An 18S rDNA-Based Molecular Phylogeny of Aphidiinae (Hymenoptera: Braconidae)

Ana Sanchis,*,† Amparo Latorre,*,‡ Fernando González-Candelas,*,‡ and José Manuel Michelena*,†

*Institut Cavanilles de Biodiversitat i Biologia Evolutiva, †Department de Biologia Animal, and ‡Departament de Genètica, Universitat de València, Apdo. Correos 22085, Valencia 46071, Spain

We have obtained a molecular phylogeny of the subfamily Aphidiinae (Hymenoptera: Braconidae) by sequencing the 18S rDNA in 37 aphidiine taxa. Approximately 1857 nucleotides were sequenced in each species. Evolutionary relationships were established by comparing the results of maximum-parsimony, maximum-likelihood, and distance analyses. The most variable region of this gene, V4 (approx 403 nucleotides), was employed to establish the basality of the tribe Ephedrini within this subfamily. All phylogenetic reconstructions yielded trees with very similar topologies that confirmed the existence of two of the four traditionally accepted tribes, Ephedrini and Praini, but questioned the existence of Trioxini and Aphidiini. To better ascertain the status of some groups, the same analyses were repeated with a reduced taxonomic sample in which some species that produced systematic errors in the former phylogenetic reconstructions had been removed. The results from this second analysis favor either the three-tribes hypothesis (Ephedrini, Praini, and Aphidiini) or a new classification with at least five tribes (Ephedrini, Praini, Monoctonini, Trioxini, and Aphidiini). The 18S rDNA gene is a useful marker to recover relationships not only at the tribe but also at the subtribe and genus levels in this group. The natural status of some traditionally accepted clusters is also corroborated with the present data whereas the placement of other clusters is questioned or remains unresolved.   © 2000 Academic Press

Key Words: Aphidiinae; 18S rDNA; maximum parsimony; neighbor-joining; maximum likelihood.

INTRODUCTION

Aphidiines (Braconidae: Aphidiinae) are endoparasitic Hymenoptera whose specific hosts are aphids (Homoptera: Aphidoidea). They are considered an independent group within the family Braconidae. Because of their importance as agents for biological pest control, much attention has been paid to this relatively small group (Mackauer, 1968; Starý, 1970, 1976, 1979). Cur-

rently, there seems to be enough evidence for their monophyly not only from morphological and behavioral information but also from molecular and embryological data (Mackauer, 1961; Tremblay, 1967; Tremblay and Calvert, 1971; Chou, 1984; Gärdenfors, 1986; Quicke and van Achterberg, 1990, 1992; Whitfield, 1992; Belshaw and Quicke, 1997; Smith *et al.,* 1999). However, the phylogenetic relationships within this subfamily remain unestablished as the different data sets are usually incomplete because of the difficulties in studying all relevant taxa at the same time. This is the case of the poorly represented and hardly available Aclitini, which has not been included in any previous molecular studies nor in this one. So, even though most authors accept the existence of four natural groups, Ephedrini, Praini, Trioxini, and Aphidiini, there is no agreement on their taxonomic status. Depending on the criterion adopted, the last two clades either have been treated as independent tribes (Tremblay and Calvert, 1971; O'Donnell, 1989; Finlayson, 1990; Belshaw and Quicke, 1997) or have been grouped into the same tribe, thus postulating a three-tribes hypothesis: Ephedrini, Praini, and Aphidiini (Mackauer, 1961, 1968; Tobias, 1967; Smith *et al.,* 1999). On the basis of their internal and external characters, Aphidiinae may be divided into two main complexes, one showing generally primitive braconid features (Ephedrini + Praini), such as complex wing venation and long ovaries, and the other appearing to have evolved toward higher specialization (Aphidiini + Trioxini), especially regarding their highly derived reproductive system (LeRalec, 1993), their embryology (Tremblay and Calver, 1971), and a tendency toward reduction in wing venation.

In our analysis, representative species of most major Aphidiinae groups have been included, preferentially using taxa from the European fauna. The subfamily is dominated by the large number of species in the hypothetical tribes Trioxini and Aphidiini. Two subtribes are established within Trioxini (Monoctonina and Trioxina) and five within Aphidiini (Paralipsina, Lysiphlebina, Archaphidina, Protaphidina, and Aphi-

diina). The other two tribes included in the subfamily, Praini and Ephedrini, have a widespread distribution but are poorly diversified.

Currently, some molecular information about the Aphidiinae is available (Belshaw and Quicke, 1997; Dowton *et al.,* 1998; Smith *et al.,* 1999). The first two papers focused mainly on their position as a group with respect to other Braconidae subfamilies. In particular, Belshaw and Quicke (1997) studied the relationships among Cyclostome subfamilies of Braconidae using partial sequences from the second expansion segment of 28S rDNA, cytochrome *b,* and elongation factor 1-α. The 28S rDNA data supported the four-tribes hypothesis (Ephedrini + (Praini + (Trioxini + Aphidiini))), with the Ephedrini as basal. However, Dowton *et al.* (1998) included some Aphidiinae species in their work dealing with Braconidae and using partial 16S rDNA gene sequences and found the Praini to be basal. Smith *et al.* (1999) found the same result in their work, restricted to Aphidiinae, with 468 bp of the mitochondrial NADH-1 dehydrogenase gene. Furthermore, the Smith *et al.* results seemed to favor the three-tribes hypothesis (Praini + (Ephedrini + Aphidiini)). Therefore, our aim in this work was to use a different molecular marker (1) to check the basality within the Aphidiinae, (2) to test the hypothesis for the existence of three or four main clades within this subfamily, and (3) to study the evolutionary relationships of conflicting taxa that were not included previously in molecular studies and whose features seem to be controversial and the evolutionary relationships of taxa that were previously included but whose placement still remains unclear.

Consequently, we chose the 18S rDNA gene, which has been successfully employed in the phylogenetic reconstruction of other arthropods at different clustering levels (Carmean *et al.,* 1992; Dowton and Austin, 1994; Black *et al.,* 1997; Vogler *et al.,* 1997), to study the relationships among 37 Aphidiinae species. The conserved and variable regions of this gene fit perfectly with our goal of recovering both ancient and recent divergences in the Aphidiinae. Additionally, the existence of secondary structures in ribosomal genes, in general, makes easier the detection of homologous positions and the removal of those that might be suffering from the effects of homoplasy (Tautz *et al.,* 1988).

MATERIALS AND METHODS

Sampling of Taxa

Table 1 lists the 37 aphidiine species, comprising 17 genera, analyzed in this study. Most species were sampled in Comunidad Valenciana (Spain), where the Mediterranean fauna is well represented. The main differences in the distribution of the European fauna are found at the species level; therefore, the most

relevant genera are all present in this work. The remaining species were gently provided from other regions (Table 1). Two or more representatives of each group were selected, trying to include species whose morphological and behavioral features seemed to be plesiomorphic as well as species with derived characters relative to the Braconidae. This was done in order to avoid making phylogenetic inferences based on single representatives of a group or based on members with highly derived characters, as both factors can affect the topology of the derived trees (Lecointre *et al.,* 1993).

DNA Preparation, PCR Amplification, and Primers

Mummies from aphid hosts were collected in the field and stored until aphidiine emergence. Subsequently, aphidiines were stored in 70% ethanol for identification.

DNA was extracted from single individuals by a modification of the method of Latorre *et al.* (1986). The protocol was as follows: each specimen was softly ground in 160 μl of buffer I (10 mM Tris, 60 mM NaCl, 5% sucrose, 10 mM EDTA, pH 7.8). After adding 200 μl of buffer II (300 mM Tris, 1.25% SDS, 5% sucrose, 10 mM EDTA, pH 8.0), the homogenate was incubated at 65°C for 30 min. A total of 60 μl of 3 M sodium acetate was then added and the tube was cooled at −20°C for 15 min and centrifuged for 15 min. The supernatant was removed and the DNA was precipitated with an equal volume of isopropanol. After centrifugation for 10 min the pellet was rinsed with 70% ethanol, vacuum dried, and resuspended in 7–20 μl of sterile TE buffer (Tris–HCL 10 mM, EDTA 1 mM, pH 8.0). PCRs were carried out, in a Perkin–Elmer 2400 thermal cycler, in a 50-μl volume containing 1–5 μl of DNA, 1.25 U of *Taq* polymerase (Pharmacia), 1 μl 200 nM primers, 10 μl 200 μM dNTPs (Pharmacia), and 5 μl 10× buffer. Amplification conditions were 1 cycle, 95°C (5 min); 35 cycles, 95°C (10 s), 55°C (30 s), 72°C (2 min); 1 cycle, 72°C (2 min). All reactions were subjected to electrophoresis on 0.8% agarose gels and visualized with ethidium bromide. Amplifications generated one single, strong 1.8-kb product. Most sequences were obtained by cloning the PCR products in pBluescript II SK plasmid (Marchuk *et al.,* 1990). In some cases PCR products were purified with the Qiaquick PCR purification kit (Qiagen) and then sequenced directly. DNA sequencing was performed in a PE/ABI 373 automated sequencer using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer). A minimum of two clones was read from each species to check *Taq* polymerase-induced errors or intraindividual polymorphisms. Occasionally, third clones or direct PCR products were sequenced to verify some nucleotide positions. Primers used in amplification and sequencing of the 18S rDNA gene are listed and located in Fig. 1. Primers 18S.up1/18S.lo1 and 18SV4.up1/18SV4.lo1 were derived from 18S rDNA sequences belonging to

TABLE 1

Aphidiinae Species Included in This Study

| Taxa	Aphid host	Sampling location and date	Accession no.	Length[a]
Tribe Ephedrini				
Ephedrus niger Gaut., Bon. & Gau., 1939	*Macrosiphoniella* sp.	Arcos de las Salinas:23.v.97	AJ009328	1754
# *Ephedrus persicae* Froggatt, 1904	*Brachyungis tamaricis*	Torrevieja: 27.iv.97	AJ009329	1752
Tribe Praini				
# *Dyscritulus planiceps* (Marshall, 1896)	*Drepanosiphum oregonensis*	Font Roja:09.v.97	AJ009340	1765
# *Praon dorsale* (Haliday, 1833)	*Uroleucon* sp.	Arcos de las Salinas:23.v.97	AJ009341	1755
Praon volucre (Haliday, 1833)	*Acyrtosiphon pisum*	La Yesa: 22.iv.96	AJ009347	1763
Tribe Trioxini				
Subtribe Trioxina				
# *Trioxys* (*Binodoxys*) *angelicae* (Haliday, 1833)	*Aphis gossypii*	Villarreal: 04.iv.97	AJ009349	1768
Trioxys (*Binodoxys*) *brevicornis* (Haliday, 1833)	*Hyadaphis phoeniculi*	Siete Aguas:19.iii.97	AJ009350	1762
Trioxys (*Trioxys*) *cirsii* (Curtis, 1831)	*Drepanosiphum oregonensis*	Font Roja: 09.v.97	AJ009352	1770
Trioxys (*Trioxys*) *pallidus* (Haliday, 1833)	*Hoplocallis picta*	Valencia: 03.iv.97	AJ009351	1766
# *Lipolexis gracilis* Förster, 1862	*Aphis ruborum*	Vallanca:17.vi.97	AJ009334	1820
Subtribe Monoctonina				
# *Monoctonia vesicarii* Tremblay, 1991	*Pemphigus spirotecae*	Alpuente:25.vii.97	AJ009337	1754
# *Monoctonus* (*Monoctonus*) sp.	*Myzus cerasi*	León: 1997	AJ009336	1788
Tribe Aphidiini				
Subtribe Aphidiina				
Aphidius ervi Haliday, 1834	*Acyrtosiphon pisum*	La Yesa: 23.iv.96	AJ009321	1769
Aphidius eadyi Starý, González & Hall., 1980	*Acyrtosiphon pisum*	La Torre: 23.iv.96	AJ009320	1774
# *Aphidius colemani* Viereck, 1912	*Hyalopterus pruni*	Enguera: 15.iv.96	AJ009318	1775
Aphidius matricariae Haliday, 1834	*Myzus cerasi*	Enguera: 14.v.97	AJ009324	1768
Aphidius salicis Haliday, 1834	*Cavariella aegopodii*	Bugarra: 02.v.97	AJ009326	1774
Aphidius rosae Haliday, 1834	*Macrosiphum rosae*	Tuéjar:28.v.97	AJ009325	1782
Aphidius funebris Mackauer, 1961	*Uroleucon sonchi*	El Palmar:05.iii.97	AJ009322	1776
# *Diaeretiella rapae* (M'Intosch, 1855)	*Xerophyllaphis suaedae*	Torrevieja:25.iv.97	AJ009323	1769
# *Lysaphidus santolinae* Michelena & Sanchis, 1997	*Coloradoa* sp.	La Yesa: 23.v.97	AJ009333	1779
Subtribe Protaphidina				
# *Pauesia* (*Paraphidius*) *cupresobii* (Starý)	*Cinara juniperi*	Tuéjar:23.v.97	AJ009339	1781
Pauesia (*Paraphidius*) *sylvestris* (Starý)	*Cinara* sp.	Valdelinares:05.viii.97	AJ009342	1780
Pauesia (*Paraphidius*) *pini* (Haliday)	*Cinara* sp.	Barracas:10.vi.97	AJ009344	1780
Pauesia (*Paraphidius*) *ahtanumensis* Pike & Starý, 1996	*Cinara ponderosae*	USA (Pacific NW)	AJ009338	1794
Pauesia (*Paraphidius*) *silana* Tremblay, 1969	*Cinara maritimae*	Font Roja:09.v.97	AJ009345	1780
Pauesia (*Paraphidius*) *jezoensis* (Watanabe, 1941)	*Cinara maritimae*	Burjassot: 18.v.96	AJ009343	1791
# *Protaphidius wissmannii* Ratzenburg, 1848	*Stomaphis* sp.	Puebla de San Miguel: 27.vii.97	AJ009348	1775
# *Pseudopauesia prunicola* Halme, 1986	*Myzus cerasi*	Bayreuth (Germany)	AJ009346	1805
# *Xenostigmus bifasciatus* (Ashmead)	*Cinara* sp.	USA (Pacific NW)	AJ009353	1819
# *Diaeretus leucopterus* (Haliday, 1834)	*Eulachnus rileyi*	Valencia: 13.iv.96	AJ009327	1782

TABLE 1—*Continued*

Taxa	Aphid host	Sampling location and date	Accession no.	Length[a]
Subtribe Lysiphlebina				
Adialytus salicaphis (Fitch, 1855)	*Chaitophorus leuco- melas*	Villamarchante: 16.v.97	AJ009319	1771
# *Adialytus ambiguus* (Haliday, 1834)	*Sipha* (*Runqsia*) *maydis*	La Torre: 24.vi.97	AJ009317	1776
Lysiphlebus (*Phlebus*) *cardui* (Starý)	*Aphis fabae*	Bayreuth (Germany)	AJ009330	1774
Lysiphlebus (*Phlebus*) *confuses* Tremblay & Eady,	*Brachycaudus cardui*	Benissanó:11.vi.97	AJ009331	1776
# *Lysiphlebus* (*Phlebus*) *fabarum* (Marshall, 1896)	*Aphis urticata*	Negrón:28.v.97	AJ009332	1774
Lysiphlebus (*Phlebus*) *testaceipes* (Cresson, 1880)	*Aphis gossypii*	Valencia: 03.v.96	AJ009335	1770

Note. We have followed the four-tribes nomenclature without further implications.

\# Species chosen as representatives of the Aphidiinae genera in the analyses with the variable region V4.

[a] Positions used in the analyses from 123 to 1948 referred to the *D. melanogaster* 18S rDNA sequence.
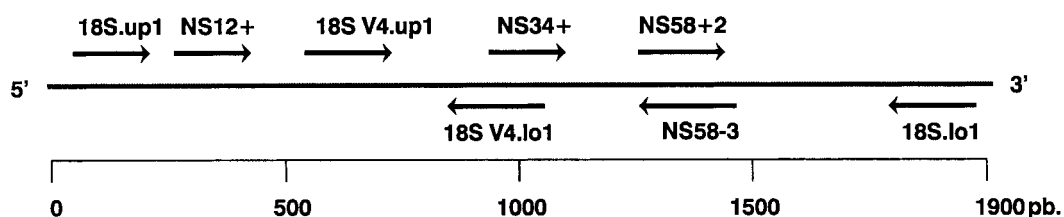
Primer name	Primer Sequence	Use[a]
18S.up1	5'-TGG TTG ATC CTG CCA GTA G-3'	A,S
18S.lo1	5'-CTT CYG CAG GTT CAC CTA C-3'	A,S
18SV4.up1	5'-CAG CCG CGG TAA TTC CAG C-3'	A,S
18SV4.lo1	5'-CRT HYT YGG CAA ATG CTT TCG C-3'	A,S
NS12+	5'-CAA ATG TCT GCC TTA TCA ACT-3'	S
NS34+	5'-GGG ACA GAT GGG GGC ATT CGT-3'	S
NS58+2	5'-TCC GAT AAC GAA CGA GAC TC-3'	S
NS58-3	5'-GAG TCT CGT TCG TTA TCG GA-3'	S

[a] A, used in PCR amplification; S, used for sequencing

FIG. 1. Primers used in the amplification and sequencing of the 18S rDNA gene in Aphidiinae.

several organisms, mainly from insects (White *et al.,* 1990; Carmean *et al.,* 1992). Primers NS12+, NS34+, NS58+2, and NS58−3 were derived from Black *et al.* (1997) but were slightly modified as sequence data were gathered from aphidiine taxa.

Amplified fragments, excluding primers 18S.up1 and 18S.lo1, corresponded from almost the beginning (position 25) to the end (position 1962) of the 18S rDNA gene in the *Drosophila melanogaster* sequence (Tautz *et al.,* 1988). The nucleotide sequence data reported in this paper have been deposited in the EMBL database (see Table 1).

Sequence Alignment and Phylogenetic Analyses

Sequence reliability was checked by reading chromatograms in the SEQUENCHER 3.0 program (Genecodes Co.); CLUSTALW 1.5 (Thompson *et al.,* 1994) was used to obtain multiple alignments based on sequence similarity under different gap-penalty conditions. All these alignments were basically identical, except for the most variable regions of the gene. Therefore, we finally aligned these regions by visual comparison with the current consensus model for secondary structure in the 18S rRNA (Neefs *et al.,* 1991; Van de Peer *et al.,* 1993, 1994). The secondary structure of some stems in the variable regions could not be objectively ascertained. In these cases, we used the programs MFOLD and PLOTFOLD in the GCG package (Genetics Computer Group, 1994) and RNASTRUCTURE V2.52 (Jaeger *et al.,* 1989; Walter *et al.,* 1994; Zucker, 1989) to derive their putative secondary structures. Consequently, clearly homoplasic positions were discarded from the analysis. Informative sites in the final alignment are shown in Table 2. The complete alignment used in the analyses is available upon request.

Three different methods of phylogenetic reconstruction were used. First, we used character state analysis (maximum parsimony, MP), both considering deletions as a fifth character state and without considering them, using PAUP V3.1.1. (Swofford, 1993). Statistical support for each node was evaluated by bootstrap analysis (Efron, 1982; Felsenstein, 1985) with 1000 random replicates. Decay indices were estimated with the program Autodecay V3.0 (available from T. Eriksson, Stockholm University) using the REVERSE CONSTRAINT option in PAUP. Second, we used a distance-based method, for which, following Nei (1991), we employed Jukes–Cantor correction for superimposed mutations (Jukes and Cantor, 1969) and the neighbor-joining algorithm (NJ) (Saitou and Nei, 1987) for obtaining a minimum-evolution tree from the corresponding pairwise nucleotide divergence matrix, as implemented in MEGA V1.01 (Kumar *et al.,* 1993). Given the nature of rDNA evolution with unequal rates for different sites, we also used Kimura two-parameter (Kimura, 1980) and Tamura–Nei (Tamura and Nei, 1993) distances with gamma correction. The α shape

parameter was estimated using PAML v1.3b (Yang, 1997). Bootstrapping evaluation of each node was performed as above. Third, we employed maximum-likelihood (ML) as implemented in DNAML in the PHYLIP package (Felsenstein, 1993).

Statistical tests based on MP (Templeton, 1983) and ML (Kishino and Hasegawa, 1989), both implemented in PHYLIP, were employed to compare the different topologies obtained.

RESULTS

To establish the basality in the subfamily Aphidiinae, in the absence of a complete 18S rDNA sequence closer to Aphidiinae than *Polistes annularis,* a first analysis was carried out with only the V4 variable region, comprising 403 nucleotides. This region starts at position 532 in our alignment and ends at position 934. We considered 17 aphidiine taxa as representative of the genera included in the present study (Table 1) and three Apocrita outgroups: one Vespidae (*P. annularis:* X74762), one Ichneumonidae (*Ichneumon* sp.: L10178), and one Formicidae (*Camponotus ligniperda:* X73270). Analyses with the three reconstruction methods (MP, NJ, and ML) gave similar topologies (Fig. 2) and established the Ephedrini tribe (genus *Ephedrus*) as the most basal within Aphidiinae. Additionally, when these trees were treated as unrooted, there was always a single branch partitioning the ingroup from the outgroup taxa, molecularly reasserting the monophyly of the Aphidiinae (Belshaw and Quicke, 1997; Dowton *et al.,* 1998; Smith *et al.,* 1999).

### Analysis of the Whole Taxonomic Sample

Once the basality of genus *Ephedrus* was established, we decided to root our phylogenetic reconstructions using Ephedrini taxa to not distort the inferred topologies by including a distant outgroup. From the 1857 positions in the final alignment (from 123 to 1948 referred to *D. melanogaster* sequence), we considered 1751 positions, after discarding some positions of ambiguous homology, of which 317 were variable and 147 informative (Table 2). We also tested whether the exclusion of loop positions could improve the phylogenetic reconstruction; in this case, we worked with 1412 nucleotides. Additionally, we tested whether loop regions alone (342 positions, after excluding the clearly homoplasic ones) or variable regions on their own were useful for phylogenetic reconstruction. None of these analyses was able to improve the phylogenetic signal obtained with the complete data set. (data not shown).

The large dataset prevented an exhaustive search for one most-parsimonius tree. Hence, we had to employ heuristic search, performing 100 replicates with 10 random additions each time. First, we treated gaps as missing data and used accelerated transformation. The tree bisection–reconnection (TBR) was used as branch

TABLE 2

Partial Alignment of Aphidiinae 18S rDNA Sequences Showing 147 Informative Positions

```
                                                                                              111111  1111111111
                 11111  1111111111  1112222345  5555555555  5556666666  6666666666  6666667777  7899011222  2333333333
                566811112  2222347778  8993358554  5555667889  9990022222  3333444455  5668893334  6001006122  6122222223
               5938646783  4567812891  2126749504  0156187783  5698946789  0237234523  6791930166  4392650614  9934567890
A. ambiguus    TTAGTGTA-G  TACCTATTTT  AGAGTAGACC  GCCGCTAGGA  TGGACTTAT-  -TAAACTACG  GCTGGATTTC  ACATGGCCGG  TTGGCTATAT
A. colemani    C.........-  .........-.  ....C..G.T  ..........  .........T  ....GT.T..  .T...G....  .......G..  A.........
A. salicaphis  C.........  .........-.  ....C.AG..  ...A......  .........T  .A...A....  A....G....  ..........  AC........
A. eadyi       C......GAA  .........-.  ....CT.G.T  ..........  ...G.....T  ....GT.T..  .T...G....  .......G..  A.........
A. ervy        C......T.-  .........-.  ....C..G.T  ..........  ...G.....T  ....GT.T..  .T...G....  .......G..  A.........
A. funebris    C........A  .........-.  ....C..G.T  ..........  .........T  ....GT.T..  .T...G....  .......G..  A.........
D. rapae       C......-.-  .........-.  ....C..G.T  ..........  ...G.....T  ....GT.T..  .T...G....  .......G..  A.........
A. matricariae C.........  .........-.  ....C..G.T  ..........  .........T  ....GT.T..  .T...G....  .......G..  A.........
A. rosae       C.....GAA  ......C.-.  ....C.AG.T  ..........  .........T  ....GT.T..  .T...G....  ..........  -.........
A. salicis     C........A  .........-.  ....C....T  ..........  ...G.....T  ....GT.TT.  .T...G....  ..GC...G..  A.........
D. leucopterus C..A.A...-  A.T-......  ....C..G..  ....T.....  .....AT.A  A.........  .T...GA...  ....C.....  A..AT.....
E. niger       CCG.C.C-.C  CG...G.C-.  ....C..G..  .....CG...  C.......--  .A.G.....A  .T...GA.C.  G.......G.  A...CGCTC
E. persicae    CCG.C.C-.C  CG...G.C-.  ....C.AG..  .....CG...  C....C.--  .G.G.....A  .....GA.C.  G.......G.  A...CGCTC
L. cardui      ..........  ..........  ..........  ..........  .........T  ..........  .....T....  ..........  A.........
L. confusus    ..........  ..........  ..........  ..........  .........T  ..........  ....T..G.  ..........  A.........
L. fabarum     ..........  ..........  ..........  ..........  .........T  ..........  .....T....  ..........  A.........
L. santolinae  C..A.....-  .........-.  ....C..G.T  ..........  .........T  ....GT.T..  .T...G....  ....C.G..  A.........
L. gracilis    ..........  ..........  ...A...GAT  CATCTCG.AT  GA.....T.T  TA......-.  .TC..G--..  ......TG..  AC.AT...TA
L. testaceipes ..........  ..........  ..........  ..........  .........T  ..........  .....T....  .......G..  A.A.......
Monoctonus sp. C.....--.C  ..T.....-.  .A..C.G..  ..........  .....AT  A.GG......  .T...G....  .......G..  A.A.GCTATA
M. vesicarii   CC...A.-.C  .G.TAGC...  G...C.AG..  .....CG.A.  .....GT-.  .A.G......  .T...GA.C.  ..........  A...TC...-
P. ahtanumensis C......TTT  .........C  ....C..G..  ..........  ....A...A  T.G.....TA  .T.A.GA...  ..........  A..AT.....
P. cupresobii  C......TTT  .........C  ....C..G..  ..........  ....A...A  T.G.....TA  .T.A.GA...  ..........  A..TTC...-
D. planiceps   CC...A.-.C  .G.TAGC...  G...C..G..  .....CG...  ........--  .A.......A  .A...GA.CT  GT.....TT  A....GTA.C
P. dorsale     CCG.C.C-.C  .G...G..-.  ....C..G..  .....CG...  ......--.  .A.......A  .A...GA.CT  GT.....TT  ....GTA.C
P. volucre     CCG.C.C-.C  .G...G..-.  ....C..G.T  ..........  ...G.G--.  .A.......A  .A...GA.CT  G.........  A....GT.TC
P. sylvestris  C......?T?  .?......?  ....C..G..  ..........  ....A...A  T.G.......  .T...GA...  .......G..  A...T.....
P. jezoensis   C......TTT  .........C  ....C..G..  ..........  ....A...A  T.G.....TA  .T.A.GA...  .......G..  A...T.....
P. pini        C.........  ..........  G...C..G..  ..........  ....A...A  T.G.......  .T...GA...  .......G..  A...T.....
P. silana      C.........  ..........  ....C..G..  ..........  ....A...A  T.G.......  .....GA...  .......G..  A...T.....
P. prunicola   C....AT..  ..T.......  ....C..G..  ..........  .....A.AT  A.G.......  .T...G....  .......G..  A..AA.....
P. wissmannii  C......TA  .........A.  ....C..G..  ..........  ....A...T  .G.......  .T...GAC.  .......G..  A..AT.....
T. angelicae   C.GAC.C-.C  .G...G....  .TTACT.GAT  CTTAT.GA.C  .AA.T.--.  .A...TC.T.  .TCAAG.G..  ....CCT...  ACA..CTATA
T. brevicornis C.GAC.C-.C  .G...G....  .TTACT.GAT  CTTAT.GA.C  .AA.T.--.  .A...TC.T.  .TCAAG.G..  ......TG..  ACA..-TATA
T. pallidus    C.GAC.C-.C  .G...G....  .TTACT.GAT  CTTAT.GA.C  .AA.T.--.  .A...TC.T.  .TCAAG.G.T  ......TG..  ACA..CTATA
T. cirsii      C.GAC.C-.C  .G...G..A.  GATA.T.GAT  CTT.T.GA.T  CAA.T.--.  .A...TC.T.  .TCAAG....  .TGC..TG..  ACA.ACT.TG
X. bifasciatus C..A...-.-  ..........  ....CT..A.  ....TCG.A.  .....A.T.T  A.......T.  AT.A.GAA..  .......G..  A..AT.....


               1111111111  1111111111  1111111111  1111111111  1111111111  1111111
               3333333333  3333333333  3444444455  5555555566  6666666667  7777777
               3333444455  5556666699  9457899012  2234489934  4677889990  0111133
               1278234506  7890123401  2277349130  4714863850  7969190341  5234617
A. ambiguus    ATTTTTCCAT  ATATAGTTT-  CCCGCTACCC  CCCCCATTCC  CTCGGAATCT  CGC-GCC
A. colemani    ........-.A  TATATAG...  ..........  .....GAC..  ........T.  ...C.A.
A. salicaphis  TATATAG...  ..........  .....GAC..  .C....GC.C  ...C...
A. eadyi       ........-.-  -ATATA....  ..........  ...A.GAC..  .C........  .......
A. ervy        ........-.A  TATATAG-..  .......T..  T....GAC..  .C......T.  .......
A. funebris    ........-.A  TATATAG...  ..........  .....GAC..  .C......T.  .......
D. rapae       ........-.A  TATATAG...  ..........  .TA..GAC..  G.......T.  ...C.A.
A. matricariae .....-.-  .G.GTAG...  ..........  .....GAC..  .........T.  .......
A. rosae       ........-.A  TATATAG..T  .GG....T..  .....GAC..  .C......T.  .......
A. salicis     ........-.A  TATATAG...  ..........  ...A.GAC..  .C......T.  .......
D. leucopterus .....-.-.-  G....TA...  .....CG...  .....GAC..  .C.A......  T.T...T
E. niger       GGC..CT-G.  CGGGT.G...  .....CG...  .....GAC..  .C......T.  .......
E. persicae    GGC..CT-G.  CGGGCT.G...  .....CG...  .....GAC..  .C......T.  .......
L. cardui      ........-.A  TATATAG...  .T........  ..T.......  ........T.  .......
L. confusus    .....-.-.A  TATATAG...  ..........  ..........  .C....GC.C  ...C...
L. fabarum     .....-.--A  TATATAG...  ..........  ..........  .C....GC.C  ...C...
L. santolinae  .C...-.--A  .GG....T..  T.A..GACG.  GG...C.T.  T..C.A.
L. gracilis    TGAGG.AT--  ----TA....  T..ATCG..T  ....AGAC..  .C.AA.GC.C  ...C...
L. testaceipes ........-.A  TATATAG...  ..........  ..........  .C........  .......T
Monoctonus sp. TA..C.-GA  TATATAG..T  ..........  .TT.......  ..A.....T.  .....A.
M. vesicarii   ----..TA--  -..G.AA...  .....CG...  .....GAC..  .C......T.  .....A.
P. ahtanumensis .......-.A  TATATAG...  .....CG...  .....GAC..  ..A.....T.  .....A.
P. cupresobii  ----..TA--  --TAT.AC..  .....CG...  ..A..GAC..  .........T.  .....A.
D. planiceps   GC.C.AT-GA  .GTA.TG...  .....CG...  .....GACG.  .C......T.  .......
P. dorsale     G..C.AT-GA  .GTA.TG...  .....CG...  .....GAC..  .C......T.  .......
P. volucre     G..C.AT-GA  CGTA.TG...  .....CG...  .....GAC..  .C......T.  .......
P. sylvestris  ........-.A  TATATAA...  .....CG..T  .....A...  .C......T.  .....A.
P. jezoensis   ........-.A  TATATAA...  .....CG...  .....GAC..  .C..G..T.  .......
P. pini        ........-.A  TATATAA...  .....CG.G.  ....GA..G  .C..G..T.  .......
P. silana      ........-.A  TATATAA...  .....CG...  .....A....  .C..G..T.  .......
P. prunicola   ..C....-GA  TATATAA..T  .....C....  .....GAC..  .CA.....T.  .....A.
P. wissmannii  ........-.A  TATATAA...  .....CG...  .....GAC..  .C..A...T.  .....A.
T. angelicae   TA...AT.G-  .....TAGC.  T...TCG...  ....AGAC..  .C.A....T.  .ATTA..
T. brevicornis TA.....G.-  .....TAGC.  T...TCG...  ....AGAC..  .C.A....T.  .AGTA..
T. pallidus    TA.....G.-  .....TAG.C  T...TCG...  ....AGAC..  .C.A....T.  .AT....
T. cirsii      .......G.-  .....TAG.C  ...ATCG...  ....AGAC.G  .C..A...T.  ..-TA..
X. bifasciatus ...GG.AA.A  TATATAA...  .....CG...  ......A...  .C......T.  .......
```

185
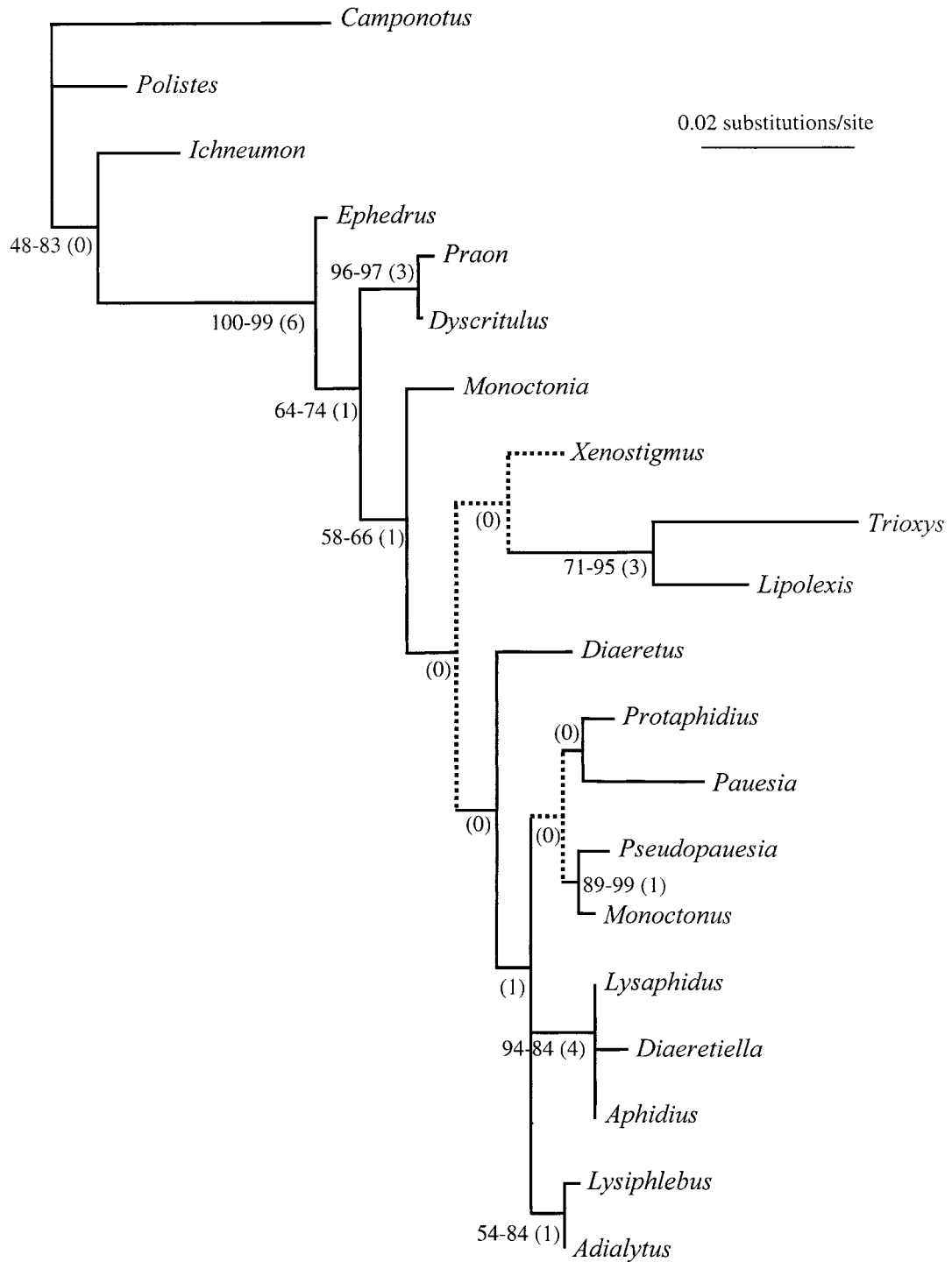
FIG. 2. Consensus tree of NJ, MP, and ML analyses of the 18S rDNA V4 region in Aphidiinae. Species on which the tree is based are indicated in Table 1. Dotted lines show the branches that changed among phylogenetic reconstructions. Bootstrap values higher than 50% are indicated by each node. The first value was estimated using the distance method and the second using parsimony. Values in parentheses represent the decay indices for internal branches. Branch lengths are proportional to nucleotide divergence. Under MP, the total length of the tree is 133 steps, CI = 0.714, H.I. = 0.286.

swapping algorithm. When our searches could not further reduce the total tree length (at 568 steps), we fixed the minimum length for the topology and then made 25 more searches by random stepwise addition with 10 replicates each time and varying the random seed. The topologies obtained from the 25 consensus trees (each obtained by strict consensus) were identical, except for the inner arrangement of some branches involving the groups Aphidiina and Lysiphlebina and within genus *Pauesia.* Therefore, we calculated a general strict consensus based on these 25 topologies as representative of MP analyses (Fig. 3). Bootstrap values were relatively low, and several terminal branches were collapsed within the Aphidiini.

Alternatively, we tested whether considering gaps as a fifth character state affected the phylogenetic reconstruction by parsimony. Consistency (C.I.) and homoplasy indices (H.I.) were similar for the two reconstructions (C.I. = 0.653, H.I. = 0.347, 568 steps, excluding gaps; C.I. = 0.643, H.I. = 0.357, 776 steps, considering gaps). The topology obtained was similar to the previous one, except for the placement of nonsupported taxa. The main differences between both trees involved the placement of three Protaphidina, *Xenostigmus bifasciatus, Diaeretus leucopterus,* and *Protaphidius wissmannii;* one recently described genus (Halme, 1986), *Pseudopauesia prunicola;* and one Trioxini, *Monoctonus* (*Monoctonus*) sp. In this case, the positioning of these taxa was coincident with that in the maximum-likelihood tree (see below). Bootstrap values in both cases (with and without gaps) were lower than 50% for the corresponding nodes, which clearly reflects the instability of these branches. Decay indices were rather low for most branches.

The ML and the NJ reconstructions produced very similar trees and only that derived by ML is shown in Fig. 4. Using the ML method, most branches were statistically significant (length different from zero with $P \leq 0.05$), with the only exceptions being two terminal branches for *Aphidius* and one *Lysiphlebus* species and three internal branches. Two of these are found within genus *Lysiphlebus,* and the last one joins *Protaphidius wissmanii,* a problematic taxon as seen above, with *Pauesia* species.

Pairwise evolutionary distances were estimated using the Jukes and Cantor method (1969) because the estimate of nucleotide substitutions per site in different sequences was rather small (Nei, 1991; Kumar *et al.,* 1993), with most being lower than 0.05 (0.075 and 0.003 are the maximum and minimum values, respectively). Distance values were determined by discarding positions with gaps in pairwise comparisons. The α parameter for the gamma distribution was small ($\alpha \cong 0.2$), indicating that most sites in the gene evolve very slowly (corresponding with the conserved secondary structure in the 18S rDNA), but a few sites have moderate to high rates (variable regions) (Uzzell and

Corbin, 1971; Wakeley, 1993; Black *et al.,* 1997). The NJ trees derived from the Kimura two-parameter and the Tamura–Nei distances under the gamma correction had exactly the same topologies and very similar bootstrap values as those obtained with the Jukes–Cantor distance. The resulting phylogenetic trees differed from that shown in Fig. 4 only in the placement of three taxa, *Adialytus ambiguus* and *Pauesia silvestris,* which occupied a different position within the same clades, and *Protaphidius wissmannii,* which in the NJ trees appeared as a sister clade to *Pauesia* species. The results of 1000 bootstrap replicates performed under NJ analysis with Jukes–Cantor distance are also shown in Fig. 4.

Considering the previous analyses, the main differences among topologies appeared associated with changes in the placement of five taxa. *Xenostigmus bifasciatus* and *Diaeretus leucopterus* appeared either paraphyletic to the Trioxini group in the MP topology (Fig. 3) or basal to the Aphidiini tribe in the ML and NJ topologies (Fig. 4). The placement of *Protaphidius wissmannii* could not be ascertained with the available information, as it changed for each reconstruction method. The cluster *Pseudopauesia prunicola + Monoctonus* (*M*) sp. appeared in the three reconstructions, but its placement differed. In NJ and ML, it was basal to the cluster formed by Lysiphlebina + Aphidiina; in MP, it constituted a nonsupported clade with *Protaphidius wissmannii,* sister to *Pauesia* species. In general, the previously mentioned differences among the topologies were consistent with the low bootstrap values and decay indices associated with those nodes.

All these topologies (MP with and without gaps, ML, and NJ) were compared by Templeton's (maximum-parsimony) and Kishino–Hasegawa's (maximum-likelihood) tests (Table 3) to investigate whether any of them was significantly better than the others and to check whether the differences among them were due to random error. In the case of MP trees, both considering and discarding gaps, the same tests were performed as previously to select the best of all the equal-length dichotomous trees on which the strict consensus was based (as collapsed branches cannot be evaluated by these tests). None of the MP topologies was significantly different from that chosen as the best one in the corresponding test (data not shown). Both tests resulted in nonsignificant differences among the four previously detailed topologies (Table 3). Hence, we rejected random error as the main cause for the instability of the problematic branches.

Alternatively, we investigated whether systematic errors could explain these results. Swofford *et al.* (1996) indicate several reasons for the appearance of this kind of error. One of them is the assumption of character independence, which is rarely satisfied by ribosomal genes (Hillis and Dixon, 1993; Wheeler and Honeycutt, 1988). Nevertheless, when we weighted characters
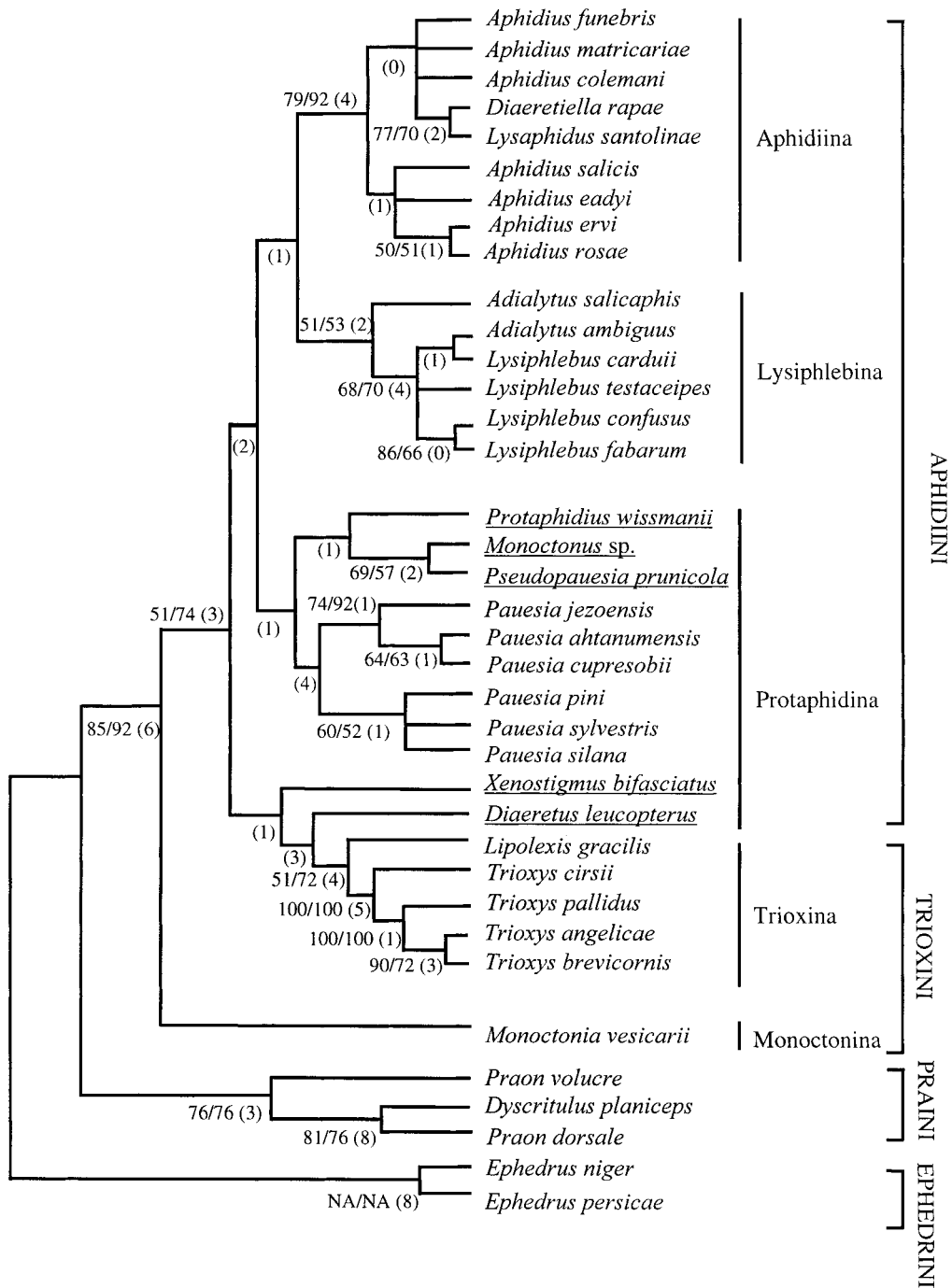
FIG. 3. Strict consensus of maximum-parsimony topologies obtained when gaps were considered as missing data for 18S rDNA in Aphidiinae species. The consensus of the reconstructions obtained considering gaps as a fifth character state is identical, except for the underlined taxa. Numbers next to each node show bootstrap values higher than 50%. The first value was obtained after applying this test when gaps were considered and the second when not. Numbers in parentheses represent decay indices. Traditionally accepted clades and the taxa that they hypothetically include are indicated on the right.

taking compensatory changes into account (data not shown), the topology obtained was similar and the bootstrap support did not change significantly. Another reason, under parsimony analyses, is that multiple changes on long unbranched lineages are not detected,

thereby creating the potential for bias if a mixture of long and short branches are present in an analysis (Felsenstein, 1978). This situation fits perfectly with our data, especially for the five more problematic taxa, and translates into homoplasic changes that are not
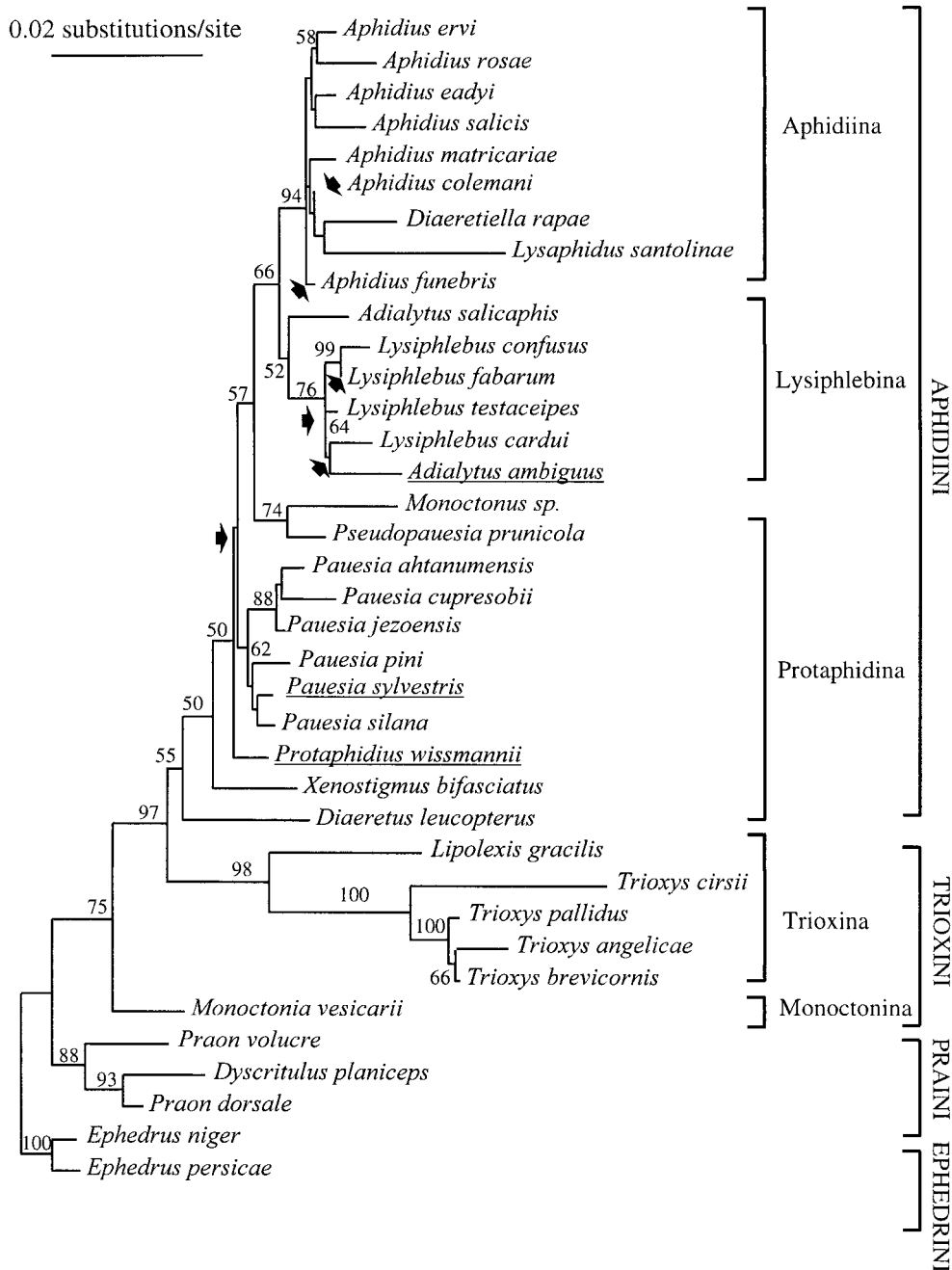
FIG. 4. Maximum-likelihood phylogenetic reconstruction of Aphidiinae from 18S rDNA gene sequences. Branch lengths are proportional to the inferred nucleotide divergence. Arrows indicate branches without statistical support. Traditionally accepted clades and the taxa that they hypothetically include are indicated on the right. Practically the same topology was obtained by the neighbor-joining clustering of Jukes–Cantor distances, with the placement of the underlined species being the only differences between them. Bootstrap values obtained by the NJ method with 1000 replicates and larger than 50% are indicated at each node.

being detected. These convergences also distort the estimations by NJ and ML, leading equally to systematic errors. Removing problematic taxa is recommended when it is suspected that they are causing systematic errors (Swofford *et al.,* 1996).

Hence, to center our discussion on firmer results, we decided to exclude from our analyses the following taxa:

Diaretus leucopterus, Xenostigmus bifasciatus, Pseudopauesia prunicola, Monoctonus sp., and *Protaphidius wissmannii,*

Analysis of the Reduced Taxonomic Sample

Next, we again performed the analyses by MP (considering gaps both as a fifth state and as missing data),

Statistical Tests Comparing the Different Topologies Obtained Applying Different Phylogeny Reconstruction Methods to the Complete Taxonomic Sample of Aphidiinae Using the 18S rDNA Gene

Method[a]		Maximum-parsimony test				Maximum-likelihood test		
	Steps[b]	Diff. steps	Standard deviation	Significantly worse?	Ln L	Diff. Ln L	Standard deviation	Significantly worse?
1 NJ	787	11	8.065	No	−6306.531	−13.707	11.502	No
2 MP	788	12	8.720	No	−6295.573	−2.749	14.984	No
3 MP	776	Best			−6331.018	−38.193	21.027	No
4 ML	787	11	8.663	No	−6292.824	Best		

[a] Phylogenetic trees obtained analyzing 1722 positions: 1, phylogeny obtained by neighbor-joining with Jukes–Cantor distance (Fig. 4); 2, maximum-parsimony topology obtained with PAUP, disregarding gaps (Fig. 3); 3, maximum-parsimony topology obtained with PAUP considering gaps as a fifth character state (Fig. 3); 4, maximum-likelihood phylogeny obtained with DNAML (Fig. 4).

[b] Number of steps estimated for each of the compared topologies when gaps were treated as a fifth character state. Templeton's test is based on this step number.

ML, and NJ with the remaining 32 taxa and using the same 1751 positions. All four topologies were very similar, showing the same patterns obtained previously with the complete taxonomic sample. The MP topology, considering gaps as missing data, showed better consistency and homoplasy indices (C.I. = 0.720, H.I. = 0.280, 475 steps) than both the topology treating gaps as a fifth state (C.I. = 0.605, H.I. = 0.395, 618 steps) and the same analyses with all the taxa (see above). The NJ and ML trees showed the same branch lengths and an arrangement of taxa very similar to that in the previous case. Hence, we chose the ML tree (Fig. 5) as a representation of these four reconstructions, on which bootstrap support values obtained from MP and NJ are also indicated.

In fact, the removal of the five conflicting taxa did not alter the main features of the topology but it resulted in a significant increase of the bootstrap support for the nodes defining monophyletic clades such as Aphidiina, *Pauesia* group, Trioxina, Monoctonina, and Praini. These were now supported in more than 70% of the bootstrap replicates and hence, according to Hillis and Bull (1993) and Berry and Gascuel (1996), were considered well supported statistically.

DISCUSSION

The 18S rDNA gene seems to be an adequate marker for confronting currently competing phylogenetic hypotheses for the Aphidiinae. Due to the recent divergence of aphidiines (their earliest known fossils date from the Oligocene; Quilis-Pérez, 1938; Schlinger, 1974), we have been able to include in our analysis information from the most variable regions. Nevertheless, the main representatives of each lineage already existed in that period. This indicates a rapid diversification after the emergence of the first aphidiine-like ancestor, which is reflected in the high sequence similarity in the variable regions of this gene. As reported by Hillis and

Dixon (1991), these seemingly functionless regions accumulate most mutational events. A detailed comparison of aphidiine sequences of the 18S rDNA gene reveals a slight tendency in these regions to increase their length from the most ancient taxa to the most evolved ones (Table 1).

The analysis of the V4 region using three non-Aphidiinae hymenoptera as outgroups indicated that the aphidiini taxa included in the analyses are monophyletic, supporting previous studies (Mackauer, 1961, 1968; Mackauer and Starý, 1967; Smith *et al.,* 1999). However, as pointed out by Smith *et al.* (1999), the inclusion of Aclitini is necessary before a final conclusion on the monophyly of the subfamily can be drawn. Most authors agree in considering Ephedrini and Praini as the most ancient clades of the Aphidiinae (Mackauer, 1961, 1968; Mackauer and Starý, 1967; Starý, 1970; Tremblay and Calvert, 1971; Gärdenfors, 1986; O'Donnell, 1989; Finlayson, 1990), as the two clades show many primitive braconid features in both the larval and the adult morphology. Our analysis recovered the Ephedrini representative as the most basal within the aphidiines. The same result was found by Belshaw and Quicke (1997) when analyzing the second expansion segment of the 28S rDNA gene. However, Smith *et al.* (1999), working with the mitochondrial NADH1 dehydrogenase gene and in a combined analysis of this and the 28S rDNA gene from 10 aphidiini taxa, also found support for *Praon* as basal to Ephedrini; the same result, but less supported, was also found by Dowton *et al.* (1998) with another mitochondrial gene, the 16S rDNA.

Apart from the basal tribe within Aphidiinae, the other traditional controversy in this subfamily relates to the taxonomic status of Trioxini. Mackauer (1961) considered this group as a subtribe (Trioxina) included within the tribe Aphidiini (a reduction in rank was necessary due to the treatment of Aphidiinae as a subfamily). However, other authors (Tremblay and
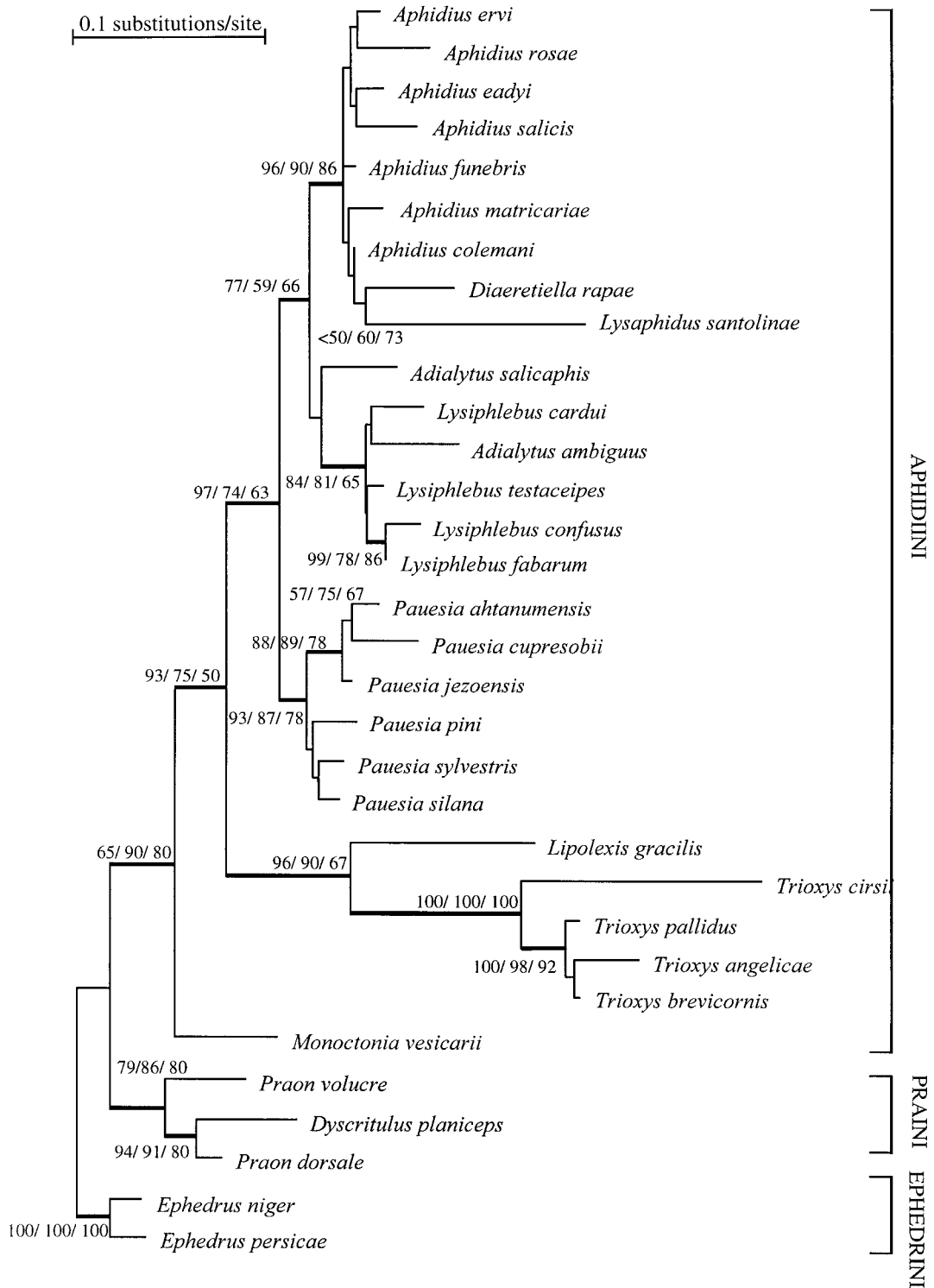
FIG. 5. Maximum-likelihood tree obtained with the reduced taxonomic sample of Aphidiinae using the 18S rDNA gene. Branch lengths are proportional to the inferred nucleotide divergence. Bold lines indicate internal branches statistically supported (length > 0). Bootstrap values are indicated next to each node. The first value corresponds to NJ, the second to MP without gaps, and the third to MP considering gaps as a fifth character state.

Calvert, 1971; O'Donnell, 1989; Finlayson, 1990), based on embryology and larval morphology, as well as several features of adult morphology, proposed raising the rank of this group to the same level as that of Ephedrini, Praini, and Aphidiini, i.e., tribe Trioxini, including subtribes Monoctonina and Trioxina. This was also supported by the molecular analyses of Belshaw and Quicke (1997).

Based on Fig. 5, our results using the 18S rDNA gene are in better agreement with the three-tribes than with the four-tribes hypothesis. Ephedrini, Praini, and Aphidiini are well supported, and, although this is also the case for Trioxina, its consideration as a fourth tribe (Trioxini) would lead to raising the other subtribe (Monoctonina) to the same rank. Nevertheless, in order to facilitate the following discussion and preserve consistency in this paper, we have maintained the four-tribes nomenclature used in Table 1.

Within tribe Aphidiini (Fig. 5), the two groups usually considered belonging to Trioxini (Monoctonina and Trioxina) occupy a basal position. However, since only one representative of Monoctonina has been retained in the reduced sample analyses, its monophyly and relationship with Trioxina should be considered cautiously, although the four analyses provide a relatively strong support for both the basality of Trioxini within Aphidiini and the basality of Monoctonina within Trioxini. Current molecular studies show different arrangements for this group: Belshaw and Quicke (1997) found one supported node defining Trioxini as a tribe and Smith *et al.* (1999) found this node to be paraphyletic. Our results are in better agreement with those of Smith *et al.* (1999). Monoctonina and Trioxina are supported as two natural clades and should be included within the tribe Aphidiini. Therefore, the existence of a monophyletic tribe Trioxini, containing two sister groups (subtribes Monoctonina and Trioxina), is not supported by these results.

Apart from Trioxina and Monoctonina, the tribe Aphidiini includes five natural groups, of which we have included representatives from the three more diversified and widespread groups: Aphidiina (represented in our study by the genera *Aphidius, Diaretiella,* and *Lysaphidus*), Lysiphlebina (*Lysiphlebus* and *Adialytus*), and Protaphidina (*Pauesia, Diaeretus, Xenostigmus, Protaphidius,* and *Pseudopauesia*). Our results show good support for the node comprising these three groups, upholding results of previous molecular research (Belshaw and Quicke, 1997; Smith *et al.,* 1999). Also, there is good support for considering the group Aphidiina as monophyletic. The group Lysiphlebina is recovered as monophyletic in our analyses but without bootstrap support (see Figs. 3–5) and as a sister clade to Aphidiina. The more conflicting group in our analysis is Protaphidina, which appears as sister clade to the two previous ones. Most of its genera, included for the first time in a molecular study, had to be removed because they were leading to systematic errors. Except for genus *Pauesia,* which is recovered as monophyletic

with strong support, the remaining Protaphidina represent independent evolutionary lineages, very poorly diversified. From the present data, we cannot discuss the status of this group, although a slight indication of nonmonophyly can be detected from the analyses with the complete taxonomic sample (Figs. 3 and 4).

At the genus level, some paraphyletic relationships have been detected (Fig. 5). In the tribe Praini, our study reveals a paraphyletic status for genus *Praon* due to the well-supported inclusion of *Dyscritulus planiceps.* However, both Belshaw and Quicke (1997) and Smith *et al.* (1999) found strong support for the monophyly of genus *Praon.* Hence, the inclusion of more *Praon* species as well as other Praini would probably translate into a better resolution for this clade. Similarly, genus *Aphidius* seems to be paraphyletic because of the presence of *Diaeretiella* and *Lysaphidus* therein. Although these branches do not have good bootstrap support, similar results were obtained for *Diaeretiella* with the NADH 1 dehydrogenase (Smith *et al.,* 1999) and the elongation factor-1α (Belshaw and Quicke, 1997) genes. Paraphyly in *Adialytus* was due to *A. ambiguus* falling inside genus *Lysiphlebus.* This supported position deserves further study because there are no previous molecular results for comparison.

Due to the strong support for the cluster formed by *Trioxys* species (Figs. 3–5) and lacking a more complete taxonomic sample, it is preferable to maintain the subgenus designations *Trioxys* (*Trioxys*) and *Trioxys* (*Binodoxys*) (Mackauer, 1960; Starý, 1976, 1979) instead of raising them to the genus level (Mackauer, 1968; Pike and Starý, 1995, 1996). Also remarkable is the strongly supported placement of *Lipolexis gracilis,* a very conflicting taxon (Belshaw and Quicke, 1997; Smith *et al.,* 1999), as a sister group to genus *Trioxys,* such as initially ascribed by Mackauer (1968).

To summarize, the present phylogeny based on 1751 bp of the 18S rDNA puts into question the existence of four distinct evolutionary lineages at the tribal rank within Aphidiinae (Ephedrini, Praini, Trioxini, and Aphidiini), thus favoring the three-tribes hypothesis, with Ephedrini, Praini, and Aphidiini. The tribe Aphidiini seems to include several successional lineages, some supported as monophyletic (Aphidiina, group *Pauesia,* Monoctonina, and Trioxina), that have evolved independently since their divergence. In view of these results, the acceptance of Trioxini as a separate tribe would lead to raising several other groups to the same rank (at least a new tribe Monoctonini). In the absence of more complete data sets, new molecular and nonmolecular studies are needed to settle this question.

ACKNOWLEDGMENTS

REFERENCES

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

Berry, V., and Gascuel, O. (1996). On the interpretation of bootstrap trees: Appropriate threshold of clade selection and induced gain. *Mol. Biol. Evol.* **13:** 999–1011.

Black, W. C., Klompen, J. S. H., and Keirans, J. E. (1997). Phylogenetic relationships among tick subfamilies (Ixodida: Ixodidae: Argasidae) based on the 18S nuclear rDNA gene. *Mol. Phylogenet. Evol.* **7:** 129–144.

Carmean, D., Kimsey, L. S., and Berbeet, M. L. (1992). 18S rDNA sequences and the holometabolous insects. *Mol. Phylogenet. Evol.* **1:** 270–278.

Chou, L.-Y. (1984). The phylogeny of Aphidiidae (Hymenoptera) of Taiwan. *J. Agric. Res. China* **33:** 437–446.

Dowton, M., and Austin, A. D. (1994). Molecular phylogeny of the insect order Hymenoptera: Apocritan relationships. *Proc. Natl. Acad. Sci. USA* **91:** 9911–9915.

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

Efron, B. (1982). "The Jackknife, the Bootstrap and Other Resampling Plans," SIAM, Philadelphia.

Felsenstein, J. (1978). Cases in which parsimony and compatibility methods will be positively misleading. *Syst. Zool.* **27:** 401–410.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39:** 783–791.

Felsenstein, J. (1993). PHYLIP manual (Phylogeny Inference Package) version 3.5c. Dept. of Genetics, Univ. of Washington, Seattle.

Finlayson, T. (1990). The systematics and taxonomy of final-instar larvae of the family Aphidiinae (Hymenoptera). *Mem. Entomol. Soc. Can.* **152:** 3–74.

Gärdenfors, U. (1986). Taxonomic and biological revision of Palearctic *Ephedrus* Haliday (Hymenoptera: Braconidae, Aphidiinae). *Entomol. Scand. Suppl.* **27:** 1–95.

Genetics Computer Group, (1994). Program Manual for the Wisconsin Package, Version 8. Genetics Computer Group, Maddison, WI.

Halme, J. (1986). Aphidiidae (Hymenoptera) of Finland. II. *Pseudopauesia prunicola* gen.n., sp. n., a new parasitoid of *Rhopalosiphum padi* (L.) (Homoptera: Aphididae). *Ann. Entomol. Fenn.* **52:** 20–27.

Hillis, D. M., and Dixon, M. L. (1991). Ribosomal DNA: Molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **66:** 411–453.

Hillis, D. M., and Dixon, M. L. (1993). Ribosomal DNA secondary structure: Compensatory mutations and implications for phylogenetic analyses. *Mol. Biol. Evol.* **10:** 256–267.

Hillis, D. M., and Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* **42:** 182–192.

Jaeger, J. A., Turner, D. H., and Zuker, M. (1989). Improved predictions of secondary structures for RNA. *Proc. Natl. Acad. Sci. USA* **86:** 7706–7710.

Jukes, T. H., and Cantor, C. R. (1969). Evolution of protein molecules. *In* "Mammalian Protein Metabolism" (H. N. de Munro, Ed.), pp. 21–132. Academic Press, New York.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16:** 111–120.

Kishino, H., and Hasegawa, M. (1989). Evaluation of the maximum likelihood estimate on the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. *J. Mol. Evol.* **29:** 170–179.

Kumar, S., Tamura, K., and Nei, M. (1993). MEGA. Molecular Evolutionary Genetic Analysis version 1.0. The Pennsylvania State Univ.

Latorre, A., Moya, A., and Ayala, F. J. (1986). Evolution of mitochondrial DNA in *Drosophila subobscura. Proc. Natl. Acad. Sci. USA* **83:** 8649–8653.

Lecointre, G., Philippe, H., Le, H. L. V., and Guyader, H. L. (1993). Species sampling has a major impact on phylogenetic inferences. *Mol. Phylogenet. Evol.* **2:** 205–224.

LeRalec, A. (1993). "Les Hymenopteres Parasitoides: Adaptations de l'Appareil Reproducteur Femelle. Morphologie et Ultrastructure de l'Ovarie, de l'Oeuf et de l'Ovopositeur," PhD thesis, Univ. de Rennes I.

Mackauer, M. (1960). Zur Systematik der Gattung *Trioxys* Haliday. *Beitr. Entomol.* **10:** 137–160.

Mackauer, M. (1961). Die Gattungen der Familie Aphidiidae und ihre verwandsschaftliche Zuordung. *Beitr. Entomol.* **11:** 792–803.

Mackauer, M. (1968). Aphidiidae. *Hymenopterorum Catalogus* (nova editio) **3:** 1–103.

Mackauer, M., and Starý, P. (1967). "World Aphidiidae (Hymenoptera: Ichneumonidea)," Le François, Paris.

Marchuk, D., Drumm, M., Saulino, A., and Collins, F. (1990). Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucleic Acids Res.* **19:** 1154.

Neefs, J. M., Van de Peer, Y., De Rijk, P., Goris, A., and De Wachter, R. (1991). Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res.* **19(Supp.):** 1987–2015.

Nei, M. (1991). Relative efficiencies of different tree making methods for molecular data. *In* "Recent Advances in Phylogenetic Studies of DNA Sequences" (M. M. Miyamoto and J. L. Cracraft, Eds.), pp. 90–128. Oxford Univ. Press, Oxford.

O'Donnell, D. J. (1989). A morphological and taxonomic study of first instar larvae of Aphidiinae (Hymenoptera: Braconidae). *Syst. Entomol.* **14:** 197–219.

Pike, K. S., and Starý, P. (1995). *Binodoxys conei* sp. N. (Hymenoptera: Braconidae: Aphidiinae), a new parasitoid of hop aphid, *Phorodon humuli* (Schrack) in Washington State. *J. Kansas Entomol. Soc.* **68:** 444–450.

Pike, K. S., Starý, P., Miller, R., Allison, D., Boydston, L., Graf, G., and Miller, T. (1996). New species and host records of aphid parasitoids (Hymenoptera: Braconidae: Aphidiinae) from the Pacific Northwest, U.S.A. *Proc. Entomol. Soc. Wash.* **98:** 570–591.

Quilis-Pérez, M. (1938). Los Aphidiidae fósiles de Wittenheim (Haut-Rhin, Francia) (Hymenoptera: Braconidae). *EOS* **14:** 23–67.

Quicke, D. L. J., and van Achterberg. C. (1990). Phylogeny of the subfamilies of the family Braconidae (Hymenoptera: Ichneumonoidea). *Zool. Verh. Leiden* **258:** 1–95.

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

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406–425.

Schlinger, E. I. (1974). Continental drift, *Notophagus* (Fagaceae) and some ecologically associated insects. *Annu. Rev. Entomol.* **19:** 323–343.

Smith, P., Kambhampati, S., Völkl, W., and Mackauer, M. (1999). A phylogeny of aphid parasitoids (Hymenoptera: Braconidae: Aphidiinae) inferred from mitochondrial NADH 1 dehydrogenase gene sequence. *Mol. Phylogenet. Evol.* **11:** 236–245.

Starý, P. (1970). "Biology of Aphid Parasites (Hymenoptera: Aphidiidae) with Respect to Integrated Control," Junk, The Hague.

Starý, P. (1976). "Aphid Parasites (Hymenoptera: Aphidiidae) of the Mediterranean Area," Junk, The Hague.

Starý, P. (1979). "Aphid Parasites (Hymenoptera: Aphidiidae) of the Central Asian Area," Junk, The Hague.

Swofford, D. L. (1993). PAUP: Phylogenetic Analysis Using Parsimony, version 3.1.1. Illinois Natural History Survey, Champaign, IL.

Swofford, D. L., Olsen, G. J., Waddell, P. J., and Hillis, D. M. (1996). Phylogenetic inference. *In* "Molecular Systematics" (D. M. Hillis, G. Moritz, and B. K. Mable, Eds.), pp. 407–425. Sinauer, Sunderland, MA.

Tamura, K., and Nei, M. (1993). Estimation of the number of nucleotides substitutions in the control region of the mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **10:** 512–526.

Tautz, D., Handcock, J. M., Webb, D. A., Tautz, C., and Dover, G. A. (1988). Complete sequence of the rRNA genes of *D. melanogaster.* *Mol. Biol. Evol.* **5:** 366–376.

Templeton, A. R. (1983). Phylogenetic inference from restriction endonuclease cleavage site maps with particular reference to the evolution of humans and apes. *Evolution* **37:** 221–224.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673–4680.

Tobias, V. I. (1967). A review of the classification, phylogeny and evolution of the family Braconidae (Hymenoptera). *Entomol. Obozr.* **56:** 646–659.

Tremblay, E. (1967). Sulla posizione sistematica degli Aphidiinae (Hymenoptera: Braconidae). *Boll. Lab. Entomol. Agr. Filippo Silvestri* **25:** 93–108.

Tremblay, E., and Calvert, D. (1971). Embryosystematics in the aphidiines (Hymenoptera: Braconidae). *Boll. Lab. Entomol. Agr. Filippo Silvestri* **29:** 223–249.

Uzzell, T., and Corbin, K. W. (1971). Fitting discrete probability distribution to evolutionary events. *Science* **172:** 1089–1096.

Van de Peer, Y., Neefs, J. M., De Rijk, P., and De Wachter, R. (1993). Reconstructing evolution from eukaryotic small-ribosomal-subunit RNA sequences: Calibration of the molecular clock. *J. Mol. Evol.* **37:** 221–232.

Van de Peer, Y., Van den Broeck, I., De Rijk, P., and De Wachter, R. (1994). Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Res.* **22:** 3488–3494.

Vogler, A. P., Welsh, A., and Hancock, J. M. (1997). Phylogenetic analysis of slippage-like sequence variation in the V4 rRNA expansion segment in Tiger beetles (Cicindelidae). *Mol. Biol. Evol.* **14:** 6–19.

Wakeley, J. (1993). Substitution rate variation among sites in hypervariable region Y of human mitochondrial DNA. *J. Mol. Evol.* **37:** 613–623.

Walter, A. E., Turner, D. H., Kim, J., Lyttle, M. H., Müller, P., Mathews, D. H., and Zucker, M. (1994). Coaxial stacking of helixes enhances binding of oligoribonucleotides and improves predictions of RNA folding. *Proc. Natl. Acad. Sci. USA* **91:** 9218–9222.

Wheeler, W. C., and Honeycutt, R. L. (1988). Paired sequence difference in ribosomal RNAs: Evolutionary and phylogenetic implications. *Mol. Biol. Evol.* **5:** 90–96.

White, T. J., Bruns, T., Lee, S., and Taylor, J. W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* "PCR Protocols" (M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 315–322. Academic Press, San Diego.

Whitefield, J. B. (1992). The polyphyletic origin of endoparasitism in the cyclostome lineages of Braconidae (Hymenoptera). *Syst. Entomol.* **17:** 273–286.

Yang, Z. (1997). PAML: A program package for phylogenetic analysis by maximum likelihood. *Comput. Appl. Biosci.* **13:** 555–556.

Zucker, M. (1989). On finding all suboptimal foldings of an RNA molecule. *Science* **244:** 48–52.