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Phylogenetic Signal in the COI, 16S, and 28S Genes for Inferring Relationships among Genera of Microgastrinae (Hymenoptera; Braconidae): Evidence of a High Diversification Rate in This Group of Parasitoids

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The subfamily Microgastrinae is a highly diversified group of parasitoid wasps that attacks all of the different groups of Lepidoptera. We explore here the phylogenetic signal in three gene (mitochondrial COI and 16S, and nuclear 28S) fragments as an assessment of their utility in resolving generic relationships within this species-rich insect group. These genes were chosen because their level of sequence divergence is thought to be appropriate for this study and because they have resolved relationships among other braconid wasps at similar taxonomic levels. True phylogenetic signal, as opposed to random signal or noise, was detected in the 16S and 28S data sets. Phylogenetic analyses conducted on each microgastrine data set, however, have all resulted in poorly resolved trees, with most clades being supported by low bootstrap values. The phylogenetic signal, if present, is therefore concentrated on a few well-supported clades. Some rapidly evolving sites may be too saturated to be phylogenetically useful. Nonetheless, the sequence data (nearly 2300 nucleotides) used here appear to exhibit the appropriate level of variation, theoretically, to resolve the relationships studied. Moreover, the clades that are well supported by the data are usually supported by more than one data set and represent different levels of sequence divergence. We suggest that the lack of phylogenetic signal observed is an indication of the presence of many short internal branches on the phylogeny being estimated, which in turn might be the result of a rapid diversification of the taxa examined. Relative specialization of diet, which is typically associated with parasitic behavior, is believed to result in high radiation rates, which may have been especially high in microgastrine wasps because of the great diversity of their lepidopteran hosts. This hypothesis of a rapid diversification caused by an abundance of host species remains speculative and more data will be needed to test it further.

Key Words: **16SrDNA; 28SrDNA; COI; Microgastrinae; Braconidae; phylogenetic signal.**

The subfamily Microgastrinae is a large group of parasitoid wasps with over 1400 described species worldwide, divided into 55 genera, and an estimated number of species ranging between 5000 and 10,000 (Whitfield, 1997a). The adult females deposit their eggs inside their larval lepidopteran hosts, within which the parasitoid larvae feed and develop (Shaw and Huddleston, 1991). The Microgastrinae attack essentially all groups of Lepidoptera and many species are used as biological control agents of agricultural pests (Whitfield, 1995, 1997a).

Morphological characters have been used to estimate the phylogeny of microgastrines in two previous studies (Mason, 1981; Walker *et al.*, 1990). The results are strongly discordant, largely due to extensive conflict between character systems and how the analyses were conducted. Other surveys of morphological characters are proceeding (Whitfield *et al.*, in review); yet they are unable to resolve many relationships within the subfamily and it has become clear that molecular characters are needed to further resolve this phylogeny.

Because of the high number of species included, estimating a phylogeny of this subfamily is a complex task. To date, relatively few molecular phylogenetic studies have concentrated at the generic level in such species-rich insect groups. The speciose Microgastrinae have clearly experienced extensive radiation events; whether their genome has retained enough historical signal to resolve all phylogenetic relationships within this group remains to be explored.

In this paper, we explore the phylogenetic signal present in three genes, the mitochondrial (mt) cytochrome oxidase I (COI), the mt large subunit (16S) rDNA, and the nuclear large subunit (28S) rDNA, to

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assess their utility in resolving generic relationships within this highly diversified group of parasitic wasps. These three genes were chosen because they appear, from previous studies, to match the level of divergence needed to infer generic relationships. The COI and 16S genes have been used in many insect phylogenetic studies (e.g., see review in Simon *et al.*, 1994), at both lower and higher taxonomic levels, as well as in studies of diverse hymenopteran groups (including the family Braconidae) (e.g., Dowton and Austin, 1994; Dowton *et al.*, 1998). The 28S gene appears to be a promising molecular marker that has been useful for estimating relationships corresponding to generic and subfamily levels of divergence within the family Braconidae (Belshaw and Quicke, 1997; Dowton and Austin, 1999).

MATERIALS AND METHODS

Specimens Examined

Table 1 provides a list of the species belonging to 17 Microgastrinae genera, collected for this study, along with the localities and dates of sampling. Three outgroup species each belonging to a different braconid subfamily were used, corresponding to the closest relatives to the Microgastrinae, as suggested by previous morphological (Whitfield and Mason, 1994) and molecular (Whitfield, 1997b; Dowton and Austin, 1999) studies.

DNA Sequencing

For each species, we sequenced a fragment of 1235 nucleotides from the COI, 440 nucleotides from the 16S, and 618 nucleotides from the 28S, corresponding to the D2 and D3 expansion regions of the *Drosophila melanogaster* sequence (Hancock *et al.*, 1988). Insects were ground in an SDS homogenization buffer (50 mM Tris, 50 mM EDTA, 1% SDS, and 0.5% NaCl) and incubated for 1–2 h with proteinase K at 60°C. Four phenol/chloroform extractions were done, followed by ethanol precipitation and resuspension in TE buffer (10 mM Tris, 1 mM EDTA). The DNA fragments were amplified using the primers shown in Table 2. PCR conditions were as follows: after an initial denaturation step of 30 s at 94°C, 35 cycles of 60 s at 94°C, 60 s at 48–60°C, and 60 s at 68–72°C, and a final extension step of 2 min at 68–72°C. The annealing and extension temperatures were modified to optimize the reaction conditions, depending on the taxon amplified and the primers used. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega). Sequencing was conducted either manually (a portion of the COI and 16S sequences) with the *fmol* Cycle Sequencing Kit (Promega) or using an ABI automated sequencer with the PRISM Dye Terminator

TABLE 1

Specimens Collected for This Study

Taxa	Subfamily	Locality	Collection date
Alphomelon n. sp.	Microgastrinae	Santa Rosa, Costa Rica	1994
Apanteles nephoptericis (Packard)	Microgastrinae	Fayetteville, Arkansas	1995
Apanteles canarsiae Ashmead	Microgastrinae	Fayetteville, Arkansas	1996
Cotesia autographae (Mues.)	Microgastrinae	Tifton, Georgia	1994
Cotesia griffini (Viereck)	Microgastrinae	Fayetteville, Arkansas	1996
Cotesia marginiventris (Cresson)	Microgastrinae	Fayetteville, Arkansas	1996
Dasylagon n. sp.	Microgastrinae	Guanabara, Brasil	1974
Diolcogaster schizurae (Muesebeck)	Microgastrinae	Fayetteville, Arkansas	1996
Dolichogenidea lacteicolor (Viereck)	Microgastrinae	Lab culture, D. Stoltz, Nova Scotia	1993
Dolichogenidea n. sp.	Microgastrinae	Santa Rosa, Costa Rica	1990
Glyptapanteles indiensis (Marsh)	Microgastrinae	Lab culture, D. Stoltz, Nova Scotia	1993
Glyptapanteles porthetriae (Mues.)	Microgastrinae	Lab culture, D. Stoltz, Nova Scotia	1993
Hypomicrogaster ecdytolophae (Mues.)	Microgastrinae	Santa Rosa, Costa Rica	1994
Microgaster canadensis Muesebeck	Microgastrinae	Fayetteville, Arkansas	1996
Microplitis maturus Weed	Microgastrinae	Fayetteville, Arkansas	1996
Parapanteles paradoxus (Muesebeck)	Microgastrinae	Santa Rosa, Costa Rica	1990
Pholetesor bedelliae (Viereck)	Microgastrinae	Fayetteville, Arkansas	1996
Pholetesor ornigis (Weed)	Microgastrinae	Fayetteville, Arkansas	1996
Prasmodon eminens Nixon	Microgastrinae	Balbina, Amazonas, Brasil	1996
Pseudapanteles dignus (Mues.)	Microgastrinae	Tucuman, Argentina	1994
Rhygoplitis terminalis (Gahan)	Microgastrinae	Fayetteville, Arkansas	1996
Sathon falcatus (Nees)	Microgastrinae	Terhagen, The Netherlands	1987
Snellenius n. sp.	Microgastrinae	Sao Carlos, Brasil	1997
Chelonus (Microchelonus) sp.	Cheloninae	Fayetteville, Arkansas	1994
Mirax sp.	Miracinae	Mt. Ida, Ouachita Mtns, Arkansas	1994
Toxoneuron nigriceps (Viereck)	Cardiochilinae	Lab Culture, S. B. Vinson, Texas	1995

TABLE 2

Primers Used in This Study

Gene	Name	Sequence	Source
COI			
Forward	C1-J-1751	5′GGATCACCTGATATAGCATTCCC3′	Simon *et al.*, 1994
Forward	C1-J-2183	5′CAACATTTATTTTGATTTTTTGG3′	Simon *et al.*, 1994
Reverse	C1-N-2191	5′CCCGGTAAAATTAAAATATAAACTTC3′	Simon *et al.*, 1994
Reverse	L2-N-3014	5′TCCAATGCACTAATCTGCCATATTA3′	Simon *et al.*, 1994
Reverse	Mod-L2-N-3014	5′TTCATTGCATTATTCTGYCATWTTA3′	Modified from Simon *et al.*, 1994
Reverse	COI-MD	5′ATTGCAAATACTGCACCTAT3′	Dowton and Austin, 1997
Forward	COI-PM	5′CATGATACWTATTATGTWG3′	This study
28S			
Forward		5′AAGAGAGAGTTCAAGAGTACGTG3′	Modified from Belshaw and Quicke, 1997
Reverse	28S-PM	5′TAGTTCACCATCTTTCGGGTCCC3′	This study
16S			
Forward	16SWb	5′CACCTGTTTATCAAAAACAT3′	Dowton and Austin, 1994
Reverse		5′CTTATTCAACATCGAGGTC3′	Whitfield, 1997b

Cycle Sequencing Ready Reaction Kit FS (Perkin–Elmer). In each case, both strands, amplified from two different PCRs, were sequenced. Voucher specimens and unused specimen remnants of all taxa are deposited in the University of Arkansas Arthropod Museum. In addition, 28S sequences corresponding to the D2 expansion region, were provided by Mark Dowton (Dowton and Austin, 1999) for three outgroup species (*Toxoneuron nigriceps, Chelonus* sp., and *Mirax lithocolletidis* Ashmead). The 28S sequence of *M. lithocolletidis* was included in the 28S data set because we failed to sequence a portion of the 28S D2 region from *Mirax* sp. used in the 16S and COI analyses. One 16S sequence for *Cotesia glomerata* (L.) was retrieved from GenBank (Accession no. U06958, Dowton and Austin, 1994). Some 16S sequences were already available from Whitfield (1997b) (U68150, U68151, U68153–U68156). We failed to sequence the 16S fragment in the case of *Chelonus* sp., and this outgroup taxon was thus not used to root the 16S tree.

Sequence Alignment

The COI sequences were edited in the program SeqPup version 0.6 (Gilbert, 1996) and aligned manually. Sequences from the two rDNA genes were aligned based on the criterion of maximum parsimony using the program Malign 2.5 (command "build," randorderns 10, alignswap) (Wheeler and Gladstein, 1995) and specifying a gap:change cost ratio of 2, 4, and 6. Ambiguous regions of alignment were identified by comparing the different alignments produced for each ribosomal gene, and the effect of excluding those regions on the phylogenetic results was tested. Because the secondary structure of the 16S is well documented for insects in general and Hymenoptera in particular, we have also aligned the 16S sequences to the secondary structure using the method described in Whitfield and Cameron (1998). A similarly well established model of secondary structure is not available for the 28S gene in Hymenoptera. Gaps were coded as missing characters. Base frequencies for each gene and uncorrected pairwise sequence divergences based on the nucleotide data were calculated with PAUP*4 (test versions 4.0d63; Swofford, 1998). A χ^2 test of homogeneity of base frequencies across taxa was also performed using PAUP*4.

Phylogenetic Signal

The phylogenetic signal in the three nucleotide data sets was explored using the methods described below. We also compared the DNA data with a set of morphological characters described in detail in Whitfield *et al.* (in review). Here, we look at a restricted morphology data set containing only the taxa examined in this study (Table 1).

Prior to phylogenetic analyses, the nucleotide data were explored using Relative Apparent Synapomorphy Analysis (RASA, Lyons-Weiler *et al.*, 1996). This regression-based statistical method tests for the presence of phylogenetic signal (i.e., character covariation) in a data set by inferring a slope that depicts the covariation between the apparent cladistic similarity and the phenetic similarity observed between pairs of taxa and by comparing this slope with a null slope expected under an equiprobable model (Student's *t* distribution). It has the advantage of being tree independent, thus avoiding potential circular reasoning (a tree-based statistic may be misleading if the tree that is estimated from the data and then used to calculate that statistic is very different from the "true" phylogeny) (Lyons-Weiler, 1998). We used RASA to test for the presence of phylogenetic signal in each of the three DNA data sets and to compare the amount of phylogenetic signal present in the different alignments of each ribosomal gene.

To explore the conflict between data sets, pairwise incongruence among the three genes and the morphology data set was estimated, after removing invariant characters (Cunningham, 1997b), using the incongru-

ence length difference test of Farris *et al.* (1994) with PAUP*4 (1000 replicates). Also, partitioned Bremer supports (Baker and DeSalle, 1997; Baker *et al.*, 1998) were computed after a "total evidence" tree was estimated from the combined data sets (DNA + morphology) (see below). These Bremer supports (Bremer, 1988) are calculated by comparing the topologies obtained for each separate data set with the combined topology and show how each data source contributes to the branch support of this "total evidence" tree (Baker and DeSalle, 1997).

In order to visualize the distribution of the phylogenetic signal in each data set among the different possible clades, we inferred distance bipartition spectra, performing a Hadamard transformation on the distance data (Hendy and Penny, 1993; Lento *et al.*, 1995; Lockhart *et al.*, 1995), including (1) p distances, (2) Tamura–Nei distances (Tamura and Nei, 1993), and (3) LogDet distances using the program Spectrum 2.0 (Charleston and Page, 1997). These distances were chosen because evidence of highly unequal base frequencies, unequal frequencies of different substitution types, and significant variation of base frequencies across taxa was observed in part of the data set (see Results). Because a maximum of 18 taxa can be analyzed by this program, we first discarded all outgroup taxa, plus *Pholetesor ornigis*, *Dasylagon*, *Sathon*, and *Diolcogaster* from the data set.

Phylogeny Estimation

All phylogenetic analyses were performed using PAUP*4 (versions d63 and d64). Unweighted parsimony analyses were conducted on the three DNA data sets and on the morphology data set (heuristic search, 100 random addition sequences, TBR swapping). Mean uncorrected sequence divergences were calculated for each clade on the inferred phylogenetic trees. To estimate the level of homoplasy in the different genes, we generated charts of the distribution of number of steps per character with MacClade 3.07 (Maddison and Maddison, 1992), using the unweighted most parsimonious trees as a reference. Because the characterization of the nucleotide variation in the different studied DNA fragments suggests the occurrence of saturation of substitutions for at least a fraction of the sites (see Results), we have conducted the following additional analyses.

(1) Weighted parsimony analyses. Some types of substitutions have a higher frequency of occurrence than others and are therefore more likely to result in homoplasy in the data set. If saturation of substitution occurs in the data set, this may justify the differential weighting of different substitution types (Milinkovitch *et al.*, 1996; Swofford *et al.*, 1996). We performed six-parameter weighted parsimony analyses (heuristic search, 100 random addition sequences, TBR swapping) which have been shown to increase accuracy and congruence among data sets in certain cases (Cunningham, 1997a). In these analyses, a specific weight was defined for each one of the six substitution classes, based on their observed frequencies. These frequencies were estimated from the unweighted parsimony trees with MacClade and the different weights were then calculated following Wheeler (1990).

(2) Minimum evolution analyses. These (Rzhetsky and Nei, 1992) were conducted on each gene fragment (heuristic search, TBR swapping). Two distances were used: the distance (referred to as F84 in PAUP*) of Tateno *et al.* (1994) corresponding to Felsenstein's (1993) model of sequence evolution that allows unequal base frequencies and defines two substitution types (transitions and transversions) (Swofford *et al.*, 1996); and the LogDet distance (Lockhart *et al.*, 1994) which corrects for unequal base composition among taxa. These distances were chosen because highly unequal base frequencies and significant variation of base frequencies across taxa were observed in part of the data set (see Results).

(3) Maximum likelihood analyses. A maximum likelihood (ML) analysis, which incorporates an empirically derived model of substitutional change and takes branch length into account, was also conducted on each data set (heuristic search, as is addition sequence, model of Hasegawa *et al.*, 1985). A successive approximations strategy was used. Different parameters of the model (Ti/Tv ratio, gamma shape parameter, and proportion of invariable sites) were first estimated, using the trees obtained from the unweighted parsimony analyses as a reasonable first estimate of the phylogeny. These parameters were then fixed in a ML heuristic search and the topology found was used to reoptimize the parameter's values. The alternation between parameters estimation and tree searching was continued until the same tree was found in successive iterations (Swofford *et al.*, 1996).

A "total evidence" parsimony analysis was conducted on the combined DNA and morphology data sets. When a taxon present in one data set was not available in another, its characters were coded as missing for the corresponding data in the combined data set. Because the two *Cotesia* species available in the 16S data set are different from the two *Cotesia* present in the other data sets, they were excluded from the combined analysis.

In all the parsimony and minimum evolution analyses, the support for each clade was estimated by performing a bootstrap analysis (400 replicates, heuristic search, 10 random addition sequence).

Branch Length Estimates

To investigate the hypothesis that the presence of short branches on the phylogeny being estimated are responsible for the low resolution provided by our data (see below), we estimated branch lengths for the most parsimonious topologies (unweighted analyses) obtained for each gene. The most parsimonious trees were

chosen to allow the comparison between branch length and branch support (bootstrap analyses could not be performed with maximum likelihood due to computational limitations). Branch lengths were estimated by maximum likelihood with PAUP*4, using the same settings used for the ML heuristic searches described above. The maximum likelihood method was used because it is more effective than parsimony for estimating branch lengths, since a model correcting for multiple substitutions at a given site is incorporated into the method. To test for the association between branch length and branch support, we examined the degree of correlation between those two variables, using a nonparametric procedure, the Kendall rank-correlation coefficient (Kendall, 1955).

RESULTS

Alignment

For the 28S data set, two most parsimonious (MP) alignments were found when specifying a gap:change cost ratio of 2 and one different MP alignment each when specifying a ratio of 4 and 6. For the 16S data set, one different MP alignment was found for each one of the three gap:change cost ratios and another one based on the secondary structure of the gene. In the alignments, 40–51 gaps were introduced in the 28S, 38–39 gaps in the 16S, and 2 gaps (3 nucleotides in length) in the COI. All sequences are available from GenBank, under Accession nos. AF102700–AF102765. Aligned sequences are available from the authors or from TreeBase (Sanderson *et al.*, 1994).

Characterization of the Nucleotide Data

Uncorrected pairwise divergences between ingroup taxa ranged from 3 to 22% for the COI, 0.7 to 12% for the 28S, and 6 to 20% for the 16S. The base composition for each gene is shown in Table 3, separately for the three codon positions of the COI. Insect mt DNA generally tends to display a high A-T content, and this trend seems even greater in Hymenoptera (Simon *et al.*, 1994; Dowton and Austin, 1997; Whitfield and Cameron, 1998). In our data, the A-T bias is particularly extreme in the 16S and in the third codon positions of COI. This is important because with essentially only two nucleotides available at many sites, homoplasy will occur much more frequently at those sites. Moreover, many COI third position and 16S sites exhibit multiple changes on our MP trees (Fig. 1) and the more variable ones are likely to be too saturated to be phylogenetically useful. On the other hand, a substantial proportion of the variable 28S sites have experienced only a small number of changes (one to three), which should make this gene a more useful marker for recovering older relationships. A large number of slowly evolving sites are also observed in the first and second positions of the COI gene, although a large proportion of those positions are invariable. The four partitions of characters presented in Fig. 1 show different levels of variation, indicating that each one should be useful for estimating a different portion of the Microgastrinae phylogeny. The χ^2 test of homogeneity of base frequencies across taxa reported *P* values of 1.0 for the 16S, 1.0 for the 28S, and 0.999, 1.0, and 0.000 for

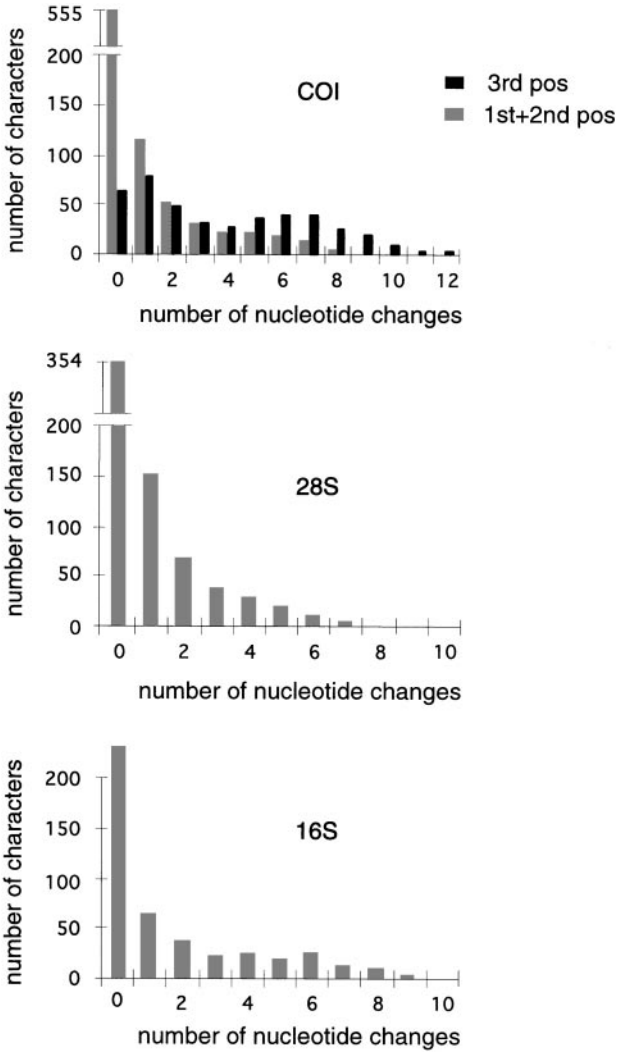

FIG. 1. Distribution of the number of changes per character for the COI, 28S, and 16S sequences on the MP trees.

TABLE 3

Mean Nucleotide Frequencies

	A	C	G	T
16S	44.23	6.43	8.99	40.35
COI (1st)	32.11	9.84	22.64	35.41
COI (2nd)	18.72	18.86	15.94	46.48
COI (3rd)	41.82	1.96	3.21	53.01
28S	25.9	18.28	22.83	32.99

the 1st, 2nd, and 3rd positions of COI, respectively. Significant differences in base frequencies between taxa is therefore observed in the 3rd position sites of COI. These differences were taken into account by performing minimum evolution analyses on the sequence data using the LogDet distance (see below).

RASA

RASA analyses were conducted on each aligned data set. For the 28S sequences, the two highest *t*-RASA values (2.53 and 2.18) were observed for the two alignments obtained using a gap:change cost ratio of 2, and those values, although relatively low, are statistically significant. The two other alignments are characterized by *t*-RASA of 1.41 (gap:change cost ratio of 4) and 1.54 (gap:change cost ratio of 6). Both values are not significant. Excluding ambiguous regions resulted in a *t*-RASA of 2.32 (significant). For the 16S, *t*-RASAs of 8.99, 7.11, and 7.31 were observed for the alignments obtained using a gap:change cost ratio of 2, 4, and 6, respectively. The 16S alignment based on the secondary structure resulted in a *t*-RASA of 3.60. Excluding ambiguous regions of alignment resulted in a *t*-RASA of 3.78. All *t*-RASA values for the 16S alignments are statistically significant. Finally, a *t*-RASA of −3.07 (all positions) and −1.53 (3rd positions excluded) were found for the COI data set, suggesting a lack of phylogenetic signal in this data set.

Phylogenetic Analyses

For the 16S and 28S, parsimony analyses were conducted on each different alignment separately. However, only the results obtained for the alignment characterized by the highest *t*-RASA (2.53 for the 28S and 8.99 for the 16S), i.e., identified as maximizing the amount of true phylogenetic signal as opposed to noise or random signal, are shown. The other alignments produced equally or less resolved trees and all clades supported by a reasonably high bootstrap value (>60%) in those trees are also present in the trees shown here. Minimum evolution and maximum likelihood analyses were performed on the alignments associated with the highest *t*-RASA only.

The results of the unweighted parsimony analyses conducted for each gene are shown separately in Fig. 2. They resulted in poorly resolved trees with only a few clades supported by a BV above 50%. The COI MP strict consensus tree resulting from the analysis of the third codon positions (3rd pos) alone (Fig. 3C) supports (with a BV > 50%) only three clades, which are characterized by a mean 3rd pos sequence divergence below 15%. This, along with the extreme A-T richness observed for those sites (Table 3) and the multiple substitutions exhibited by most of them (Fig. 1), suggests that saturation of substitutions occurs above this threshold divergence level. A similar explanation cannot be used to account for the low support values observed on the COI 1st + 2nd MP consensus tree (Fig. 2B), however. Indeed, the mean sequence divergences shown by the well-supported clades on that tree range between 0.5 and 11.0%, spanning the entire spectrum of divergence values observed. The 28S MP consensus tree (Fig. 2D) is equally poorly resolved and supports clades (with a BV above 50%) showing a range of mean sequence divergences of 0.7–11.0%, again providing no obvious evidence for saturation of substitutions at those sites. Both the 28S and the COI MP trees indicate support for the following clades: (*Microplitis* + *Snellenius*), (*Prasmodon* + *Pseudapanteles*), and a clade grouping the two *Cotesia* species. The first clade is supported even by third COI positions (although with a low BV), despite displaying a mean sequence divergence (3rd pos) above the 15% threshold value. The 16S MP tree shows resolution only for the clades characterized by a mean sequence divergence equal to or below 11% and seems to show saturation of substitutions above this threshold value. The clade (*Glyptapanteles ind.* + *Glyptapanteles por.* + *Sathon*) is also found on the COI MP tree, the clade (*Parapanteles* + *Hypomicrogaster*) is also found on the 28S MP tree, and the clade grouping *Cotesia* species is supported by all three data sets. Thus, the few clades that are well supported by the data are usually supported by at least two data sets.

Large differences in the frequencies of the different types of changes were observed in each data set. As expected from the high AT richness of the mitochondrial genome, A-T transversions dominate in COI and 16S, representing almost 80% of all substitutions. The weighted parsimony analyses, in which we attempted to take into account these differences, resulted in MP trees that were very similar to the unweighted MP trees (results not shown). The COI MP tree shows slightly higher BVs for clades already well supported (BV > 50%) by the unweighted analysis and more closely resembles the 16S tree: the clade (*Glyptapanteles indiensis* + *Sathon*) appears, supported with a BV of 74%, as well as the clade (*Apanteles can.* + *Apanteles nephoptericis*) (BV of 51%). The 28S and 16S weighted MP trees remain similar to the unweighted MP trees, at least with respect to the clades supported by a BV > 50%. The weighted analyses did not increase the number of clades showing high BVs; most clades in each tree were still supported below 20–30%, with a high proportion of them showing BVs below 5%.

The minimum evolution analyses using the F84 and LogDet distances implemented in PAUP* resulted in topologies that were highly similar to the parsimony analyses. The clades that were well supported by the parsimony analyses were also highly supported in the ME trees; no additional clades were found. The maximum likelihood analyses resulted in one ML tree for the COI, one ML tree for the 16S, and three ML trees for the 28S. All the clades that were supported by a

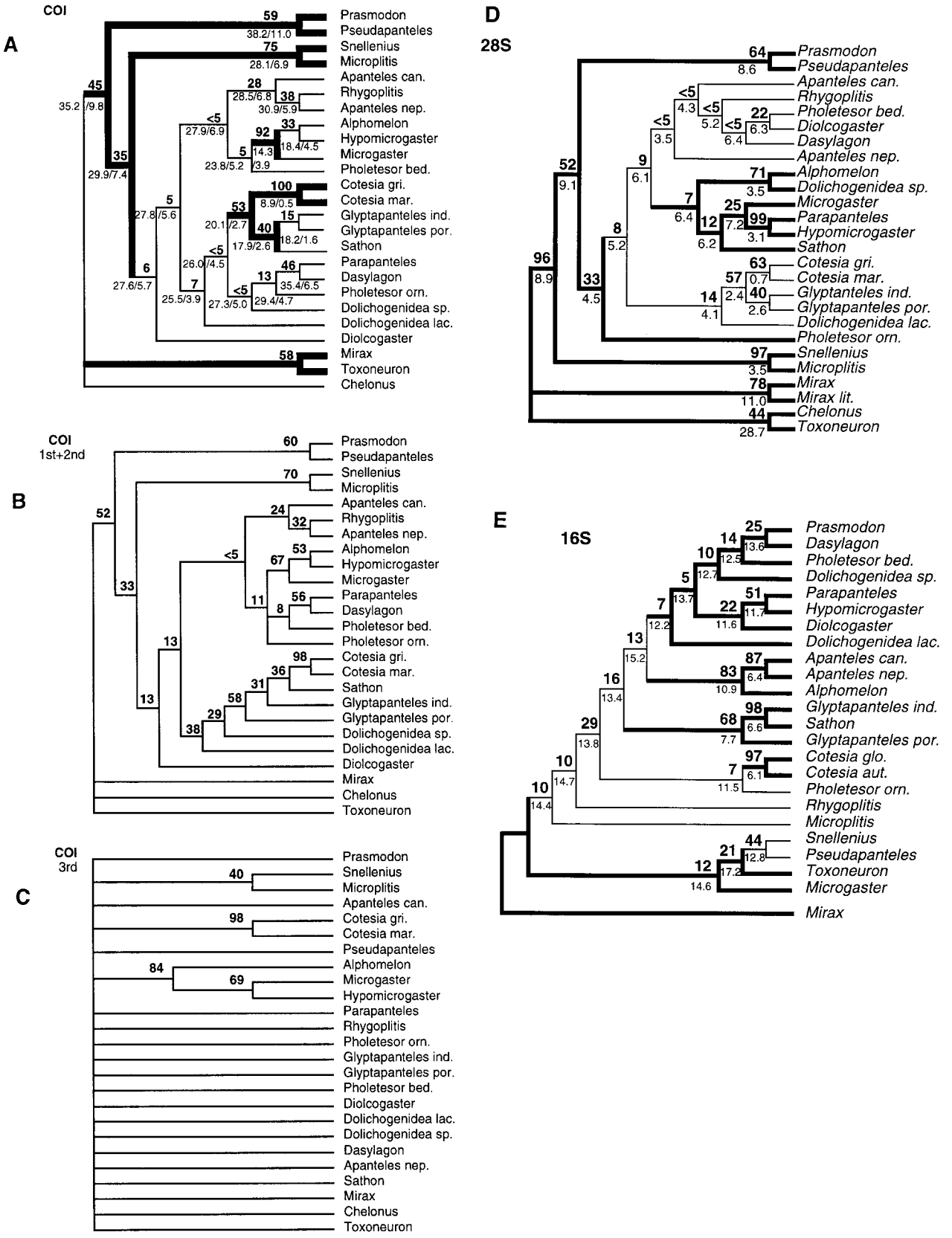

FIG. 2. MP trees inferred by unweighted parsimony analysis of the different data partitions: (A) COI, all positions included. One of the 6 MP trees. BV are shown above the branches and thick branches denote clades common to all 6 MP trees (strict consensus). Values below branches are mean 3rd-codon-position divergences/mean 1st + 2nd-codon-position divergences. (B) COI, third positions excluded. Strict

BV > 50% on the MP trees were present in the corresponding ML trees. Because the ML trees provide an estimate of branch lengths, they are presented for each gene fragment in Fig. 3.

Finally, the parsimony analysis of the morphology data set provided one fully resolved MP tree, shown in Fig. 4. The level of support for most of the clades is, however, as low as on the DNA trees.

Congruence Among Data Partitions

The incongruence length difference (ILD) test resulted in significant *P* values of 0.001 for each pairwise test involving the 16S data set. In the COI vs 28S, COI vs morphology, and 28S vs morphology tests, *P* values of 0.029, 0.110, and 0.160, respectively, were obtained.

We combined all three genes and the morphology data into one data set, upon which an unweighted parsimony analysis was performed. This analysis resulted in one MP tree shown in Fig. 5. As expected, clades that were well supported by one or more data sets and not contradicted by others are recovered with high BVs and clades that are well supported by one data set but contradicted by another show a lower level of support in the combined MP tree. A large proportion of this combined tree remains poorly supported.

Table 4 shows the partitioned Bremer supports for the combined tree. The 16S shows principally negative values and thus is responsible for most of the conflict between data sets, in agreement with the results of the ILD test. The nodes for which the 16S data conflict with the other data partitions are those characterized by a sequence divergence level above the threshold value of 11%. This further supports the case that the 16S fragment is too saturated above this divergence level. More importantly, more than one data partition usually contributes to the high total Bremer support displayed by a few clades on the combined MP tree. This corroborates the fact that well-supported clades are usually well supported by more than one data set in this study.

It is interesting to notice that although RASA failed to detect a significant level of phylogenetic signal in the COI data set, a few clades are relatively well supported (BV and partitioned Bremer support) by the COI sequences. This support is unlikely to be due to random signal because each clade is also supported by at least one of the other data partitions. It is thus not clear why RASA has detected phylogenetic signal in the 16S and 28S data sets and not in the COI data set, while the proportion of clades supported by each data partition appear very similar.

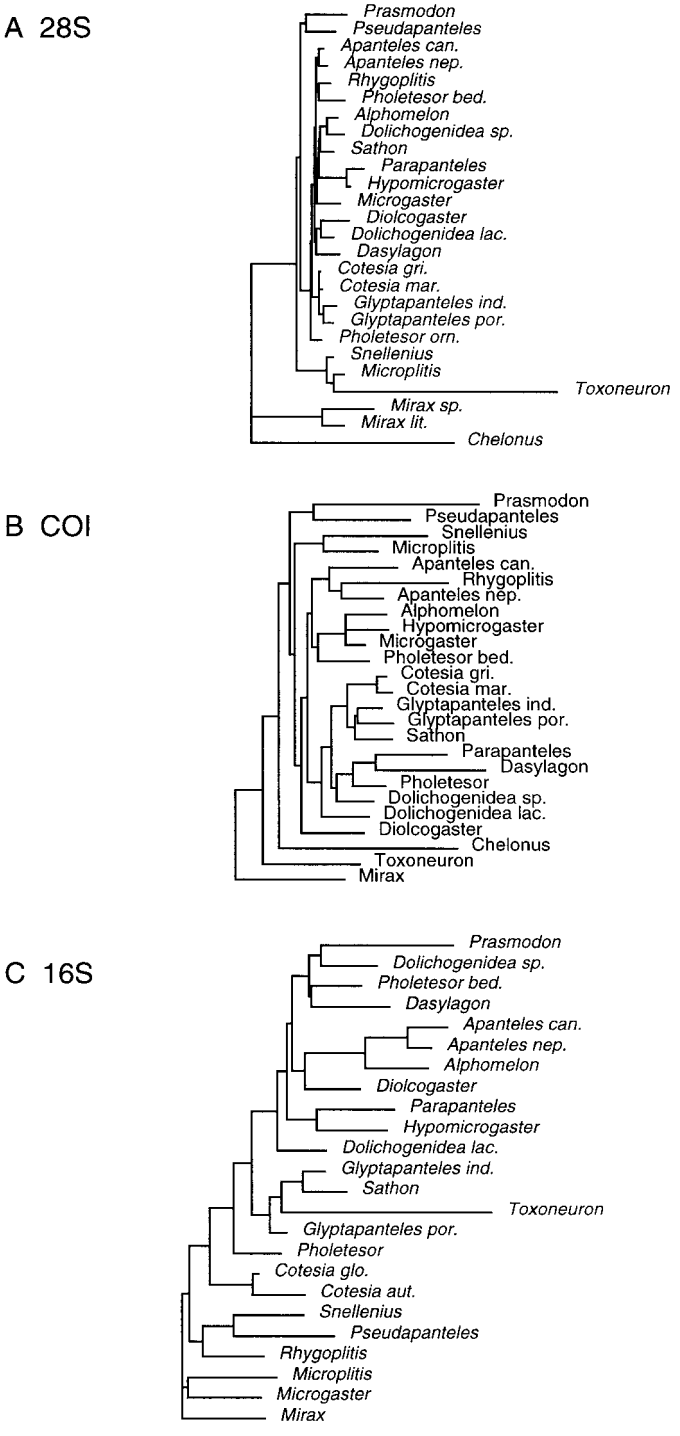

FIG. 3. Maximum likelihood trees inferred from the (A) 28S (strict consensus of three trees), (B) COI (one tree), and (C) 16S (one tree) data set. The estimated lengths of many internal branches are relatively short, especially those that correspond to weakly supported clades on the MP trees of Fig. 2.

consensus of 6 MP trees. BV are shown. (C) COI, third positions only. Strict consensus of 7 MP trees. (D) 28S. One of the 52 MP trees. BV are shown above the branches and thick branches denote clades common to all 52 MP trees. Values below branches are mean 28S sequence divergences. (E) 16S. One of the 8 MP trees. BV are shown above the branches and thick branches denote clades clades common to all 8 MP trees.

Bipartition Spectra

The distance bipartition spectra (p distances) for each gene are shown in Fig. 6. In each spectrum, we observe high support for the internal branches (each bipartition or "split" corresponds to one possible branch of a phylogenetic tree) that displayed high BVs on the MP trees of Fig. 2. The remaining splits are poorly supported, which confirms the presence of little phylogenetic signal in the data examined. The general pattern of all the Tamura–Nei and LogDet distance spectra (results not shown) are similar to the p distance spectra, in that no new well-supported split appears and all previously well-supported splits are found again.

Branch Lengths

Branch lengths inferred with parsimony (number of steps along a branch) were severely underestimated, especially for the 16S and COI data sets which contain some highly variable sites. Branch lengths were thus all estimated via maximum likelihood. For each gene fragment, a significant correlation ($P < 0.01$) was observed between bootstrap values and branch lengths on the MP trees of Fig. 2. A Kendall rank-correlation coefficients of 0.495, 0.569, and 0.428 were inferred for the COI, 28S, and 16S trees, respectively. The existence of a positive correlation between branch length and bootstrap support is hardly surprising. A short branch characterizes a clade for which few shared derived characters have evolved during the common history of the taxa involved and will thus be poorly supported.

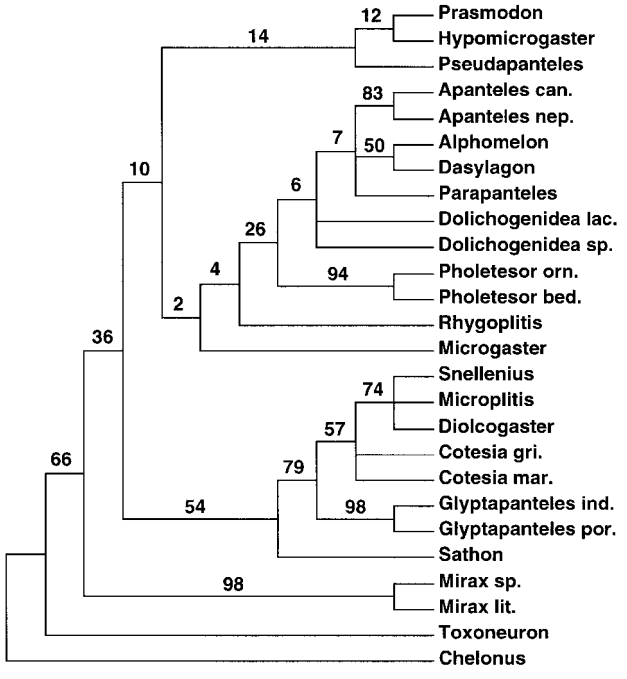

FIG. 4. Strict consensus of 36 trees resulting from the unweighted parsimony analysis of the morphology data set. BVs are shown above branches.

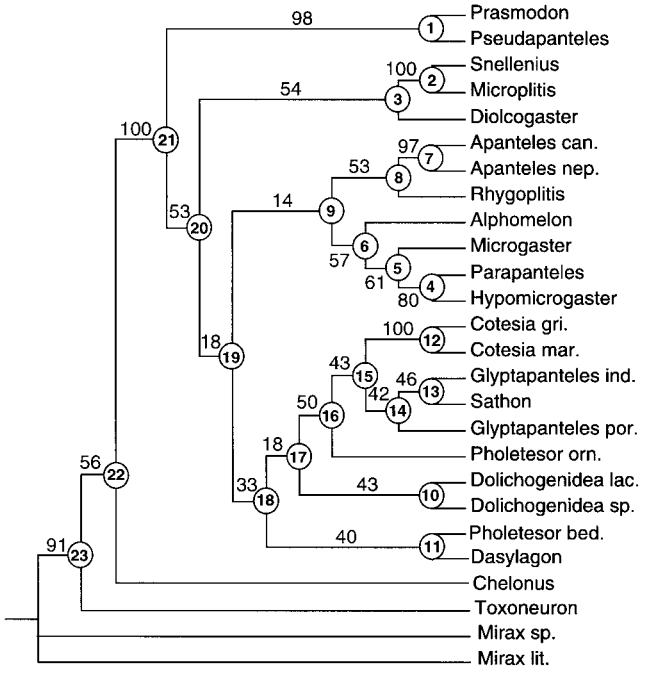

FIG. 5. MP tree inferred by unweighted parsimony analysis of the combined DNA + morphology data sets. BVs are shown above branches. Nodes are labeled (circled numbers) for identification in Table 4.

DISCUSSION

The high proportion of clades showing a low bootstrap support in the phylogenetic analyses is puzzling. Each of the genes examined in this study, representing a total of almost 2300 nucleotides, has been used previously and was proven to be phylogenetically useful for insects in general and Hymenoptera in particular at similar taxonomic levels. But many clades in our MP trees are supported by BVs under 20, 10, and 5%, revealing the presence of mostly noise (random signal produced by homoplasy) at that level. This is confirmed by the very few splits that receive high support in the inferred distance bipartition spectra. The detection of phylogenetic signal in the 28S and 16S data sets using RASA is not in contradiction with this result, as the signal detected could concern only a few clades.

How can we explain the low amount of phylogenetic signal observed in these data? One hypothesis is that the genes used here are inappropriate for the level of divergence examined. Indeed, the 16S and the third codon positions of the COI gene seem to be too saturated to be phylogenetically useful, except for those few clades exhibiting low levels of sequence divergence. Although both 16S and COI have been used at a higher taxonomic levels of analysis in Hymenoptera, it is

TABLE 4

Partitioned Bremer Support (Baker and DeSalle, 1997; Baker *et al.*, 1998) for the Total Evidence Tree (Including Morphology) Shown in Fig. 5

Node	Morphology	16S	COI	28S	Total Bremer Support
1	9.5	−7.5	5.5	3.5	11
2	2	−7.5	17	4.5	16
3	2.5	−1.5	0.5	−0.5	1
4	−2	−1.5	1	16.5	14
5	−2	−3	7	2	4
6	0	−5	9	0	4
7	9	4	9.3	−2.3	20
8	−2.9	−2.1	9.9	−1.9	3
9	3	−2	2.7	−2.7	1
10	5	−2	−2	0	1
11	3	0	−1	−1	1
12	5	2	6	2	15
13	−1	4.4	1	−3.4	1
14	−0.75	4	0.75	−3	1
15	1	−13.0	17	4	9
16	−2.5	−0.5	0.5	3.5	1
17	−1	0	2	−1	0
18	−1	−10	14	−2	1
19	−1	−2	5.7	−1.7	1
20	−0.5	−0.5	3.5	−1.5	1
21	4.5	3.5	13.5	1.5	23
22	−2	−1	7.5	−1.5	3
23	−2	6	1	−1	4
Totals	24.85	−28	125.15	14.0	136
Inform. chars.	45	146	418	154	763

Note. Values were calculated using TreeRot (Sorensen, 1996) and PAUP* (Swofford, 1998). Node numbers refer to the labeled nodes in Fig. 5. Nonintegers indicate that the values from several equally parsimonious trees were averaged.

possible that the clades studied here are located at a divergence level in which rapidly evolving sites are saturated, in part due to their extremely high AT content, while more constrained sites are not sufficiently variable yet to be phylogenetically useful. Similarly, the 28S gene resolves several clades at the tips of the tree and two basal clades. Thus, it could be argued that the level of divergence of the generic relationships examined here are for the most part located in a window in which rapidly evolving sites are too saturated and slowly evolving sites are not variable enough to provide phylogenetic signal.

The assumption of saturation of the 16S hypervariable regions and COI third codon position sites seems reasonable. However, there is no obvious evidence to suggest a lack of variation of the slowly evolving sites in

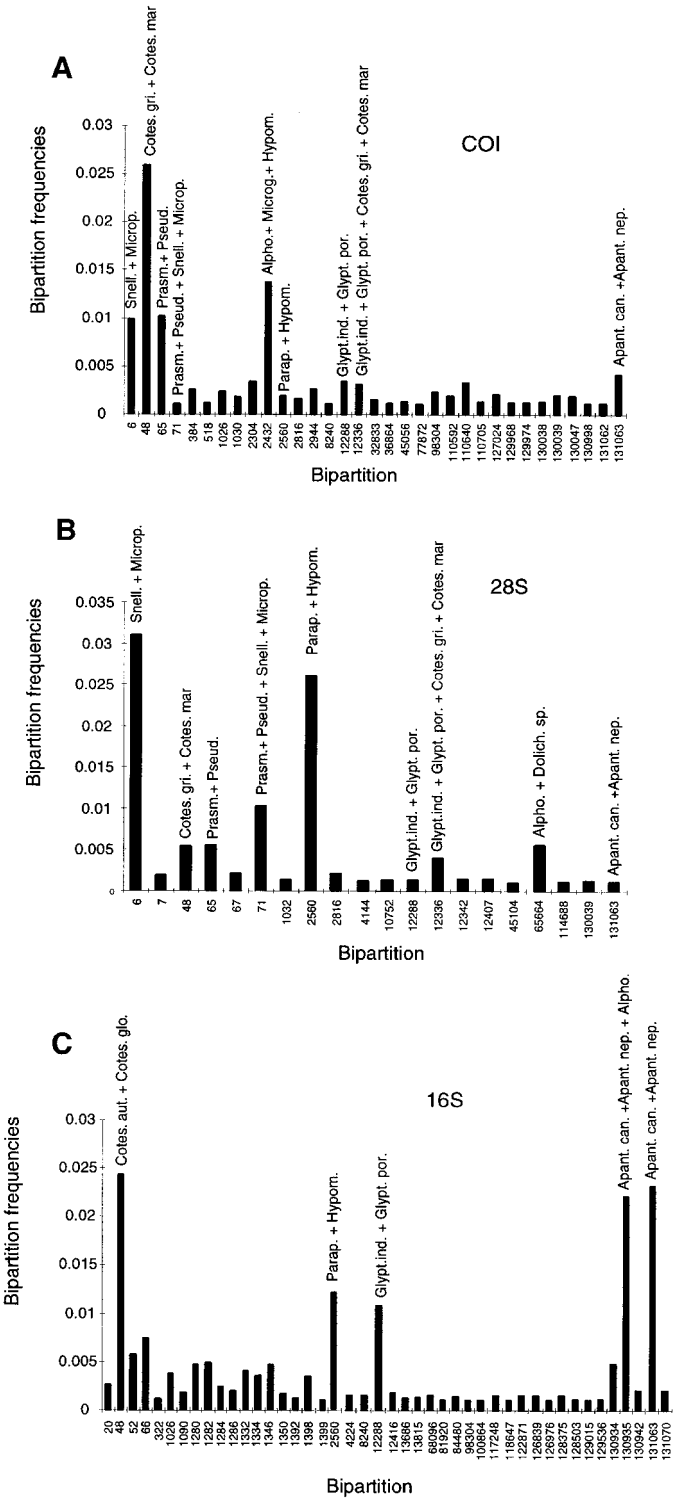

FIG. 6. Distance bipartition spectra inferred from p distances for the COI (A), 28S (B), and 16S (C) data sets. Each bar represents one bipartition or split, which corresponds to one branch of a phylogenetic tree. The frequency of each split indicates how well it is supported by the data. Only the splits corresponding to internal branches and that are supported by a frequency of at least 0.001 are shown. All splits that correspond to an internal branch supported by a BV >50% in at least one of the MP trees are identified in each spectrum, when present. All outgroup and four ingroup taxa were removed from the data set prior to the spectrum analysis, as the program Spectrum allows a maximum of 18 taxa.

our data. On the 28S MP tree (Fig. 2D), the well-supported clades (BV > 50%) display mean sequence divergence values virtually covering the entire range of observed divergences (0.7–11.0%). It is not possible to delineate a range of variation within which all clades are unresolved on the 28S tree. Similarly, for the COI MP trees (Figs. 2A–2C), first and second codon positions resolve clades exhibiting high sequence divergence and those grouping much closer taxa, including some that are independently supported by third positions. With respect to the taxonomic level examined in our study, both the 28S and the first and second positions of COI have a substantial proportion of slowly evolving sites (one to three steps, see Fig. 1). This condition is thought to be excellent for phylogenetic analysis because of the consequent low levels of homoplasy.

As already emphasized above, these three genes have already been used and have proven useful at similar taxonomic levels. Perhaps the most striking example concerns the 28S gene. The D2 expansion region of this gene (which is included in our data set) has been used with great success to resolve relationships within the subfamily Aphidiinae, as well as relationships between Aphidiinae and other braconid subfamilies, including the Microgastrinae (Belshaw and Quicke, 1997). In that study, the D2 expansion region was shown to be a promising molecular marker, exhibiting phylogenetic signal at all taxonomic levels examined. Moreover, the level of sequence divergence that characterizes the well-resolved clades within the Aphidiinae (as calculated by us after retrieving the sequences from GenBank) is similar to the level of divergence characterizing the clades within Microgastrinae. There is no a priori reason to suspect that this same gene would show a completely different pattern of evolution in two closely related subfamilies of parasitoid wasps.

Because there is no strong reason to suppose that the genes used here are inappropriate for the level of divergence examined, we suggest another hypothesis which appears more compatible with our observations. If the branch lengths estimated by the maximum likelihood analyses are correct, many of the internal branches on the trees of Fig. 3 are relatively short (branches should be compared within a given tree; for example, overall branch length of the 28S tree is smaller than that of the COI or 16S tree because the 28S gene is less variable). Moreover, a statistically significant positive correlation was shown between branch length and branch support (BVs). The low amount of phylogenetic resolution encountered in this study might therefore not be due to the inappropriateness of the gene fragments used, but rather to the peculiar topology (many short internal branches) of the tree being estimated. This is further supported by the fact that the few clades that are resolved in our analyses are often supported by more than one data set (see Results); i.e., the low resolution observed appears to be taxon, rather than character, dependent. The poor resolution offered by morphological data may also point to this same conclusion. The presence of a high proportion of short internal branches is not restricted to the trees of Fig. 2, since the spectral analysis examined all possible internal branches (or splits) and no other well-supported split than the ones present in those trees were found. Our conclusions therefore do not depend on the trees examined. The same hypothesis was invoked by Kraus and Miyamoto (1991) to explain the lack of resolution provided by complete sequences of the 12S and 16S genes to resolve phylogenetic relationships among the pecoran ruminants and by Lara *et al.* (1996) and Lessa and Cook (1998) using cytochrome *b* to estimate the phylogenies of two different groups of rodents.

The presence of many short branches on the phylogeny being estimated could be the result of a high diversification rate in this group of insects. Short periods of time between speciation events would easily explain the problems encountered with estimating their phylogeny. Is there any biological clue that would lead us to suspect a rapid diversification of these microgastrine insects that could result in the short branches that characterize most of the clades studied here?

Unfortunately, fossils provide little insight into this question. Virtually all microgastrine (amber) fossils are assignable to extant genera and are probably not old enough to recover the patterns of diversification at the generic level (Whitfield, unpublished data). Ecologically, all microgastrines are parasitoids and are relatively species specific, parasitizing only certain Lepidoptera species. They attack virtually the entire taxonomic and biological spectrum of Lepidoptera (Whitfield, 1997a). Because ecological specialization, particularly with respect to the insect's diet, is believed to result in high diversification rates (Wiegmann *et al.*, 1993), the extensive diversification of the Microgastrinae could be related to the extreme diversity of their hosts (the relatively recently evolved order Lepidoptera is one of the most diverse orders of insects, with 165,000 described species (Scoble, 1995)).

The hypothesis, although purely speculative, that microgastrine wasps have experienced a burst of speciation, resulting in the high number of extant species known today, seems reasonable and deserves further investigation. It will be necessary to accumulate additional data, from morphology and molecules, to further test this hypothesis as well as to resolve, if possible, the phylogenetic relationships within the Microgastrinae.

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