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## Molecular and Morphological Data Suggest a Single Origin of the Polydnviruses among Braconid Wasps

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Endoparasitoids exhibit some of the most intricate and intimate interspecific physiological interactions recognized among animals [1]. These typi-

cally wasplike insects feed as larvae within the bodies of other insects (often caterpillars), ultimately killing their hosts. A wide variety of endopar-

asitoid/host interactions are known [2], which in turn are mediated by diverse agents such as venoms, teratocytes, and viruslike entities. These mediating agents, introduced into the host by the female parasitoid during oviposition [3, 4], misdirect or dismantle the host's defense response and development, thereby enabling the larva(e) to develop freely inside [5].

Perhaps the most remarkable agents introduced into the hosts of some endoparasitoid wasps (Braconidae and Ichneumonidae) are viruslike entities known as polydnviruses. Polydnviruses are unusual in two respects. First, they are integrated into the wasp's chromosomal DNA and passed on from parent to offspring in mendelian fashion [6–8]. Second, within the cells of the wasp's ovarian

Table 1. Taxonomic identity and sources of 16S sequences used for the molecular phylogeny part of this study

Subfamily	Genus	Species	Polydnaviruses known *	GenBank no.	Reference	Collection information
Outgroup Ichneumonidae						
Ichneumoninae	<i>Ichneumon</i>	<i>promissorius</i>	No	UO 6960	Dowton and Austin (1994)	(Refer to original reference)
Campopleginae	<i>Venturia</i>	<i>canescens</i>	(Ichnovirus)	UO 6961	Dowton and Austin (1994)	(Refer to original reference)
Ingroup Braconidae						
Braconinae	<i>Digonogastra</i>	<i>kimballi</i>	No	Not submitted	Derr et al. (1992 a,b)	(Refer to original reference)
Braconinae	<i>Bracon</i>	<i>hebetor</i>	No	U 68 145	This study	Reared from bumble been nests in Arkansas
Agathidinae	<i>Alabagrus</i>	<i>stigma</i>	No	Not submitted	Derr et al. (1992 a,b)	(Refer to original reference)
Ichneutinae	<i>Paroligoneurus</i>	sp.	No	U 68 148	This study	Field collected in Brazil
Meteorinae	<i>Meteorus</i>	<i>pulchricornis</i>	No	U 68 146	This study	Lab colony: USDA, Newark Delaware
Neoneurinae	<i>Neoneurus</i>	<i>mantis</i>	?	U 68 147	This study	Field collected in Wyoming by S.R. Shaw
Cheloninae	<i>Chelonus</i>	<i>undetermined</i>	Yes	U 68 150	This study	Field collected in Arkansas
Cheloninae	<i>Ascogaster</i>	<i>argentifrons</i>	Yes	U 68 149	This study	Field collected in Arkansas
Cardiochilinae	<i>Toxoneuron</i>	<i>nigriceps</i>	Yes	U 68 151	This study	Lab colony: Texas A&M University (B. Vinson)
Miracinae	<i>Mirax</i>	<i>lithocolletidis</i>	Yes	U 68 152	This study	Field collected in Arkansas
Microgastrinae	<i>Pholetesor</i>	<i>bedelliae</i>	Yes	U 68 153	This study	Field collected in Arkansas
Microgastrinae	<i>Microgaster</i>	<i>canadensis</i>	Yes	U 68 154	This study	Field collected in Arkansas
Microgastrinae	<i>Microplitis</i>	sp.	Yes	U 68 155	This study	Field collected in Arkansas
Microgastrinae	<i>Cotesia</i>	<i>autographae</i>	Yes	U 68 156	This study	Field collected in Georgia by J. Ruberson
Microgastrinae	<i>Cotesia</i>	<i>congregata</i>	Yes	U 68 157	This study	Lab colony U.C. Riverside (N. Beckage)
Microgastrinae	<i>Cotesia</i>	<i>glomerata</i>	Yes	UO 6958	Dowton and Austin (1994)	(Refer to original reference)
Microgastrinae	<i>Cotesia</i>	<i>orobena</i>	Yes	U 68 158	This study	Field collected in Arkansas)
Microgastrinae	<i>Cotesia</i>	<i>rubecula</i>	Yes	UO 6959	Dowton and Austim (1994)	(Refer to original reference)

\* A “no” means that at least some members of the subfamily have been examined and none have been found to contain polydnaviruses. Additional subfamilies from the “cyclostome” and “helconoid” complexes have also been found to lack the viruses

calyx, polydnaviral DNA is replicated and packaged into nucleocapsids and envelopes in the form of multiple (and nonequimolar) circlets of double-stranded DNA [9]. In this form the polydnaviruses are introduced into host insects. While they do not replicate within the host [9], they express some of their genes with profound physiological and developmental effects on the host [10]. Thus the target of polydnaviral gene expression is a different organism than that within which the virus reproduces [9].

Although the currently favored hypothesis is that the polydnaviruses are at least largely viral in origin, their unusual life “cycle” has led to the suggestion that the polydnaviruses may not be viruses at all but instead are an ingeniously engineered way for a wasp to manipulate its host (reviewed in [11]). Insights into the origin of these putative viruses can best be obtained with knowledge of the phylogenetic history of the wasps. A phylogeny would, for example, reveal whether the virus association arose

once or multiple times in the evolution of the wasps.

Recent work indicates that there are two morphologically dissimilar (possibly unrelated) groups of polydnaviruses. One occurs only within campoplegine and related ichneumonid wasps (the ichnoviruses), and the other occurs within several subfamilies of Braconidae (the bracoviruses) [10, 12]. Although Braconidae and Ichneumonidae are sister-groups among extant wasp lineages [13], their respective virus groups are un-

likely to have a common origin because each occurs only within relatively derived subfamilies within its respective wasp family [14, 15]. However, within each wasp family the polydnviruses could be monophyletic, as they appear to be distributed only among groups of wasps that are closely related. Preliminary support for this hypothesis comes from examination of the known taxonomic distributions of the viruses among the wasps [12] and from preliminary examinations of genetic [12, 16] and serological similarity [17] among the polydnviruses. The data suggest that the closer the relationship among the wasps within each family, the more genetically similar their polydnviruses. However, this hypothesis can be tested only with a rigorous analysis of the monophyly of the polydnvirus-bearing wasps, which has hitherto not been available. This study was undertaken to clarify the phylogenetic relationships among the bracovirus-bearing wasps in order to test whether the virus association has one or multiple origins within the Braconidae.

In a recent phylogenetic analysis Whitfield and Mason [15] used 21 morphological characters to infer relationships among the braconids of the "microgastroid complex," and to assess the relationship of this complex to other more distantly related Braconidae. They concluded that the "microgastroid complex" (Adeliinae + Cheloniinae + Dirrhopinae + Mendesellinae + Cardiochilinae + Khoikhoiinae + Miracinae + Microgastrinae) is monophyletic. This lineage includes all braconid taxa known to carry bracoviruses [12] and none of the included groups are known to lack the viruses (Table 1). Several other broad analyses of braconid phylogeny based on morphological characters [18, 19] also corroborate the monophyly of the microgastroid complex. Although the morphological results suggested that the bracovirus-bearing wasps form a single lineage, it was important to further test the monophyly of this complex with molecular data. The large ribosomal (16S) subunit of the mitochondrial genome was selected because this region had been used successfully to estimate relationships among families [20] and tribes [21] within other Hymenoptera.

A list of taxa used in the DNA sequence analysis, their current classification, collection source, and GenBank accession numbers or publication sources is provided in Table 1. An analysis of all subfamilies within Braconidae was not considered practical; hence a subset of taxa was selected to represent a range of putative relationship to microgastroids, including groups that at one time were considered to be related to some of the microgastroid taxa (e.g., *Neoneurus*, *Paroligoneurus*).

The new sequences were obtained from fresh, frozen, ethanol-preserved and dried specimens. Cellular DNA was extracted using minor modifications of standard procedures for phenol-chloroform extraction [22]. Fragments of the 16S gene were amplified by the polymerase chain reaction (PCR) using modified primers developed initially from sequences of *Apis mellifera* [20]. One primer was tailored to improve PCR efficiency with braconid wasps (a reverse primer with the sequence 5' CTTTAATTCAA-CATCGAGGTC 3'). PCR was performed as described [23] with several temperature modifications to adjust stringency, depending on the preservation quality of the original sample (lower annealing temperatures were required for dried specimens). Amplified mtDNA was purified by polyacrylamide (3.5%) gel electrophoresis, and the desired-size fragment was excised and electroeluted from the gel slices. Amplified double-stranded mtDNA was sequenced directly and manually by dideoxy chain termination [24] using a CircumVent sequencing kit (New England Biolabs). From each taxon approximately 450 bp could be read when both primers were used in separate sequencing reactions. This procedure also allowed for the examination of complementary sequences from both strands.

All DNA sequences were entered unaligned into the sequence manager program SeqApp Version 1.9 [25]. Alignment of sequences was performed by three methods: (a) distance-based multiple alignment using CLUSTAL W [26]; (b) parsimony-based multiple alignment using MA-LIGN version 2.7 [27]; and (c) align-

ment by eye with reference to secondary structure using a fit to the model of Gutell [28, 29]. Regions that were length-variable (usually corresponding to variable-sized loops) or of uncertain alignment were excluded from some of the phylogenetic analyses. These corresponded to 104 bp of the total average aligned length of 438 bp. The total sequences correspond to bp 13470–13894 in the total mtDNA genome sequence of *Apis mellifera* [30]. Alignment gaps in conserved regions were treated as a fifth character state in the analyses.

Aligned sequences for the 20 taxa under consideration were analyzed using both maximum parsimony and maximum likelihood. Maximum parsimony has been the favored approach to estimating phylogenies from character data such as DNA sequences. Parsimony performs well under a broad range of conditions [31], although it can prove misleading when one or more assumptions of the method are violated [32, 33]. Parsimony analysis was implemented in PAUP\* version 4.0d48 [34]. All characters were equally weighted, and the tree-bisection-and-reconnection (TBR) strategy of tree search was used for analyses performed using all 20 taxa [35]. Additionally, analyses were performed using sampled subsets of the taxa to compensate for the large number of Microgastrinae in the data set. This allowed for more even representation of taxa among subfamilies, and for the use of the branch-and-bound algorithm of tree search to guarantee finding all most parsimonious trees [35]. Bootstrap analyses, implemented in PAUP\* (100 replicates, TBR branch swapping), were applied as heuristic methods to obtain a measure of relative branch support for particular clades.

Maximum likelihood methods are a useful alternative to parsimony and have been shown to outperform other methods, including parsimony, with various models of sequence evolution [31, 33, 36, 37]. This is true even with relatively short sequences such as those employed here. However, Gaut and Lewis [38] showed that maximum likelihood estimation can become inconsistent when significant site-to-site variation in rate of substi-

Table 2. Summary of phylogenetic analyses performed in this study

Included taxa	Alignment	Nucleotide sites excluded	Analysis	Search strategy	Bracovirus-bearing group monophyletic
16S analyses					
All 20	MALIGN	None	Parsimony	Heuristic/TBR	Depends on alignment parameters
All 20	CLUSTAL W	None	Parsimony	Heuristic/TBR	Depends on alignment parameters
All 20	Fit to secondary structure	None	Parsimony	Heuristic/TBR	Yes, but low bootstrap value
14 (balanced)	MALIGN	None	Parsimony	Branch-and-bound	Depends on alignment parameters
14 (balanced)	CLUSTAL W	None	Parsimony	Branch-and-bound	Depends on alignment parameters
14 (balanced)	Fit to secondary structure	None	Parsimony	Branch-and-bound	Yes, but low bootstrap value
All 20	MALIGN	104 length-variable	Parsimony	Heuristic/TBR	Yes, but low bootstrap value
All 20	CLUSTAL W	104 length-variable	Parsimony	Heuristic/TBR	Yes, but low bootstrap value
All 20	Fit to secondary structure	104 length-variable	Parsimony	Heuristic/TBR	Yes, but low bootstrap value
14 (balanced)	MALIGN	104 length-variable	Parsimony	Branch-and-bound	Yes, but low bootstrap value
14 (balanced)	CLUSTAL W	104 length-variable	Parsimony	Branch-and-bound	Yes, but low bootstrap value
14 (balanced)	Fit to secondary structure	104 length-variable	Parsimony	Branch-and-bound	Yes, but low bootstrap value (Fig. 1A)
14 (balanced)	MALIGN	104 length-variable	Max. likelihood	Branch-and-bound	Yes
14 (balanced)	CLUSTAL W	104 length-variable	Max. likelihood	Branch-and-bound	Yes
14 (balanced)	Fit to secondary structure	104 length-variable	Max. likelihood	Branch-and-bound	Yes (Fig. 1B)
Morphology					
All analyses result in monophyly – does not depend on number of included taxa					Yes, high bootstrap value (Fig. 1C)
Combined-data					
14 (balanced)	MALIGN	104 length-variable	Parsimony	Branch-and-bound	Yes, rel. high bootstrap value
14 (balanced)	CLUSTAL W	104 length-variable	Parsimony	Branch-and-bound	Yes, rel. high bootstrap value
14 (balanced)	Fit to secondary structure	104 length-variable	Parsimony	Branch-and-bound	Yes, high bootstrap value (Fig. 1D)

tutional change exists. Any such variation should be taken into account.

To adjust for site-to-site variation in the 16S sequences, the gamma distribution [39, 40] was incorporated into the maximum likelihood procedure implemented in PAUP\* using four substitution-rate categories and a shape parameter of 0.4 (a range of other shape parameter values up to 2.5 were also tried, with little effect on the resulting topology). This figure is consistent with estimates of the shape parameter produced by Yang [41] for 16S rRNAs in eukaryotes. The base composition and transition/transversion ratio were estimated from the empirical data. TBR branch swapping was employed in heuristic searches using 20 randomized taxon-input orders.

To obtain a measure of the phylogenetic agreement between trees resulting from morphological data and those estimated from the 16S analyses outlined above, the morphological data from Whitfield and Mason [15] were coded for the specific exemplar taxa used in the molecular analysis (Table 1). Each data set (morphological and molecular) was analyzed sepa-

rately with branch-and-bound parsimony searches and heuristic bootstrap analyses using PAUP\*. As a measure of heterogeneity among data sets, the incongruence length difference [42, 43] within and among the data sets was calculated and tested for significance using the implementation in DADA version 1.0 [44]. The two data sets were insignificantly heterogeneous ( $P \gg 0.05$ ), and the morphological data were used with the molecular data in a combined branch-and-bound parsimony analysis [45] after recoding the morphological matrix into DNA data form.

The results of the analyses testing the monophyly of the bracovirus-bearing wasps are summarized in Table 2. The phylogenetic trees resulting from the most taxonomically balanced (14-taxon) molecular, morphological, and combined analyses excluding nucleotides from length-variable regions are depicted in Fig. 1. Essentially all molecular analyses resulted in trees showing the bracovirus-bearing lineage to be monophyletic (Table 2). However, analyses that included all nucleotide sites gave this result with some combinations of alignment pa-

rameters but not others. In general, analyses including the length-variable (and difficult-to-align) sites tended to have a high noise-to-signal ratio, and often resulted in trees strongly discordant with the morphological results, especially in basal branching order. Taxon representation (20-taxa vs. 14-taxa) had no effect on results; both sets of analyses supported the monophyly of the bracovirus-bearing wasps, although bootstrap values for this clade were low (Table 2, Fig. 1A). In general, bootstrap support was higher in analyses using hand alignments to secondary structure. This suggests that incorporation of the secondary structure information improved the alignment, as has been indicated recently in other studies [46].

The morphological data, despite the relatively smaller size of the data set (21 characters versus 118 informative 16S characters), strongly supports the monophyly of the same bracovirus-bearing group (Fig. 1C). When combined with the molecular data, the morphological data tended to strengthen the result of monophyly, while shifting relationships within the “mi-

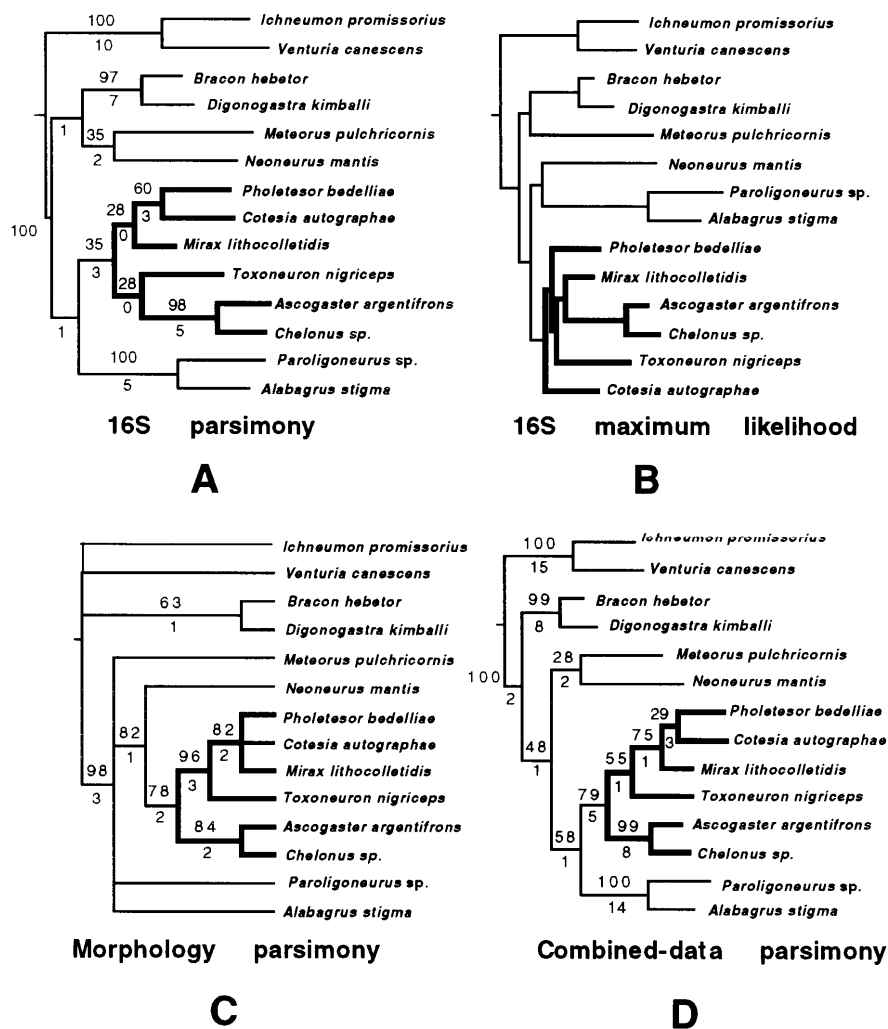


Fig. 1 A–D. Phylogenetic trees resulting from the 14-taxon analyses. Numbers above branches on the trees are the proportion of bootstrap replicates resolving that clade; those below the branches on the parsimony trees are Bremer [54] support (decay indices) calculated using TreeRot [55]. The polydnvirus-bearing groups are highlighted using thicker branches. **A** Treelength 409; consistency index (excluding uninformative characters) 0.52. **B** Ln likelihood 2112.29. **C** Treelength 26; consistency index 0.90. **D** Treelength 439; consistency index 0.53

crogastrid assemblage” to resemble those obtained using only the morphological data. This result, in which a small morphological data set contributes significantly to phylogenetic resolution when combined with a larger molecular data set, is not uncommon [47, 48], and emphasizes the advantages of using available data from multiple sources when practical. Interestingly, the data within the length-variable 16S regions often corroborated the morphological data within the microgastrids, but inclusion of these data produced mostly phylogenetic noise at higher taxo-

mic levels (among outgroups and more distantly related subfamilies). This probably results from the fact that data within those regions are easily aligned among closely-related taxa, but far more difficult to align unambiguously when more distant taxa are compared. Thus, as Simon et al. [49] suggested for the 12S gene, the 16S gene may contain regions useful for analysis at relatively high taxonomic levels (more conserved sites) and low levels (the length-variable regions), but use of the entire sequence may be inappropriate for higher level analyses.

These results indicate that the polydnvirus-bearing lineage of braconid wasps is monophyletic. The molecular data are consistent with morphological data under the criteria of both maximum parsimony and maximum likelihood, and when the data sets are combined, the support for monophyly is most robust. The results are also consistent with other molecular analyses of braconid relationships using other genes but of different scope [50, 51]. Furthermore, use of the secondary structure of the 16S molecule in aligning the DNA sequences leads to increased stability of results.

Preliminary evidence has been presented that the association between braconids and their viruses extends back in time at least as far as the initial divergence of the microgastrid complex of subfamilies (at least 60 mya based on fossils assignable to extant microgastrid genera collected from Eocene and Oligocene deposits [52, 53]). Given this probable long association between the wasps and viruses, it is not surprising that together they have evolved a rich variety of interactions with their caterpillar hosts. Further molecular investigations into the phylogeny of the viruses themselves are likely to provide additional insights into the origin and history of this fascinating association.

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## Poison Gland of Queen Fire Ants (*Solenopsis invicta*) is the Source of a Primer Pheromone

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The use of pheromones in animal communication is ancient and widespread. These semiochemicals are generally divided into two main groups – releaser pheromones which act on the nervous system to elicit rapid behavioral responses, and primer pheromones which act physiologically to modify the endocrine or reproductive system [1]. Both releaser and primer pheromones play a critical role in regulating the behavior and physiology of social insects. Whereas the glandular source and chemical structure of dozens of social insect releaser pheromones have been documented [2], the glands producing