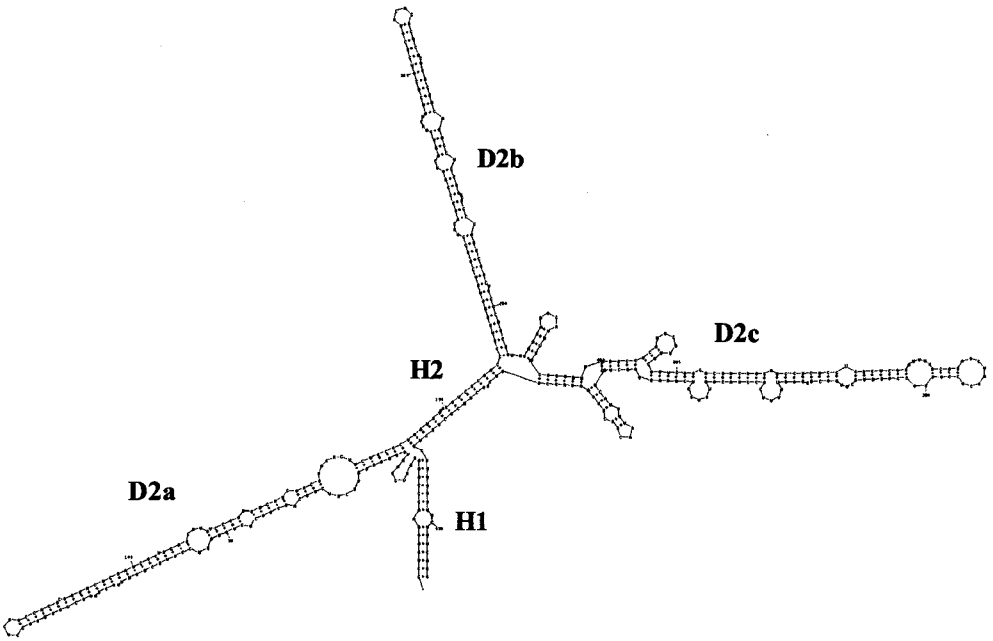

Figure 2 Secondary structure model for D2 expansion segment of 28S rRNA of *Psilochalcis* sp. (Chalcididae: Haltichellinae). Overall structure inferred from thermodynamic folding of the full sequence of the D2 region using *mfold* version 2.3. Individual substructures within subdomains were confirmed by folding respective nucleotide sequences using the RNA folding program in MacDNASIS and examination of compensatory substitutions (Roussett *et al.* 1991). Nomenclature of subdomains and helices are according to Michot & Bachellerie (1987).

Some groups, such as Aphelinidae, Eupelmidae and Pteromalidae are not supported by morphological synapomorphies (Heraty *et al.* 1997; Gibson *et al.* 1999). Thus, absence of monophyly for these groups in an analysis using molecular data is not unwarranted, although Eupelmidae should have been grouped together at least as a paraphyletic assemblage (Gibson 1995). Generally, tribes of Aphelinidae are monophyletic except for Pteroptricini, which was paraphyletic at the base of the tree. Only Aphytini and Aphelinini were monophyletic. A sister group relationship between *Eretmocerus* and Trichogrammatidae was supported in all molecular analyses to date, including a separate analysis using the highly variable region synonymous to the E21 helix of 18S rRNA (Campbell & Heraty unpublished). *Eretmocerus* has usually been placed in the Aphelininae, recently within a separate tribe, the Eretmocerini (Hayat 1998). However, Shaffee and Rizvi (1990) proposed a classification in which *Eretmocerus* was closer to Trichogrammatidae, and Heraty *et al.* (1997) found shared characteristics of the mesofurca between the two groups. We are unaware of any morphological support for a sister group relationship between *Ablerus* (Azotinae) and *Eretmocerus* + Trichogrammatidae as inferred on the molecular tree. Azotinae is usually placed as closer to Signiphoridae (Woolley 1988), Coccophaginae (Hayat 1994) or Aphelininae (Heraty *et al.* 1997).

Analysing the 28S-D2 data within a more restricted taxonomic range may be better for addressing relationships among some of the problematic taxa. For example, Chalcididae are a demonstrably monophyletic group sharing several derived characteristics, including a plate-like labrum, non-overlapping clypeus, reduced prepectus, lateral scutellar arch and enlarged hind

femora (Wijesekara 1997). Few systematists would doubt the monophyly of included members, yet none of the subfamilies formed a coherent 'chalcidid' assemblage in our molecular based analysis including all chalcidoids. However, monophyly of Haltichellinae was supported, and relationships of included genera match those based on morphological hypotheses (Wijesekara 1997). Dirhininae was represented by only one species in this analysis. The three species of *Conura* in our analysis are members of the side-group of species within Chalcidini and are not representative of the diversity within the tribe. *Brachymeria* and *Acanthochalcis* were previously treated as the Brachymeriinae, but were recently allocated to Chalcidinae as Brachymeriini and Cratocentrini (Boucek 1988). Recently each was elevated to subfamily status by Wijesekara (1997). Results of these analyses suggest *Acanthochalcis* and *Brachymeria* are closely related, but their relationship to Chalcidini is uncertain.

An effort was made to analyse the chalcidid taxa independent of other chalcidoids, but including traditionally accepted sister groups of Eurytomidae and Leucospidae. In this analysis, the 28S-D2 sequences were aligned so that homologous base positions were matched to the greatest extent possible. This alignment was based on homology of secondary structure and substructures of rRNA synonymous to the D2 region. Secondary structures were inferred based upon thermodynamic folding using *mfold* v.2.3 (Zucker *et al.* 1999) via the *mfold* server (http://mfold1.wustl.edu/~mfold/rna/form1.cgi). Confirmation of certain inferred substructures using shorter sequences was made with the RNA-folding subprogram of MacDNASIS® (Hitachi Software). The overall secondary structure of the chalcidid D2 region (Fig. 2) corresponded to three subdomains inferred for *Drosophila* (Linares *et al.* 1991; Rousset *et al.* 1991) and aphidiines (Belshaw & Quicke 1997). The fewer number of taxa examined in this subset of the chalcidoid dataset enabled a more robust alignment of homologous nucleotide positions. These positions could be rigorously ascertained according to homology of subdomain structure and retention of certain substructures (bulges, loops, etc), which show much broader variation among all chalcidoids. Moreover, it was determined that alignment based on 'similarity' using *ClustalW* did not always align homologous positions according to secondary structure.

Despite this independent rigor given to sequences of Chalcididae, Leucospidae and Eurytomidae, the same problems of non-monophyly occurred. Eurytomids were polyphyletic and the haltichellines were the sister to all other chalcids and the leucopsid. When these taxa were scored according to the morphological character matrix presented by Wijesekara (1997) a monophyletic eurytomid was sister to Leucospidae + Chalcididae, with internal arrangement of Chalcididae almost concordant with that of Wijesekara (1997) and Haltichellinae as the distal lineage. Character analysis of the morphological and nucleotide datasets provided some explanation for the different topologies generated. While the morphological dataset provided three synapomorphies supporting Chalcididae, there were zero in the nucleotide dataset. The morphological dataset provided one synapomorphy to support Leucospidae + Chalcididae and the nucleotide dataset provided zero. Conversely, the nucleotide dataset provided three molecular synapomorphies supporting *Brachymeria* + *Acanthochalcis* (Brachymeriinae *sensu lato*), while there were no morphological synapomorphies for this clade. Haltichellinae was supported by both datasets, as was also the Hybothoricini, suggesting that current haltichelline taxonomic groupings are probably accurate. Interestingly, when both datasets were combined a currently preferred set of relationships (except for equivocal placement of *Brachymeria* and *Acanthochalcis*) was resolved (Campbell & Heraty unpublished).

The observed congruence between the molecular hypotheses generated from the 28S-D2 region and the accepted morphological-based classifications for some of the included taxa increase our faith in this region as a reasonable estimator of relationships for some families and almost all

subfamilies. However, we must regard this analysis as preliminary. Better resolution of some groups, such as Chalcididae, Eurytomidae and Torymidae, will be necessary before we can begin to accept relationships postulated for higher level taxa. Is it a case of adding more molecules (new regions), adding morphological data, or adding more taxa? Additional genes, based on the same limited sampling of taxa, are likely to duplicate inadequacies of the 28S data set. Adding morphological characters to the matrix is obviously a necessary step towards fully understanding the relationships of this group. However, less than 45% of chalcidoid subfamilies are represented in the current analysis. For Pteromalidae, only five of 32 subfamilies are included. Within some groups, representation is poor. For example, sequences of Chalcidini are based only on members of one clade, the *Conura* side-group, and Perilampidae is represented only by two species of the *Perilampus fulvicornis*-group. Other studies demonstrate that an adequate sampling of taxa is most important to provide a proper estimate of the phylogeny (Graybeal 1998; Poe 1998). Hence, future emphasis on increased taxon sampling, particularly for the Pteromalidae, Chalcididae, Eurytomidae and Torymidae may be the best approach for further resolving higher-level relationships of Chalcidoidea.

Summary

Based on cladistic analysis of nucleotide sequences of the 28S-D2 expansion region we find:

1) Chalcidoidea are monophyletic, and Mymaridae are the sister group to the remaining Chalcidoidea. This relationship agrees with recent morphology-based hypotheses (Gibson *et al.* 1999).

2) Eulophidae (including *Elasmus*) are monophyletic. This is consistent with morphological data, although hypotheses of monophyly are based only on reductions or losses of characters (number of tarsal segments and flagellomeres and reduced fore tibial spur).

3) Elasmidae are closest to the Eulophinae within the Eulophidae.

4) *Eretmocerus* and Trichogrammatidae are sister groups. This relationship was proposed by Shafee & Rizvi (1990) without supporting character evidence, and again more recently by Heraty *et al.* (1997) using similar structure of the mesofurca.

5) The families Mymaridae, Eucharitidae and Trichogrammatidae, and the subfamilies Aphelininae (excluding Eretmocerini), Cleonyminae, Haltichellinae and Brachymeriinae (*sensu lato*) are all supported as monophyletic using more than one generic exemplar.

6) Encyrtidae is rendered paraphyletic by the genus *Chartocerus* (Signiphoridae) in all analyses. The *Chartocerus* sequence has been checked with other specimens, but an additional sequence from another genus of signiphorid is needed to verify its placement.

7) Aphelinidae is never supported as monophyletic. Aphelininae (excluding *Eretmocerus*) are monophyletic in all analyses, but the monophyly of the Coccophaginae (as represented by *Encarsia, Coccophagoides, Coccobius* and *Coccophagus*) is transient. No morphological evidence supports the monophyly of Aphelinidae (Gibson *et al.* 1999).

8) Pteromalidae are not monophyletic. However, only five of 32 subfamilies are represented.

9) Chalcididae, as represented by Brachymeriinae (*Brachymeria, Acanthochalcis*), Chalcidinae (*Conura*) and Haltichellinae (*Psilocharis, Hockeria, Schwarzella*), is not monophyletic. This was a surprising result considering strong morphological evidence supporting this

assemblage (Wijesekara 1997). The combination of molecular and morphological data sets resolved relationships to current consensus.

10) Agaonidae is polyphyletic in accordance with recent findings of Rasplus *et al.* (1998).

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References

Belshaw, R. & Quicke, D. L. J. (1997) A molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae). *Molecular Phylogenetics & Evolution* **7:** 281-293.

Bouček, Z. (1988) *Australasian Chalcidoidea (Hymenoptera): A Biosystematic Revision of Genera of Fourteen Families, with a Reclassification of Species.* C.A.B. International, Wallingford.

Bouček, Z. & Noyes, J. S. (1987) Rotoitidae, a curious new family of Chalcidoidea (Hymenoptera) from New Zealand. *Systematic Entomology* **12:** 407-412.

Breeuwer, J. A. J., Stouthamer, R., Burns, S. M., Pelletier, D. A., Weisberg, W. G. & Werren, J. H. (1992) Phylogeny of cytoplasmic incompatibility microorganisms in the parasitoid was genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. *Insect Molecular Biology* **1:** 25-36.

Campbell, B., Steffen-Campbell, J. D. & Werren, J. H. (1993) Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed spacer ITS2 and 28S rDNA sequences. *Insect Molecular Biology* **2:** 225-237.

Carmean, D., Kimsey, L. S. & Berbee, M. L. (1992) 18S rDNA sequences and the holometabolous insects. *Molecular Phylogenetics & Evolution* **1:** 270-278.

Carpenter, J. (1988) Choosing among equally parsimonious cladograms. *Cladistics* **4:** 291-296.

Derr, J. N., Davis, S. K., Woolley, J. B. & Wharton R. A. (1992a) Variation and the phylogenetic utility of the large ribosomal subunit of mitochondrial DNA from the insect order Hymenoptera. *Molecular Phylogenetics & Evolution* **1:** 136-147.

Derr, J. N., Davis, S. K., Woolley, J. B. & Wharton R. A. (1992b) Reassessment of the 16S rRNA nucleotide sequence from members of the parasitic Hymenoptera. *Molecular Phylogenetics & Evolution* **1:** 338-341.

Dowton, M. & Austin, A. D. (1994) Molecular phylogeny of the insect order Hymenoptera: Apocritan relationships. *Proceedings of the National Academy of Sciences, USA* **91:** 9911-9915.

Dowton, M. & Austin, A. D. (1995) Increased genetic diversity in mitochondrial genes is correlated with the evolution of parasitism in the Hymenoptera. *Journal of Molecular Evolution* **41:** 958-965.

Frenk, J. P. M., Silva, I., Schilthuizen, M., Pinto, J. D., & Stouthamer, R. (1996) Use of DNA-based methods for the identification of minute wasps of the genus *Trichogramma*. *Proceedings of Experimental and Applied Entomology N.E.V. Amsterdam* **7:** 233-237.

Gauthier, N., LaSalle, J., Quicke, D. L. J. & Godfray, H. C. J. (2000) Phylogeny of the Eulophidae (Hymenoptera: Chalcidoidea), with a reclassification of the Eulophinae and the recognition that the Elasmidae are derived eulophids. *Systematic Entomology* **25**: 1-20.

Gibson, G. A. P. (1986) Evidence for monophyly and relationships of Chalcidoidea, Mymaridae, and Mymarommatidae (Hymenoptera: Terebrantes). *Canadian Entomologist* **118:** 205-240.

Gibson, G. A. P. (1989) Phylogeny and classification of Eupelmidae, with a revision of the world genera of Calosotinae and Metapelmatinae (Hymenoptera: Chalcidoidea). *Memoirs of the Canadian Entomological Society* **149:** 121 pp.

Gibson, G. A. P. (1990). A word on chalcidoid classification. *Chalcid Forum (newsletter)* **13:** 7–9.

Gibson, G. A. P. (1995) Parasitic wasps of the subfamily Eupelminae: Classification and revision of World genera (Hymenoptera: Chalcidoidea: Eupelmidae). *Memoirs of Entomology, International* **5:** 1-421.

Gibson, G. A. P., Heraty, J. M. & Woolley, J. B. (1999) Phylogenetics and classification of Chalcidoidea and Mymarommatoidea – a review of current concepts (Hymenoptera, Apocrita). *Zoologica Scripta* **28:** 87-124.

Gordh, G. (1979) Encyrtidae. pp. 890-966. *In* Krombein, K. V., Hurd, B., Smith, D. R. & Burks, B. D. (Eds), *Catalog of Hymenoptera in America North of Mexico. Volume 1.* Smithsonian Institution Press, Washington.

Goulet, H. & Huber, J. T. (1993) *Hymenoptera of the World: An Identification Guide to Families.* Agriculture Canada Publication 1894/E.

Graybeal, A. (1998) Is it better to add taxa or characters to a difficult phylogenetic problem? *Systematic Biology* **47:** 9-17.

Hayat, M. (1994) Notes on some genera of the Aphelinidae (Hymenoptera: Chalcidoidea), with comments on the classification of the family. *Oriental Insects* **28:** 81-96.

Hayat, M. (1998) Aphelinidae of India (Hymenoptera: Chalcidoidea): A taxonomic revision. *Memoirs on Entomology, International* **13:** 416 pp.

Heraty, J. M. & Schauff, M. E. (1998) Mandibular teeth in Chalcidoidea: function and phylogeny. *Journal of Natural History* **32:** 1227-1244.

Heraty, J. M., Woolley, J. B. & Darling, D. C. (1994) Phylogenetic implications of the mesofurca and mesopostnotum in Hymenoptera. *Journal of Hymenoptera Research* **3:** 241-277.

Heraty, J. M., Woolley, J. B. & Darling, D. C. (1997) Phylogenetic implications of the mesofurca and mesopostnotum in Chalcidoidea (Hymenoptera), with emphasis on Aphelinidae. *Systematic Entomology* **22:** 45-65.

Kozlov, M. A. & Rasnitsyn, A. P. (1979) On the limits of the Serphitidae (Hymenoptera, Proctotrupoidea). *Entomologicheskoe Obozrenie* **58:** 402–416. [in Russian]

LaSalle, J. (1993) Parasitic Hymenoptera, biological control and the biodiversity crisis. pp. 197-216. *In* LaSalle, J. & Gauld, I. D. (Eds), *Hymenoptera and Biodiversity.* C.A.B. International: Wallingford.

LaSalle, J. & Gauld, I. D. (1991) Parasitic Hymenoptera and the biodiversity crisis. *Redia* **74:** 315-334.

LaSalle, J. & Noyes, J. S. (1985) New family placement for the genus *Cynipencyrtus* (Hymenoptera: Chalcidoidea: Tanaostigmatidae). *Journal of the New York Entomological Society* **93:** 1261-1264.

LaSalle, J., Polaszek, A., Noyes, J. S. & Zolnerowich, G. (1997) A new whitefly parasitoid (Hymenoptera: Pteromalidae: Eunotinae), with comments on its placement, and implications for classification of Chalcidoidea with particular reference to the Eriaporinae (Hymenoptera: Aphelinidae). *Systematic Entomology* **22:** 131-150.

Linares, A. R., Hancock, J. M. & Dover, G. A. (1991) Secondary structure constraints on the evolution of *Drosophila* 28S ribosomal RNA expansion segments. *Journal of Molecular Biology* **219:** 381-390.

Machado, C. A., Herre, E. A., McCafferty, S. & Berminham, E. (1996) Molecular phylogenies of fig pollinating and non-pollinating wasps and the implications for the origin and evolution of the fig-fig wasp mutualism. *Journal of Biogeography* **23:** 531-542.

Michot, B. & Bachellerie, J. P. (1987) Comparison of large subunit rRNAs reveal some eukaryote-specific elements of secondary structure. *Biochimie* **69:** 11-23.

Noyes, J. S. (1978) On the numbers and species of Chalcidoidea (Hymenoptera) in the world. *Entomologist's Gazette* **29:** 163-164.

Noyes, J. S. (1990) A word on chalcidoid classification. *Chalcid Forum* (newsletter) **13:** 6-7.

Noyes, J. S. & Hayat, M. (1994) *Oriental Mealybug Parasitoids of the Anagyrini (Hymenoptera: Encyrtidae).* CAB International, Wallingford.

Patterson, C., Williams, D. M. & Humphries, C. J. (1993) Congruence between molecular and morphological phylogenies. *Annual Review of Ecology & Systematics* **24:** 153-188.

Poe, S. (1998) Sensitivity of phylogeny estimation to taxonomic sampling. *Systematic Biology* **47:** 18-31.

Rasplus, J. Y., Kerdelhué, C. & Mondor, G. (1998) Molecular phylogeny of fig wasps (Hymenoptera). Agaonidae is not monophyletic. *Compte Rendu de l'Académie des Sciences de Paris* **321:** 21-31.

Rasnitsyn, A. P. (1980). Origin and evolution of hymenopterous insects. *Trudy Paleontologicheskogo Instituta Akademiya Nauk SSSR* **174:** 1–192. [in Russian]

Rousset, R., Pélandakis, M. & Solignac, M. (1991) Evolution of compensatory substitutions through G•U intermediate state in *Drosophila* rRNA. *Proceedings of the National Academy of Sciences, USA* **88**: 10032-10036.

Shafee, S. A. & Rizvi, S. (1990) Classification and phylogeny of the family Aphelinidae (Hymenoptera: Chalcidoidea). *Indian Journal of Systematic Entomology* **7:** 103–115.

Trjapitzyn, V. A. & Gordh, G. (1978) Review of the genera of Nearctic Encyrtidae (Hymenoptera: Chalcidoidea). II. *Entomological Review* **57:** 437-448.

Wheeler, W. C., Cartwright, P. & Hayashi, C. (1993) Arthropod phylogeny: a combined approach. *Cladistics* **9:** 1-39.

Wijesekara, A. (1997) A phylogenetic analysis of the Chalcididae (Hymenoptera: Chalcidoidea). *Memoirs of the American Entomological Institute* **29:** 1-61.

Woolley, J. B. (1988) Phylogeny and classification of the Signiphoridae (Hymenoptera: Chalcidoidea). *Systematic Entomology* **13:** 465-501.

Zuker, M., Matthews, D. H. & Turner, D. H. (1999) *Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide in RNA Biochemistry and Biotechnology.* Barciszewski, J. & Clark, B. F. C. (Eds), NATO ASI Series, Kluwer Academic Publishers.

Appendix 1

List of species used in this study showing their family/subfamily placement.

Agaonidae

Agaoninae

Seres Waterston

Otitisellinae

Aepocerus Mayr

Aphelinidae

Coccophaginae

Coccophagus rusti Compere
Coccophagus scutellaris Dalman
Coccophagoides Girault
Encarsia formosa Gahan
Encarsia lutea Masi
Encarsia aurantii Howard
Encarsia protransvena Viggiani
Encarsia luteola Howard
Encarsia pergandiella Howard
Encarsiella Hayat

Aphelininae

Aphelinus varipes Foerster
Aphelinus albipodus Hayat & Fatima
Aphelinus asychis Walker
Aphytis melinus DeBach
Aphytis yanonensis Debach & Rosen
Eretmocerus Haldeman

Azotinae

Ablerus Howard

Calesinae

Cales noacki Howard

Chalcididae

Chalcidinae

Conura Spinola

Brachymeriinae

Brachymeria Westwood
Acanthochalcis Cameron

Dirhininae

Dirhinus Dalman

Haltichellinae

Hockeria Walker
Psilochalcis Kieffer
Schwarzella Ashmead

Elasmidae

Elasmus Westwood

Encyrtidae

Metaphycus Mercet
Copidosoma Ratzeburg
Comperiella Howard
Aenasius Walker
Ooencyrtus Ashmead
Zaomma Ashmead

Eucharitidae

Oraseminae

Orasema Cameron

Eucharitinae

Obeza Heraty

Eulophidae

Entedoninae

Chrysocharis Foerster
Horismenus Walker
Entedon Dalman

Eulophinae

Cirrospilus Westwood
Elachertus Spinola
Deutereulophus Schulz

Tetrastichinae

Melittobia Westwood
Aprostocetus Westwood
Henryana Yoshimoto

Eupelmidae

Eupelminae

Brasema Cameron
Eusandalum Ratzeburg
Eupelmus Dalman

Calosotinae

Calosota Curtis

Eurytomidae

Eurytominae

Eurytoma Illiger
Sycophila Walker

Rileyinae

Rileya Ashmead

Leucospidae
Leucospis F.

Mymaridae
Polynema Haliday
Gonatocerus Nees

Perilampidae
Perilampinae
Perilampus Latreille
Chrysolampinae
Chrysomalla Foerster

Pteromalidae
Asaphinae
Asaphes Walker
Colotrechninae
Colotrechnus Thomson
Eunotinae
Scutellista Motschulsky
Eunotus Walker
Idioporus LaSalle & Polaszek
Eutrichosomatinae
Eutrichosoma Ashmead
Spalangiinae
Spalangia Latreille
Pteromalinae
Trichomalopsis Crawford
Nasonia vitripennis Walker
Nasonia longicornis Darling
Nasonia giraulti Darling
Muscidifurax Girault & Sanders
Pachycrepoideus Ashmead
Cleonyminae
Cleonymus Latreille
Epistenia Westwood

Signiphoridae
Chartocerus Motschulsky

Tanaostigmatidae
Tanaostigmodes Ashmead

Tetracampidae
Epiclerus Haliday

Torymidae
Toryminae
Torymus Dalman
Podagrion Spinola
Glyphomerus Foerster
Megastigminae
Megastigmus Dalman

Trichogrammatidae
Trichogramma pretiosum Riley
Trichogramma platneri Nagarkatti
Trichogramma fuentesi Torre
Aphelinoidea Girault
Oligosita Walker
Uscana Girault

Outgroups

Scelionidae
Trissolcus Ashmead
Gryon Haliday

Cynipidae
Diplolepis rosae (L.)

Eucoilidae
Leptopilina boulardi (Barbotin, Carton, & Kelner-Pillault)

Figitidae
Xyalophora Kieffer