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Systematics of the cyclostome subfamilies of braconid parasitic wasps (Hymenoptera: Ichneumonoidea): A simultaneous molecular and morphological Bayesian approach

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Abstract

Phylogenetic relationships among 95 genera collectively representing 17 of the 18 currently recognized cyclostome braconid wasp subfamilies were investigated based on DNA sequence fragments of the mitochondrial COI and the nuclear 28S rDNA genes, in addition to morphological data. The treatment of sequence length variation of the 28S partition was explored by either excluding ambiguously aligned regions and indel information (28SN) or recoding them (28SA) using the 'fragment-level' alignment method with a modified coding approach. Bayesian MCMC analyses were performed for the separate and combined data sets and a maximum parsimony analysis was also carried out for the simultaneous molecular and morphological data sets. There was a significant incongruence between the two genes and between 28S and morphology, but not for morphology and COI. Different analyses with the 28SA data matrix resulted in topologies that were generally similar to the ones from the 28SN matrix; however, the former topologies recovered a higher number of significantly supported clades and had a higher mean Bayesian posterior probability, thus supporting the inclusion of information from ambiguously aligned regions and indel events in phylogenetic analyses where possible. Based on the significantly supported clades obtained from the simultaneous molecular and morphological analyses, we propose that a total of 17 subfamilies should be recognized within the cyclostome group. The subfamilial placements of several problematic cyclostome genera were also established.

Keywords: Braconidae; Phylogeny; Simultaneous analysis; Fragment-level alignment; Ambiguously aligned regions

1. Introduction

With approximately 15,000 described species, and that almost certainly only a fraction of the real number (Dolphin and Quicke, 2001; Quicke and Baumgart, submitted), the parasitic wasp family Braconidae is the second largest family in the Hymenoptera (Wharton, 1997; Wharton and van Achterberg, 2000). These wasps are mostly larval parasitoids of other holometabolous insects (Quicke, 1997; Shaw and Huddleston, 1991). However, while most species are ecto or endoparasitic, a few are known to be phytophagous, usually producing galls (e.g., Austin and Dangerfield, 1997, 1998; Infante et al., 1995; Marsh, 2002; Wharton and Hanson, 2005), though recently seed eating (Flores et al., in press) and brood predation (Stanton et al., submitted) have also been discovered.

The Braconidae traditionally has been divided into two major groups, the cyclostomes and the non-cyclostomes, based in most cases on whether the lower part of the clypeus is sharply recessed exposing a concave labrum (Wharton, 1997). Some morphological studies have suggested that the cyclostomes form a paraphyletic basal grade leading to the non-cyclostomes (van Achterberg and Quicke, 1992; Quicke and van Achterberg, 1990). More recently, however,

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molecular and combined studies have consistently revealed that the cyclostomes actually form a clade with the inclusion of a few subfamilies whose members have secondarily lost the cyclostome condition (viz. Alysiinae, Aphidinae, many Betylobraconinae, Gnamptodontinae, Mesostoinae, and most Opiinae; Belshaw et al., 1998; Dowton et al., 2002), with the remaining non-cyclostomes subfamilies (with the possible exception of the Trachypetinae) forming its sister clade. Whereas members of the non-cyclostome clade are all koinobiont (wasps that allow the recovery and further development of the host after this is attacked; Askew and Shaw, 1986; Godfray, 1993; Quicke, 1997) endoparasitoids, both ecto and endoparasitoids (many of them idiobionts) and all known phytophagous braconid species are found among the cyclostomes at different taxonomic levels, making them an appealing model system for behavioral, ecological, and evolutionary studies.

Although the Braconidae has received considerable taxonomic attention in recent years, there is still considerable confusion over the definitions and extents of several subfamilies, especially among the cyclostomes. This particularly concerns several groups that are morphologically intermediate between the large subfamilies Doryctinae and Rogadinae (viz. Exothecinae, Hormiinae, Lysiterminae, Pambolinae, and Rhysipolinae; Wharton, 1993). This uncertainty is mainly due to a scarcity of diagnostic morphological features for higher level taxa as well as high levels of homoplasy. As a result, there has been considerable disagreement regarding whether to split these taxa into several small subfamilies (e.g., van Achterberg, 1995; Quicke and van Achterberg, 1990) or to amalgamate them into few morphologically heterogeneous groups (e.g., Wharton, 1993, 2000; Whitfield and Wharton, 1997).

Several higher taxonomic level phylogenies have been reconstructed for the Braconidae based on morphological (e.g., van Achterberg, 1984; Quicke and van Achterberg, 1990; Quicke and Belshaw, 1999; Whitfield, 1992; Zaldivar-Riverón et al., 2004), molecular (e.g., Belshaw et al., 1998, 2000; Belshaw and Quicke, 2002; Dowton, 1999; Dowton et al., 1998), and simultaneous (e.g., Dowton et al., 2002; Shi et al., in press) analyses. Among the few relationships that have been firmly established within the cyclostome group is the recognition of a clade containing the morphologically underived Rhyssalinae along with the Histeromerinae as the sister group to the remaining cyclostomes, and a clade comprising the Braconinae, Gnamptodontinae, Exothecinae, Opiinae, and Alysiinae. However, the relationships among the latter group and between the remaining subfamilies remain unresolved and the monophylies of many the problematic groups have not been tested. One of the main limitations of the above phylogenetic studies has been the restricted number of taxa sampled. None of the molecular analyses have included members of all the putative subfamilies, and in many cases even larger subfamilies have only been represented by one or two terminal taxa. On the other hand, the morphological analyses have traditionally employed summary terminal taxa, thus making assumptions of monophyly.

Recent studies, based on both simulated and real data, have claimed that increased taxon sampling may be important for increasing overall phylogenetic accuracy (Graybeal, 1998; Hillis, 1996, 1998; Pollock et al., 2002; Soltis et al., 1999; Zwickl and Hillis, 2002). Therefore, in this study we attempted to incorporate a wide range of terminal taxa including, where possible, multiple members of all subfamilies and tribes. The taxa examined were sequenced for two different genetic markers and scored for 81 morphological characters. These data were analyzed using the Bayesian MCMC method. In addition, we also explored the treatment of sequence length variation of the 28S gene by either excluding its ambiguously aligned regions (and indel information) or recoding them to retain all their potential phylogenetic information contained. As a result, the subfamilial classification within the cyclostome group is revised and the subfamilial placement of several problematic genera is established.

2. Materials and methods

2.1. Taxon sampling

A total of 95 terminal taxa were selected for this study, including representatives from all cyclostome subfamilies and most tribes that have been recognized in recent classifications. The only exceptions at subfamily level were the monotypic, poorly known Apozyginae and Vaepellinae, the former potentially being extremely basal (Quicke et al., 1999; Sharkey and Wahl, 1992; Sharkey, 1997) and the latter now being considered a member of the Braconinae (Tobias, 1988). Also missing is the Ypsistocerini, composed of three genera previously regarded as constituting a subfamily in their own right but currently placed as a tribe of the Doryctinae (van Achterberg, 1995; Belokobilskij et al., 2004; Quicke et al., 1992a,c). Four species of *Aleiodes* representing its three recognized subgenera (*sensu* van Achterberg, 1991) were included among the members of the Rogadinae (*sensu* van Achterberg, 1995) as this is the largest and morphologically most diverse genus of the subfamily. The terminal taxa also comprised several genera belonging to the group of small, problematical subfamilies Exothecinae, Hormiinae, Lysiterminae, Pambolinae, Rhyssalinae, and Rhysipolinae, as well as seven genera of uncertain subfamilial placement (viz. *Anachyra*, *Andesipolis*, *Conobregma*, *Doryctomorpha*, *Leptorhaconotus*, *Monitoriella*, and *Pentatermus*; van Achterberg, 1995; Belokobilskij et al., 2004; Quicke, 1996; Wharton, 1993; Whitfield et al., 2004).

Representatives of two non-cyclostome subfamilies, *Helcon* of the Helconinae and *Meteorus* of the Euphorinae, were included as outgroups, with *Helcon* itself used for rooting the trees. A simultaneous molecular (using 28S and 16S rDNA genes) and morphological phylogenetic study recovered *Helcon* as one of the most basal non-cyclostomes not counting the Trachypetinae, which appears to be the sister group of all other braconids (Dowton et al., 2002; Quicke et al., 1999). On the other hand, another study based only on the 16S rDNA gene recovered *Meteorus* at the base

of a grade composed first by the non-cyclostomes and then by the cyclostomes (Dowton et al., 1998). Moreover, a recent simultaneous molecular and morphological analysis recovered *Meteorus* as part of the Euphorinae, with the latter appearing as the sister taxon of the Aphidiinae within a major clade comprising the remaining non-cyclostome subfamilies (Shi et al., in press).

Taxa included in the present study, their provenances, and EMBL/GenBank accession numbers are detailed in Table 1.

2.2. DNA sequence data

Two gene fragments were examined. These were approximately 700 bp of the second and third domains (D2-3) of the nuclear 28S rDNA gene and a 603 bp region of the mitochondrial cytochrome oxidase I (COI) gene at the 5′ end. The D2-3 28S gene has been one of the most commonly used gene fragments in phylogenetic analyses within the Braconidae at higher taxonomic levels due to its relatively slow substitution rate (e.g., Belshaw et al., 1998, 2000, 2003; Dowton et al., 2002). On the other hand, COI has been widely employed within the Hymenoptera (e.g., Danforth et al., 2003; Dowton and Austin, 1995, 1997; Mardulyn and Whitfield, 1999; Niehuis and Wägele, 2004; Rokas et al., 2002) to resolve relationships at various taxonomic levels, though due to its higher substitution rate it is considered to perform better for resolving lower-level relationships [see Lin and Danforth (2004) for a review of this subject].

Of 174 sequences used here, 157 were newly generated (Table 1). Genomic DNA was extracted from either 95% ethanol preserved or dry pinned specimens (up to 15 years old). DNA extractions were carried out by placing the dried samples in 50 μl of 5% (w/v) Chelex (Bio-Rad) containing 12 μg/ml proteinase K, followed by digestion for approximately 2 h at 55–60 °C. Proteinase K was then heat-inactivated at 96 °C for 15 min. Samples were vortexed for approximately 10 s and the Chelex pelleted by centrifugation at 13,000*g* for approximately 30 s prior to removal of 2 μl of supernatant as template for PCRs. 28S primers were designed by Belshaw and Quicke (1997) (fwd: 5′GCG AAC AAG TAC CGT GAG GG3′) and Mardulyn and Whitfield (1999) (rev: 5′TAG TTC ACC ATC TTT CGG GTC CC′3). COI primers were designed by Folmer et al. (1994) (LCO 5′GGT CAA CAA ATC ATA AAG ATA TTG G3′; HCO 5′TAA ACT TCA GGG TGA CCA AAA AAT CA3′). PCRs were carried out in a 25 μl final volume using pure *Taq* ready to-go PCR beads (Amersham Biosciences). The PCR program for both 28S and COI amplifications had an initial 3 min denaturation at 80 °C, followed by 40 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min. A 10 min extension period following the final cycle was added in all cases. PCR products were purified using the wizard SV gel and PCR clean up system (Promega) and then sequenced in both directions using dideoxy terminator cycle sequencing (Applied Biosystems) with an ABI 3700 automated DNA sequencer.

2.3. Sequence alignment

Sequences for both markers were edited and manually aligned. Three of the COI sequences had three or six base pair deletions, but their alignment could be established by examining the translated amino acids. The manual alignment for 28S was performed by superimposing the highly conserved areas and then delimiting the regions of ambiguous alignment (RAAs) based on the criterion proposed by Lutzoni et al. (2000).

Inspection of the 28S manual alignment showed that several RAAs involve length variation in only a few isolated taxa, whereas others define clusters of potentially closely related taxa with conserved sequence length and often apparent sequence homology. Therefore, in order to preserve as much potential phylogenetic information as possible while still using objective criteria, we explore here the effect of excluding and including the RAAs and indel information using the 'fragment-level' alignment method (*sensu* Lee, 2001b) but with a modified coding approach. The 28S data set with the RAAs and indel information excluded is referred to as 28SN and the one with them included as 28SA. Indels of identical length that were potentially phylogenetically informative were treated as aligned blocks and indels of different length were scored as question marks for all other taxa. An additional non-additive 'morphological' character was added for each indel region, assigning indels of each particular length the same character state and again treating uninformative indel lengths as missing data (Fig. 1). Different from previous fragment-level alignment coding approaches (Kjer et al., 2001; Lutzoni et al., 2000; Wheeler, 1999), the coding approach used herein conserves all the nucleotide variation that is not ambiguous due to sequence length variation.

2.4. Morphological data

A total of 81 characters from adult and larval external morphology, male genitalia, venom apparatus, and ovipositor structure were scored (see characters list and their states scored in Appendix A, respectively). Most of these characters have proven to be informative at various higher taxonomic levels in previous studies (Belokobilskij et al., 2004; Quicke and van Achterberg, 1990; Quicke et al., 1992a,b, 1995; Wharton, 1993; Zaldivar-Riverón et al., 2004). Adult external morphological characters were scored from the DNA voucher specimens, whereas data on the remaining character systems were mainly scored from previous published surveys (see references in character list). All morphological characters were treated as unordered.

2.5. Bayesian analyses

Bayesian MCMC analyses were performed for the separate and the simultaneous data sets using MrBayes version 3.0b4 (Ronquist and Huelsenbeck, 2003). To reduce the

Table 1
Localities and EMBL/GenBank accession numbers of the taxa included

Taxon	28S	COI
Alysiinae		
Asobara tabida, Silwood culture, UK[b]	AY935421	AY935342
Conalysia sp., Sabah, Malaysia[e]	AY935422	—
Cratospila sp., Tana Rata, West Malaysia[e]	AY935420	AY935341
Dacnusa sibirica, ex culture[e]	AY935425	AY935345
Exotela sp., UK[e]	AY935426	AY935346
Gnathopleura sp., Costa Rica[b]	AY935423	AY935343
Phaenocarpa sp., no data[b]	AY935424	AY935344
Aphidinae		
Ephedrus californicus, culture, UK[e]	AJ302938[f]	AY935416
Monoctonus pseudoplatani, Ascot, UK[e]	Z83589[f,g]	AY935417
Betylobraconinae		
Betylobracon waterhousi, Australia[e]	AJ245686[f]	—
Mesocentrus sp., Canberra, Australia[b]	AY935461	—
Braconinae		
Aspidobracon sp., Taiwan[b]	AY935437	AY935357
Bracon sp., Kibale, Uganda[b]	AY935436	AY935353
Coeloides sordidator, Switzerland[b]	AJ231529[f]	AY935355
Merinotus sp., Kibale, Uganda[a]	AY296649[f]	AY935358
Megacoeloides sp., Colombia[d]	AY935439	AY935360
Pseudoshirakia sp., S. Korea[d]	AY935438	AY935359
Spinadesha sp., West Malaysia[d]	AY935440	AY935361
Sylvibracon sp., Cameroun[b]	AY296645[f]	AY935356
Vipio sp., Bolum, Turkey[b]	AJ296045[f]	AY935354
Doryctinae		
Aivalykus arawak, Costa Rica[a]	AY935471	AY935398
Caenophanes sp., Africa[a]	AY935465	—
Caenopachys hartigii, Corsica, France[a]	AY935474	AY935401
Doryctes leucogaster, Israel[a]	AY935472	—
Doryctes sp., no data[e]	—	AY935399
Hecabolus sulcatus, Ascot, UK[a]	AY935473	AY935400
Heterospilus prosopidis, Silwood culture, UK[e]	AY935469	AY935396
Labania sp., Costa Rica[e]	AY935470[g]	AY935397
Liobracon sp., Costa Rica[e]	AY935467	AY935394
Leptorhaconotus sp., Madagascar[a]	AY935479	AY935406
Megaloproctus sp., Colombia[a]	AY935466	AY935393
Monitoriella sp., Costa Rica[a]	AY935457	AY935387
Notiospathius sp., Caucagua, Venezuela[b]	AY935477	AY935404
Odontobracon sp., Costa Rica[a]	AY935468	AY935395
Rhaconotus menippus, Benin[a]	AY935464	AY935392
Schlettereriella sp., Kibale, Uganda[a]	AY935478	AY935405
Spathius sp., Tollara Province, Madagascar[b]	AY935476	AY935403
Stenocorse sp., Belize[a]	AY935475	AY935402
Exothecinae		
Colastes sp., Ascot, UK[b]	AY935431	AY935350
Pseudophanomeris unicolor Primorskii Krai, Russia[a]	AY935433	AY935351
Shawiana orientalis, Primorskii Krai, Russia[a]	AY935432	—
Xenarcha abnormis, Primorskii Krai, Russia[a]	AY935434	AY935352
Gnamptodontinae		
Gnamptodon pumilo, Ascot, UK[e]	Z93662[f,g]	—
Gnaptogaster astrachanica, Astrachanskaya region, Russia[a]	AY935441	AY935363
Pseudognaptodon sp., no data[e]	AJ296059[f]	AY935362
Hormiinae		
Hormius sp., Mahajanga Province, Madagascar[b]	AY935455	AY935385
Parahormius sp., Mt. Coupe, Cameroun[a]	AY935456	AY935386
Pentatermus sp., Benin[a]	AY935453	AY935383

(*continued on next page*)

Table 1 (*continued*)

Taxon	28S	COI
Lysiterminae		
Acanthormius sp., Madagascar[a]	AJ302883[f]	AY935381
Cedria sp., Madagascar[a]	AY935460	AY935390
Tetratermus sp., Kibale, Uganda[a]	AY935452	AY935382
Mesostoinae		
Andesipolis sp., Flor del Lago, Chile[a]	AY935485	AY935411
Aspilodemon sp., Colombia[a]	AY935487	AY935413
Hydrangeocola sp., Flor del Lago, Chile[a]	AY935486	AY935412
Mesostoa kerri, Australia[b]	AJ302930[f]	AY935415
Proavga sp., Canberra, Australia[e]	AJ416977[f]	AY935414
Opiinae		
Ademon sp., Bangalore, India[b]	AY935429	—
Bitomus sp., no data[e]	AY935428	AY935348
Diachasmimorpha sp., no data[b]	AY935430	AY935349
Opius sp., Ascot, UK[b]	AY935427	AY935347
Pambolinae		
Notiopambolus depressicauda, Canberra, Australia[b]	AY935459	AY935389
Pambolus sp., Choroni, Venezuela[b]	AY935458	AY935388
Pseudorhysipolis sp., Costa Rica[b]	AY935450	AY935377
Rhyssalinae		
Acrisis brevicornis, Primorskii Krai, Russia[a]	AY935483	AY935410
Dolopsidea sp., Ascot, UK[e]	AJ302920[f,g]	—
Histeromerus mystacinus, Ascot, UK	Z83601[f]	—
Oncophanes rugosus, Ascot, UK[e]	AY935481	AY935407
Rhyssalus clavator, Poland, Kazimierz[a]	AY935482	AY935409
Thoracoplites sp., Kenya[b]	AJ302920[f]	—
Tobiason pronotalis, Vietnam[a]	AY935480	AY935408
Rhysipolinae		
Noserus flavicoxa, Russia[b]	AY935454	AY935384
Rhysipolis temporalis, Primorskii Krai, Russia[a]	AY935449	AY935376
Rogadinae		
Aleiodes dispar, Ascot, UK[e]	AJ784935	AY935365
Aleiodes ruficornis, Ascot, UK[e]	AY935443	AY935367
Aleiodes seriatus, France[e]	AY935444	AY935368
Aleiodes sp., Cuyagua, Venezuela[a]	AY935442	AY935366
Anachyra sp., Malaysia[b]	AY935463	—
Artocella sp., Zaragoza, Spain[c]	AY935451	AY935379
Bulborogas sp, Colombia[e]	AJ784930	—
Bulborogas sp., French Guyana	—	AY935372
Choreborogas sp., Colombia[b]	AY935447	—
Clinocentrus sp., Ascot, UK[b]	AJ784962	AY935378
Colastomion concolor, Benin[b]	AY935446	AY935370
Cystomastax sp., Caucagua, Venezuela[b]	AY935445	AY935369
Polystenidea sp., Magdalena, Colombia[b]	AY935448	AY935374
Pseudoyelicones limonensis, Costa Rica[d]	AJ784929	—
Rogas sp., Amani, Tanzania[a]	**AJ784931**	AY935364
Spinaria sp., Malaysia[a]	**AJ784960**	AY935371
Stiropius sp., Costa Rica[a]	**AJ784961**	AY935373
Tebennotoma sp., Taiwan	AJ784933	AY935380
Yelicones spectabile, Tollara Province, Madagascar[d]	AJ784319	AY935375
Telengainae		
Telengaia ventralis, Turkmenia[a]	AY935435	—
Unplaced taxa		
Allobracon sp., Brazil[e]	**AJ302886**[f]	AY935391
Conobregma sp., Sabah, Malaysia[b]	AY935462	—
Doryctomorpha sp., New Zealand[e]	**AY935484**	—
Euphorinae		
Meteorus corax, Finland[d]	**AY935488**	AY935418

Table 1 (*continued*)

Taxon	28S	COI
Helconinae		
Helcon sp., Sabah, Malaysia[d]	**AJ302815**[f]	AY935419

Subfamilial classification based on the results obtained in this study. *Note*. Between 6 and 91 bp near either the 5′ or 3′ end of the fragment could not be obtained in 17 and 4 sequences for 28S and COI, respectively.

[a] Zoological Institute, St. Petersburg, Russia.
[b] Nationaal Natuurhistorisch Museum, Leiden, Netherlands.
[c] National Museums of Scotland, Edinburgh, UK.
[d] Natural History Museum, London, UK.
[e] No voucher.
[f] Sequences obtained from previous works.
[g] D3 region of the 28S gene fragment not sequenced.

	AAR1	AAR1a	AAR1b	Indel
Ademon	T[TACTACTTGTAG----]TA	????????????????	?????????	2
Cratospila	T[TACTACTTGTAG----]TA	????????????????	?????????	2
Merinotus	T[TACTACTTATGTAG--]TA	????????????????	?????????	3
Aspidobracon	T[TACTATTTCGGTAG--]TA	????????????????	?????????	3
Sylvibracon	T[TACTACTTGTAG----]TA	????????????????	?????????	2
Aleiodes dispar	T[TACTACTTGTAG----]TA	????????????????	?????????	2
Cystomastax	T[TACTACTTGTAG----]TA	????????????????	?????????	2
Rhysipolis	T[TACTACTTGTAG----]TA	????????????????	?????????	2
Caenophanes	T[TACCCTCGGG------]TA	????????????????	TACCCTCGG	1
Labania	T[TACTTTCGAG------]TA	????????????????	TACTTTCGA	1
Doryctes	T[TACTCTTGTAG-----]TA	????????????????	?????????	4
Leptorhaconotus	T[TACTCTTGTAG-----]TA	????????????????	?????????	4
Histeromerus	T[TACTCTTGAG------]TA	????????????????	TACTCTTGA	1
Rhyssalus	T[TACTCTCGAG------]TA	????????????????	TACTCTCGA	1
Acrisis	T[TACTCTTTGAG-----]TA	????????????????	?????????	4
Doryctomorpha	T[TATCCTTTTNGGGA--]TA	????????????????	?????????	3
Andesipolis	T[TGTCCTCTCACGGGGA]TA	TGTCCTCTCACGGGG	?????????	0
Hydrangeocola	T[TATCCTCTCACGGGGA]TA	TATCCTCTCACGGGG	?????????	0
Aspilodemon	T[TATCCTCTCACGGGGA]TA	TATCCTCTCACGGGG	?????????	0
Proavga	T[ACTCTTCTCACGAGGA]TA	ACTCTTCTCACGAGG	?????????	0
Mesostoa	T[TGTTCTCTCACGAGAA]TA	TGTTCTCTCACGAGA	?????????	0

Fig. 1. Illustration of the coding method implemented in this study for the ambiguously aligned regions and indel characters.

chance of analyses becoming trapped in local optima, two independent analyses were run for each data set, each consisting of four chains from random starting trees and using uniform priors. Chains were run for 2 million generations, sampling trees every 1000 generations. Modeltest version 3.06 (Posada and Crandall, 1998) was used to determine the most appropriate model of sequence evolution for the two gene markers examined. The GTR+I+Γ (general time reversible; Lanave et al., 1984) model was used for the DNA sequence data and the Mk + Γ (Markov *k*; Lewis, 2001) model for the indel characters and the morphological data. The morphological (including indel characters) and 28S data sets were each treated as single partitions, whereas COI data were treated as comprising three separate partitions based on codon positions. The burn-in phases for the different analyses were discarded (see Table 2). The relationships obtained from the remaining sampled trees were highly correlated between the two analyses run for each data set. Therefore, the topologies, branch lengths, and Bayesian posterior probabilities (BPPs) for each of the separate and combined data sets were constructed with a 50% majority rule consensus tree that pooled the trees sampled from both

Table 2
Attributes and analysis results for the separate molecular and morphological and the combined data sets

	28SN	28SA	COI	Morph.	28SN + COI	28SA + COI	28SN + COI + M	28SA + COI + M
No. of taxa included	95	95	79	95	95	95	95	95
No. of characters	559	957	603	81	1162	1560	1243	1641
% variable characters	68.7	70.8	64.5	100	64.5	68.8	66.7	70.3
% parsimony inf. characters	47.8	5.9	52.4	100	49.3	52.2	52.3	54.5
% A/T base composition	0.512	0.544	0.735	—	—	—	—	—
Burn-in phase (generations)	90,000	100,000	150,000	70,000	300,000	100,000	160,000	130,000
Mean − ln score	10385.9	15320.7	17,260	2145.6	28715.3	33244.9	30853.5	35669.5
No. of clades with BPP ⩾ 0.95	29	35	16	12	35	44	47	51
Mean BPP value[a]	68.97	74.3	56.8	50.27	76.19	82.56	83.02	84.04
Mean BPP value (RC)[b]	82.44	84.85	—	—	81.64	85.47	88.05	89.48
No. of clades BTP ⩾ 70%	—	—	—	—	—	—	27	32

[a] Mean BPP value for all the recovered clades.
[b] Mean BPP value considering only clades in common between data sets with 28SN and 28SA.

independent analyses. Clade support was regarded as significant if the clade was present in at least 95% of the sampled trees. The molecular and morphological data matrices and their simultaneous molecular and morphological Bayesian topologies obtained can be downloaded from the *TreeBase* web page (study Accession No. S1374, matrix Accession No. M2440).

2.6. Parsimony-based tests

Data set attribute calculations and statistical analyses were carried out using PAUP* version 4.0b10 (Swofford, 2002). Congruence among data partitions was assessed using the incongruence length difference test (ILD test; Farris et al., 1994) with 1000 replicates each with 50 random stepwise additions holding no more than one tree. All parsimony uninformative characters were excluded and tests were carried out excising those taxa that were not scored for one of the data partitions (Cunningham, 1997; Lee, 2001a).

In addition to the Bayesian analyses, an equally weighted maximum parsimony analysis considering all characters as unordered was also performed for the two simultaneous morphological and molecular data sets (with 28SN and 28SA) using PAUP*. A total of 10,000 random additions with TBR branch swapping holding only one tree was used. Clade support was evaluated using a non-parametric bootstrap (Hillis and Bull, 1993) with 1000 replicates and 10 random additions each holding only one tree.

2.7. Test of alternative hypotheses

Several clades in our simultaneous molecular and morphological phylogenies contradicted the current classification for some of the included terminal taxa. However, maximum likelihood based tests of topologies could not be implemented for these phylogenies because of the inclusion of morphological characters. Therefore, the Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999) was employed in order to test whether some relationships recovered in the molecular (using 28SN) phylogeny were significantly better explanations of the data than phylogenetic hypotheses that constrained taxa according to their subfamilial placement accepted prior this study. The SH tests were carried out using 1000 replicates and the RELL sampling. Heuristic MP searches as mentioned above were carried out to obtain the alternative topologies. The GTR+I+Γ model of evolution was used to calculate the maximum likelihood values of the resulting MPTs for the SH tests.

3. Results

3.1. Data attributes

The main attributes for the separate and combined data sets and their resulting Bayesian trees are presented in Table 2. For the 28S rDNA fragment, a total of 13 RAAs with lengths varying between 3 and 22 bp and a further eight parsimony informative unambiguously alignable indels (all consisting of a single position) were delimited after manual alignment. Implementation of the coding method added 398 characters to this data set, of which 378 were from the RAAs sequence clusters and 21 from the indel characters. A/T base composition was found to be considerably higher for COI compared with 28S data (Table 2) and was especially so for third codon position where 91.8% of bases were A or T.

ILD tests revealed a high degree of incongruence between the two genes examined and between the 28SA and the morphological data sets (28SN/COI and 28SA/COI: $P < 0.001$; 28SA/Morphology: $P = 0.007$). However, incongruence between 28SN and morphology was only marginally significant ($P = 0.02$), and the COI and morphology partitions were not significantly incongruent ($P = 0.125$).

3.2. Separate analyses

The 28SN and 28SA phylogenies both recovered the cyclostomes as a strongly supported monophyletic group as well as all the subfamilial relationships supported in previous molecular studies (see introduction). The 28SN and 28SA Bayesian topologies were similar in the composition of most of the recovered clades (only the 28SN tree is shown; Fig. 2A). Differences involved only in few weakly supported clades and four relationships that were only supported significantly in the 28SA tree (see Table 2). The latter were a *Dacnusa* + *Exotela* clade (Dacnusini: BPP = 0.99), a clade with all the included exothecine genera except for *Colastes* (BPP = 0.95), a clade with six doryctine genera together with *Monitoriella* (BPP = 0.99), and *Monitoriella* and *Labania* as sister taxa (BPP = 0.95). Addition of RAAs and indel characters to the 28S data increased the number of significantly supported clades from 29 to 35, and the mean BPP value was increased by 5.33% (and by 2.4% when considering only relationships in common with the 28N phylogeny; Table 2).

The COI Bayesian topology (Fig. 2B) differs widely from the 28S ones and has far fewer significantly supported clades (Table 2), which were located mainly towards the tips. However, as in the 28S phylogenies, the mesostoine and aphidiine genera together with *Andesipolis* were recovered at the base of the cyclostomes.

The morphological Bayesian phylogeny only recovered 12 significantly supported clades (Fig. 3B). Several relationships in this topology, however, resembled those found in the 28S and the combined molecular Bayesian topologies (see below). Among the significantly supported relationships were a monophyletic cyclostome clade (BPP = 1.0), a clade containing the members of the Rogadina + *Pseudoyelicones* (BPP = 1.0), with the latter genus as sister taxon to *Bulborogas* (BPP = 1.0), a Doryctinae clade with the exclusion of both *Monitoriella* and *Leptorhaconotus* (BPP = 1.0), a monophyletic Dacnusini (*Exotela* + *Dacnusa*; BPP = 1.0), and an Alysiinae + Opiinae clade (BPP = 0.95).

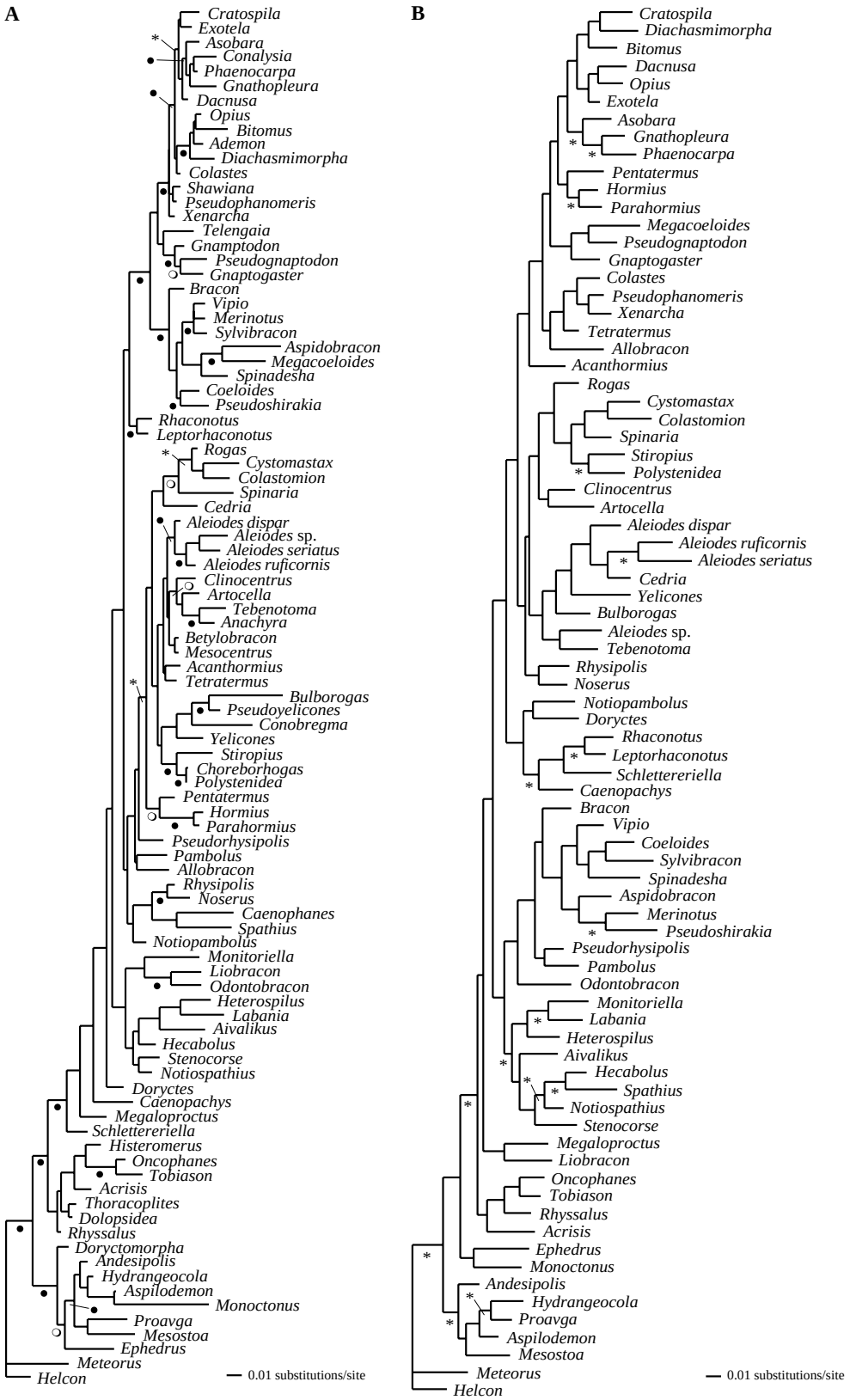

Fig. 2. Bayesian phylogenetic trees for the 28S and COI data sets. (A) Majority rule phylogram resulting from Bayesian analysis of the 28S data set excluding RAAs and indel information based on a combined 3.82 million postburn-in generation under the GTR+I+Γ model of evolution. Branches with a black circle were supported by posterior probabilities $\geqslant 95\%$ in both the 28SN and 28SA analyses, branches with an open circle were supported by posterior probabilities $\geqslant 95\%$ only in the 28SA analysis, and branches with an asterisk were supported by posterior probabilities $\geqslant 95\%$ only in the 28SN analysis. (B) Majority rule phylogram resulting from Bayesian analysis of the COI data set based on a combined 3.7 million postburn-in generation under the GTR+I+Γ model of evolution. Branches with an asterisk were supported by posterior probabilities $\geqslant 95\%$.

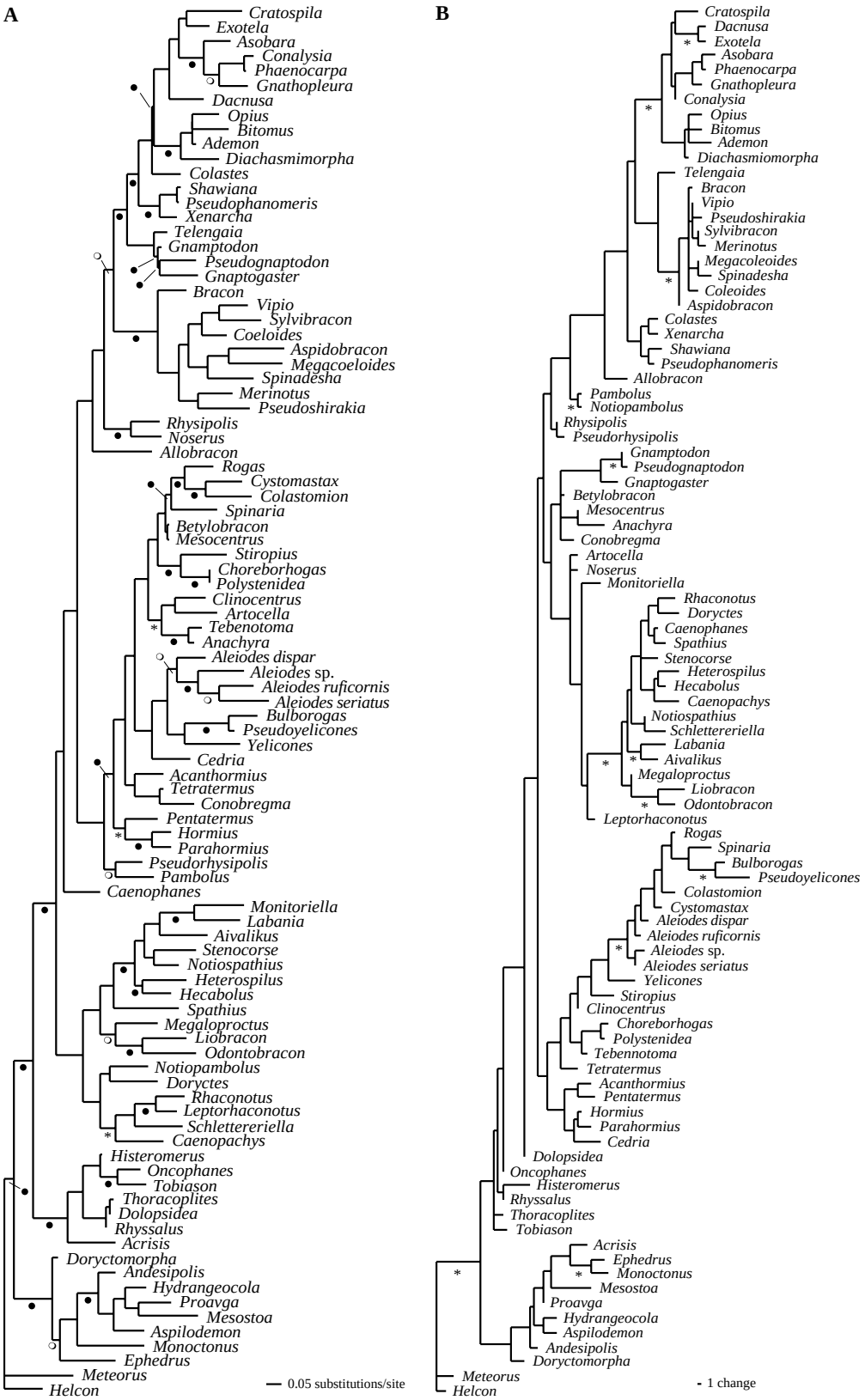

Fig. 3. Bayesian phylogenetic trees for the molecular and morphological data sets. (A) Majority rule phylogram resulting from Bayesian analysis of the two genes combined (28SN + COI) based on a combined 3.4 million postburn-in generation under the GTR+I+Γ model of evolution. Branches with a black circle were supported by posterior probabilities ⩾95% in the analyses using the 28SA and 28SN data sets, branches with an open circle were supported by posterior probabilities ⩾95% only in the analysis including 28SA, and branches with an asterisk were supported by posterior probabilities ⩾95% only in the analysis including 28SN. (B) Majority rule phylogram resulting from Bayesian analysis of the morphological data set based on a combined 3.86 million postburn-in generation under the Mk + Γ model of evolution. Branches with an asterisk were supported by posterior probabilities ⩾95%.

3.3. Combined analyses

All the combined molecular and the simultaneous molecular and morphological Bayesian topologies (Figs. 3A and 4, respectively) resemble the two 28S phylogenies, suggesting that this latter data set provides the strongest phylogenetic signal. Nevertheless, addition of the COI and morphological information to the 28S data considerably increased the support for several clades (Table 2) and lead to the recovery of some of the higher taxonomic level taxa recognized in current classifications. This same pattern was observed when the RAAs and indel characters were included in the simultaneous molecular and molecular and morphological data sets, where the number of significantly supported clades and mean BPP values also increased in comparison with data sets that excluded them (Table 2). Moreover, as with the 28S phylogenies, most of the relationships that differed between the simultaneous analysis topologies excluding and including RAAs and indel information appeared weakly supported.

A comparison of selected relationships recovered by the different separate and combined data sets analyzed is given in Table 3. All data sets recovered the cyclostomes as monophyletic (BPP = 1.0), and most data sets recovered a 'Gondwanan' Mesostoinae + Aphidiinae clade (BPP = 1.0) at the base followed by a clade with the members of Rhyssalinae (BPP $\geqslant$ 0.95). Within these clades, *Doryctomorpha* and *Andesipolis* were consistently recovered within the Mesostoinae + Aphidiinae clade and, although its relationships differed among topologies, the Rhyssalinae were paraphyletic with respect to *Histeromerus* and also consistently included the genus *Acrisis*. Adding the morphological data in the simultaneous analyses led to recovery of a monophyletic Doryctinae with the inclusion of both *Leptorhaconotus* and *Monitoriella* (BPP $\geqslant$ 0.95).

None of the small subfamilies Pambolinae, Rhysipolinae, Hormiinae, Lysiterminae, and Betylobraconinae as currently recognized were recovered as monophyletic. The Rogadinae was not recovered as monophyletic in the simultaneous analysis adding RAAs and indel characters but was paraphyletic with respect to the Betylobraconinae *sensu* van Achterberg (1995), which appeared as sister group to the Clinocentrini (BPP = 0.51). Tribal relationships within the Rogadinae were not significantly supported in any of the topologies and a monophyletic Rogadini was only recovered in the morphological phylogeny. Finally, all the combined analyses recovered an Opiinae + Alysiinae + Exothecine + Braconinae + Gnampto dontinae + Telengaiinae clade, with the last two subfamilies appearing as sister taxa.

Parsimony analyses for the simultaneous molecular and morphological data including and excluding the RAAs and indel characters yielded four and seven MPTs with lengths of 7327 and 8330 and CIs of 0.197 and 0.227, respectively (trees not shown). All the strongly supported relationships (bootstrap values $\geqslant$70%; Hillis and Bull, 1993) recovered by these two MP analyses were significantly supported in the two simultaneous Bayesian analyses, and therefore we only consider the Bayesian phylogenies for further discussion.

3.4. Tests of alternative hypotheses

The SH test of the resulting alternative MP topologies that constrained the taxa according to the following currently accepted higher-level classification for the cyclostomes showed that their likelihood values are significantly lower that those of the simultaneous 28SN + COI Bayesian hypothesis ($P < 0.003$). Thus, (1) *Doryctomorpha* is not accepted as a member of the Doryctinae (*sensu* Belokobilskij, 1992); (2) *Allobracon* and *Monitoriella* are not members of the Hormiinae (*sensu* van Achterberg, 1995; Wharton, 1993); (3) the Rogadina is not monophyletic with the inclusion of *Bulborogas* and *Aleiodes* (*sensu* van Achterberg, 1991, 1995); and (4) the Rhysipolinae (viz. *Rhysipolis*, *Noserus*, and *Pseudorysipolis*) does not constitute a monophyletic group (*sensu* Belokobylskij, 1984; Scatolini et al., 2002).

Table 3
Selected relationships recovered from analyses of the separate molecular and morphological and the combined data sets

Clade	28SN	28SA	COI	Morph.	28SN + COI	28SA + COI	28SN + COI + M	28SA + COI + M
Cyclostomes	Y	Y	Y	Y	Y	Y	Y	Y
Mesostoinae (including *Andesipolis*) + Aphidinae	Y	Y	N	Y	Y	Y	Y	Y
Rhyssalinae (including *Acrisis*) + *Histeromerus*	Y	Y	—	N	Y	Y	Y	Y
Doryctinae (including *Leptorhaconotus* and *Monitoriella*)	N	N	N	Y	N	N	Y	Y
Exothecinae	N	N	Y	Y	N	N	Y	Y
Rogadinae + Betylobraconinae (excluding *Conobregma*)	N	Y	N	N	N	Y	Y	Y
Rogadinae (including *Anachyra*)	N	N	—	N	N	N	Y	N
Rogadini	N	N	N	Y	N	N	N	N
Aleiodes + Yeliconini (*Yelicones*, *Pseudoyelicones*, *Bulborogas*)	N	N	—	N	Y	Y	Y	Y
Hormiinae + Lysiterminae (including *Cedria*)	N	N	N	N	N	N	Y	Y
Teleng. + Gnaptodont. + Opiin. + Alysiin. + Exoth. + Bracon.	Y	Y	N	N	Y	Y	Y	Y
Telengainae + Gnaptodontinae	N	N	—	N	Y	Y	Y	Y
Alysiinae	Y	Y	N	Y	Y	N	Y	N

Subfamilial classification based on this study (see Table 1).

4. Discussion

4.1. Inclusion of RAAs and indel information

A great advantage of the Bayesian MCMC method of phylogenetic inference over other current maximum likelihood methods is that it can cope simultaneously with morphological and DNA sequence data (MrBayes 3.0b4; Ronquist and Huelsenbeck, 2003), and therefore, in the latter case it can also cope with some methods that include indel information. However, there remains the problem of how to generate an objective alignment for length variable rDNA genes.

Multiple alignment programs such as Clustal W (Thompson et al., 1994), although quick, usually give such unsatisfactory results that they often have to be manually 'corrected.' On the other hand, optimization alignment (as implemented in POY; Wheeler, 1996) is internally consistent at finding homology lines given its tree. However, it is very computationally intensive such that problems involving even moderate number of taxa require supercomputers. Further, with both Clustal and POY it is necessary to specify relative gap and substitution costs though it is hard to justify any particular combination of parameters. The use of information from secondary structure represents an important tool to locate RAAs within homologous positions in rRNA molecules (Kjer, 1995; Gillespie, 2004) though it does not solve the problem of how to incorporate the phylogenetic information contained in single stranded motifs. Combining all plausible alignments into a single analysis (the 'elision' method; Wheeler et al., 1995) also requires potentially huge computational and memory time and the range of the parameters (i. e., relative gap and substitution costs) employed to perform the different alignments results subjective (Lutzoni et al., 2000). Other options include simply excising all ambiguous regions, but that means potentially discarding a considerable amount of information.

Several methods have been proposed that offer a simple but objective way of coding RAAs including variants of what have been termed by Lee (2001b) as fragment-level alignment (fixed character state: Wheeler, 1999; INAASE: Kjer et al., 2001; Lutzoni et al., 2000). The principle of the fragment-level alignment method relies on the treatment of each RAA as a single multi-state character, with each distinct length or sequence variant considered as a separate character state. Moreover, some variants of this method can also include step matrices based on substitution and gap changes. This method, however, cannot be readily implemented if the RAAs, so coded, lead to the recognition of numbers of character states that are beyond the current limits of standard software packages, except for Wheeler's (1999) variant using POY (Gladstein and Wheeler, 1996).

In the present example, we implemented a variant of fragment-level alignment using an approach that codifies information about the lengths of indel events but allows nucleotide variation within inserts of identical length (whose positional homology is often established) to contribute to the final tree. We applied both MP and Bayesian MCMC techniques to various data sets including 28S rDNA, and we compared the results from simply excluding all alignment ambiguous regions and including them according to the above method. In all combinations (28S alone, 28S + COI, and 28S + COI + morphology), inclusion of information derived from RAAs led to higher levels of support (based on both total and mean BPP) and the number of clades with significant BPPs (Table 2). Thus, our results are in agreement with previous studies that support the inclusion of this kind of data as an important and reliable source phylogenetic information (reviewed in Lee, 2001b; Lutzoni et al., 2000; Simmons and Ochoterena, 2000).

Among the limitations of our coding approach is that its use loses possible substitution information that spreads across indels of different lengths, and thus is less useful for assessing relationships among more highly divergent taxa, where indel expansion and contraction is common. Moreover, the topographic identity (*sensu* Brower and Schawaroch, 1996) of the sequences is established only by their length but not by their nucleotide similarity, and thus this does not guarantee positional homology of the nucleotide positions involved even when in the clusters of sequences with similar length the number of informative sites that fulfill the criterion of correspondence of relative position appear to be greater than the number of sites that do not.

4.2. Taxonomic inferences

Based on the significantly supported relationships obtained from the simultaneous molecular and morphological Bayesian analyses including and excluding RAAs and indel information, we propose that a total of 17 subfamilies should be recognized within the cyclostome group with the inclusion of the Apozyginae (Table 1). In those cases where the relationships were weakly supported, the subfamilies involved were maintained in their currently recognized senses.

Both simultaneous analyses significantly support the existence of a basal 'Gondwananan' clade composed by the Mesostoinae + Aphidiinae, which was also recovered in some previous studies (Belshaw et al., 2000; Belshaw and Quicke, 2002; Dowton et al., 2002). This relationship differs from two recent phylogenetic analyses that weakly support the Aphidiinae as the sister group of the Euphorinae, with one of them also recovering a *Mesostoa* + *Hydrangeocola* clade at the base of a 'helconoid complex' (Shi and Chen, 2005; Shi et al., in press). Our molecular and morphological evidence clearly shows that the aberrant South American genus *Andesipolis* belongs to the Mesostoinae (*sensu* Belshaw and Quicke, 2002) though *Doryctomorpha* is left with uncertain placement until further analyses including other presumably related genera (e.g., *Caenophachyella*, *Canberra, Apoavga*) and additional genetic markers allow confirmation of its subfamilial position. The Mesostoinae

+ Aphidiinae clade and the composition of the Mesostoinae as constituted herein are supported by one and three apparent synapomorphic substitutions and insertions present in the 28S alignment, respectively (Mesostoinae + Aphidiinae clade: RAA number 3 and unambiguously alignable indels b and h; Mesostoinae: RAA number 1; see matrix in *TreeBase*).

Consistent paraphyly of the Rhyssalinae with respect to the Histeromerinae leads us to propose that the latter should be treated as a tribe within the Rhyssalinae. *Anachyra*, which was originally considered to belong to the Rhyssalinae (van Achterberg, 1995) is shown to actually belong to the Clinocentrini in the subfamily Rogadinae, indicating considerable morphological homoplasy.

Monophyly of the Doryctinae in the combined analyses is only recovered and consistently supported when morphological information is added to the molecular data. Our analyses, however, strongly support the inclusion of the little known genera *Leptorhaconotus*, from Madagascar and South Africa, and *Monitoriella*, from Central America, as members of the Doryctinae. Previous placement of these taxa has been hampered in the former case by its extreme morphology (Quicke, 1996) and in the latter by the absence of a venom apparatus (Belokobilskij et al., 2004). *Monitoriella*, which was previously considered to belong to the Hormiinae (Wharton, 1993), appears strongly supported as the sister taxon of the otherwise typical doryctine genus *Labania*, and interestingly both of these taxa are gall formers (Infante et al., 1995; Marsh, 2002; Wharton and Hanson, 2005). Relationships found within the Doryctinae are also in agreement with some found in a recent phylogenetic analysis of the group based on morphology (Belokobilskij et al., 2004), where *Labania* and *Aivalykus*, *Heterospilus* and *Hecabolus*, and *Liobracon* and *Holcobracon* were each recovered within same major clades.

In our preferred hypotheses (see Fig. 4) some putative members of the small subfamilies Rhysipolinae, Pambolinae, Lysiterminae, Betylobraconinae, and Hormiinae were intermingled or placed alone forming grades between more inclusive groups. Based on the significantly supported clades recovered and/or morphological congruence, a taxonomic arrangement for these subfamilies is presented in Table 1.

The Hormiinae and Lysiterminae were only weakly supported as being sister taxa and thus they are maintained here as separate subfamilies pending the addition of more taxa and characters in further analyses. The placements of *Allobracon* and *Conobregma* where left as uncertain. Wharton (1993) retained *Allobracon* within the Hormiinae mainly because of its desclerotized metasomal tergites. However, he recognized that other morphological features resemble those observed in members of the Rogadinae and Rhysipolinae. A placement of *Allobracon* in the latter group is more congruent with our current results. The small genus *Conobregma*, for which COI sequence data could not be obtained, was originally described in the Betylobraconinae (van Achterberg, 1995). It was recovered with only weak support within the Lysiterminae in our simultaneous phylogenies, though it was nested within the Yeliconini (viz. *Yelicones*, *Pseudoyelicones*, and *Bulborogas*) based on 28S data alone. The latter placement is also supported by their shortened foretarsi, and in addition, some *Conobregma* species have a triangular midbasal area at the base of the second tergite, which is very similar to that of most members of the Rogadinae and therefore we presume that this is probably where it belongs.

The extent and tribal composition of the Rogadinae as currently recognized is maintained despite its apparent paraphyly with respect to the Betylobraconinae in the simultaneous analysis including RAAs and indel information and the surprising significantly supported clade containing the Yeliconini and *Aleiodes*. A polyphyletic Rogadini was also found in a recent phylogenetic analysis based on the D2 region of the 28S rDNA gene (Chen et al., 2003), which included 9 rogadine genera and 11 species of *Aleiodes*. These findings contrast with a study of rogadine venom apparatus (Zaldivar-Riverón et al., 2004), where a cone of filaments located inside the secondary venom duct was proposed as a synapomorphy for the members of the Rogadini with the inclusion of *Aleiodes*. However, *Aleiodes* (including *Cordylorhogas*) possesses a soft secondary venom duct with well-defined internal filaments, whereas in the remaining Rogadini genera (including *Bulborogas*) the secondary duct is evidently thickened and hardened and the filaments are less evident.

4.3. Transitions in the mode of parasitism

Our hypothesis of relationships among the cyclostome subfamilies confirms previous hypotheses that suggested that endoparasitism has evolved independently in the Alysiinae + Opiinae clade and in the Rogadinae (Belshaw et al., 1998; Dowton et al., 2002; Quicke, 1993; Whitfield, 1992) and further confirms that it has also evolved independently within the Braconinae in the Aspidobraconina. Future investigation of the actual taxonomic placement of the enigmatic doryctine genus *Sericobracon*, which is known to be an endoparasitoid (of web spinners: Embioptera; Shaw and Edgerly, 1985), and confirmation of the mode of parasitism in the Gnamptodontinae (for which ovipositor morphology indicates endoparasitoidism; Belshaw et al., 2003) and of the Betylobraconinae may reveal additional endoparasitoid lineages within the Braconidae.

Phytophagy on the other hand is known to occur within the Braconidae in a small number of genera belonging to the Doryctinae and Mesostoinae (see Wharton and Hanson, 2005 for review) and has recently been discovered in the Braconinae (Flores et al., in press). Members of these doryctine and mesostoine genera whose biologies have been confirmed are all known to be gall inducers, but others are suspected to be inquilines that feed on the plant tissue of galls induced by cynipids or cecidomyiids (Wharton and Hanson, 2005). In our study, phytophagy appears to have originated at least three

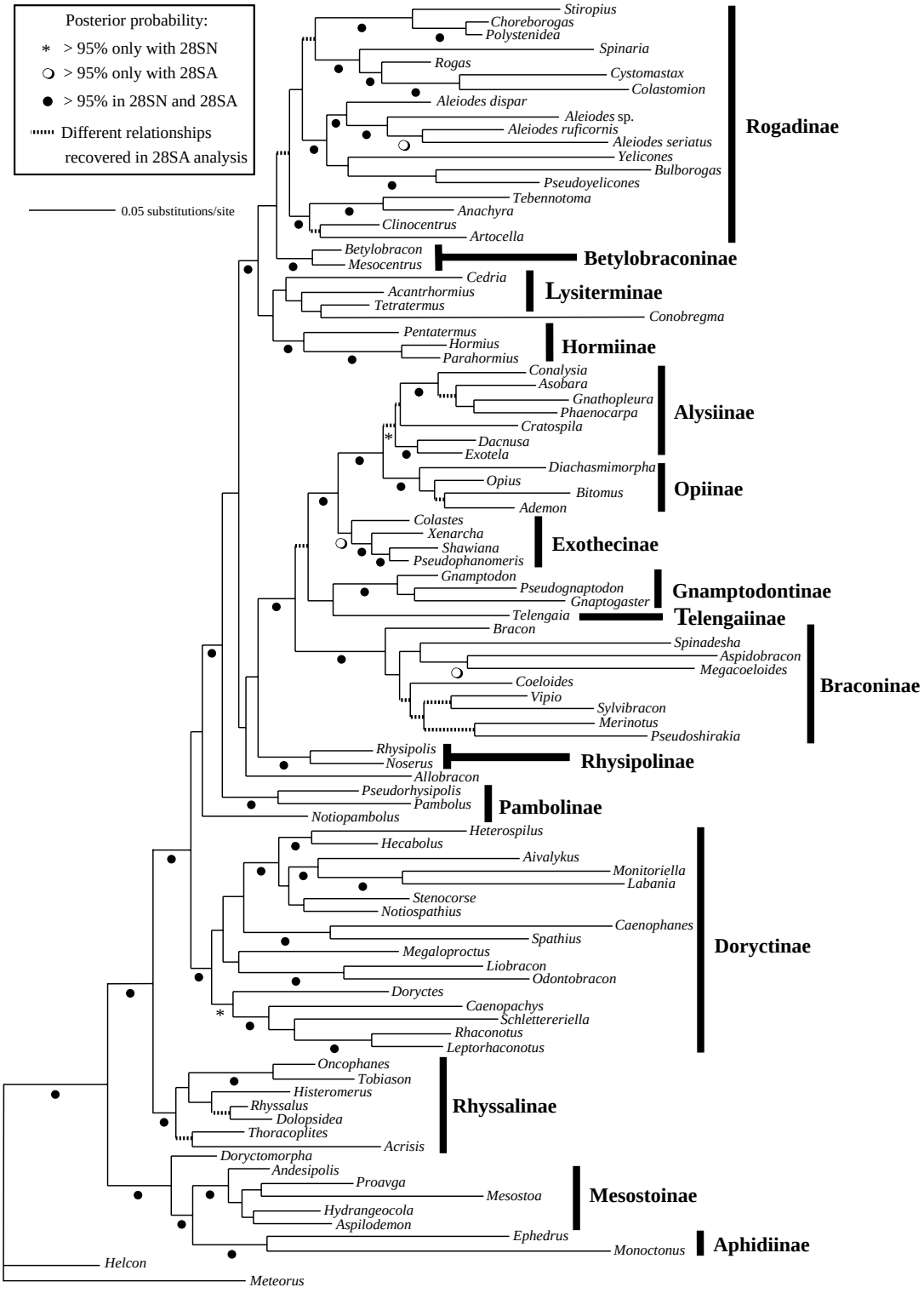

Fig. 4. Majority rule phylogram resulting from the simultaneous Bayesian analysis of the molecular (using 28SN) and morphological data sets based on a combined 3.68 million postburn-in generation under the GTR+I+Γ model of evolution. Branches indicated with a black circle were supported by posterior probabilities ⩾95% in the analyses using the 28SA and 28SN data sets, branches with an open circle were supported by posterior probabilities ⩾95% only in the analysis including 28SA, and branches with an asterisk were supported by posterior probabilities ⩾95% only in the analysis including 28SN. Broken lines indicate clades for which a different relationship was recovered with the simultaneous analysis using 28SA. Subfamilial classification based on the relationships recovered is indicated at the right of the tree.

times within the cyclostome group, once within the Mesostoinae, once within the doryctine *Monitoriella* + *Labania* clade, and once more within the Braconinae. However, the presence of other phytophagous genera (*Allorhogas*, *Psenobolus*, and *Mononeuron*) within the Doryctinae suggests that this type of biology has probably arisen in other separate lineages, and future molecular phylogenetic analysis will help to reveal the actual number of lineages with phytophagous species within this subfamily.

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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.2005.08.006.

References

van Achterberg, C., 1984. Essay of the phylogeny of Braconidae (Hymenoptera: Ichneumonoidea). Entomol. Tidskr. 105, 41–58.

van Achterberg, C., 1991. Revision of the genera of the afrotropical and w. palearctic rogadinae foerster (Hymenoptera: Braconidae). Zool. Verh. Leiden 273, 1–102.

van Achterberg, C., 1995. Generic revision of the subfamily Betylobraconinae (Hymenoptera: Braconidae) and other groups with modified fore tarsus. Zool. Verh. Leiden 298, 1–342.

van Achterberg, C., Quicke, D.L.J., 1992. Phylogeny of the subfamilies of the family braconidae: A reassessment assessed. Cladistics 8, 237–264.

Askew, R.R., Shaw, M.R., 1986. Parasitoid communities: their size, structure, and development. In: Waage, J., Greathead, G. (Eds.), Insect Parasitoids. Academic Press, London, pp. 225–264.

Austin, A.D., Dangerfield, P.C., 1997. A new species of Jarra Marsh and Austin (Hymenoptera: Braconidae) with comments on other parasitoids associated with the eucalypt longicorn Phoracantha semipunctata (F.) (Coleoptera: Cerambycidae). Austr. J. Entomol. 36, 327–331.

Austin, A.D., Dangerfield, P.C., 1998. Biology of *Mesostoa kerri* (Insecta: Hymenoptera: Braconidae: Mesostoinae), an endemic Australian wasp that causes stem galls on *Banskia marginata*. Austr. J. Bot. 46, 559–569.

Belokobylskij, S.A., 1984. On the division of the tribe Exothecini s.l. (Hymenoptera, Braconidae) in two with description of the new genus and subgenus. Zool. Zhurn. 63, 1019–1026 (in Russian).

Belokobilskij, S.A., Zaldivar-Riverón, A., Quicke, D.L.J., 2004. Phylogeny of the genera of the parasitic wasps subfamily Doryctinae (Hymenoptera: Braconidae) based on morphological evidence. Zool. J. Linn. Soc. 142, 369–404.

Belshaw, R., Quicke, D.L.J., 1997. A molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae). Mol. Phylogenet. Evol. 7, 281–293.

Belshaw, R., Quicke, D.L.J., 2002. Robustness of ancestral state estimates: evolution of life History strategy in ichneumonoid parasitoids. Syst. Biol. 51, 450–477.

Belshaw, R., Grafen, A., Quicke, D.L.J., 2003. Inferring life history ovipositor morphology in parasitoid wasps using phylogenetic regression and discriminant analysis. Zool. J. Linn. Soc. 139, 213–228.

Belshaw, R., Dowton, M., Quicke, D.L.J., Austin, A.D., 2000. Estimating ancestral geographic distributions: a Gondwanan origin for aphid parasitoids. Proc. R. Soc. Series B 267, 491–496.

Belshaw, R., Herniou, E., Gimeno, C., Fitton, M.G., Quicke, D.L.J., 1998. Molecular phylogeny of the Ichneumonoidea (Hymenoptera) based on D2 expansion region of 28S rDNA. Syst. Entomol. 23, 109–123.

Brower, A.V.Z., Schawaroch, V., 1996. Three steps of homology assessment. Cladistics 12, 265–272.

Capek, M., 1970. A new classification of the Braconidae (Hymenoptera) based on the cephalic structures of the final instar larva and biological evidence. Can. Entomol. 102, 846–875.

Chen, X., Piao, M., Whitfield, J.B., He, J.H., 2003. A molecular phylogeny of the subfamily Rogadinae (Hymenoptera: Braconidae) based on the D2 variable region of 28S ribosomal RNA. Acta Entomol. Sin. 46, 209–217.

Cunningham, C.W., 1997. Is congruence between data partitions a reliable predictor of phylogenetic accuracy? Empirically testing an iterative procedure of choosing among phylogenetic methods. Syst. Biol. 46, 464–478.

Danforth, B.N., Conway, L., Ji, S., 2003. Phylogeny of eusocial *Lasioglossum* reveals multiple losses of eusociality within a primitively clade of bees (Hymenoptera: Halictidae). Syst. Biol. 52, 23–36.

Dolphin, K., Quicke, D.L.J., 2001. Estimating the global species richness of an incompletely described taxon: an example using parasitoid wasps. Biol. J. Linn. Soc. 73, 279–286.

Dowton, M., 1999. Relationships among the cyclostome braconid (Hymenoptera: Braconidae) subfamilies inferred from a mitochondrial tRNA rearrangement. Mol. Phylogenet. Evol. 11, 283–287.

Dowton, M., Austin, A.D., 1995. Increased genetic diversity in mitochondrial genes is correlated with the evolution of parasitism in the Hymenoptera. J. Mol. Evol. 41, 958–965.

Dowton, M., Austin, A.D., 1997. Evidence for AT-T transversion bias in wasp (Hymenoptera: Symphyta). J. Mol. Evol. 44, 398–405.

Dowton, M., Austin, A.D., Antolin, M.F., 1998. Evolutionary relationships among the Braconidae (Hymenoptera: Ichneumonidea) inferred from partial 16rDNA gene sequences. Insect Mol. Biol. 7, 129–150.

Dowton, M., Belshaw, R., Austin, A.D., Quicke, D.L.J., 2002. Simultaneous molecular and morphological analysis of braconid relationships (Insecta: Hymenoptera: Braconidae) indicates independent mt-tRNA gene inversions within a single wasp family. J. Mol. Evol. 54, 210–226.

Edson, K.M., Vinson, S.B., 1979. A comparative morphology of the venom apparatus of female braconids (Hymenoptera: Braconidae). Can Entomol. 111, 1013–1024.

Farris, J.S., Käjersjö, M., Kluge, A.G., Bult, C., 1994. Constructing a significance test for incongruence. Cladistics 10, 315–319.

Flores, S., Nassar, J.M., Quicke, D.L.J. Reproductive phenology and predispersal predation in *Protium tovarense* (Burseraceae), with description of the first known phytophagous *Bracon* species (Hymenoptera: Braconidae: Braconinae). J. Nat. Hist., in press.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mithochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Gillespie, J.J., 2004. Characterizing regions of ambiguous alignment caused by the expansion and contraction of hairpin-stem loops in ribosomal RNA molecules. Mol. Phylogenet. Evol. 33, 936–943.

Gladstein, D., Wheeler, W., 1996. POY: Software for direct optimisation of DNA and other data, American Museum of Natural History (ftp://ftp.amnh.org/pub/molecular/poy).

Godfray, H.C.J., 1993. Parasitoids: behavioural and evolutionary ecology. Princeton Univ. Press, Princeton, NJ.

Graybeal, A., 1998. Is it better to add taxa or characters to a difficult phylogenetic problem? Syst. Biol. 47, 9–17.
Hillis, D.M., 1996. Inferring complex phylogenies. Nature 383, 130–131.
Hillis, D.M., 1998. Taxonomic sampling, phylogenetic accuracy, and investigator bias. Syst. Biol. 47, 3–8.
Hillis, D.M., Bull, J.J., 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42, 182–192.
Infante, F., Hanson, P., Wharton, R., 1995. Phytophagy in the genus *Monitoriella* (Hymenoptera: Braconidae) with description of new species. Ann. Entomol. Soc. Am. 88, 406–415.
Kjer, K.M., 1995. Use of rRNA secondary structure in phylogenetic studies to identify homologous positions: an example of alignment and data presentation from the frogs. Mol. Phylogenet. Evol. 4, 314–330.
Kjer, K.M., Blahnik, R.J., Holzenthal, R.W., 2001. Phylogeny of Trichoptera (Caddisfishes): characterization of signal and noise within multiple data sets. Syst. Biol. 50, 781–816.
Lanave, C.G., Preparata, C., Saccone, C., Serio, G., 1984. A new method for calculating evolutionary substitution rates. J. Mol. Evol. 20, 86–93.
Lee, M.S.Y., 2001a. Uninformative characters and apparent conflict between molecules and morphology. Mol. Biol. Evol. 18, 676–680.
Lee, M.S.Y., 2001b. Unalignable sequences and molecular evolution. Trends Ecol. Evol. 16, 681–685.
Lewis, P.O., 2001. A likelihood approach to estimating phylogeny from discrete morphological character data. Syst. Biol. 50, 913–925.
Lin, C.P., Danforth, B.N., 2004. How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from Bayesian analyses of combined data sets. Mol. Phylogenet. Evol. 30, 686–702.
Lutzoni, F., Wagner, P., Reeb, V., Zoller, S., 2000. Integrating ambiguously aligned regions of DNA sequences in phylogenetic analysis without violating positional homology. Syst. Biol. 4, 628–651.
Mardulyn, P., Whitfield, J.B., 1999. Phylogenetic signal in the COI, 16S, and 28S genes for inferring relationships among genera of Micrograstrinae (Hymenoptera; Braconidae): evidence of a high diversification rate in this group of parasitoids. Mol. Phylogenet. Evol. 12, 282–294.
Marsh, P.M., 2002. The Doryctinae of Costa Rica (excluding the genus *Heterospilus*). Mem. Am. Entomol. Inst. 70, 1–319.
Niehuis, O., Wägele, J.H., 2004. Phylogenetic analysis of the mitochondrial genes LSU rRNA and COI suggests early adaptive differentiation of anal teeth in chrysidine cuckoo wasps (Hymenoptera: Chrysididae). Mol. Phylogenet. Evol. 30, 615–622.
Pollock, D.D., Zwickl, D.J., McGuire, J.A., Hillis, D.M., 2002. Increased taxon sampling is advantageous for phylogenetic inference. Syst. Biol. 51, 664–671.
Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.
Quicke, D.L.J., 1993. The polyphyletic origin of endoparasitism in the cyclostome lineages of Braconidae (Hymenoptera): a reassessment. Zool. Med. Leiden 67, 159–177.
Quicke, D.L.J., 1996. First record of *Leptorhaconotus* Granger (Hymenoptera: Braconidae) from South Africa, with the description of a remarkable new species and a discussion of the subfamilial placement of the genus. Afr. Entomol. 4, 111–116.
Quicke, D.L.J., 1997. Parasitic Wasps. Chapman & Hall, London.
Quicke, D.L.J., van Achterberg, C., 1990. Phylogeny of the subfamilies of the family Braconidae (Hymenoptera). Zool. Verh. Leiden 258, 1–95.
Quicke, D.L.J., van Achterberg, C., Godfray, H.C.J., 1997. Comparative morphology of the venom gland and reservoir in opiine and alysiine braconid wasps (Insecta, Hymenoptera, Braconidae). Zool. Scr. 26, 23–50.
Quicke, D.L.J., Belshaw, R., 1999. Incongruence between morphological data sets: an example from the evolution of endoparasitism among parasitic wasps (Hymenoptera: Braconidae). Syst. Biol. 48, 436–454.
Quicke, D.L.J., Ficken, L., Fitton, M.G., 1992a. New diagnostic ovipositor characters for doryctine wasps (Hymenoptera, Braconidae). J. Nat. Hist. 26, 1035–1046.
Quicke, D.L.J., Fitton, M.G., Harris, J., 1995. Ovipositor steering mechanisms in braconid wasps. J. Hym. Res. 4, 110–120.
Quicke, D.L.J., Fitton, M.G., Ingram, S., 1992b. Phylogenetic implications of the structure and distribution of ovipositor valvilli in the Hymenoptera. J. Nat. Hist. 26, 587–608.
Quicke, D.L.J, Basibuyuk, H.H., Fitton, M.G., Rasnitsyn, A.P., 1999. Morphological, palaentological and molecular aspects of ichneumonoid phylogeny (Hymenoptera: Insecta). Zool. Scr. 28, 175–202.
Quicke, D.L.J., Tunstead, J., Falco, J.V., Marsh, P.M., 1992c. Venom gland and reservoir morphology in the Doryctinae and related braconid wasps (Insecta, Hymenoptera, Braconidae). Zool. Scr. 21, 403–416.
Quicke, D.L.J., Fitton, M.G., Tunstead, J.R., Ingram, S.N., Gaitens, P.V., 1994. Ovipositor structure and relationships within the Hymenoptera, with special reference to the Ichneumonoidea. J. Nat. Hist. 28, 635–682.
Rahman, H., Fitton, M.G., Quicke, D.L.J., 1998. Ovipositor internal microsculpture in the Braconidae (Insecta, Hymenoptera). Zool. Scr. 27, 319–331.
Rokas, A., Nylander, J.A.A., Ronquist, F., Stone, G.N., 2002. A maximum-likelihood analysis of eight phylogenetic markers in gallwasps (Hymenoptera: Cynipidae): implications for insect phylogenetic studies. Mol. Phylogenet. Evol. 22, 206–219.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Scatolini, D., Penteado-Dias, A.M., van Achterberg, C., 2002. *Pseudorhysipolis* gen. nov. (Hymenoptera: Braconidae: Rhysipolinae), with nine new species from Brazil, Suriname and Panama. Zool. Med. Leiden 76, 109–131.
Sharkey, M.J., Wahl, D.B., 1992. Cladistics of the Ichneumonoidea (Hymenoptera). J. Hymenopt. Res. 1, 15–24.
Sharkey, M.J., 1997. Apozyginae. In: Wharton, R.A., Marsh, P.M., Sharkey, M.J. (Eds.), Manual of the New World Genera of the Family Braconidae (Hymenoptera). International Society of Hymenopterists, Special Publ. 1, Washington, DC, pp. 133–134.
Shaw, S.R., Edgerly, J.S., 1985. A new braconid genus (Hymenoptera) parasiting webspinners (Embiidina) in Trinidad. Psyche 92, 505–511.
Shaw, M.R., Huddleston, T., 1991. Classification and biology of braconid wasps (Hymenoptera: Braconidae). Handbks. Ident. Br. Insect 7, 1–126.
Shi, M., Chen, X., 2005. Molecular phylogeny of the Aphidiinae (Hymenoptera: Braconidae) based on DNA sequences of 16S rRNA, 18S rDNA and ATPase 6 genes. Eur. J. Entomol. 102, 133–138.
Shi, M., Chen, X., Achterberg, C. van. Phylogenetic relationships among the Braconidae (Hymenoptera: Ichneumonoidea) inferred from partial 16S rDNA, 28S rDNA D2, 18S rDNA gene sequences and morphological characters. Mol. Biol. Evol., in press.
Shimodaira, H., Hasegawa, M., 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. Mol. Biol. Evol. 16, 1114–1116.
Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence based phylogenetic analyses. Syst. Biol. 49, 369–381.
Soltis, P.S., Soltis, D.E., Wolf, P.G., Nickrent, D.L., Chaw, S.M., Chapman, R.L., 1999. The phylogeny of land plants inferred from 18S rDNA sequences: pushing the limits of rDNA signal? Mol. Biol. Evol. 16, 1774–1784.
Swofford, D.L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), Version 4.0b10. Sinauer, Sunderland, MA.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position, specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Tobias, V.I., 1988. A new subfamily of braconids (Hymenoptera, Braconidae) from Australia. Entomol. Obozr. 67, 644–648.
Wharton, R.A., 1993. Review of the Hormiini (Hymenoptera: Braconidae) with a description of new taxa. J. Nat. Hist. 27, 107–171.
Wharton, R.A., 1997. Introduction. In: Wharton, R.A., Marsh, P.M., Sharkey, M.J. (Eds.), Manual of the New World Genera of the Family Braconidae (Hymenoptera). International Society of Hymenopterists, Special Publ. 1, Washington, DC, pp. 1–18.
Wharton, R.A., 2000. Can braconid classification be restructured to facilitate portrayal of relationships. In: Austin, A., Dowton, M. (Eds.),

Hymenoptera: evolution, biodiversity, and biological control. CSIRO, Melbourne, pp. 143–153.
Wharton, R.A., van Achterberg, C., 2000. Family group names in Braconidae (Hymenoptera: Ichneumonoidea). J. Hym. Res. 9, 254–270.
Wharton, R.A., Hanson, P.E., 2005. Gall wasps in the family Braconidae (Hymenoptera). In: Raman, A., Schaefer, C.W., Withers, T.M. (Eds.), Biology, Ecology, and Evolution of Gall-inducing Arthropods. Science, New Hampshire, USA.
Wheeler, W.C., 1996. Optimization alignment: the end of multiple sequence alignment in phylogenetics? Cladistics 12, 1–9.
Wheeler, W.C., 1999. Fixed character states and the optimization of molecular sequence data. Cladistics 15, 379–385.
Wheeler, W.C., Gatesy, J., DeSalle, R., 1995. Elision: a method for accomodating multiple molecular sequence alignments with alignment ambiguous sites. Mol. Phylogenet. Evol. 4, 1–9.
Whitfield, J.B., 1992. The polyphyletic origin of endoparasitism in the cyclostome lineages of Braconidae (Hymenoptera). Syst. Entomol. 17, 273–286.
Whitfield, J.B., Wharton, R.A., 1997. Hormiinae. In: Wharton, R.A., Marsh, P.M., Sharkey, M.J. (Eds.), Manual of the New World Genera of the Family Braconidae (Hymenoptera). International Society of Hymenopterists, Special Publ. 1, Washington, DC, pp. 291–307.
Whitfield, J.B., Won-Young, C., Kyong-In, S., 2004. *Andesipolis*, a puzzling new genus of cyclostome Braconidae (Hymenoptera) from the Chilean Andes, with descriptions of three new species. Zootaxa 438, 1–15.
Zaldivar-Riverón, A., Areekul, B., Shaw, M.R., Quicke, D.L.J., 2004. Comparative morphology of the venom apparatus in the braconid wasp subfamily Rogadinae (Insecta, Hymenoptera, Braconidae) and related taxa. Zool. Scr. 33, 223–237.
Zwickl, D.J., Hillis, D.M., 2002. Increased taxon sampling greatly reduces phylogenetic error. Syst. Biol. 51, 588–598.