

Phylogenetic relationships of egg parasitoids (Hymenoptera: Eulophidae) and correlated life history characteristics of their Neotropical Cassidinae hosts (Coleoptera, Chrysomelidae)

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Abstract

Egg parasitoids in the family Eulophidae (Hymenoptera) are an important part of the community of insects attacking neotropical leaf beetles in the subfamily Cassidinae. We present a phylogeny of 24 species of oophagous Eulophidae, using the 28S rDNA, the ITS2 rDNA and the cytochrome *b* genes, applying the NJ, MP, ML and Bayesian tree reconstruction methods on each data set. We ask whether the phylogenetic relationships of the parasitoids are linked with the life history characteristics of their beetle hosts. We show that cladogenesis in the oophagous Eulophidae does correlate with ovipositional behaviour and, to a lesser extent, diet and tribal affinities of their hosts. Additionally using two methods of simultaneous analysis of several gene sets: the Total Evidence method, and the construction of a “supertree” by Matrix Representation Parsimony (MRP), we substantiate the same major phylogenetic relationships within the Eulophidae.

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1. Introduction

The Cassidinae (*sensu stricto*, i.e., “tortoise beetles” not including “hispine beetles”) is one of the most food-specialized subfamilies of Chrysomelidae (Jolivet, 1988). The association of Cassidinae with their host plants is characterized by the fact that most species feed on one or a small number of closely related host plant species, usually within the same plant family. Further, the subfamily as a whole is associated with a remarkably small percentage of available plant families (Vencl and Morton, 1999). For example, the approximately 130 species of Panamanian Cassidinae are known to feed on only 8 of approximately 150 plant families, and at a specific level almost all species are monophagous or narrowly oligophagous (Windsor et al., 1992). Brazilian Cas-

sidinae feed on only 15 plant families (Buzzi, 1994). Consequently, the continual occurrence of particular tortoise beetles species on the same family and species of host makes them predictable targets for predators and parasitoids (Cox, 1994). It has been proposed that, in response to this selective pressure, Cassidinae evolved numerous defense mechanisms at all stages of their development (Eisner, 1967; Hilker, 1994; Olmstead, 1996; Windsor et al., 1992; Cuignet et al., unpublished manuscript). Despite these defensive adaptations, the Cassidinae are one of the most parasitized subfamilies within the Chrysomelidae (Cox, 1994). In a previous study, we collected and identified the parasitoid guild of Neotropical Cassidinae in Panama (Cuignet et al., submitted). This guild was dominated by hymenopteran egg parasitoids in the family Eulophidae. As the largest family of Chalcidoidea, containing an estimated 3980 species in 283 genera (Gibson et al., 2000), the Eulophidae comprise four subfamilies: the Euderinae, Eulophinae, Tetrastichinae and Entedoninae (Cox, 1994). All four

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subfamilies are characterized by both molecular and morphological synapomorphies (Gauthier et al., 2000). Due to the diversity of their biology as well as the wealth of literature on the group, the Eulophidae are a valuable model system for investigating a variety of questions in ecology and evolution (Godfray, 1994; Gauthier et al., 2000). The goal of this study was to reconstruct the phylogenetic relationships of an assemblage of Eulophidae parasitoids and to investigate how they are related to Cassidinae of different taxonomic ranks (tribes and genera), diet and ovipositional behaviour. We additionally evaluate two methods of tree reconstruction from different data sets: the combination of the different datasets into a single matrix (Total Evidence) as opposed to the supertree reconstruction by Matrix Representation Parsimony (MRP). Interspecific variation in egg deposition among Cassidinae is considerable with some species laying their eggs in masses or solitarily, some species covering their eggs with faeces or enclosing them in an ootheca made of secretions, or even sometimes actively guarding egg masses (Windsor, 1987; Selman, 1994; Windsor et al., 1992). We test whether host ovipositional behaviour determines accessibility to egg parasitoids.

2. Materials and methods

2.1. Species

Our study focuses on the minute Eulophidae egg parasitoids of Neotropical Cassidinae species from Panama (Cuignet et al., submitted). Except for one individual Tetrastichinae (*Aprostocetus* sp.), all species reared from Cassidinae eggs fall within the subfamily Entedoninae (genera *Horismenus* and *Emersonella*). Eulophidae species, including seven newly described species (Hansson, 2002) were identified by Christer Hansson (Department of Zoology, Lund University). Secondary voucher specimens were deposited at the Natural History Museum of London and at the Smithsonian Tropical Research Institute in Panamá. The species studied and their associated Cassidinae host are listed in Table 1.

2.2. Morphological analysis

Sixty-seven morphological characters for the *Emersonella* species (except *E. nr. carballoï*) were coded and submitted to a parsimony analysis. All the information available was included based on the morphological description of Hansson (2002) and independent of the evolutionary significance of each character. Characters were weighted evenly. A listing of the morphological characters can be consulted online as [supplementary material](#).

2.3. DNA amplification and sequencing

DNA sequences were obtained from 24 species of Eulophidae. Sequence fragments from three genes dis-

playing an increasing degree of variability were analyzed: the conserved D2 expansion of the 28S nuclear gene, the more variable nuclear internal transcribed spacer 2 (ITS2), and the highly variable mitochondrial cytochrome *b* gene. DNA from single individuals previously stored in 95° ethanol was extracted with CTAB (Sigma) according to Navajas et al. (1998). Standard 25 µl PCR reactions were performed using 0.625 U *Taq* polymerase (Roche), 2.5 µl *Taq* buffer (Roche, 10 × Cc, 1.5 mM MgCl₂), 1 µl MgCl₂ 25 mM (Perkin-Elmer), 0.8 µl BSA 6.25 mg/ml and 1.25 µl (0.5 µM) of each primers. Primers sequences for the 28S rDNA D2 and the ITS2 were from Campbell et al. (1993). Primers for the cytochrome *b* were modifications of primers designed by Crozier et al. (1991), kindly provided by A. Beckenbach (Simon Fraser University). Forward primer sequence was 5'-GTT CTA CTT TGA GGN CAA ATR TC-3'; reverse primer sequence was 5'-AAC TCC TCC TAG TTT ATT NGG-3'. PCR conditions for the 28S D2 and the ITS2 were: 35 cycles of 94 °C denaturation (30 s), 55 °C annealing (30 s) and 72 °C elongation (30 s) with an initial 94 °C denaturation (60 s) and a final 72 °C extension (7 min). The denaturation and extension phases were similar for the cytochrome *b* but cycling conditions were: 35 cycles of 94 °C denaturation (30 s), 50 °C annealing (30 s) and 72 °C elongation (60 s). PCR products (100 ng of DNA or more) were purified using the Exosap-IT purification kit (Amersham–Pharmacia Biotech) and sequenced in the forward direction using the same primer for the PCR reactions (5 pmol) and the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham–Pharmacia Biotech). Weak PCR products were purified and reamplified. When the PCR signal gave multiple bands, the DNA bands were individually gel-extracted at 4 °C in 100 to 200 µl of water, the DNA diffusing overnight from the gel extract to the surrounding water. A few microliters of the water containing the diffused DNA were then used in a new PCR reaction after purification.

2.4. Phylogeny reconstruction

2.4.1. Analysis of each individual gene

Cytochrome *b* allowed resolution to the species level. A preliminary parsimony tree was constructed using all the individual cytochrome *b* sequences to group specimens by taxon. Different molecular subgroups were identified for some species. To reduce the data set, and consequently the length of the computational analyses, individual sequences were then assembled into a consensus sequence at the species or at the molecular subgroup level, for every gene. Almost all sequences reported are the consensus of at least three specimens for each species or, when applicable, for molecular subgroups.

Nucleotide sequences were aligned manually and parts of the data were excluded from the analysis where the alignment was questionable. For the ITS2 gene, 13 indels were recoded according to the 'simple indel coding' method

Table 1
Host–parasitoid relationships and host life-history information used in this paper

Eulophidae species	Phoresy habit	Cassidinae host species	Tribe of the host	Host oviposition	Chorion type of the host eggs	Diet of the host
<i>Aprostocetus</i> sp.	Not observed	<i>Discomorpha salvini</i>	<i>Omocerini</i>	Clumped	2	Boraginaceae
<i>Emersonella albicoxa</i>	Not observed	<i>Hilarocassis evanida</i>	<i>Stolaini</i>	Clumped	3	Convolvulaceae
<i>Emersonella carballoi</i>	Yes	<i>Deloyala guttata</i>	<i>Cassidini</i>	Solitary	1	Convolvulaceae
<i>Emersonella cuignetae</i>	No	<i>Chelymorpha alternans</i>	<i>Stolaini</i>	Clumped	3	Convolvulaceae
<i>Emersonella horismenoides</i>	Not observed	<i>Cistudinella foveolata</i>	<i>Ischyrosomychini</i>	Clumped	2	Boraginaceae
<i>Emersonella niveipes</i>	No	<i>Chelymorpha alternans</i> , <i>Stolas pictilis</i> , <i>Hilarocassis evanida</i>	<i>Stolaini</i>	Clumped	3	Convolvulaceae
<i>Emersonella</i> nr. <i>carballoi</i>	Yes	<i>Xenocassis ambita</i>	<i>Cassidini</i>	Solitary	3	Convolvulaceae
<i>Emersonella</i> nr. <i>hastata</i>	Not observed	<i>Hybosa mellicula</i>	<i>Cassidini</i> (?)	Clumped	2	Bignoniaceae
<i>Emersonella planiceps</i>	Yes	<i>Microctenochira</i> sp., <i>Charidotella sinuata</i> , <i>C. sexpunctata</i>	<i>Cassidini</i>	Solitary	3	Convolvulaceae
<i>Emersonella planiscuta</i>	Yes	<i>Stolas lebasi</i>	<i>Stolaini</i>	Loosely aggregated	3	Asteraceae
<i>Emerson ella pubennis</i>	Yes	<i>Acromis sparsa</i> , <i>Paraselenis tersa</i>	<i>Stolaini</i>	Clumped	3	Convolvulaceae
<i>Emersonella reticulata</i>	Not observed	<i>Polychalma multicava</i>	<i>Goniocheniini</i>	Clumped	2	Boraginaceae
<i>Emersonella rotunda</i>	Yes	<i>Agroiconota</i> sp., <i>Charidotella</i> sp., <i>Microctenochira</i> sp., <i>Deloyala guttata</i>	<i>Cassidini</i>	Solitary	3	Convolvulaceae
<i>Emersonella</i> sp. 1	No	<i>Chelymorpha alternans</i>	<i>Stolaini</i>	Clumped	3	Convolvulaceae
<i>Emersonella</i> sp. 2	No	<i>Stolas lebasi</i>	<i>Stolaini</i>	Loosely aggregated	3	Asteraceae
<i>Emersonella</i> sp. 3	Not observed	<i>Deloyala guttata</i> , <i>Metrionella erratica</i>	<i>Cassidini</i>	Solitary	1	Convolvulaceae
<i>Emersonella</i> sp. 4	Not observed	<i>Cistudinella foveolata</i>	<i>Ischyrosomychini</i>	Clumped	2	Boraginaceae
<i>Emersonella tanigaster</i>	Not observed	<i>Charidotis abrupta</i>	<i>Cassidini</i>	Clumped	2	Bignoniaceae
<i>Emersonella varicolor</i>	Not observed	<i>Tapinaspis wesmaeli</i>	<i>Cassidini</i>	Solitary	3	Asteraceae
<i>Emersonella windsori</i>	Not observed	<i>Omaspides</i> sp.	<i>Stolaini</i>	Clumped	3	Convolvulaceae
<i>Horismenus</i> sp. 1	Not observed	<i>Polychalma multicava</i>	<i>Goniocheniini</i>	Clumped	2	Boraginaceae
<i>Horismenus</i> sp. 2	Not observed	<i>Discomorpha salvini</i>	<i>Omocerini</i>	Clumped	2	Boraginaceae
<i>Horismenus</i> sp. 3	Not observed	<i>Discomorpha salvini</i>	<i>Omocerini</i>	Clumped	2	Boraginaceae
<i>Horismenus</i> sp. 4	Not observed	<i>Cistudinella foveolata</i>	<i>Ischyrosomychini</i>	Clumped	2	Boraginaceae
<i>Horismenus</i> sp. 5	Not observed	<i>Spaethiella</i> sp.	<i>Hemisphaerotini</i>	Solitary	1	Heliconiaceae
<i>Horismenus</i> sp. 6	Not observed	<i>Spaethiella</i> sp.	<i>Hemisphaerotini</i>	Solitary	1	Heliconiaceae
Signiphoridae species 1	Not observed	<i>Charidotis abrupta</i>	<i>Cassidini</i>	Clumped	2	Boraginaceae
Signiphoridae species 2	Not observed	?	?	?	?	?

Phoresy habit: “not observed” means that too few adults were observed to infer a reliable conclusion concerning the phoretic habit of the parasitoid species. Type of chorion of the host egg: 1 = egg directly in contact with the leaf, covered by a thin membrane; 2 = eggs without a hard chorion, protected in a membranous ootheca or extrachorion; 3 = presence of a hard brittle coat.

(Simmons and Ochoterena, 2000). Each data set was analyzed with Paup* v.4.0.b.10 (Swofford, 2002) using three reconstruction methods: the Neighbor-Joining distance method (with distances corrected for multiple hits using the LogDet/paralinear transformation), maximum parsimony (MP) and maximum likelihood (ML). In the ML and MP heuristic search, the 300 starting-trees for TBR branch-swapping were obtained by stepwise addition using a random addition-sequence process. For the ML analysis, the substitution model best fitting each data set was assessed from the Akaike Information Criterion output scores produced with Modeltest 3.0. (Posada and Crandall, 1998). The level of statistical support for the different clades was assessed by bootstrapping (min. 100 replicates) (Felsenstein, 1985) or by fast-bootstrapping (min. 5000 replicates) depending on the complexity of the data set. A Bayesian analysis was conducted on each data set using Metropolis-coupled Markov chains Monte-Carlo (MCMC) implemented in the MrBayes software (Huelsenbeck and Ronquist, 2001). Each Bayesian analysis consisted of four

chains, random starting trees, a uniform prior distribution of parameters, and the GTR + I + Γ model of nucleotide substitution. The chains were run for 2 million generations, and trees sampled every 100 generations. Stationarity was determined by visual examination of the log-likelihood plots and the burn-in trees were discarded. For the cytochrome *b* gene, the parameters of the nucleotide substitution model were unlinked between the three codon positions.

As the MP and ML analyses usually gave multiple trees, we reduced the set of trees to one consensus tree. Semi-strict consensus was calculated when the initial set of trees issued from multiple tree islands or when the consensus tree was composed of only a few trees. Otherwise, the majority-rule consensus was calculated and only the branches common to at least 70% of the trees were retained. The consensus for the Bayesian method is automatically a majority-rule consensus, where the number of times a bipartition is found in the initial set of trees gives an approximation of the posterior probability (statistical support) of the bipartition.

Within each gene, the consensus trees obtained by the four methods were compared by a Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999). If not statistically different, a majority-rule consensus based on the consensus trees obtained for each methodology was calculated. Bipartitions of this consensus gene tree were kept whenever they were present in at least three of the four source trees. The Eulophidae have been shown to be a monophyletic group (LaSalle and Schauff, 1995; LaSalle et al., 1997; Gibson et al., 2000), and we used two Signiphoridae sequences to root our trees. Signiphoridae have been shown to be monophyletic (Woolley, 1988) and both Eulophidae and Signiphoridae belong to the Chalcidoidea superfamily. However, the relationship of Signiphoridae with other families is problematic and the Signiphoridae have sometimes been

treated as a subfamily in Aphelinidae, Encyrtidae or Eulophidae (Gibson et al., 2000). Depending on the author, Signiphoridae are sometimes included in the “pteromalid” lineage (Gibson et al., 2000), and sometimes in the “eulophid” lineage, as a sister family to the Eulophidae (Noyes, 1990). Nevertheless, Signiphoridae were close enough phylogenetically to the Eulophidae to be used as an outgroup in our study.

2.4.2. Analysis of the entire dataset

The entire dataset was analysed in two ways: (1) The different data sets were combined after the completion of a partition-homogeneity test (Farris et al., 1995). The resulting combined data set was submitted to a Bayesian analysis, with unlinked parameters between the different gene partitions

Table 2
Characteristics of the data set and trees found, along with the parameters of the DNA substitution models

	28S rDNA	ITS2 rDNA	Cyt <i>b</i> mtDNA	Combined data set
Nb of taxa	25	30	37	39
Nb of characters included in the analysis	490	377	126 + 126 + 125 ^a	1243
Nb of variable sites	55	120	92 + 51 + 94 ^a	410
Nb of parsimony-informative sites	25	97	188	315
<i>Parameters of the ML analysis</i>				
Rate distribution	γ	γ	γ	—
$r(G \rightarrow T)$	1	1	1	—
$r(C \rightarrow T)$	1.64	3.681	11.573	—
$r(C \rightarrow G)$	0.603	0.549	2.305	—
$r(A \rightarrow T)$	1.594	2.118	2.305	—
$r(A \rightarrow G)$	1.64	2.287	7.445	—
$r(A \rightarrow C)$	0.366	0.786	1	—
Freq (A)	0.178	0.197	0.334	—
Freq (C)	0.28	0.3	0.177	—
Freq (G)	0.312	0.291	0.058	—
Freq (T)	0.23	0.212	0.43	—
α	0.1715	0.6877	0.6345	—
Pinv	0	0	0.2887	—
<i>Parameters of the Bayesian analysis</i>				
Rate distribution	γ	γ	γ	γ
Mean tree length	2.585	0.931	10.036	8.657
$r(G \rightarrow T)^a$	1	1	1; 1; 1	1; 1; 1; 1
$r(C \rightarrow T)^a$	34.679	4.716	56.93; 5.35; 3.55	59.84; 4.48; 58.83; 2.59; 2.51
$r(C \rightarrow G)^a$	0.419	0.541	3.29; 6.13; 1.82	0.29; 0.64; 2.48; 3.90; 1.22
$r(A \rightarrow T)^a$	21.193	3.016	9.05; 3.28; 0.68	10.76; 2.48; 7.54; 1.34; 0.46
$r(A \rightarrow G)^a$	1.591	2.868	8.97; 4.82; 11.55	0.73; 3.12; 8.50; 3.43; 10.11
$r(A \rightarrow C)^a$	3.765	1.03	5.50; 3.14; 0.36	1.85; 1.19; 7.00; 1.28; 0.35
Freq (A) ^a	0.174	0.192	0.358; 0.231; 0.326	0.182; 0.192; 0.346; 0.251; 0.328
Freq (C) ^a	0.272	0.303	0.139; 0.244; 0.178	0.278; 0.293; 0.134; 0.223; 0.169
Freq (G) ^a	0.333	0.288	0.144; 0.096; 0.017	0.328; 0.289; 0.141; 0.083; 0.015
Freq (T) ^a	0.222	0.217	0.356; 0.429; 0.480	0.212; 0.225; 0.379; 0.444; 0.488
α^a	0.054	0.519	0.323; 0.122; 2.154	0.842; 0.144; 0.341; 0.144; 1.665
Nb of trees in parsimony	5	15	2	—
Score of the besttree(s) found in MP	67	194	973	—
Nb of trees in ML	2	1	2	—
Score of the besttree(s) found in ML (–InL)	1130.8	1262.18	4382.42	—
Mean score of the Bayesian trees (–InL)	1209.09	1389	4320.67	7297.34
Consensus type in MP	Semi-strict	Majority-rule	Semi-strict	—
Consensus type in ML	Semi-strict	—	Semi-strict	—

^a Values are given separately for the different data partitions. Partitions for the Cyt *b* mtDNA gene correspond to the first, second and third codon positions, respectively. Partitions for the combined data set correspond to the 28S D2, the ITS2, and the first, second and third codon positions of the Cyt *b* mtDNA, respectively.

and between the codon positions of the cytochrome *b* gene. We applied the Bayesian method because of its speed and its ability to apply different evolutionary parameters to the different data partitions. (2) A “supertree” based on the three consensus gene trees was constructed using Matrix Representation Parsimony (Sanderson et al., 1998). The 28S D2, ITS2 and cytochrome *b* trees were combined and recoded into a binary matrix suitable for a parsimony analysis according to Baum (1992) and Ragan (1992), without the Purvis modification (Purvis, 1995) using the software Rad-Con (Thorley and Page, 2000). To take into account the confidence level of each node, characters of this matrix were weighted according to their bootstrap support (Ronquist, 1996). The bootstrap support for a node of one consensus gene tree was calculated as the mean of the four bootstrap values obtained for this node with the NJ, MP, ML and Bayesian analysis. The resulting MRP matrix was analysed by parsimony using Paup as described before.

3. Results

For each gene, details concerning the characteristics of the data set, parameters of the substitution models for the ML and the Bayesian analysis, the number of trees found and their score, and the consensus type are summarized in Table 2. Genbank accession numbers can be consulted in Table 3.

3.1. Analysis of the 28S D2 rDNA gene

P-values for the SH two by two comparisons of the trees obtained with the four tree reconstruction methods ranged between 0.109 and 0.441. Trees were thus combined into a unique consensus tree (Fig. 1). The 28S D2 rDNA gene was not variable enough (435 of the 490 sites were constant) to resolve the relationships within the Eulophidae with confidence.

Table 3
Genbank accession numbers

Parasitoid species	Genbank Accession Nos.		
	28SD2	ITS2	Cytochrome <i>b</i>
<i>Aprostocetus species</i>	/	/	AY820879
<i>Emersonella albicoxa</i>	AY771681	AY772786	AY820845
<i>Emersonella carballoi</i>	AY771698	AY772785	AY820853
<i>Emersonella cuignetae</i> subtype 1	AY771696	AY772802	AY820846
<i>Emersonella cuignetae</i> subtype 2	AY771696	AY772802	AY820849
<i>Emersonella horismenoides</i> subtype 1	AY771682	AY772798	AY820847
<i>Emersonella horismenoides</i> subtype 2	AY771682	AY772799	AY820848
<i>Emersonella niveipes</i> subtype 1	AY771683	AY772787	AY820850
<i>Emersonella niveipes</i> subtype 2	AY771683	AY772787	AY820851
<i>Emersonella nr carballoi</i>	AY771685	AY772788	AY820854
<i>Emersonella nr hastata</i>	AY771684	AY772800	AY820852
<i>Emersonella planiceps</i> subtype 1	AY771686	AY772789	AY820855
<i>Emersonella planiceps</i> subtype 2	AY771686	AY772789	AY820856
<i>Emersonella planiscuta</i>	AY771687	/	AY820857
<i>Emersonella pubipennis</i> subtype 1	AY771688	AY772803	AY820858
<i>Emersonella pubipennis</i> subtype 2	AY771688	AY772803	AY820859
<i>Emersonella reticulata</i>	/	AY772807	AY820860
<i>Emersonella rotunda</i> subtype 1	AY771689	AY772790	AY820861
<i>Emersonella rotunda</i> subtype 2	AY771689	AY772791	AY820862
<i>Emersonella rotunda</i> subtype 3	AY771689	AY772792	AY820863
<i>Emersonella rotunda</i> subtype 4	AY771689	AY772793	AY820864
<i>Emersonella rotunda</i> subtype 5	AY771689	AY772794	AY820865
<i>Emersonella species 1</i>	AY771690	AY772804	AY820843
<i>Emersonella species 2</i>	AY771691	AY772805	AY820844
<i>Emersonella species 3</i> subtype 1	AY771697	AY772797	AY820866
<i>Emersonella species 3</i> subtype 2	AY771697	AY772797	AY820867
<i>Emersonella species 4</i>	AY771692	AY772801	AY820868
<i>Emersonella tanigaster</i>	AY771693	AY772795	AY820869
<i>Emersonella varicolor</i>	AY771694	AY772796	AY820870
<i>Emersonella windsori</i>	AY771695	AY772806	AY820871
<i>Horismenus species 1</i>	AY771701	AY772810	AY820875
<i>Horismenus species 2</i>	/	AY772812	AY820874
<i>Horismenus species 3</i>	/	/	AY820878
<i>Horismenus species 4</i>	/	AY772811	/
<i>Horismenus species 5</i>	AY771699	AY772808	AY820872
<i>Horismenus species 6</i>	AY771700	AY772809	AY820873
Signiphoridae species 1	AY771702	AY772813	AY820876
Signiphoridae species 2	/	AY772814	AY820877

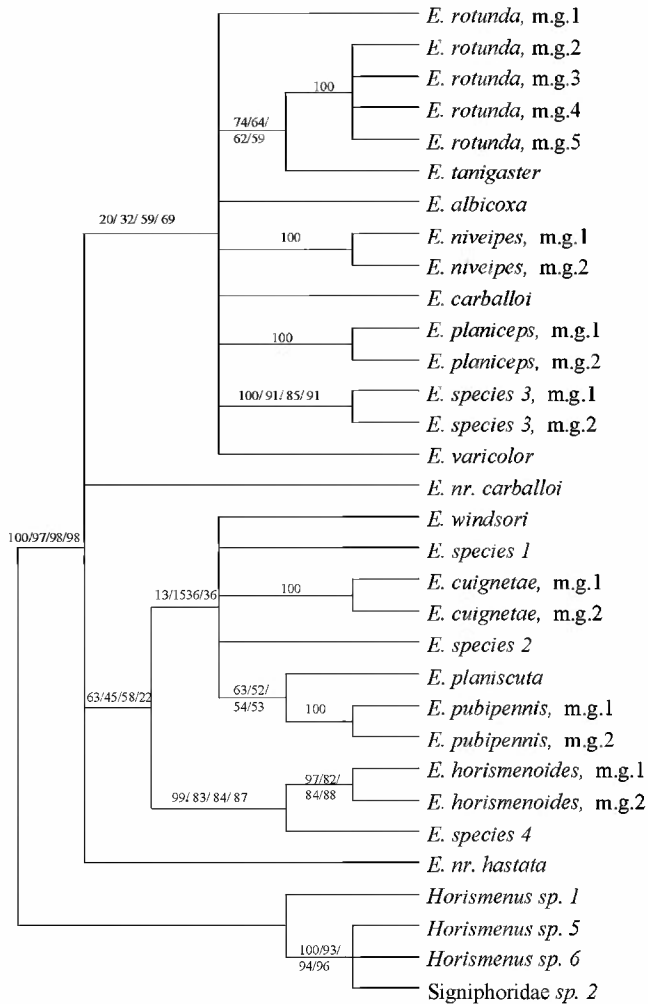


Fig. 1. Consensus tree based on the 28S rDNA. “m.g.” stands for “molecular group.” Support values are indicated above branches for the Bayesian, ML, MP and NJ analyses, respectively.

3.2. Analysis of the ITS2 rDNA gene

More than fifty percent of the ITS2 nucleotide data had to be ignored due to questionable homology of characters. Nevertheless, the trees obtained with the four reconstruction methods were well resolved and the nodes were supported by high bootstrap values. *P*-values for the SH test varied between 0.249 and 1. The four trees were combined into a single consensus tree (Fig. 2).

3.3. Analysis of the Cytb mtDNA gene

The first and third codon positions of the cytochrome *b* sequence were saturated (73 and 75.2% of the positions were variable, respectively), but a separate analysis of those positions did not show conflicting relationships compared with the results obtained for the two other genes, suggesting that their inclusion would not introduce spurious relationships into the tree. The resolved tree was obtained by analyzing simultaneously the three

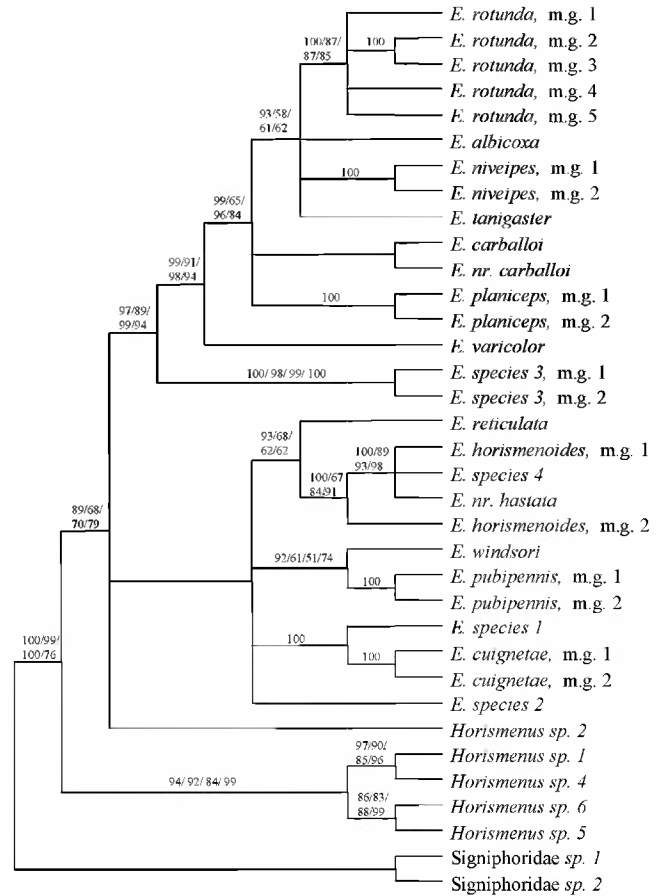


Fig. 2. Consensus tree based on the ITS2 rDNA. “m.g.” stands for “molecular group”. Support values are indicated above branches for the Bayesian, ML, MP and NJ analyses, respectively.

codon positions, equally weighted. An analysis of the first and second codon positions only gave numerous most parsimonious trees belonging to a great number of different tree islands, resulting in a largely unresolved consensus tree (result not shown), and downweighting transitions on the third codon position did not change that situation much. Except for the Bayesian tree, trees were resolved but no deep nodes were supported by high bootstrap values. The ML, MP and NJ trees were identical (*p*-values for the SH test varied between 0.103 and 0.398), but the Bayesian tree was different from every other tree (*p*-values = 0.001). Visual examination revealed that the deep parts of the Bayesian tree were largely unresolved compared to the other trees, with no separation of taxa except for the Signiphoridae and the *Aprostocetus* species. However, the relationships between very close species displayed in the ML, MP and NJ trees were also present in the Bayesian tree, so that the latter did not conflict with the former. As the lack of deep resolution, rather than conflicting relationships between species, seemed to be responsible for the statistical difference observed, we included the Bayesian tree in the consensus gene tree for the cytochrome *b* (Fig. 3).

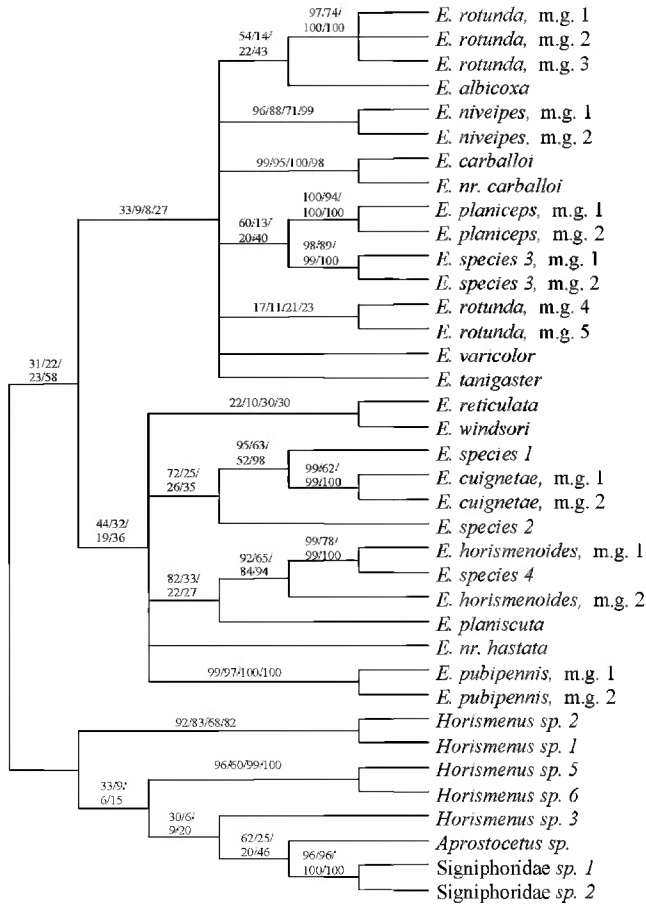


Fig. 3. Consensus tree based on the cytochrome *b* mtDNA. “m.g.” stands for “molecular group.” Support values are indicated above branches for the Bayesian, ML, MP and NJ analyses, respectively. Bootstrap values for branches marked with an asterisk are: *1, 97/74/100/100; *2, 100/94/100/100; *3, 98/89/99/100; *4, 95/63/52/98; *5, 99/62/99/100; *6, 92/65/84/94; *7, 99/78/99/100; *8, 96/60/99/100; *9, 30/06/09/20; *10, 62/25/20/46; *11, 96/96/100/100.

3.4. Analysis of the combined data sets

The *p*-value for the partition homogeneity test was 0.97, indicating that the three gene data sets were highly congruent and could be combined into a single data set. The Bayesian tree for the whole dataset is presented in Fig. 4. The tree was well resolved and supported by high posterior probabilities.

3.5. The MRP analysis

The MRP matrix contained 39 taxa and 65 characters. The parsimony analysis of the weighted matrix representation of the combined 28S, ITS2 and Cyt *b* consensus trees gave only one most parsimonious tree of a score of 5811 (Fig. 5). This supertree was well resolved but the bootstrap values were generally low. The MRP supertree was compared to the Bayesian tree elaborated from the combined data sets. The *p*-value for the SH test was 0.057 indicating that the two trees were not statistically different, but neither were they very similar.

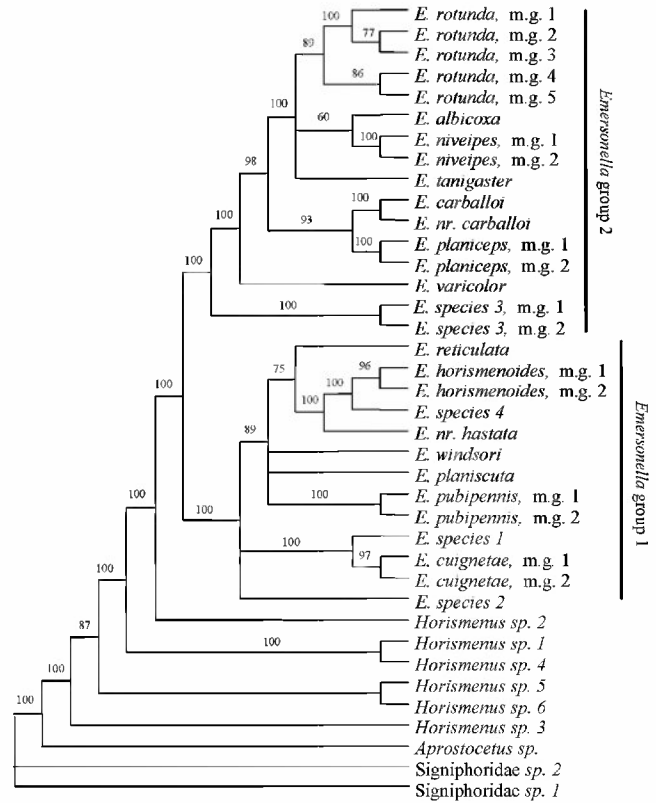


Fig. 4. Total Evidence tree resulting from Bayesian analysis. Posterior probabilities are indicated above branches. “m.g.” stands for “molecular group.”

3.6. Morphological analysis

The matrix contained a relatively high proportion of missing data, and the parsimony analysis gave numerous trees from multiple islands. Although the consensus was not well resolved, relationships could be highlighted between *E. carballoi* and *E. planiceps* (grouped together with *E. varicolor*), between *E. pubipennis* and *E. windsori*, and between *E. sp. 1* and *E. sp. 2*.

4. Discussion

To produce a confident and well resolved phylogenetic tree of the Eulophidae, fragments of two nuclear genes and one mitochondrial gene were sequenced and analyzed using a range of current phylogenetic methods. Special care was taken in investigating the congruence between the trees resulting from those analyses and in consensus trees. All three genes allowed us to identify a group of *Horismenus* species and a group of *Emersonella* species divided into two subgroups. The ITS2 gene was the most informative, and deep nodes were supported by high bootstrap values only in its case. However, relationships not supported by high bootstrap values are not necessarily false. Poorly supported clades are unreliable because they may have been recovered by chance (Erixon et al., 2003). In cases of low bootstrap

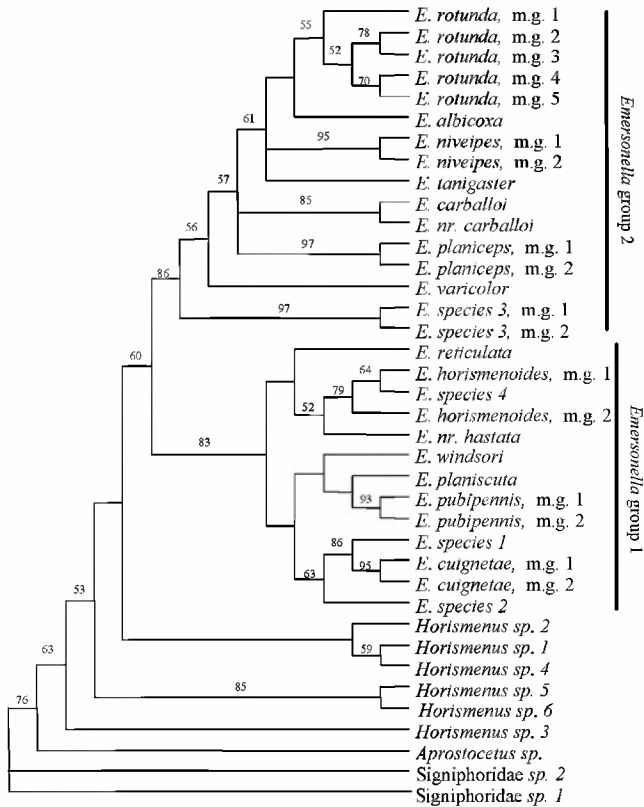


Fig. 5. MRP supertree. Bootstrap values are indicated above branches. “m.g.” stands for “molecular group”.

values, sequencing several (congruent) genes increases confidence in uncertain clades. Topological congruence between phylogenies supported by independent data partitions is considered as one of strongest support for phylogenetic relationships (Kim, 1993; Hillis, 1995; Miyamoto and Fitch, 1995; Adoutte et al., 2000; but see Cunningham, 1997).

A synthesis based on trees from different data partitions or from several genes responding to different evolutionary patterns is expected to be as close as possible to the natural species tree (Huelsenbeck and Crandall, 1997). Accordingly, we used two approaches to integrate our data: (1) Total Evidence, where sequences from the different genes are concatenated into a single matrix and analyzed simultaneously; and (2) Matrix Representation Parsimony (MRP), which implies the construction of a consensus tree (called a “supertree”) that summarizes the topological features shared among the trees resulting from the separate analyses (Chippindale and Wiens, 1994).

The Total Evidence tree and the MRP supertree (Figs. 4 and 5) inferred the same main relationships among Eulophidae parasitoids of Cassidinae. The genus *Aprostocetus* (Eulophidae: Tetrastichinae) is basal to the tree. According to LaSalle and Schauff (1995), the Eulophinae appear to be the most basal subfamily since they are less morphologically specialized. Eulophinae are closer to Entedoninae than to the two other subfamilies. However, Boucek (1988) considers Entedoninae (to which *Emersonella* and *Horisme-*

nus belong) as the most derived subfamily of Eulophidae. Molecular data (Gauthier et al., 2000) supported Eulophinae as a derived group and suggests that Euderinae may be the most primitive. Euderinae and Tetrastichinae appear closely related to each other (Graham, 1987; Gibson et al., 2000). Knowing that, the genus *Aprostocetus* (Tetrastichinae) can be considered to be primitive in regards to the genera *Horismenus* and *Emersonella*. Among the Entedoninae, the genus *Horismenus* is basal to the genus *Emersonella*. The genus *Horismenus* appears to be paraphyletic. There was no autapomorphy characterizing the genus, suggesting that its taxonomy should be reviewed, and that the *Horismenus* genus should probably be split into several monophyletic genera. The only study addressing phylogenetic relationships among Eulophidae on a large scale (Gauthier et al., 2000) included only one *Horismenus* species, thereby leaving monophyly of that genus totally unresolved. On the other hand, monophyly of the genus *Emersonella* is well supported by molecular data, except in the ITS2 tree, where *Horismenus sp. 2* was placed within the *Emersonella* genus, thereby disrupting their presumed monophyly. An analysis of the ITS2 sequences alignment revealed the uniqueness of the *Horismenus sp. 2* sequence, with most nucleotidic substitutions characteristic of the *Horismenus* genus, but including indels common to the *Emersonella* species. We suspect that gene transfer occurred between *Horismenus sp. 2* and *Emersonella* species. This emphasizes the importance of sequencing several genes when working with molecular phylogenies. The cytochrome *b* analysis clearly identifies the problematic taxa within *Horismenus* species.

We can hypothesize that the *Horismenus* species attacking Cassidinae (Figs. 4 and 5) originated as a *Horismenus* parasitoid of egg masses of *Discomorpha salvini*, a beetle feeding on Boraginaceae or other similar Cassidinae species. Two groups of *Horismenus* species evolved after differentiation of *Horismenus sp. 3*. Species in the first group may have lost the oophagous habit to become gregarious parasitoids of larvae or pupae from the genus *Spaethiella*, a basal group of Cassidinae feeding on several monocot families (Windsor et al., 1992; Hsiao and Windsor, 1999). The other *Horismenus* group remained oophagous on *Discomorpha salvini* or became specialized on more derived Cassidinae, like *Cistudinella foveolata* and *Polychalma multicava* (respectively from the tribes *Ischyrosomychini* and *Goniocheniini*), which additionally feed on Boraginaceae. This second group of *Horismenus*, as well as the basal *Horismenus* and the *Aprostocetus* species, parasitize egg masses whose eggs lack a resistant extrachorion but are embedded in an ootheca made of colleterial gland secretions (Hilker, 1994; Selman, 1994). Either *Horismenus* species parasitizing *C. foveolata* and *P. multicava* or one *Horismenus* species parasitizing *D. salvini* could be at the base of the genus *Emersonella*. The first hypothesis is more plausible, since some parasitoids of *C. foveolata* and *P. multicava* are included in the genus *Emersonella*.

We found no evidence that *Horismenus* species exhibited phoretic behaviour. However, greater sampling is desirable

on this point. The adaptive significance of phoresy remains unclear, it seems reasonable that it may facilitate host location when the eggs are rare or do not give off chemical clues, as for solitary eggs or when the eggs are not enclosed within ootheca of secretions. Phoresy may have appeared several times independently among the *Emersonella* species, however additional observations are needed to test this possibility.

The genus *Emersonella* is divided into two groups (Figs. 4 and 5). The first group includes *E. reticulata*, *E. nr. hastata*, *E. horismenoides*, *E. sp. 1*, *E. sp. 2*, *E. sp. 4*, *E. cuignetae*, *E. windsori*, *E. planiscuta* and *E. pubipennis*, and the second is composed of *E. sp. 3*, *E. varicolor*, *E. carballoi*, *E. nr. carballoi*, *E. planiceps*, *E. tanigaster*, *E. niveipes*, *E. albicoxa* and *E. rotunda*. This result corroborates the morphological study by Hansson (2002), which established three artificial groups among the *Emersonella* species he described; the species from our second and first groups belong to his “rotunda” and to his “unplaced species” groups, respectively. *Emersonella* group 1 contains exclusively parasitoids of egg masses while *Emersonella* group 2 includes primarily parasitoids of solitary eggs, except *E. tanigaster*, *E. niveipes* and *E. albicoxa* which parasitize egg masses. *E. tanigaster* is also an oophagous parasitoid of a host in the tribe *Cassidini* like the others taxa of the group, but its host, *Charidotis abrupta*, feeds on Bignoniaceae and places its eggs in a secreted ootheca. *E. niveipes* and *E. albicoxa* parasitize tortoise beetles in the tribe *Stolaini* (*Chelymorpha alternans*, *Stolas pictilis* and *Hilarocassis evanida*), which deposit eggs in masses and enclose them in an individual extrachorion. Finding *E. niveipes* and *E. albicoxa* in this second group is unexpected. Based on their ecological characteristics, one might expect them to be included with the other *Emersonella* species parasitizing the *Stolaini* egg masses. Their position, however, is unequivocal since all three gene trees agree in this placement, and they were included in the “rotunda” group by Hansson (2002). *Emersonella sp. 3* appears to be at the base of the second *Emersonella* group. One of its two hosts, *Deloyala guttata*, deposits eggs on the leaf surface which lack a shell and are covered by a thin membrane, resembling the eggs of the basal *Spaethiella* species. Parasitism of this type of host egg might have constituted the intermediate step between the parasitism of eggs enclosed in an ootheca and solitary eggs enclosed in a resistant extrachorion.

Topological differences are present in the total evidence tree and the MRP supertree within the two *Emersonella* clades. Within the first *Emersonella* group (exclusively parasitoids of egg masses), the clade constituted by *E. pubipennis*, *E. planiscuta* and *E. windsori* is grouped with *E. cuignetae*, *E. sp. 1* and *E. sp. 2* in the MRP supertree (Fig. 5), but with *E. horismenoides*, *E. nr. hastata* and *E. reticulata* in the Total Evidence tree (Fig. 4). The MRP supertree is more consistent with host relationships, even if it is not supported by good bootstrap values, as *E. cuignetae*, *E. sp. 1* and *E. sp. 2* are parasitoids of beetles from the tribe *Stolaini*, as are *E. pubipennis*, *E. planiscuta* and *E.*

windsori, whereas *E. horismenoides*, *E. sp. 4*, *E. nr. hastata* and *E. reticulata* are parasitoids from other tribes that do not feed on Convolvulaceae (Table 1). Further, of the three genes, only the 28S D2 gave us information about the position of the subgroup, placing it in agreement with the MRP supertree. Empirical studies have shown that in trees based on combined data, relationships can appear that are absent in the trees resulting from the separate analysis of the different data partitions (Chippindale and Wiens, 1994). The Total Evidence tree presents a relationship that does not appear in the separate analysis of two genes and is also in disagreement with the third one. Thus, the MRP supertree may be more acceptable than the Total Evidence tree.

Unlike the Total Evidence tree, the MRP supertree links the morphologically similar species, *E. pubipennis* and *E. windsori*, both of which parasitize subsocial Cassidinae—e.g., Cassidinae that offer maternal care to their progeny (Windsor et al., 1992). However, the position of *E. planiscuta* (whose host is not subsocial) between *E. windsori* and *E. pubipennis* remains unexplained in the MRP supertree. The MRP supertree also groups the two morphologically similar species, *E. sp. 2* and *E. sp. 1*. Within the first *Emersonella* group, the phylogeny revealed by the MRP supertree seems more natural than the one proposed by the Total Evidence tree. According to the MRP supertree (Fig. 5), *Emersonella* group 1 is divided into a clade constituted by parasitoids of *Stolaini* beetles (1.a. Fig. 5), that feed on Convolvulaceae and Asteraceae, and another clade (1.b. Fig. 5) parasitizing beetles from Cassidinae tribes feeding on Boraginaceae and on Bignoniaceae. In the *Stolaini*, eggs are individually protected by an extrachorion, whereas in the second clade host eggs are enclosed in an ootheca.

Concerning the *Emersonella* group 2 (Figs. 4 and 5), the Total Evidence tree gives this time more natural results, placing *E. niveipes* and *E. albicoxa* together whereas *E. albicoxa* is placed near *E. rotunda* in the MRP supertree, corresponding little to biological characteristics of those species (see Table 1). In the same way, *E. carballoi*, *E. nr. carballoi* and *E. planiceps* which are morphologically close are clustered together in the Total Evidence tree.

We can not argue for one method of simultaneous analysis over an other. Depending on which part of the tree is considered (*Emersonella* group 1 or 2), either Total Evidence or the MRP supertree present relationships that appear to be more natural. Both methods present “probably correct” and “probably incorrect” relationships. Bootstrap values of the MRP supertree are much lower than the corresponding posterior probabilities of the Bayesian tree, but such low values can be explained partly by the fact that the weighting applied to the characters of the MRP matrix is ignored by the bootstrapping process. Moreover, the MRP matrix data contained a smaller number of characters, since the genetic information is reduced to a single character per node, which has a negative effect on bootstrap values. On the other hand, posterior probabilities in Bayesian methods overestimate the branch support (Huelsenbeck et al., 2001). Suzuki et al. (2002) showed that the method

often gives high support values even with completely uninformative data (Erixon et al., 2003).

Opinions concerning the Total Evidence approach range from “never combine” (Miyamoto and Fitch, 1995) to “always combine” (Chippindale and Wiens, 1994; Wenzel and Siddall, 1999) as several problems are raised using this approach. First, it can be difficult to combine different data sets into a common matrix because they may not include the same taxa or character set. Reducing the common data set such that the same terminal taxa are represented in each data partition may be too restrictive (Kitching et al., 1998), and encoding the missing gene sequences as missing characters can lead to the generation of multiple equally most parsimonious cladograms, spurious theories of character evolution, and a lack of resolution (Kitching et al., 1998; Kennedy and Page, 2002; but see Wiens and Reeder, 1995). Further, data partitions of different nucleotide numbers will have different weight on the final result (Slowinski and Page, 1999). Some authors have argued that use of combined analysis alone will obscure some patterns of congruent and discordant characters that can only be discovered by using separate analyses of data set partitions (Chippindale and Wiens, 1994). This is why most authors usually agree to combine data sets only if they are not heterogeneous (Slowinski and Page, 1999). But the partition homogeneity test (also called ILD test) used to test the heterogeneity of the different data partitions has been criticized because it fails under some circumstances (Wiens, 1998; Dolphin et al., 2000). We suggest that combining the data sets because of the congruence of the phylogenetic signal does not negate running separate analyses of each gene data set. One claim of the simultaneous analysis approach is that the resulting cladogram is nearly always more resolved than is a consensus of separate cladograms (Kitching et al., 1998). Relationships that appear in Total Evidence trees and not in the separate analysis of the data partitions must be taken with caution, particularly when one of the individual partition tree diverges.

The Matrix Representation Parsimony (MRP) method has the advantage that the source trees can be combined into a MRP matrix whether they have the same taxa set or not. MRP has gained popularity due to its ease of applicability, but its scientific underpinnings remain still to be discovered (Racheli, 2004). Hackett et al. (1995) suggested, however, that combining trees resulting from different phylogenetic methods into a consensus tree could be misleading, because it is not clear why to give as much weight to the results of “weak” methods than to more consistent, robust, and efficient ones. Similar reasoning can be applied concerning the quality of the source trees for the MRP analysis, since the supertree method by default gives equal weight to the source trees. However, it is possible to weight source trees differentially according to the quality of the data set, but it is less clear how to evaluate a “good” data set and which relative weight it should be given. Within a source tree, characters can also be weighted according to the degree of support for the corresponding nodes (Sanderson

et al., 1998), thus objectively reflecting the quality of each data set. In our case, this greatly improved the number of most parsimonious trees found by the MRP analysis. Problems arise when some relationships are conflicting and strongly supported in different data sets, and the MRP method should not be applied in this case. If source trees are mutually compatible, as in our study, then no relationship strongly supported by ‘good’ data from one source tree will be contradicted by ‘bad data’ from another source tree, and the signal in each data set will be seen in the super-tree(s) (Sanderson et al., 1998). However, even if computation is much faster for a MRP analysis than for a Total Evidence, the MRP supertree method necessitates many more tree manipulations (source of errors!) and so is time-consuming for the researcher. This is in part due to the lack of relevant software, for instance to weight the nodes according to their bootstrap values.

5. Conclusion and perspectives

When Total Evidence and MRP trees are compared, the two methods agree in separating a *Horismenus* genus ancestral to the *Emersonella* genus, and two groups of *Emersonella* species. No single ecological trait of the host accounts for the observed phylogeny of the oophagous Eulophidae. The different egg laying behaviours of the host Cassidinae would seem to be an important factor that has influenced the diversification of their egg parasitoids. However, we can not rule out the influence of the diet the tribe to which the host belong.

Within the two *Emersonella* groups, the Total Evidence and the MRP supertree methods posed different—and sometimes contradictory—hypotheses. We believe that running both methods is helpful in highlighting uncertainties. It is then necessary to consider each hypothesis in the light of morphological and ecological characteristics. Whatever the method chosen, we think that it is absolutely necessary to carry out separate analysis of each gene and to discuss the results obtained for the total tree in the light of the individual gene trees.

Knowing the phylogeny of one of the actors in the host–parasitoid relationship can be useful to interpreting the phylogeny of the other, and to accept or reject uncertainties. A well supported phylogeny of the Cassidinae will permit testing hypotheses about the evolutionary relationships within the different tribes suggested by the phylogeny of their parasitoids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2006.09.005](https://doi.org/10.1016/j.ympev.2006.09.005).

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