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Molecular phylogeny of the insect order Hymenoptera: Apocritan relationships

(Symphyta/molecular systematics/mitochondrial DNA/16S ribosomal RNA)

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ABSTRACT **Phylogenetic relationships among the major groups of hymenopteran insects were investigated by using comparative sequence information from the mitochondrial 16S rRNA gene. The placement of the ectoparasitic Stephanidae as the sister group to the remaining Apocrita confirmed ectoparasitism as the ground plan biology for the Apocrita. Endoparasitism evolved at least eight times within the Apocrita, and the consequent association with polydnaviruses and virus-like particles evolved at least three times. The Evaniomorpha were consistently placed as basal to the remaining Apocrita but were not resolved as monophyletic. The Gasteruptiidae were resolved as the sister group to the Evaniidae, but the relationship between the Trigonalyoidea and the Evanioidea was unclear. The Proctotrupomorpha (sensu Rasnitsyn) was resolved by topology-dependent permutation tail probability (T-PTP) testing as monophyletic, with strong evidence for a sister group relationship between the Platygastroidea and the Chalcidoidea. Strong evidence was found for the monophyly of the Ichneumonomorpha (Ichneumonidae + Braconidae) and the sister-group relationship between the Aculeata (Vespomorpha) and the Ichneumonomorpha.**

The Hymenoptera (ants, bees, and wasps) comprise one of the largest (115,000 described species) (1) and most biologically diverse group of insects (2) containing both eusocial and parasitic groups. Apart from the termites, the order Hymenoptera contains most other eusocial insects, while the parasitic Hymenoptera are the most important group of biological control agents for insect and weed pests (3). Although our understanding of the evolution of these complex behaviors depends upon a robust phylogeny (4–6), historical relationships among the Hymenoptera are currently poorly understood. Molecular systematic studies are likely to provide important evidence to resolve these histories (4). For example, evidence that advanced eusociality in bees has multiple origins was provided recently after comparison of mitochondrial 16S rRNA gene sequences (5). However, no comparable molecular systematic study has been performed on the parasitic Hymenoptera to evaluate the current hypothesis that endoparasitoids and their association with symbiotic polydnaviruses arose multiple times from ectoparasitic ancestors (6, 7).

The first fully resolved phylogenetic hypothesis concerning the Hymenoptera was advanced by Rasnitsyn (8). Within the suborder Apocrita (which contains all of the parasitic Hymenoptera apart from the ectoparasitic Orussidae), he proposed four major lineages; the Ichneumonomorpha, the Vespomorpha (Aculeata), the Proctotrupomorpha, and the Evaniomorpha, with sister-group relationships between the former and latter pairs (Fig. 1 *Left*). Although there is strong morphological evidence for the monophyly of the Ichneumonomorpha (the Ichneumonidae and Braconidae; refs. 8–12), most remaining apocritan relationships are contentious (6) (Fig. 1 *Right*). More generally, any phylogeny of the apocritan wasps that is based on morphology suffers from problems associated with reductional synapomorphies because of the extremely small size of many members of the Proctotrupomorpha and Ceraphronoidea (≈1 mm; refs. 8 and 11).

Molecular systematic studies have thus far been unable to provide a robust systematic framework for the Hymenoptera. One study (13, 14) using the 16S rRNA gene reemphasized the sister-group relationship between the Ichneumonidae and the Braconidae, but the omission of most of the major groups precluded the placement of the Ichneumonoidea within an apocritan phylogeny. A survey of a wider range of hymenopteran groups used 18S rRNA sequences (15, 16). However, the level of variation was too low to resolve any relationships. In the present study, we investigate the evolution of the parasitoid life-style in the Hymenoptera using molecular sequence data from the 16S rRNA gene; we survey 10 of the 14 superfamilies of the Apocrita and 4 of the 7 superfamilies of the Symphyta (sawflies) in an attempt to lay the foundations for a phylogenetic understanding of the entire order.*

MATERIALS AND METHODS

DNA Amplification and Sequencing. Sequences were obtained either from the literature or generated in our laboratory from the following 14 superfamilies: Tenthredinoidea [*Perga condei* (Benson), *Phylacteophaga froggatti* (Riek), and Tenthredinidae indet. (14)]; Siricoidea [*Tremex columba* (L.) (14)]; Cephoidea [*Hartigia trimaculata* (Say)]; Orussoidea [*Orussus terminalis* (Newman)]; Stephanoidea [*Schlettererius cinctipes* (Cresson)]; Ichneumonoidea [*Cotesia glomerata* (L.), *Cotesia rubecula* (Marshall), *Cotesia flavipes* Cameron (14), *Digonogastra kimballi* (Kirkland) (14), *Alabagrus stigma* (Brulle) (14), *Ichneumon promissorius* (Erichson), *Venturia canescens* (Gravenhorst), and *Xanthopimpla stemmator* (Thunberg) (14)]; Vespoidea [*Myrmecia forficata* (F.) and *Polistes versicolor* (Olivier) (14)]; Apoidea [*Apis mellifera* (L.) (17)]; Platygastroidea [*Scelio fulgidus* (Crawford) and *Trissolcus basalis* (Wollaston)]; Chalcidoidea [*Aphytis melinus* (De Bach), *Encarsia formosa* (Gahan), and *Pteromalus puparum* (L.)]; Proctotrupoidea [*Ropronia garmani* (Ashmead) and *Vanhornia eunemidarum* (Crawford)]; Cynipoidea [*Ibalia leucospoides* (Hochenwarth)]; Trigonalyoidea [*Poecilogonalos costalis* (Cresson) and *Orthogonalys pulchella* (Cresson)]; and Evanioidea [*Eufoenus* sp., *Gasteruption* sp., and *Evania* sp.]. Dipteran outgroups were *Drosophila yakuba* (Burla) (18) and *Aedes*

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Abbreviation: T-PTP, topology-dependent permutation tail probability.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U06953–U06975).

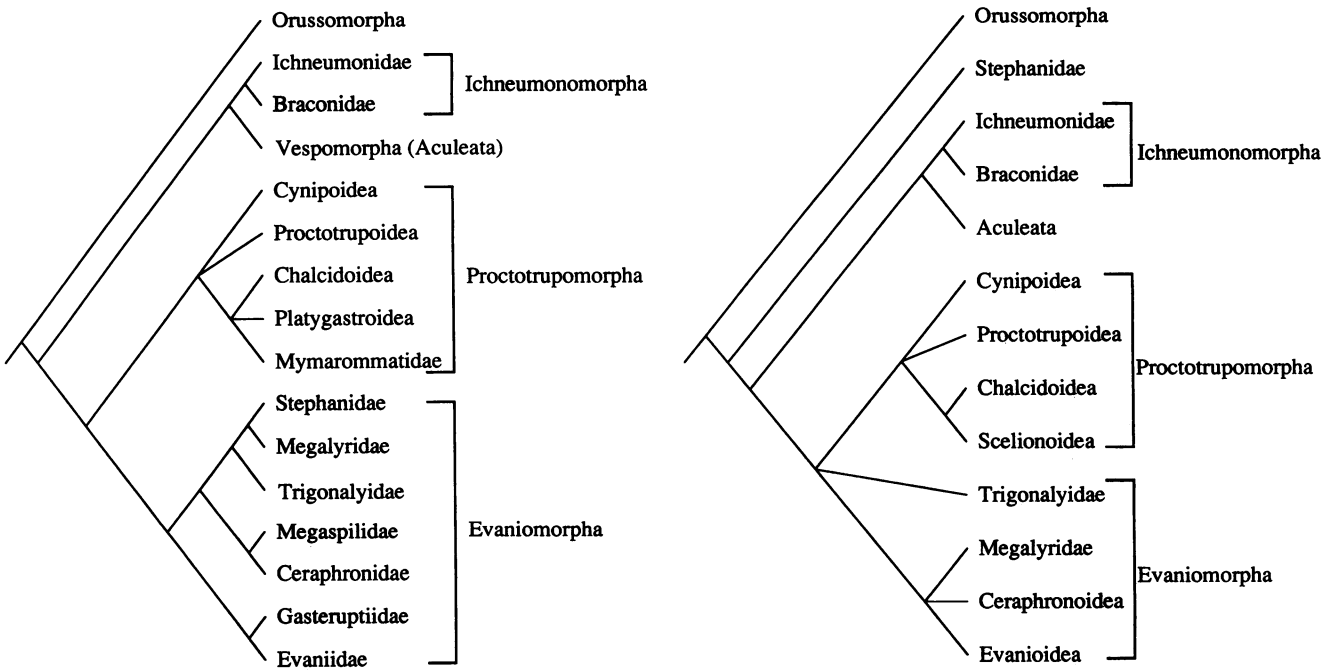

FIG. 1. Cladogram of the Hymenoptera as proposed by Rasnitsyn (8) as it appears in ref. 6 (*Left*) and as revised in the review by Whitfield (6) (*Right*). (Reproduced from ref. 6 with permission.)

aegypti (L.) (19). Genomic DNA was extracted from frozen (−80°C) or 100% ethanol-stored specimens as described (20). Those specimens that were held in 100% ethanol were washed three times for 15 min in 10 mM Tris·HCl, pH 8/100 mM NaCl/1 mM $MgCl_2$ before extraction (21). A portion of the 16S gene was then amplified with primers as described (22) but edited according to the honeybee sequence (17). The primers used were: 16SWa (5′-CGTCGATTTGAACTCAAATC-3′; anneals to nucleotides 1146–1165 of the honeybee 16S rRNA gene) and 16SWb (5′-CACCTGTTTATCAAAAACAT-3′; anneals to nucleotides 616–635). Where amplifications were unsuccessful under a variety of conditions, one of the following primers was used in place of 16SWa: 16S.Sh (5′-AGATTTTAAAAGTCGAACAG-3′; anneals to nucleotides 1121–1140) or 16Wa.1 (5′-GACTTACGTCGATTTGAACT-3′; anneals to nucleotides 1152–1171). The PCR product was thus in the range of 480–550 base pairs. PCR reactions were optimized as described (23). Reactions were carried out in 67 mM Tris·HCl buffer, pH 8.8/16.6 mM $(NH_4)_2SO_4$/0.2 mg of gelatin per ml/0.45% Triton X-100 containing also 2–4 mM $MgCl_2$, 25 μM dNTPs, 0.05–0.20 μM each primer, and 0.5 units of *Taq* DNA polymerase in a total volume of 20 μl. Reaction mixtures were overlaid with 20 μl of mineral oil and heated to 75°C before the addition of genomic DNA. The conditions for amplification were as follows: five cycles of (*i*) denaturation at 94°C for 1 min, (*ii*) annealing at 50°C for 1 min, and (*iii*) extending at 72°C for 1 min, followed by 25 cycles with the annealing temperature at 55°C. Double-stranded PCR products were purified by either using the Spinbind (FMC) procedure or treating with mung bean nuclease (New England Biolabs) to remove primers (24) prior to sequencing. *Taq* cycle sequencing reactions were performed with the *Taq* Dye Deoxy Terminator Cycle Sequencing Kit from Applied Biosystems, using one of the PCR primers to initiate the sequencing reaction. In some cases, the sequencing reactions were optimized as described (25). All reported sequences are the consensus obtained after sequencing both strands from two individuals.

Sequence Analysis. Sequences, corresponding to nucleotides 663–1114 of the honeybee gene (17) were aligned by using CLUSTAL V (26), based on the secondary structure model of the *Drosophila yakuba* 16S gene (27). This strategy has been used to align other 16S sequences of varying length (28). Gaps were coded as single characters, irrespective of length, as described (29). Briefly, gaps were coded as missing data, and an additional "gap" data matrix was constructed. Where overlapping gaps occurred, each size gap was assigned a particular character state. Phylogenetic analysis was performed by maximum parsimony using PAUP version 3.1.1 (29), with transitions and transversions weighted equally. The number of informative characters was 386. Both most parsimonious as well as near-most parsimonious trees were examined to investigate the cladistic structure of the sequence data (30). Analyses were also performed with transitions weighted relative to transversions at 1:2, 1:3, 1:4, and 1:5. One-hundred thousand trees were drawn at random from the set of all possible trees, and the number of trees was plotted as a function of the number of steps in the tree. This plot showed a left-skewed distribution ($g_1 = -0.53$), indicating that the data contains a strong phylogenetic signal ($P < 0.01$; ref. 31). The degree of support for various nodes was further assessed by the T-PTP (Topology-dependent Permutation Tail Probability) test (32) and by bootstrapping (33). For the T-PTP test, 100 randomized data sets were generated by character permutation (outgroups are not randomized). The number of shorter trees (compared with the actual data set) in each of the randomized data sets that contain the node in question was then determined. The node was not supported where P (the probability of observing a particular node in the randomized data sets) ≥ 0.05.

RESULTS

Maximum parsimony analysis of the aligned sequences using an heuristic search found six most parsimonious trees having a length of 2333 steps, a consistency index (excluding uninformative characters) of 0.324, and a retention index of 0.458. A strict consensus of these six trees is shown in Fig. 2. The included families and superfamilies of the Apocrita were consistently resolved in each of the six shortest trees, although the Ichneumonoidea were not unequivocally sup-

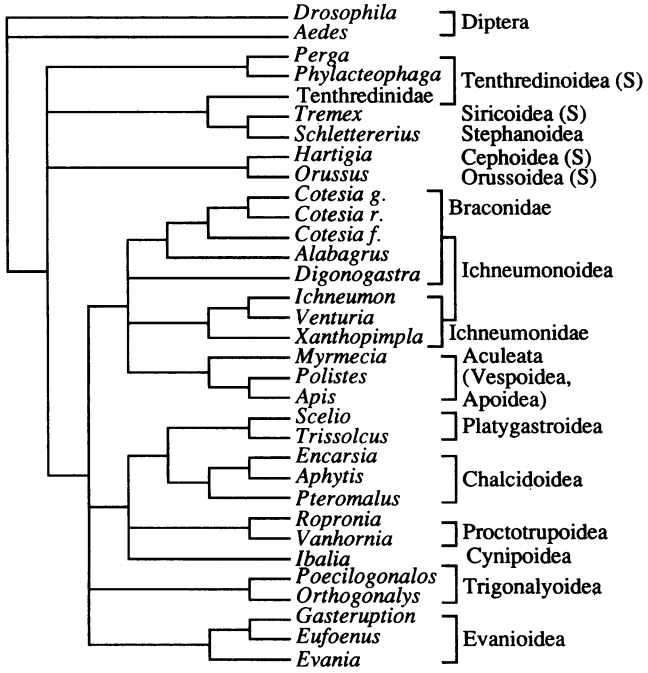

FIG. 2. Strict consensus of the six most parsimonious trees. Parsimony analysis was performed with PAUP version 3.1.1 (29) by heuristic search; taxa were added by using the simple option with the MULPARS option in effect and with branch swapping by the tree bisection–reconnection algorithm, 10 trees being held at each step and all trees ≤ 2335 steps being held. These trees were then filtered to find the six shortest trees. The same six shortest trees were found when taxa were added randomly, with 20 replications. (S) denotes that the superfamily is in the Symphyta.

ported. The mostly phytophagous symphytan groups were not well supported—e.g., the three Tenthredinoidea (*Perga*, *Phylacteophaga*, and Tenthredinidae indet.) were not resolved as monophyletic, although the Pergidae (*Perga* and *Phylacteophaga*) were. This may indicate that the degree of variation in the 16S gene is too great to resolve the basal hymenopteran groups.

The Evaniomorpha were proposed by Rasnitsyn (8) as a monophyletic lineage and included the Stephanidae, Megalyridae, Trigonalyidae, Gasteruptiidae, Evaniidae, Megaspilidae, and Ceraphronidae. However, the representatives of this group that we surveyed (Stephanidae, Trigonalyidae, Gasteruptiidae, and Evaniidae) were not supported as monophyletic. The placement of the ectoparasitic stephanid (*Schlettererius*) among the Symphyta in each of the six shortest trees (Fig. 2) was surprising, as there is strong morphological evidence that the Stephanidae are part of the Apocrita (e.g., refs. 6, 8, 11, and 34). This relationship was also held in each of the near-most parsimonious trees (72 trees were found within two steps of the shortest trees). T-PTP testing (Fig. 3) also indicated that the stephanid wasp fell outside of the remaining Apocrita (T-PTP < 0.01), as did bootstrap resampling (Fig. 3; 91%). This is consistent with the proposal that the Stephanidae are the sister group to the remaining Apocrita (6, 11, 35). Their placement within the Symphyta in this analysis may be due to the variation in the 16S gene being too great to accurately resolve the relationships among the basal groups of the Hymenoptera.

The relative placement of the remaining Evaniomorpha (Gasteruptiidae, Evaniidae, and Trigonalyidae) was not resolved in the six shortest trees. However, most parsimonious and near-most parsimonious analysis suggested that they were placed basally compared with the remaining Apocrita (56%), as did T-PTP testing (Fig. 3; T-PTP < 0.01) and bootstrap analysis. The relationship between the Trigonalyoidea and the Evanioidea was not consistently supported, with three of the shortest trees suggesting monophyly and the other three suggesting paraphyly (Fig. 2). Although a monophyletic relationship was supported in 59% of the near-most parsimonious trees, the competing hypothesis (paraphyly) was present in each of the remaining near-most parsimonious trees, making it difficult to distinguish between these two hypotheses. T-PTP testing supported neither monophyly nor paraphyly. Similarly, bootstrapping did not confidently resolve this relationship. However, within the Evanioidea, the Evaniidae and the Gasteruptiidae were resolved as monophyletic in each of the six shortest trees (Fig. 2), in each of the near-most parsimonious trees, and in each of the bootstrap resampled data matrices. T-PTP testing similarly indicated monophyly (T-PTP < 0.01).

The Proctotrupomorpha have been proposed as a distinct hymenopteran lineage (8) and include the Platygastroidea (= Scelionoidea, sensu Whitfield), Chalcidoidea, Proctotrupoidea, and Cynipoidea (Fig. 1). All six shortest trees supported this group (Fig. 2), as did 93% of the near-most parsimonious trees. T-PTP testing (Fig. 3) similarly supported this group as monophyletic (T-PTP < 0.01), although bootstrap resampling (Fig. 3) less confidently supported this lineage (34%).

Within the Proctotrupomorpha (sensu Rasnitsyn), the Platygastroidea and Chalcidoidea were supported as sister groups in each of the six shortest trees (Fig. 2), and in every near-most parsimonious tree. T-PTP testing (Fig. 3) similarly indicated monophyly (T-PTP < 0.01). Bootstrap analysis (Fig. 3) also supported this relationship (68%). Conversely, monophyly of the Proctotrupoidea and Cynipoidea was not well supported, with five of the six shortest trees suggesting monophyly and the other supporting paraphyly. Of the near-most parsimonious trees, 67% supported these two groups as sister groups, with the remainder supporting them as paraphyletic. Bootstrapping (Fig. 3) suggested monophyly (55%), but T-PTP testing did not (T-PTP > 0.05). The inability to resolve this relationship is perhaps not surprising, given that the Proctotrupoidea are probably not monophyletic (8, 34). In addition, we did not extensively survey these two superfamilies, analyzing only two of the nine proctotrupoid families and one of the four cynipoid families.

The Ichneumonidae + Braconidae + Aculeata were supported as a distinct apocritan lineage in each of the six shortest trees. The Braconidae and Ichneumonidae were supported as sister groups in 78% of the near-most parsimonious trees, and the Aculeata was supported as the sister group to the Braconidae + Ichneumonidae in 85% of these trees. The monophyly of this lineage was further assessed with the T-PTP test (30) (Fig. 3), which supported the Ichneumonidae + Braconidae as monophyletic (T-PTP < 0.05) and the Ichneumonidae + Braconidae + Aculeata as monophyletic (T-PTP < 0.01). Bootstrap resampling (Fig. 3) similarly placed the Braconidae and Ichneumonidae as sister groups (58%) and the Aculeata as the sister group to this clade (57%). The maximum parsimony analysis thus consistently resolved the (Braconidae + Ichneumonidae) + Aculeata as monophyletic.

DISCUSSION

Evolution of Parasitism. To test hypotheses concerning the evolution of parasitism within the Hymenoptera, it is necessary to resolve the basal apocritan group. Several authors place the ectoparasitic Stephanidae as the sister group to the remaining Apocrita (6, 11, 35) in contrast to their earlier placement within the Evaniomorpha (8). The results of the present study confirm that the Stephanidae are the most basal apocritan. The placement of the Stephanidae with the Siricoidea and Tenthredinidae indet. is undoubtedly question-

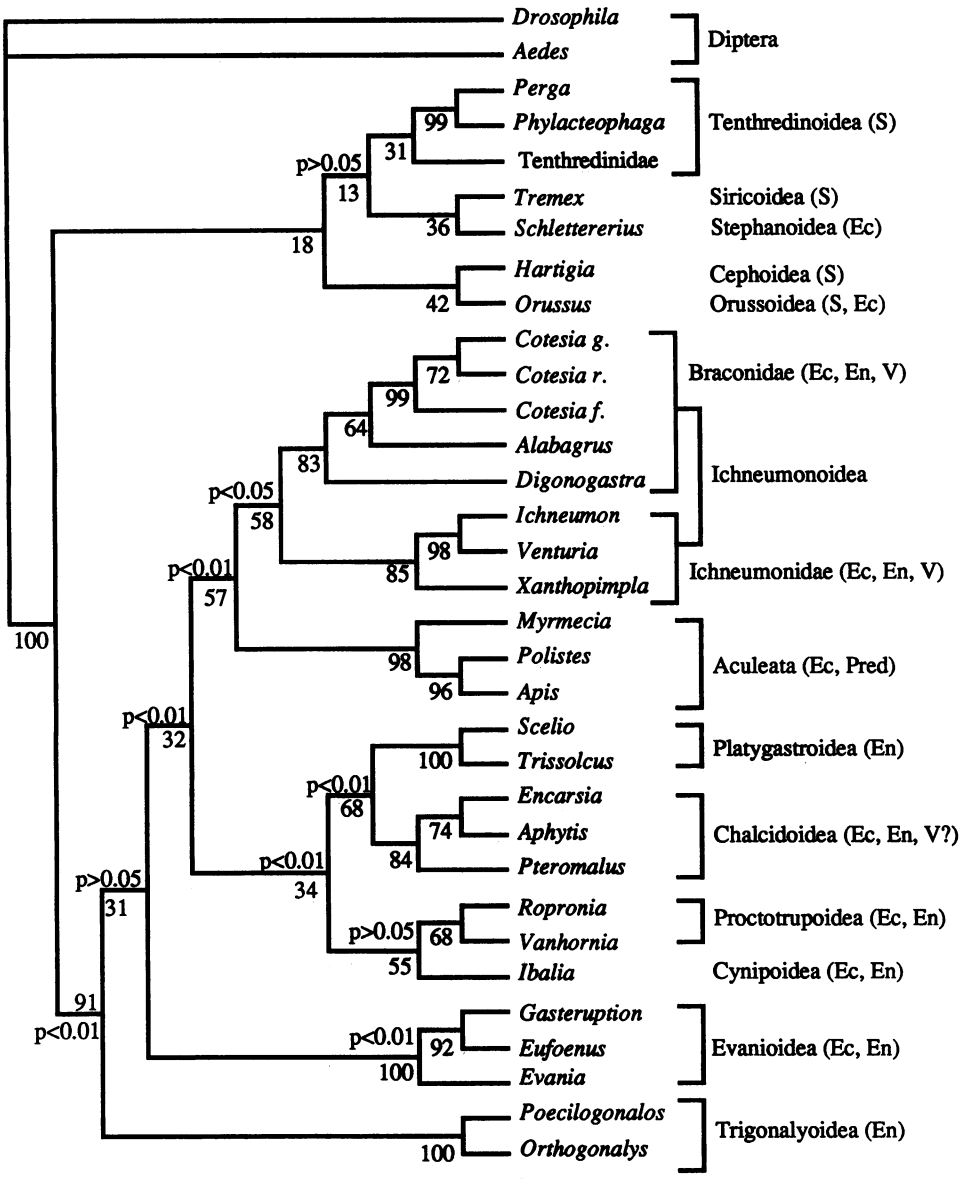

FIG. 3. Statistical analysis of hymenopteran relationships. Nodes that were assessed by the T-PTP test (30) are indicated (p) above the line and to the left of the node. Bootstrap values (below the line) were calculated after generation of 100 bootstrap resampled data matrices. Parsimony analysis was performed with PAUP version 3.1.1 (29) by heuristic search with the MULPARS option in effect and with branch swapping by the tree bisection–reconnection algorithm, 1 tree being held at each step. Parsimony analyses with transitions/transversions weighted 1:2, 1:3, 1:4, and 1:5 yielded congruent topologies. (S), superfamily is from the Symphyta; (Ec), family/superfamily contains some ectoparasitoids; (En), family/superfamily contains some endoparasitoids; (Pred), family/superfamily contains some predators; (V) family/superfamily contains some kind of associated viruses. The information on the biology of the various families/superfamilies (i.e., Ec, En, V, Pred) is from Whitfield (6).

able and was not supported by the T-PTP test or bootstrapping. As there is strong morphological evidence to support its placement within the Apocrita (e.g., refs. 6, 8, 11, and 34), it realistically can be considered to be the most basal apocritan, particularly as there was strong support for the monophyly of the remaining apocritan groups surveyed. This is consistent with the notion that the ectoparasitic lifestyle of the Stephanidae is the ground-plan state for the Apocrita (6), the superfamilies of which mostly contain ectoparasitic basal groups (Fig. 3). Further, the results of both the present and previous (6) analyses suggest that endoparasitism evolved a number of times within the Apocrita (Fig. 3), and the consequent independent association with polydnaviruses and virus-like particles evolved at least three, possibly four, times. The only apocritan groups that do not contain ectoparasitic stem groups are the Platygastroidea, Trigonalyoidea, and possibly the Ceraphronoidea. The outstanding question is whether the ectoparasitic Orussidae are the sister group to the Apocrita, which would distinguish between a single or multiple origin for ectoparasitism within the Hymenoptera.

Monophyly of the Evaniomorpha. The Evaniomorpha (excluding the Stephanidae) were consistently placed as basal to the rest of the Apocrita, (most and near-most parsimonious trees, T-PTP test, and bootstrapping). The groups surveyed (Evanioidea and Trigonalyoidea) show very different parasitic lifestyles to both the more basal Stephanidae as well as the remaining Apocrita. The Trigonalyidae are predominantly hyperparasitic (36), while the Evaniidae are parasitoids of cockroach oothecae and the Gasteruptiidae are ectoparasitic or cleptoparasitic in the nests of wasps and bees, respectively (37). However, it is not clear how these distinct life histories are related to the basal placement of the Trigonalyoidea and the Evanioidea within the Apocrita.

The relative placement of the Trigonalyidae and the Evanioidea was less clearly resolved. Rasnitsyn (8) postulates that

these two groups are members of the monophyletic Evaniomorpha, while Whitfield (6) placed the Trigonalyidae as an unresolved polytomy between the Evaniomorpha and the Proctotrupomorpha. Our analyses variously suggested both monophyly and paraphyly. The inability of our data to resolve these relationships may be due to the level of variation in the 16S gene, given that the various symphytan groups (the most basal Hymenoptera) were also not well resolved. With respect to the Evanioidea, most authors place the Evaniidae and Gasteruptiidae as sister groups (e.g., ref. 10). However, Gibson (11) found no support for their monophyly. Our analysis strongly supports the monophyly of these two groups.

Monophyly of the Proctotrupomorpha. This lineage was first proposed by Rasnitsyn (8) but was not based on cladistic principles, as the data were not treated computationally to search for the most parsimonious topology (6). Nevertheless, our cladistic analysis supports the monophyly of the Proctotrupomorpha, exclusive of both the Trigonalyoidea and the Evanioidea (T-PTP < 0.01). Within the Proctotrupomorpha, the Platygastroidea and the Chalcidoidea have been postulated as sister groups by various authors (e.g., refs. 6, 8, and 38). However, because of their small size, the grouping is reliant upon a number of reductional synapomorphies. Our findings independently demonstrate the sister-group relationship between the Platygastroidea and the Chalcidoidea and the monophyly of the Proctotrupomorpha (sensu Rasnitsyn) as a whole. It should be noted, however, that the Proctotrupoidea are probably not monophyletic (8, 34). We did not examine this issue, surveying only two of the nine proctotrupoid families.

Monophyly of the (Ichneumonidae + Braconidae) + Aculeata. There is little doubt from morphological evidence that the entirely parasitic Ichneumonidae and Braconidae are sister groups (8–12). Our analysis strongly supported this hypothesis. However, the placement of the Aculeata as the sister group to the Ichneumonidae + Braconidae is more contentious. The Apocrita have traditionally been classified into the Parasitica and the Aculeata, implying that the Aculeata are outside the remaining Apocrita. This was reflected by the earlier placement of the Aculeata as relatively unrelated to the Ichneumonidae + Braconidae (34), while Rasnitsyn (10) stated that "The relationship between Ichneumonomorpha and Vespomorpha (Aculeata). . .remains obscure." However, Rasnitsyn (8) later placed the Aculeata as the sister group to the Ichneumonomorpha, in agreement with other studies (9, 39). Our data consistently placed the Aculeata as the sister group to the Ichneumonidae + Braconidae. Taken together, these findings strongly suggest that the division of the Apocrita into the Parasitica and the Aculeata is not realistic.

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