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EVOLUTION OF THE MAJOR LINEAGES OF TAPEWORMS (PLATYHELMINTHES: CESTOIDEA) INFERRED FROM 18S RIBOSOMAL DNA AND *ELONGATION FACTOR-1α*

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ABSTRACT: The interrelationships of the tapeworms (Platyhelminthes: Cestoidea) were inferred by analysis of complete gene sequences (~2,200 bp) of 18S small subunit ribosomal DNA (18S) and partial gene sequences (~900 bp) of *elongation factor-1α* (*Ef-1α*). New collections were made of 23 species representing each of the 14 currently recognized orders of tapeworms, including the Amphilinidea, Gyrocotylidea, and the 12 orders of the Eucestoda. Sequences were determined directly from polymerase chain reaction (PCR) products by either manual or automated methods. Nucleotide sequences of platyhelminth species outside of the Cestoidea were obtained for rooting the resulting trees. The 18S sequences were aligned with reference to the secondary structural features of the gene and the *Ef-1α* sequences were aligned with reference to their corresponding amino acid residues. Significant length variation among taxa was observed in the V2, V4, and V7 variable regions of the 18S gene. Such positions where sequences could not be aligned confidently were excluded from the analyses. Third codon positions of the *Ef-1α* gene were inferred to be saturated at an ordinal level of comparison. In addition, a short (~35 bp) intron region of the *Ef-1α* gene was found to be shared only among the eucestode taxa, with the exception of *Spathebothrium simplex* (Spathebothriidea), which lacked the intron. Complete alignments showing structural features of the genes and sites excluded from analysis are provided as appendices. The sequence data were partitioned into 7 data sets in order to examine the effects of analyses on different subsets of the data. Analyses were conducted on the 2 genes independently, different codon positions of *Ef-1α*, amino acid sequences of Ef-1α, and combinations thereof. All subsets of the data were analyzed under the criterion of maximum parsimony as well as minimum evolution using both maximum-likelihood estimated, and LogDet-transformed distances. Results varied among the different data partitions and methods of analysis. Nodes with strong character support, however, were consistently recovered, and a general pattern of evolution was observed. Monophyly of the Cestoidea (Amphilinidea + Gyrocotylidea + Eucestoda) and Eucestoda and the traditionally accepted positions of the Amphilinidea and Gyrocotylidea as sister lineages to the Eucestoda were supported. Within the Eucestoda, the Spathebothriidea was found to be the sister of all other eucestodes. The remaining orders generally formed a diphyletic pattern of evolution consisting of separate difossate and tetrafossate lineages. This pattern was not universally observed among the analyses, primarily because the trypanorhynch and diphyllidean taxa showed instability in their phylogenetic position. Additional relationships that showed high levels of nodal support included a sister relationship between the Pseudophyllidea and Haplobothriidea and a clade uniting the Cyclophyllidea, Nippotaeniidea, and Tetrabothriidea. The Tetraphyllidea, as currently defined, was found to be paraphyletic without the inclusion of the orders Proteocephalidea and, possibly, Lecanicephalidea. Ordinal status of a monophyletic Litobothriidea, currently classified within the Tetraphyllidea, was found to be supported from a phylogenetic perspective.

The Cestoidea Rudolphi, 1808 (Amphilinidea + Gyrocotylidea + Eucestoda) is a diverse group of platyhelminth parasites of vertebrates that, together with monogeneans, forms a derived clade within the Neodermata (Ehlers, 1986; Rohde, 1990; Littlewood et al., 1999). Differences in opinion regarding the membership of the major groups within the Cestoidea have resulted in the recognition of as few as 7 (Fuhrmann, 1931) and as many as 21 (Wardle et al., 1974) orders in the class. Such taxonomic instability has, in part, hindered previous attempts to elucidate the interrelationships of the group. In the most recent treatment of the group (Khalil et al., 1994), 14 orders, consisting of the Amphilinidea, Gyrocotylidea, plus 12 orders within the Eucestoda, were recognized. Although further study is necessary in some cases to circumscribe strictly monophyletic groups, wider acceptance of the most recent classification of the tapeworms has resulted in greater consistency among phylogenetic studies aimed at elucidating higher level relationships (e.g., Hoberg et al., 1997; Mariaux, 1998), which in turn has allowed for more meaningful comparisons among alternate hypotheses.

Only recently have the interrelationships among the cestodes been investigated by formal phylogenetic analysis. In the past, evolutionary studies of the cestodes often concentrated on single aspects of the biology of the group, such as host relationships (Fuhrmann, 1931; Euzet, 1959), life history (Freeman, 1973; Jarecka, 1975), or ultrastructure of the spermatozoa (Euzet et al., 1981; Justine, 1998). Previous hypotheses of ordinal-level interrelationships were reviewed recently by Hoberg et al. (1997), and systematic progress stemming from different classes of characters was discussed by Mariaux (1996). The first cladistic analysis of the group was that of Brooks et al. (1991) and was based on a suite of putatively homologous ontogenetic and morphological characters derived from the literature. However, only 5 of the 12 currently accepted orders of eucestodes were recognized in their analysis; thus, the phylogenetic positions of a majority of the groups now accepted as orders were not examined. A more recent cladistic analysis of the group by Hoberg et al. (1997) used the orders recognized by Khalil et al. (1994) as the basis for the terminal taxa. The Hoberg et al. (1997) study increased the number of morphological characters used in the analysis beyond that of Brooks et al. (1991). Parsimony analysis of the data resulted in the single strictly bifurcating tree (Fig. 1A). The following year, a molecular phylogenetic analysis by Mariaux (1998), based on partial sequences of 18S small subunit ribosomal DNA (18S rDNA) resulted in a somewhat different hypothesis, but was itself only partially resolved by strict consensus (Fig. 1B). However, this analysis did not examine the position of the orders Gyrocotylidea, Haplobothriidea, and Lecanicephalidea. The phylogenetic hypotheses derived from these studies show conflict as well as congruence between morphological and molecular evidence, and it is clear that additional data must be brought to bear on the issue

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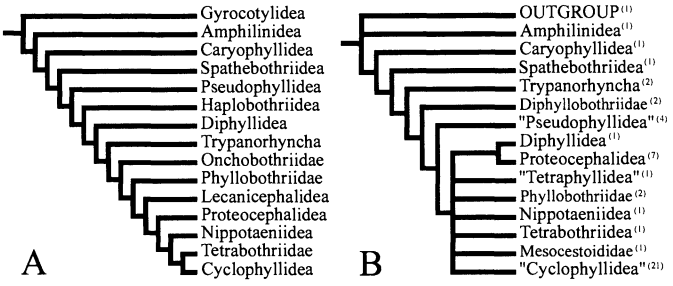

FIGURE 1. Two recent hypotheses of the ordinal interrelationships of the Cestoidea. Quotations indicate a lack of support for monophyly of the taxonomic group. (A) Most parsimonious tree of Hoberg et al. (1997, 1999a) based on an analysis of morphological characters. (Note: potential paraphyly was tested only for the order Tetraphyllidea, for which the families Onchobothriidae and Phyllobothriidae were coded separately; other terminals were coded at the ordinal level and were therefore assumed a priori to represent monophyletic groups; but see B.) (B) Ordinal-level interpretation based on a strict consensus of 480 trees from the analysis of partial 18S rDNA sequences by Mariaux (1998). Number of taxa representing each higher clade in his analysis is shown parenthetically. No representative of the orders Gyrocotylidea, Haplobothriidea, or Lecanicephalidea was included. Note that the orders Cyclophyllidea, Pseudophyllidea, and Tetraphyllidea were found to be paraphyletic as currently defined (Khalil et al., 1994).

in order to achieve greater congruence between these classes of characters.

As in the work of Mariaux (1998), the present study was undertaken to examine phylogenetic relationships among the orders of tapeworms using data independent from morphology. To this end, sequence data were generated from 2 independent gene loci; that is, complete sequences of 18S rDNA (~2,200 bp) and partial sequences of *elongation factor-1α* (*Ef-1α*; ~900 bp). At the outset of the project, only the 18S rDNA gene had been sampled broadly among platyhelminth taxa (Baverstock et al., 1991; Barker et al., 1993; Blair, 1993; Rohde et al., 1993; Blair and Barker, 1994), and complete 18S rDNA sequences were available from GenBank only for medically important cyclophyllidean tapeworms, such as *Echinococcus* spp. A more comprehensive taxonomic representation of tapeworm diversity, therefore, required the collection of fresh specimens. New collections also afforded the opportunity to examine the phylogenetic utility of regions of the genome not previously examined from tapeworms.

Systematic studies aimed at recovering deep-level divergences have relied heavily on the information encoded by the nuclear ribosomal gene array. Protein-coding genes used for phylogenetic reconstruction commonly have been those of the mitochondria, which have been shown to evolve at a rate of evolution too fast for most studies involving distantly related taxa (Simon et al., 1994). Thus, efforts have recently been aimed at identifying slowly evolving, protein-coding genes from the nuclear genome. Friedlander et al. (1992), for example, identified 14 nuclear protein-coding genes as potential candidates for systematic studies at higher taxonomic levels. Phylogenetic utility was evaluated, in part, by the base identity of the genes among published insect and mammalian sequences. Among the 14 genes, *Ef-1α* was found to have the highest level of nucleotide conservation and was thus suggested as a promising candidate gene. In comparison with 18S rDNA, however, the phylogenetic utility of the *Ef-1α* gene remains largely unexplored, and only a handful of such studies are found in the literature (e.g., Cho et al., 1995; Kamaishi et al., 1996; Kobayashi et al., 1996; Mitchell et al., 1997; Regier and Shultz, 1997). Recent publications (e.g., Moreira et al., 1999; Roger et al., 1999), however, show that the *Ef-1α* gene is receiving greater attention among molecular systematists. The application of *Ef-1α* to the question of the interrelationships of tapeworm orders represents the first wide sampling of this gene within an early metazoan lineage.

MATERIALS AND METHODS

Collection of specimens

Fresh specimens of 23 species representing the 14 orders of tapeworms recognized by Khalil et al. (1994) were collected or obtained for DNA analysis and stored in 95% EtOH at 20 C. A systematic listing of the taxa sequenced, their hosts, and collection localities is given in Table I. Additional individuals of most taxa were preserved in 10% neutral buffered formalin and stored in 70% EtOH for identification and voucher deposition. Formalin-preserved specimens were stained with hematoxylin, dehydrated in a graded ethanol series, cleared in xylene, and mounted in Canada balsam for identification by light microscopy. Voucher specimens have been deposited in the Connecticut State Museum of Natural History in the Department of Ecology and Evolutionary Biology at the University of Connecticut, except for specimens of *Diphyllobothrium stemmacephalum* and *Tetrabothrius forsteri,* which have been deposited in the United States National Parasite Collection (Beltsville, Maryland) under accession numbers 86992 and 86991, respectively.

DNA isolation, PCR amplification, and gene sequencing

Genomic DNA of whole worms ≤1 cm long was extracted following the method of Coen et al. (1982). For a few of the smaller taxa, such as *Echinobothrium fautleyae* and *Tetrabothrius forsteri,* it was necessary to pool multiple individuals in order to extract sufficient quantities of template DNA for polymerase chain reaction (PCR) amplification. Genomic DNA from worms larger than 1 cm in length was extracted from either partial or entire specimens using the CTAB/DTAB protocol of Gustincich et al. (1991). Prior to extraction, all specimens were rinsed thoroughly in 95% EtOH and lyophilized to facilitate grinding of the tissue.

The entire 18S rDNA gene was amplified by PCR in 2 overlapping fragments, a 1,100-bp fragment using primers 18S-E and 18S-A27 and a 1,500-bp fragment using primers 18S-8 and 18S-Cestode-6 (Table II). Robust, high-fidelity, double-stranded amplifications were obtained with a Perkin-Elmer 9600 thermocycler using 2.5 mM MgCl buffer (Pääbo, 1990) and the following thermocycling profile: 3 min denaturation hold at 97 C; 36 cycles of 1 min at 96 C, 1 min at 54 C, 1 min at 72 C; and 7 min extension hold at 72 C.

Approximately 900 bp of the *Ef-1α* gene were generated by PCR using the M44-1 and rcM53-2 primer combination of Cho et al. (1995). The same thermocycling profile was used as above, except for the annealing temperature and buffer MgCl concentration which were 60 C and 1.5 mM, respectively. These differences in the PCR profile helped compensate for the degeneracy of the *Ef-1α* primers. The production of multiple, nontarget PCR products was common when either lower annealing temperatures or higher MgCl concentrations were used. Secondary PCR amplifications using gel-excised PCR products had to be performed for some taxa in order to obtain amplifications of sufficient quantity for sequencing.

Unincorporated PCR primers and nucleotides were removed from the PCR products prior to sequencing by either enzymatic degradation using a combination of shrimp alkaline phosphotase and exonuclease-I (USB™, United States, Biochemical Corp., Cleveland, Ohio), or by agarose gel excision followed by centrifugation of the excised band using a 0.45-μm Millipore® Ultrafree®-MC filter unit (Millipore Corp., Bedford, Massachusetts) or a Qiagen® QIAQuick™ spin tube (Qiagen Inc., Santa Clara, California).

Nucleotide sequences were determined directly from PCR products by either dideoxy manual sequencing (Sanger et al., 1977) using Sequenase™ version 2.0 (USB™) or by automated sequencing using ABI

TABLE I. List of taxa sequenced, their hosts and site of collection, and accession numbers and lengths of the 18S rDNA and *Ef-1α* sequences.

Order Family *Species* (Host [common name], collection locality)	Sequence accession numbers and lengths (bp)						
	18S rDNA				*Ef-1α*		
	GenBank no.	Total	V4	V7	GenBank no.	Total	Intron
Amphilinidea Poche, 1922							
Schizochoeridae Poche, 1922							
Schizochoerus liguloideus (Diesing, 1850) Poche, 1922 (Ex. *Arapaima gigas* [pirarucú], Itacoataria, Brasil)	AF124454	2,382	585	352	AF124793	827	*
Gyrocotylidea Poche, 1926							
Gyrocotylidae Benham, 1901							
Gyrocotyle rugosa Diesing, 1850 (Ex. *Hydrolagus collei* [spotted ratfish], Gulf of Alaska, Alaska)	AF124455	2,209	455	174	†	‡	*
Spathebothriidea Wardle and McLeod, 1952							
Spathebothriidae Yamaguti, 1934							
Spathebothrium simplex Linton, 1922 (Ex. *Liparis atlanticus* [Atlantic snailfish], Atlantic Ocean, Rye Beach, Rye, New Hampshire)	AF124456	1,976	366	204	AF124795	836	*
Caryophyllidea van Beneden *in* Carus, 1863							
Caryophyllaeidae Leuckart, 1878							
Hunterella nodulosa Mackiewicz and McCrae, 1962 (Ex. *Catostomus commersoni* [carp sucker], Illinois)	AF124457	2,048	361	259	AF124794	870	35
Diphyllidea van Beneden *in* Carus, 1863							
Echinobothriidae Perrier, 1897							
Echinobothrium fautleyae Tyler and Caira, 1999 (Ex. *Rhinoptera steindachneri* [golden cownose ray], Sea of Cortéz, Santa Rosalia, Baja, Mexico)	AF124464	1,844	372	181	†	†	†
Macrobothridiidae Khalil and Abdul-Salam, 1989							
Macrobothridium sp. (Ex. *Rhinobatus typus* [giant shovelnose ray], Timor Sea, Shoal Bay, Darwin, NT Australia)	AF124463	1,935	358	181	AF124801	875	31
Trypanorhyncha Diesing, 1863							
Tentaculariidae Poche, 1926							
Tentacularia sp. (Ex. *Prionace glauca* [blue shark], Atlantic Ocean, Montauk, New York)	AF124461	1,933	360	179	AF124799	859	36
Hepatoxylidae Dollfus, 1940							
Hepatoxylon sp. (Ex. *Prionace glauca* [blue shark], Atlantic Ocean, Montauk, New York)	AF124462	1,973	366	207	AF124800	814	34
Tetraphyllidea Carus, 1863							
Litobothriidae Dailey, 1969							
Litobothrium alpoias Dailey, 1969 (Ex. *Alopias superciliosus* [bigeye thresher shark], Sea of Cortéz, Santa Rosalia, Baja, Mexico)	AF124468	1,971	359	198	AF124807	853	36
Renyxa amplifica Kurochkin and Slenkis, 1973 (Ex. *Alopias pelagicus* [pelagic thresher shark], Sea of Cortéz, Santa Rosalia, Baja, Mexico)	AF124467	1,941	365	198	AF124806	853	36

TABLE I. Continued.

Order **Family** *Species* (Host [common name], collection locality)	Sequence accession numbers and lengths (bp)						
	18S rDNA				*Ef-1α*		
	GenBank no.	Total	V4	V7	GenBank no.	Total	Intron
Onchobothriidae Braun, 1900							
Calliobothrium sp.	AF124469	1,947	364	174	AF124812	867	38
(Ex. *Mustelus canis* [smooth dogfish], Long Island Sound, Connecticut)							
Platybothrium auriculatum Yamaguti, 1952	AF124470	1,943	364	173	AF124811	856	39
(Ex. *Prionace glauca* [blue shark], Atlantic Ocean, Montauk, New York)							
Phyllobothriidae Braun, 1900							
Anthobothrium laciniatum Linton, 1890	AF124471	1,945	356	178	AF124810	855	38
(Ex. *Prionace glauca* [blue shark], Atlantic Ocean, Montauk, New York)							
Rhinebothrium maccallumi (Linton, 1924) Campbell, 1970	AF124476	1,946	364	173	AF124813	866	37
(Ex. *Dasyatis americana* [southern stingray], Gulf of Mexico, U.S.A.)							
Lecanicephalidea§ Wardle and McLeod, 1952							
Cephalobothrium cf. *aetobatidis* Shipley and Hornell, 1906	AF124466	1,977	367	194	AF124808	853	36
(Ex. *Aetobatus narinari* [spotted eagle-ray], Gulf of Thailand, Bangsaray, Thailand)							
Eniochobothrium gracile Shipley and Hornell, 1906	AF124465	2,036	391	221	AF124809	856	39
(Ex. *Rhinoptera* sp. [cownose ray], Timor Sea, Fog Bay, NT Australia)							
Pseudophyllidea Carus, 1863							
Diphyllobothriidae Luhe, 1910							
Diphyllobothrium stemmacephalum Cobbold, 1858	AF124459	2,015	380	216	AF124796	873	38
(Ex. *Lagenorhynchus acutus* [Atlantic white-sided dolphin], Wellfleet Bay, Cape Cod, Massachusetts)							
Schistocephalus solidus (Müller, 1776)	AF124460	2,034	377	232	AF124797	873	38
(Ex. *Gasterosteus aculeatus* [3-spined stickleback], Hidden Lake, Matanuska-Susitna Valley, Alaska)							
Haplobothriidea Joyeux and Baer, 1961							
Haplobothriidae Cooper, 1917							
Haplobothrium globuliforme Cooper, 1914	AF124458	1,928	362	181	AF124798	868	33
(Ex. *Amia calva* [bowfin], Lake Ontario, Hay Bay, Canada)							
Nippotaeniidea Yamaguti, 1939							
Nippotaeniidae Yamaguti, 1939							
Amurotaenia decidua Hine, 1977	AF124474	1,953	371	175	AF124804	826	33
(Ex. *Gobiomorphus cotidianus* [sleeper fish], Mouth of Kuratan River, Lake Taupo, New Zealand)							
Proteocephalidea Mola, 1928							
Proteocephalidae La Rue, 1911							
Proteocephalus perplexus La Rue, 1911	AF124472	1,919	363	160	AF124805	854	37
(Ex. *Amia calva* [bowfin], Lake Ontario, Hay Bay, Canada)							
Tetrabothriidea Baer, 1954							
Tetrabothriidae Linton, 1891							
Tetrabothrius forsteri (Kreft, 1871) Fuhrmann, 1904	AF124473	2,183	417	295	AF124803	846	53
(Ex. *Lagenorhynchus acutus* [Atlantic white-sided dolphin], Wellfleet Bay, Cape Cod, Massachusetts)							

TABLE I. Continued.

Order **Family** *Species* (Host [common name], collection locality)	Sequence accession numbers and lengths (bp)						
	18S rDNA				*Ef-1α*		
	GenBank no.	Total	V4	V7	GenBank no.	Total	Intron
Cyclophyllidea van Beneden *in* Braun, 1900							
Hymenolepididae Ariola, 1899							
Hymenolepis diminuta (Ruldophi, 1819) Weiland, 1858 (Ex. *Rattus norvegicus* [Norway rat], Laboratory colony, University of Nebraska-Lincoln)	AF124475	2,054	416	222	AF124802	829	36

* Intron not present.
† Sequence not available.
‡ Sequence length could not be determined due to large regions of missing data.
§ *Cephalobothrium* is listed as genus inquirenda by Euzet (1994a) due to insufficient description. If valid, it is suggested to belong to the family Lecanicephalidae by Euzet (1994a). *Eniochobothrium gracile* is listed as incertae sedis within the order Lecanicephalidea (Euzet, 1994a).

TABLE II. Oligonucleotide primers used for amplifying and sequencing the 18S rDNA gene.

Name	Sequence	D*	Position†	Notes
18S-E	5′ CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT 3′	→	1–35	Eukarya-specific
18S-F	5′ CAA GCT TGA TCC TTC TGC AGG TTC ACC TAC 3′	←	‡	Designed to be universal at 3′ end
18S-2	5′ ATA ACA GGT CTG TGA TGC CCT TAG A 3′	→	2,218–2,242	
18S-3	5′ TCT AAG GGC ATC ACA GAC CTG TTA T 3′	←	2,242–2,218	Reverse compliment of 18S-2
18S-4	5′ AGC GAC GGG CGG TGT GTA C 3′	←	2,446–2,428	
18S-5	5′ GGT ACC CTT TGT ACA CAC CGC CCG TCG CT 3′	→	2,418–2,446	Offset reverse compliment of 18S-4
18S-7	5′ GCC CTA TCA ACT GTC GAT GGT A 3′	→	423–445	Reverse compliment of 18S-10
18S-8	5′ GCA GCC GCG GTA ATT CCA GC 3′	→	685–707	Reverse compliment of 18S-Pace-A
18S-9′	5′ TTT GAG TGC TCA AAT CAG 3′	→	1,215–1,233	Modified for tapeworms
18S-10	5′ TAC CAT CGA CAG TTG ATA GGG C 3′	←	445–423	Reverse compliment of 18S-7
18S-11	5′ AAC GGC CAT GCA CCA CCA CCC 3′	←	1,774–1,752	
18S-11F	5′ GGG TGG TGG TGC ATG GCC GTT 3′	→	1,752–1,774	Reverse compliment of 18S-11
18S-A27′	5′ CCA TAC AAA CGT CCC CGC CTG 3′	←	1,374–1,354	Modified for tapeworms
18S-Pace-A	5′ GTG TTA CCG CGG CTG CTG 3′	←	707–685	Reverse compliment of 18S-8
18S-Pace-B	5′ CCG TCA ATT C(A/C)T TT(A/G) AGT TT 3′	←	1,641–1,622	
18S-Pace-BF	5′ AAA CTT AAA GGA ATT GAC GG 3′	→	1,622–1,641	Reverse compliment of 18S-Pace-B
18S-Cestode-1	5′ TTT TTC GTC ACT ACC TCC CC 3′	←	592–573	Based on tapeworm sequences
18S-Cestode-2	5′ GTA AAC GTG CCA TCC GC 3′	←	1,204–1,183	Based on tapeworm sequences
18S-Cestode-3	5′ GGT TGG CTT CTG ATC TAA TAA 3′	←	278–262	Based on tapeworm sequences
18S-Cestode-4	5′ CAC CAC AGA CAT GGC TGA AAG G 3′	←	1,025–1,006	Based on tapeworm sequences
18S-Cestode-6	5′ ACG GAA ACC TTG TTA CGA CT 3′	←	2,596–2,575	Designed to avoid misannealing by 18S-F

* Direction of priming: →, 5′–3′ (forward); ←, 3′–5′ (reverse).
† Annealing site of primer based on alignment in Appendix A.
‡ Misannealing in cestode rDNA genes results in a ~400-bp PCR product when used in conjunction with 18S-E.

BigDye™ Terminator Cycle Sequencing Ready Reaction mix and an ABI PRISM™ 377 automated sequencer (Perkin-Elmer Applied Biosystems, Norwalk, Connecticut). Sixteen internal primers, as well as the 4 PCR primers listed above, were used to sequence the 18S gene (Table II), and 5 primers were used to sequence the *Ef-1α* gene (listed in Cho et al., 1995). The sequences were determined for all or most sites in both the 5′–3′ and 3′–5′ directions. The 18S rDNA and *Ef-1α* sequences were deposited with GenBank under accession numbers AF124454-76 and AF124793-813, respectively (Table I).

Selection of outgroup taxa

Multiple outgroup taxa were chosen based on their hypothesized affinities to the ingroup taxa in existing phylogenetic hypotheses (e.g., Ehlers, 1986; Rohde, 1990; Littlewood et al., 1999) and on the availability of appropriate sequence data. Different outgroup taxon sequences were selected for each of the genes due to the unavailability of both 18S rDNA and *Ef-1α* sequences for any single outgroup taxon. Sequences of 2 monogenean taxa (*Pseudomurraytrema* sp., EMBL AJ228793, and *Polystomoides malayi,* EMBL AJ228792) were used as outgroups for analyses of the 18S rDNA data, and sequences of a turbellarian (*Dugesia japonica,* GenBank 1389621), a schistosome (*Schistosoma mansoni,* GenBank 1619613), and a monogenean (*Neomicrocotyle pacifica*) were used as outgroups for analyses of the *Ef-1α* sequences.

Sequence alignment

Contiguous sequences were assembled by hand from the sequence fragments generated by the various forward and reverse primers used in the enzymatic sequencing reactions. The contiguous sequences were imported into the SeqLab editor of the Wisconsin Package™ (Genetics Computer Group, 1996) and aligned by eye. The 18S rDNA sequences were aligned with reference to the secondary structural model of Neefs et al. (1990), with the exception of the loop regions between stems 10 and E10-1 of the V2 variable region and the V4 and V7 regions for which sequence composition and length variation were too great to conform to a single model and could not be aligned with accuracy. Homologous positions in these hypervariable regions were determined only for the bases at the distal ends of each region, and the central positions were removed prior to analysis. The complete 18S rDNA alignment is shown in Appendix A. Secondary structural features (stem regions) are highlighted and numbered on the alignment according to the model of Neefs et al. (1990). Variable regions are designated by bars, and sites excluded from analysis are denoted by asterisks. Coding regions of the *Ef-1α* nucleotide sequences were aligned by reference to their corresponding amino acid codons. One intron region and 2 regions possessing indels in the *Ef-1α* alignment were removed prior to analysis. Aligned *Ef-1α* nucleotide sequences are shown in Appendix B. The intron region is designated by a bar and sites excluded from analysis are highlighted.

Data analysis

Sequence format, data partitioning, and rooting: NEXUS-formatted sequence data files were created using the SeqLab program. Mask sequences (text strings of 0s and 1s) were used in the SeqLab global alignment file in order to designate sites for removal upon exporting the alignments. Regions of the 18S rDNA gene where gaps were greater than 2 bp in length, or which contained missing data for 1 or more taxa were also removed. Likewise, a noncoding (intron) region of the *Ef-1α* gene and regions where gaps were greater than 2 amino acids in length (i.e., 6 bp), were removed from the alignment prior to analysis. The SeqLab editor was used to translate the *Ef-1α* nucleotide data into the corresponding amino acid residues using the standard nuclear eukaryotic amino acid translation table.

Both parsimony and distance-based phylogenetic analyses were performed using PAUP* version 4.01b (Swofford, 1998). The sequence data were divided into 7 different partitions in order to examine the effects on tree topologies of the 2 different genes, the 3 codon positions of *Ef-1α,* and combinations thereof. The 7 partitions analyzed were: (1) 18S rDNA only, (2) all codon positions of *Ef-1α,* (3) first and second codon positions of *Ef-1α,* (4) second codon positions of *Ef-1α,* (5) amino acid residues of Ef-1α, (6) 18S rDNA combined with first and second codon positions of *Ef-1α,* and (7) 18S rDNA combined with Ef-1α amino acid residues. Each data partition was examined by chi-square analysis for the possibility of erroneous groupings of taxa due to among-taxon base frequency heterogeneity. Different outgroup taxa were used to root the branching networks depending on the data set being analyzed (see above). *Gyrocotyle rugosa* was removed from all analyses that included *Ef-1α* data because of the excessive amount of undetermined sequence for this taxon (see Appendix B). Because of the lack of both 18S rDNA and *Ef-1α* sequence data for any 1 outgroup taxon, combined analyses had to be rooted using the functional outgroup (Watrous and Wheeler, 1981) taxon, *Spathebothrium simplex,* based on the basal position of this taxon in prior independent analyses using nontapeworm outgroup taxa.

Parsimony analyses: The nucleotide and amino acid character data were analyzed under the optimality criterion of maximum parsimony. Heuristic searches were performed on each data partition using the random addition sequence and tree bisection reconnection branch-swapping options in replicates of 1,000 in order to maximize the chances of finding the most parsimonious topological arrangement of the taxa. Analyses were run with all characters treated as unordered and unweighted. Alignment gaps were treated as missing data. Nodal support was assessed by both bootstrap resampling (Felsenstein, 1985) and decay analyses (Bremer, 1994). Bootstrap values were generated using 100 resampling replicates, with 10 heuristic searches per replicate. Decay indices were generated with the nonproprietary software program AutoDecay, version 3.0.3 (Eriksson and Wikström, 1995). Ten heuristic searches were run for each topological constraint defined by the AutoDecay command file.

Minimum evolution analyses: Pairwise distance data estimated by the method of maximum likelihood and log-determinant (LogDet, Lockhart et al., 1994) or paralinear transformations were generated and analyzed by the method of minimum evolution (ME) as a means of examining possible systematic error influencing the analyses by parsimony. These analyses were restricted to the nucleotide sequence data partitions; no attempt was made to analyze the amino acid sequences using distance methods. For each data partition, the previously determined, single most parsimonious tree or 1 of the set of previously determined most parsimonious trees was used as an unrooted topology with which to test the fit of each nucleotide substitution model implemented by PAUP*. These models were: Jukes-Cantor, Kimura 2-parameter, Felsenstein, 84/Hasegawa, Kashino and Yano, 85, and General time-reversible (GTR). In addition to testing each of the 4 models alone, estimates of among-site rate variation were incorporated using (1) an invariant-sites model (I), (2) a gamma model (Γ), and (3) both an invariant-sites and a gamma model (I + Γ). Log-likelihood scores for the 16 possible combinations were compared by chi-square analysis (Page and Holmes, 1998). For each data partition analyzed, the GTR substitution model, including estimates of invariant sites and gamma (GTR + I + Γ), was found to be the best fit and was subsequently used for all maximum likelihood estimates of genetic distance.

The LogDet transformation was used in conjunction with the value for proportion of invariable sites estimated by maximum likelihood (LogDet + I) as an additional means of estimating genetic distances for the ME analyses, even though only 1 of the data partitions was found to have significant nucleotide bias among the taxa (see results). It was useful to use LogDet because it has a lower variance (although the GTR model fit the data best). Bootstrap values were generated by performing ME analysis on 100 resampled replicates of each data set. Minimum evolution analyses, based on maximum-likelihood estimated distances using the GTR + I + Γ model of nucleotide substitution, and those based on LogDet-transformed distances incorporating the estimate of invariant sites (LogDet + I), are referred to in the text simply as GTR and LogDet, respectively.

RESULTS

Primary structure of the 18S rDNA sequences

The primary structure of the 18S rDNA sequences showed considerable variation in length among the ingroup taxa. Whereas the average sequence length was 1,986 bp, the length of sequences among the ingroup taxa ranged from 1,844 bp in *Echinobothrium fautleyae* (Diphyllidea) to 2,382 bp in *Schizo-*

TABLE III. Summary statistics of 18S rDNA and *Ef-1α* sequence length variation.

Sequence data	Mean	Min–max	Range	SD
18S rDNA				
All taxa	1,985.9	1,831–2,382	551	109.2
Cestoidea only*	1,996.2	1,844–2,382	538	107.2
Eucestoda only†	1,976.2	1,844–2,183	339	68.8
V4 only				
All taxa	393.7	355–597	242	48.5
Cestoidea only*	396.3	365–597	232	49.8
Eucestoda only†	383.4	365–429	64	17
V7 only				
All taxa	200.3	96–402	306	56.9
Cestoidea only*	207.7	160–402	242	52.8
Eucestoda only†	200.0	160–295	135	32.5
Ef-1α‡				
All taxa	825.3	793–857	64	17.4
Cestoidea only*	821.3	793–844	51	14.8
Eucestoda only†	821.0	793–844	51	15.1
Intron region only	37	31–52	22	4.4

* Calculations exclude outgroup taxa.
† Calculations exclude outgroup and cestodarian (*Schizochoerus liguloideus* and *Gyrocotyle rugosa*) taxa.
‡ Sequence lengths based only on the coding regions of the molecule.

choerus liguloideus (Amphilinidea). The majority of this variation was contained in the V4 and V7 regions (Table III), although the V2 region also showed considerable variation in length among certain taxa (Appendix A). *Schizochoerus liguloideus* had particularly long inserts in both the V4 and V7 regions. In the V2 region, however, *Tetrabothrius forsteri* (Tetrabothriidea) possessed unique inserts in the loop regions of stems 10 and E10-1. Outside of the variable regions, the sequences were highly conserved and a majority of sites was found to be invariant among the ingroup taxa. The 2 cestodarian taxa, *Gyrocotyle rugosa* (Gyrocotylidea) and *S. liguloideus,* possessed a unique insert in the V4 region spanning positions 1,025–1,075. However, there was no apparent homology between the sequences of *G. rugosa* and *S. liguloideus* in this region, and the alignment was considered tentative. Average nucleotide composition of the sequences showed a slight bias of purines (26 and 27% of adenine and guanine and 23 and 24% of cytosine and thymine, respectively). However, chi-square analysis of base frequencies did not indicate significant base frequency heterogeneity among taxa.

Primary structure of the *Ef-1α* sequences

Sequence length of the coding region of the portion of the *Ef-1α* gene determined was 825 bp on average, ranging from 793 bp in *Hepatoxylon* sp. to 844 bp in *Macrobothridium* sp. among the ingroup taxa (Table III). Relative to the 18S rDNA sequences, length variation of the region determined for *Ef-1α* was low. A 31–52-bp intron (positions 583–634, indicated by a bar in Appendix B) was found only among the ingroup taxa, with the exception of the cestodarians, *Gyrocotyle rugosa* and *Schizochoerus liguloideus,* and the eucestode species *Spathebothrium simplex* (Spathebothriidea), which, like the outgroup taxa, lacked this intron. Outside of the intron region, length variation was found in only 2 regions, a 6-bp insertion or deletion at positions 280–285, and a larger region spanning positions 451–508, for which potential homology among sites was not apparent. In both cases, however, the length variation corresponded to complete losses or gains of amino acids (i.e., the length of inserted alignment gaps was divisible by 3).

Over 98% of third codon positions were found to vary among the taxa, accounting for half of the total amount of variability in the region of *Ef-1α* analyzed. First codon positions accounted for 28.7% and second codon positions 19.7% of the total sequence variability. Nucleotide composition of the region was nearly equal for each of the 4 bases when all codon positions were considered and averaged among the taxa. However, first and second codon positions together showed a bias of purine bases and second positions alone were biased for adenine and thymine, on average. Base frequency heterogeneity was not found to be significant in either data partition. In the data partition including all codon positions, chi-square analysis indicated significant heterogeneity of base frequencies among the taxa, with *Amurotaenia decidua* and *Schizochoerus liguloideus* being outliers on either extreme. Separate chi-square analyses of among-taxon base frequency heterogeneity of the 3 codon positions indicated that only the third codon positions showed significant heterogeneity among taxa.

Phylogenetic analyses

General comments: A numerical summary of the results of analyses by parsimony is shown in Table IV. Phylogenetic estimates differed among the 2 genes, the different data partitions of the *Ef-1α* gene, and the different methods of analysis. However, nodes with high levels of character support were recovered from most or all data partitions and methods of analysis. Results of the various analyses are discussed below and dendrograms are shown for some analyses (Figs. 2–4). Support for monophyly of specific subgroups of taxa by the different data partitions and methods of analysis is summarized in Table V.

Analyses of 18S rDNA: Parsimony analysis of 1,338 total sites of the 18S rDNA gene resulted in a single tree (Fig. 2A) 965 steps long with a consistency index (CI) of 0.62 and a retention index (RI) of 0.5. Using the monogenean outgroup taxa *Pseudomurraytrema* sp. and *Polystomoides malayi,* the monophyly of the ingroup was strongly supported, as were the positions of the amphilinidean and gyrocotylidean taxa as basal in position between the outgroup and eucestode taxa. *Spathebothrium simplex* (Spathebothriidea) was found to be the most basal of the ingroup taxa. With the exception of the trypanorhynch taxon, *Tentacularia* sp., the remaining eucestode taxa formed 2 sister clades: a clade including the caryophyllidean, diphyllidean, haplobothriidean, and pseudophyllidean taxa, as well as the other trypanorhynch taxon (*Hepatoxylon* sp.), and a clade including the cyclophyllidean, lecanicephalidean, nippotaeniidean, proteocephalidean, tetrabothriidean, and tetraphyllidean taxa. Bootstrap support was low for most nodes in this tree. Exceptions included the nodes separating the outgroup from the Cestoidea and the cestodarians from the Eucestoda and the nodes supporting the 2 pseudophyllidean taxa, *Diphyllobothrium stemmacephalum* and *Schistocephalus solidus,* the litobothriidean taxa *Litobothrium alopias* and *Renyxa amplifica,* and a clade uniting members of the orders Cyclophyllidea, Nip-

TABLE IV. Summary of analyses by maximum parsimony.

Data partition	No. taxa	Root†	No. characters* Total	Constant (%)	Parsimony informative (%)	No. EPTs‡	Length (steps)	CI	RI
18S rDNA	25	OUT	1,338	879 (66)	207 (15)	1	965	0.62	0.5
Ef-1α (all codon positions)	24	OUT	748	271 (36)	391 (52)	2	2,686	0.34	0.33
Ef-1α (1st and 2nd codon positions)	24	OUT	499	268 (54)	150 (31)	2	778	0.46	0.45
Ef-1α (2nd codon positions)	24	OUT	249	155 (62)	58 (23)	40	295	0.5	0.48
Ef-1α (amino acids)	24	OUT	247	105 (43)	94 (38)	2	593	0.57	0.48
18S rDNA + *Ef-1α* 1st and 2nd codons	20	F-O	1,872	1,333 (71)	323 (17)	6	1,759	0.45	0.37
18S rDNA + *Ef-1α* amino acids	20	F-O	1,599	1,159 (72)	205 (13)	2	1,146	0.63	0.45

* Based only on characters included in the analyses.
† Method of rooting tree(s): OUT, outgroup comparison method; F-O, functional outgroup (see text).
‡ Equally parsimonious trees.

potaeniidea, and Tetrabothriidea. In addition, all analyses supported the position of the proteocephalidean *Proteocephalus perplexus* within a paraphyletic Tetraphyllidea.

Results of ME analyses of the 18S rDNA data partition varied between the 2 methods of estimating the genetic distances. LogDet analysis yielded a topology (Fig. 2B) largely consistent with that resulting from analysis by parsimony and had greater bootstrap support. The parsimony and LogDet analyses differed in that some taxa formed sister pairs in the LogDet analysis, whereas they were "ladderized" in the parsimony analysis (e.g., compare the positions of *Spathebothrium simplex* and *Tentacularia* sp.). GTR analysis, however, produced a topology inconsistent with the results of either the parsimony or LogDet analyses, in that the lecanicephalidean and litobothriidean (tetraphyllidean) taxa were found at the base of the ingroup clade, and the gyrocotylidean, rather than the amphilinidean, taxon was basal to the remaining ingroup taxa. Otherwise, the topologies resulting from both GTR and LogDet were congruent. Branch lengths of internal nodes (Fig. 2B) were estimated to be considerably shorter than those of terminal branches.

Analyses of Ef-1α*:* Analyses of the *Ef-1α* data gave differing results depending on the data partition analyzed. In each analysis, the trees were rooted using the turbellarian *Dugesia japonica* and also included the digenean *Schistosoma mansoni* and the monogenean *Neomicrocotyle pacifica* as outgroup taxa. Parsimony analysis of all codon positions (748 characters) resulted in only 2 equally parsimonious trees, but had the lowest CI and RI of any of the data partitions (0.34 and 0.33, respectively). Unlike the other *Ef-1α* data partitions analyzed, monophyly of the Eucestoda was not supported by analyses of all codon positions because of the placement of *Hunterella nodulosa* (Caryophyllidea) among the outgroup taxa and the placement of *Schizochoerus liguloideus* (Amphilinidea) among the eucestode taxa. Extremely high levels of homoplasy attributable to saturation at the third codon position suggested that these data were not phylogenetically informative, and results from this data partition were given little consideration. Parsimony analysis of second codon positions alone (249 characters) resulted in 40 equally parsimonious trees (EPTs), a strict consensus of which left resolution only among the lecanicephalidean, proteocephalidean, and tetraphyllidean taxa and a sister relationship between the tetrabothriidean and cyclophyllidean taxa. Opposite of the third codon positions, second codon positions were, in isolation, too conserved to provide an adequate number of variable sites.

Parsimony analysis of first and second codon positions (Fig. 3A; 499 characters) supported the monophyly of the Eucestoda and the basal position of *Schizochoerus liguloideus* as the sister taxon to the eucestode clade. Monophyly of the trypanorhynch taxa, *Hepatoxylon* sp. and *Tentacularia* sp., was supported by parsimony analysis but not by ME analyses, in which case the Trypanorhyncha was found to be paraphyletic. In the LogDet analysis (Fig. 3B), *Tentacularia* sp. was intermediate between a clade consisting of *Hepatoxylon* sp. plus the caryophyllidean, haplobothriidean, and pseudophyllidean taxa and a clade consisting of the remaining eucestode taxa. Although both ME analyses supported the basal position of *Spathebothrium simplex* within the eucestode clade, parsimony analysis supported a more derived position of the spathebothriidean taxon. Similar to the 18S rDNA analyses, the topology of the ingroup taxa showed a largely diphyletic pattern of evolution in which the "difossate" and "tetrafossate" orders formed separate clades. Also congruent with the results of the 18S rDNA analyses was the support for a clade including the caryophyllidean, haplobothriidean, and pseudophyllidean taxa and a clade including the cyclophyllidean, nippotaeniidean, and tetrabothriidean taxa. In addition, the proteocephalidean taxon was placed within a paraphyletic Tetraphyllidea.

Parsimony analysis of the amino acid translation (Fig. 4A; 247 characters) resulted in the highest CI and RI (0.57 and 0.48, respectively) of the 4 *Ef-1α* data partitions (Table IV). Results were congruent with those from analysis of first and second codon positions combined, except for the following differences: the amphilinidean was placed among the outgroup taxa, and the spathebothriidean was placed as the sister taxon, followed by the caryophyllidean, to the remaining eucestodes. Parsimony analysis of both the amino acid, as well as the first and second codon data partitions, supported a sister group relationship between the lecanicephalidean taxa and a clade including the cyclophyllidean, nippotaeniidean, and tetrabothriidean taxa. Most other data partitions supported a position of the lecanicephalidean taxa closer to the tetraphyllidean taxa.

Analyses of 18S rDNA and Ef-1α *combined:* Parsimony

TABLE V. Unambiguous support for monophyly among data partitions for specific groups of taxa.

Group	Data partition*						
		Ef-1α†				18S rDNA and *Ef-1α*†	
	18S rDNA	All	1st and 2nd	2nd	Amino acids‡	1st and 2nd	Amino acids‡
Cestoidea	P, M, L	M, L	P, M, L	M	§	§	§
Gyrocotyle rugosa + *Schizochoerus liguloideus*	—	§	§	§	§	§	§
Eucestoda	P, M, L	—	P, L	M, L	P	§	§
Diphyllidean taxa	P, M, L	§	§	§	§	§	§
Lecanicephalidean taxa	P, L	—	P, M, L	M, L	—	P, M, L	P
Pseudophyllidean taxa	P, M, L	P, M, L	P, M, L	—	P	P, M, L	P
Tetraphyllidean taxa	—	—	—	—	—	—	—
Tetraphyllidea: litobothriid taxa	P, M, L	P, M, L	P, M, L	P, M, L	P	P, M, L	P
Tetraphyllidea: onchobothriid taxa	M, L	—	—	—	P	—	—
Tetraphyllidea: phyllobothriid taxa	—	—	—	—	—	—	—
Trypanorhynch taxa	—	—	P	P, M	P	—	—
Haplobothriidean + pseudophyllidean taxa	—	P, M, L	P, M, L	P, M, L	P	P, M, L	P
Caryophyllidean + haplobothriidean + pseudophyllidean taxa	P, M, L	L	P, M, L	P, M, L	—	—	P
Cyclophyllidean + nippotaeniidean + tetrabothriidean taxa	P, M, L	M, L	P, M, L	P, M, L	P	P, M, L	P
Proteocephalus perplexus + *Anthobothrium laciniatum* + *Calliobothrium* sp. + *Platybothrium auriculatum*	M, L	P	P, L	M, L	P	M, L	P
"Tetraphosates" (cyclophyllidean + lecanicephalidean + nippotaeniidean + proteocephalidean + tetraphyllidean + tetrabothriidean taxa)	P, L	—	M	—	—	M, L	P

* Support by data partition is indicated by type of analysis: P, maximum parsimony; M, minimum evolution based on distances derived estimated by the GTR + I + Γ substitution model; L, minimum evolution based on LogDet-transformed distances. Cases in which monophyly was neither supported nor refuted are not listed.
† Ef-1α data partitioned by codon position.
‡ Data partitions including amino acid data were analyzed by maximum parsimony only.
§ Not tested.

analysis of the 18S rDNA data combined with the first and second codon positions of *Ef-1α* (1,872 total characters) resulted in 6 EPTs 1,759 steps long (CI = 0.45, RI = 0.37). Parsimony analysis of the 18S rDNA data combined with the Ef-1α amino acid data (1,599 total characters) resulted in 2 EPTs 1,146 steps long (CI = 0.63, RI = 0.45). Strict consensus of the 2 EPTs is shown in Figure 4B. Trees resulting from the analyses of the combined data partitions were highly congruent, although some differences were found. For example, analysis of 18S rDNA combined with first and second codon positions of *Ef-1α* supported a position of the caryophyllidean taxon at the root of the tree, whereas the 18S rDNA combined with the Ef-1α amino acid data supported its position at the base of a clade including the haplobothriidean and pseudophyllidean taxa. The trypanorhynch and diphyllidean taxa showed the greatest instability in placement. Monophyly of the trypanorhynch taxa was either not supported or was ambiguous. Both GTR and LogDet analyses of the nucleotide data supported the position of *Hepatoxylon* sp. as a member of a clade including the haplobothriidean and pseudophyllidean taxa, whereas parsimony analyses of both combined data partitions supported a more derived position of this trypanorhynch species.

DISCUSSION

Relationships among the cestodarians, caryophyllideans, and spathebothriideans

A few researchers have suggested that the Amphilinidea may belong within the Eucestoda based on a superficial similarity between the morphology of adult amphilinideans and that of free segments of the polyzoic eucestodes (Fuhrmann, 1931; Llewellyn, 1965). Most morphologists, however, have agreed that the cestodarians form the sister lineages to the Eucestoda, with the amphilinideans commonly thought to be more closely related to the eucestode clade than the gyrocotylideans (Fuhrmann, 1931; Bychowsky, 1957; Freeman, 1973; Ehlers, 1986; Brooks, 1989; Rohde, 1990; Hoberg et al., 1997). Previous studies based on 18S rDNA have found either ambiguous placement (Baverstock et al., 1991; Rohde et al., 1993) or placement of the 2 groups within the Eucestoda (Campos et al., 1998), but these studies included relatively few representative tapeworm species. The more comprehensive molecular analyses of Mariaux (1998) and Littlewood et al. (1999) support the traditional position of the cestodarian taxa as sister to the Eucestoda. Neither study, however, included representatives of both cestodarian groups; therefore, the question of the position of amphilinideans and gyrocotylideans relative to one another was not addressed.

Monophyly of the 2 cestodarian taxa was examined using only the 18S rDNA data partition due to a lack of *Ef-1α* sequence data for the gyrocotylidean *Gyrocotyle rugosa*. All methods of analysis supported the position of these 2 taxa between the more basal monogenean outgroup taxa and the eucestode taxa (Fig. 2A, B). However, monophyly of the "Cestodaria" (Amphilinidea + Gyrocotylidea) was not supported. Nodal support separating the ingroup taxa from the outgroup taxa and separating the eucestode taxa, including the caryophyl-

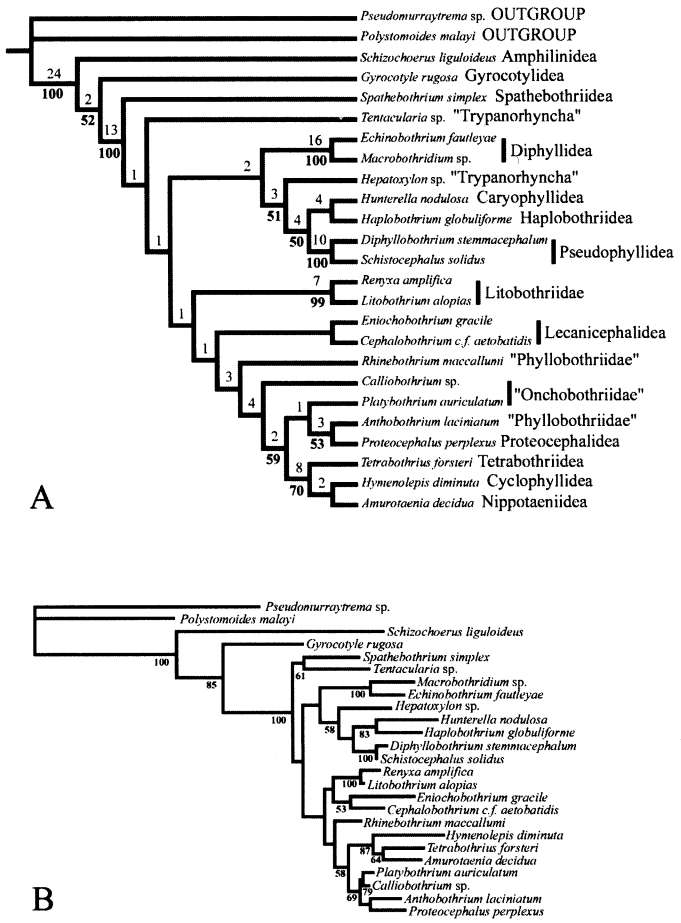

FIGURE 2. Phylogenetic analyses of 18S rDNA. (**A**) Maximum parsimony. (**B**) Minimum evolution based on LogDet-transformed genetic distances (branch lengths are proportional to the estimated distances between taxa). Decay indices are shown in plain font above nodes, and bootstrap values ≥50% are shown in bold beneath nodes. Quotations indicate a lack of support for monophyly of the taxonomic group.

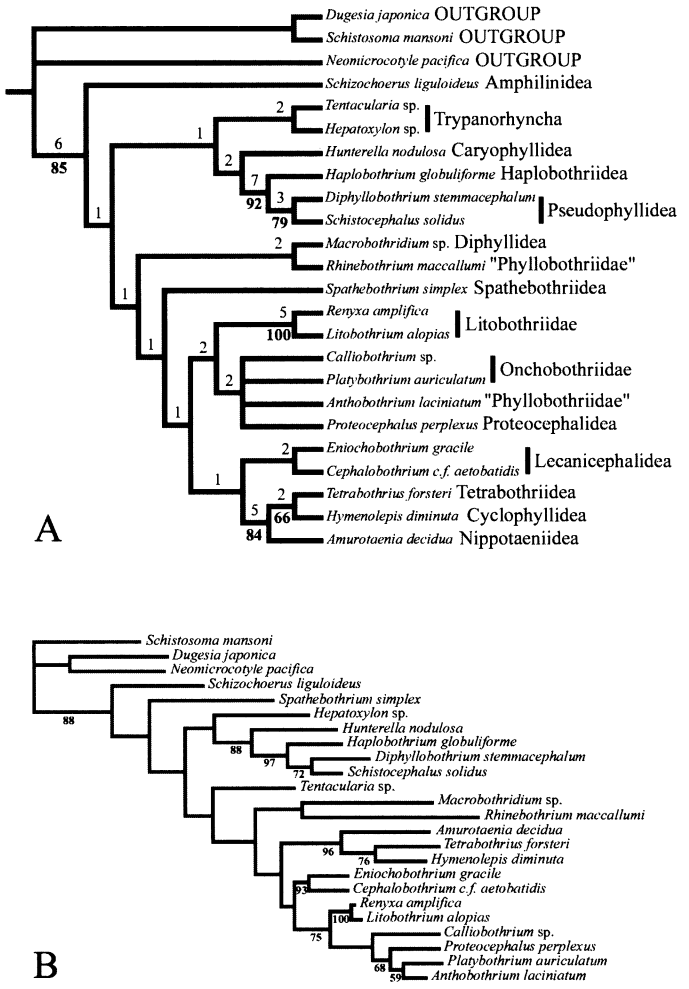

FIGURE 3. Phylogenetic analyses of the first and second codon positions of *Ef-1α*. (**A**) Maximum parsimony. (**B**) Minimum evolution based on LogDet-transformed genetic distances (branch lengths are proportional to the estimated distances between taxa). Decay indices are shown in plain font above nodes, and bootstrap values ≥50% are shown in bold beneath nodes. Quotations indicate a lack of support for monophyly of the taxonomic group.

lidean and spathebothriidean, from the amphilinidean and gyrocotylidean was strong. However, the relative positions of the amphilinidean and gyrocotylidean taxa with respect to one another was weakly supported and differed among analyses. Trees resulting from both analysis by parsimony and by LogDet placed the amphilinidean *Schizochoerus liguloideus* basal to *G. rugosa,* but GTR supported the opposite arrangement (i.e., the gyrocotylidean was basal to the amphilinidean).

The position of the amphilinidean was also examined by analyses of the *Ef-1α* data. Like the 18S rDNA data, analyses of *Ef-1α* supported a position of the amphilinidean between the outgroup taxa and the eucestode taxa (Fig. 3A, B), except in the analyses in which third codon positions were included. The position of the cestodarian orders outside of the Eucestoda is further supported by the lack of an intron in the *Ef-1α* sequences of *S. liguloideus, G. rugosa,* and the outgroup taxa (although the intron was also found to be lacking in the eucestode taxon *Spathebothrium simplex*).

The basal position of the Amphilinidea relative to the Gyrocotylidea found herein has not been hypothesized previously. This result was not well supported, however, and may have been influenced by the extreme divergence of the 18S rDNA gene of *Schizochoerus liguloideus* relative to the other taxa. Such divergence can lead to problems associated with long-branch attraction (Felsenstein, 1978). As can be seen in Figure 2B, the relative length of the terminal branch leading to *S. liguloideus* is as long as those of the more distantly related outgroup taxa, whereas the terminal branches of the eucestode taxa are comparatively short. The GTR + I + Γ model of sequence evolution attempts to "correct" for such extremes in evolutionary rate heterogeneity among taxa (Sullivan et al., 1995; Swofford et al., 1996), and indeed, the GTR analysis of the 18S rDNA data supported a basal position of the Gyrocotylidea relative to the Amphilinidea (just the opposite of the results of parsimony and LogDet analyses). Although the method of parsimony may be subject to systematic error in situations in which long branches are shared by distantly related taxa (termed the "Felsenstein zone"), likelihood-based approaches are known to repel long branches and are similarly subject to systematic error in situations in which long branches are shared by closely related taxa, recently termed the "Farris zone" (Siddall, 1998).

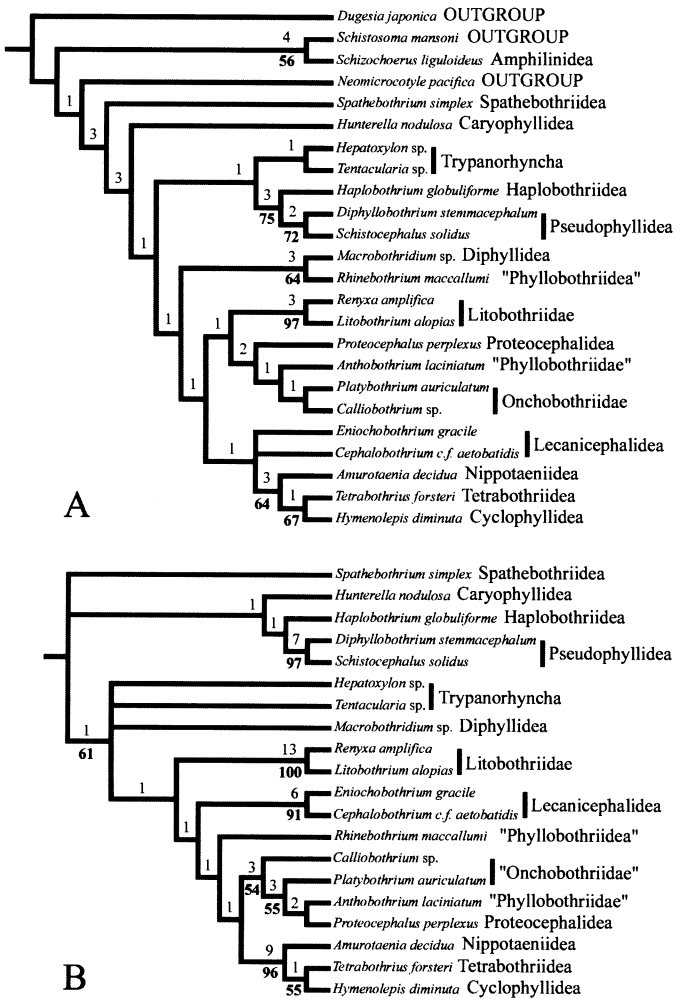

FIGURE 4. Maximum parsimony analyses of (**A**) *Ef-1α* amino acids and (**B**) *Ef-1α* amino acids combined with 18S rDNA nucleotides. Decay indices are shown in plain font above nodes, and bootstrap values ≥50% are shown in bold beneath nodes. Quotations indicate a lack of support for monophyly of the taxonomic group.

Unlike simulation studies, in which relationships have been predetermined (e.g., Hulsenbeck and Hillis, 1993; Siddall, 1998), long branches remain a problem when one cannot readily determine which "zone" the data may be subject to. Such is the case concerning the relative positions of the Amphilinidea and Gyrocotylidea as inferred from the 18S rDNA data in the present study.

The taxonomic status of the Spathebothriidea has varied considerably. Until the work of Wardle and McLeod (1952), the spathebothriideans had not been recognized as a separate order and, like the caryophyllideans, were generally regarded as an aberrant group of pseudophyllideans (e.g., Fuhrmann, 1931). Even following the work of Wardle and McLeod (1952), ordinal status of the group was not universally accepted, and some workers continued to classify them together with the pseudophyllideans (e.g., Joyeux and Baer, 1961). From a morphological perspective, the fact that they are proglottized, but not segmented, makes their phylogenetic affinities ambiguous. Freeman (1973) hypothesized an independent origin of the Spathebothriidea, together with the Caryophyllidea, from the original "protocestode" stock. Mackiewicz (1981), however, envisioned them to be intermediate between the basal Caryophyllidea and the more derived Pseudophyllidea. Recent analyses by Hoberg et al. (1997) and Mariaux (1998) supported the views of Mackiewicz (Fig. 1A, B), in that the Caryophyllidea was found to be the most basal order of the eucestode clade, followed by the Spathebothriidea. A majority of the analyses herein independently showed the spathebothriidean *Spathebothrium simplex* to form the most basal lineage of the eucestode clade. Analyses that did not support this position were inconsistent in their alternative placements. Moreover, like the outgroup and cestodarian taxa, *S. simplex* was found to lack an intron in the sequenced region of the *Ef-1α* gene. This noncoding intron region was otherwise globally observed among the eucestode taxa (see Appendix B). Considered together, these results provide strong evidence for the basal position of the order Spathebothriidea.

Segmentation is a hallmark of the Eucestoda. The basal position of *Spathebothrium simplex* on the eucestode tree indicates that the polyzoic body of spathebothriideans is primarily, rather than secondarily, nonsegmented. Thus, proglottization may be seen as the first step toward increasing fecundity through serial repetition of the sex organs. The advantages of further external subdivision are speculative, but with regard to the species richness of the extant forms, the Spathebothriidea hardly compare in number to their polyzoic kin. This fact suggests that external segmentation was a key character in the successful radiation of the more recent polyzoic lineages and, therefore, deserves further study of its underlying genetic basis and ecological significance.

The phylogenetic placement of the caryophyllidean *Hunterella nodulosa* was less consistent among analyses than was that of *Spathebothrium simplex.* Only GTR analysis of *Ef-1α* that included the highly saturated third codon positions resulted in a topology consistent with the hypothesis that the caryophyllideans form the most basal lineage within the Eucestoda (Llewellyn, 1965; Mackiewicz, 1982; Ehlers, 1985; Hoberg et al., 1997; Mariaux, 1998). Analyses of the combined data partitions were ambiguous in their results and placed the caryophyllidean taxon in a trichotomy that included the functional outgroup (*S. simplex*) and a lineage uniting the remaining eucestode taxa. In most analyses, however, the caryophyllidean was found to occupy either of 2 positions, a lineage that was between the more basal spathebothriidean and the remaining eucestode taxa (Fig. 4A) or, more commonly, in a clade with the pseudophyllideans and their kin (Figs. 2A, B, 3A, B), as has been hypothesized by Baer (1950), Joyeux and Baer (1961), and Freeman (1973). These 2 alternative positions bear directly on the question of whether the lack of segmentation in caryophyllideans represents a symplesiomorphic condition or a secondary loss. If their position is between the more basal Spathebothriidea and the more derived polyzoic orders, it then follows that proglottization first evolved in the ancestor of the Eucestoda, was lost in the Caryophyllidea, and was then reacquired, along with external segmentation, in the more derived clade of polyzoic eucestodes. Alternatively, if their position is within a clade including the Pseudophyllidea and their kin, then proglottization, followed by external segmentation, would have each evolved once and were lost together in the Caryophyllidea. Furthermore, if proglottization and segmentation became coupled genetically in the ancestor of the more derived polyzoic forms, this loss would rep-

resent a single step from the polyzoic ancestral condition of the lineage leading to the Pseudophyllidea and their kin to the plesiomorphic monozoic condition exhibited in the caryophyllideans.

Relationships among the difossate orders and their kin

The difossate orders include those groups whose members possess scolices bearing a pair of bothria and include the orders Diphyllidea, Pseudophyllidea, and Trypanorhyncha. Some pseudophyllidean and trypanorhynch species, however, exhibit variation in this general bipartite scolex morphology (and in some cases are not difossate at all). Traditional views suggest that the Pseudophyllidea represent the most basal lineage of segmented polyzoic tapeworms (Baer, 1950; Freeman, 1973; Jarecka, 1975; Brooks et al., 1991), and this has been supported more recently by the work of Hoberg et al. (1997). Mariaux's results (1998), however, suggest that the Trypanorhyncha occupy the most basal position among the polyzoic orders, followed by the Pseudophyllidea. Results herein strongly support a sister group relationship between the 2 pseudophyllidean taxa and the haplobothriidean *Haplobothrium globuliforme.* Together, these 3 taxa consistently formed the most basal clade of polyzoic tapeworms. However, results varied among analyses, and often the caryophyllidean taxon, and less frequently the diphyllidean and trypanorhynch taxa, formed a clade together with the haplobothriideans and pseudophyllideans. Thus, determining the relative positions among the difossate orders was problematic, and the results of Hoberg et al. (1997) and Mariaux (1998) can be neither refuted nor supported strongly. Consistently, the difossate orders showed affinities to one another separate from the tetrafossate orders.

Two species of pseudophyllideans were included in the analysis: *Diphyllobothrium stemmacephalum* and *Schistocephalus solidus.* Monophyly of these taxa was supported by nearly all analyses (Table V). Both species are members of the family Diphyllobothriidae, which are unique among pseudophyllideans in part because they utilize tetrapods, rather than fish, as definitive hosts. The analysis of Mariaux (1998) indicated paraphyly of the order. Specifically, he found the Diphyllobothriidae to form a lineage basal to a lineage uniting the other representatives of the Pseudophyllidea included in his analysis (Fig. 1B). This is in contrast with the works of Freeman (1973) and Dubinina (1980), who postulated the family Diphyllobothriidae to be among the most highly derived families in the order. Mariaux (1998) noted, however, that separating the Diphyllobothriidae from the other families in the order is compatible with the scheme of Brooks and McLennan (1993) and others. It is also compatible with the recent morphological analysis of the order by Bray et al. (1999) in that the diphyllobothriid genera formed a distinct clade, and they suggested separation into 2 suborders was warranted. Paraphyly of the order Pseudophyllidea was not tested either by Bray et al. (1999) or herein. It is clear from the results of Mariaux (1998) and Bray et al. (1999), however, that the taxa used herein are representative only of 1 lineage of pseudophyllideans that may have followed an evolutionary trajectory separate from other such groups in the order.

Previously, the phylogenetic position of the enigmatic Haplobothriidea has been uncertain. Adults are known only from the primitive North American bowfin (*Amia calva*), and with the exception of a solitary report in the literature of a second species from bowfins in Florida (Premvati, 1969), only the species *Haplobothrium globuliforme* is known. Superficially, the scolex tentacles of haplobothriideans are reminiscent of those of the trypanorhynchs and they have been grouped accordingly by some authors. Fuhrmann (1931), for example, considered *H. globuliforme* to represent an intermediate step in the conversion of the accessory suckers of certain tetraphyllideans (which he considered to be the progenitors of the Eucestoda) into the more complex and armed tentacles of the trypanorhynchs. However, most authors have recognized a closer relationship between the haplobothriideans and the order Pseudophyllidea based on the similarity of their proglottid and sperm morphology and common host associations (Wardle and McLeod, 1952; Euzet, 1959; Yamaguti, 1959; Dubinina, 1980; MacKinnon and Burt, 1985; Schmidt, 1986; Brooks and McLennan, 1993). This is supported by Hoberg et al. (1997) as well, where the Haplobothriidea was placed between the more basal order Pseudophyllidea and more derived order Diphyllidea. Alternatively, Freeman (1973) suggested that the haplobothriideans arose from within the order Pseudophyllidea. It seems clear from the results herein that the haplobothriideans are in fact more closely related to the pseudophyllideans than to the trypanorhynchs. Testing the hypothesis of Freeman (1973), however, would require a far more comprehensive representation of the members of the Pseudophyllidea.

Most authors have allied the trypanorhynchs with the pseudophyllideans, the haplobothriideans, or both. The phylogenetic position of the diphyllideans, however, has remained largely uncertain. Both trypanorhynchs and diphyllideans are parasites of elasmobranchs, whereas the other difossate orders parasitize teleosts (with only a few exceptions). Fuhrmann (1931) suggested that the trypanorhynchs gave rise to the pseudophyllideans and their kin, but could not draw conclusions on the proper position of the diphyllideans due to their similarity not only to pseudophyllideans and trypanorhynchs, but also to the cyclophyllideans and tetraphyllideans. Euzet's diphyletic scheme (1959) was similar in that it aligned the haplobothriideans, pseudophyllideans, and trypanorhynchs in a sister lineage to the tetrafossate orders but differed in that it showed an independent lineage leading to the Diphyllidea that stemmed from the ancestral stock common to both his difossate and tetrafossate clades. Investigation of cestode sperm morphology by Euzet et al. (1981) was also unable to resolve the phylogenetic affinities of the diphyllideans. Freeman (1973) hypothesized a sister group relationship between the pseudophyllideans and trypanorhynchs but placed the diphyllideans at the base of a clade that included both the lecanicephalideans and tetraphyllideans. Dubinina's scheme (1980) split the eucestodes strictly along difossate and tetrafossate lines and showed the trypanorhynchs to form the root of the difossate lineage, followed by the diphyllideans, haplobothriideans, and pseudophyllideans. In their cladistic analysis of the Eucestoda, Brooks et al. (1991) avoided detailed consideration of the phylogenetic position of the trypanorhynchs by combining them with members of the order Tetraphyllidea, in part on the basis of their possession of bothridia with "rigid margins." The diphyllideans appeared to be ignored altogether. The analysis of Hoberg et al. (1997) resulted in a tree entirely pectinate in form (Fig. 1A), with the orders

Diphyllidea and Trypanorhyncha occupying medial positions between the basal difossate lineages and the more derived tetrafossate lineages. In contrast, Mariaux (1998) found the Trypanorhyncha to form the most basal lineage of polyzoic tapeworms, followed by the order Pseudophyllidea. The position of the Diphyllidea was unresolved by strict consensus of the trees resulting from his analysis (Fig. 1B).

Establishing the phylogenetic position of the trypanorhynchs and diphyllideans based on present analyses was similarly problematic. Among data partitions and methods of analysis, the most consistent result was that members of these 2 orders were consistently placed between the more basal spathebothriidean taxon and a more derived tetrafossate clade. Thus, they were found to be either part of, or near, a difossate clade including the pseudophyllidean, haplobothriidean, and, in most instances, the caryophyllidean taxa. *Hepatoxylon* sp. consistently formed the basal lineage of this clade (Figs. 2A, B, 3A, B, 4A), whereas *Tentacularia* sp. was often placed in either a basal (Fig. 2A, B) or a derived (Fig. 3B) position relative to the difossate clade. Both instances refuted monophyly of the Trypanorhyncha (Table V). Parsimony analysis of the first and second codon positions (Fig. 3A) and amino acid (Fig. 4A) data partitions of *Ef-1α,* however, supported the monophyly of the 2 trypanorhynch taxa, as well as their position within the difossate clade. It is odd that the 18S rDNA data were so divergent between the taxa *Hepatoxylon* and *Tentacularia,* as recent morphological analysis of the order by Beveridge et al. (1999) showed a very close relationship between these genera. It suggests that 1, if not both, of the taxa may indeed not be broadly representative of the order with regard to the 18S rDNA gene.

Simultaneous analysis of the 2 diphyllidean taxa was tested only using 18S rDNA data because of the lack of *Ef-1α* sequence data for *Echinobothrium fautleyae.* All methods of analysis of the 18S rDNA data partition supported the monophyly of the 2 diphyllidean taxa (Table V) and their position within the difossate clade (Figs. 2A, B). However, analyses of the *Ef-1α* and combined data partitions supported a position of *Macrobothridium* sp. between the more basal difossate clade and more derived tetrafossate clade (Figs. 3A, B, 4A, B), similar to the position of the Diphyllidea hypothesized by Hoberg et al. (1997; Fig. 1A).

Relationships among the tetrafossate orders and their kin

The tetrafossate orders include those groups whose members possess scolices bearing 4 bothridia, suckers, or combinations thereof. These include the Cyclophyllidea, Lecanicephalidea, Proteocephalidea, Tetrabothriidea, and Tetraphyllidea. Members of these groups, however, exhibit considerable variation in their scolex (especially lecanicephalideans and tetraphyllideans) and proglottid morphology. Monophyly of the representatives of the tetrafossate orders listed above was generally supported in the current study (Table V), but this group generally included the nontetrafossate species *Amurotaenia decidua* (Nippotaeniidea). The tetraphyllidean species, *Rhinebothrium maccallumi,* however, exhibited considerable instability in its phylogenetic placement and was most often the sole taxon responsible for refuting the monophyly of a tetrafossate clade. Within the tetrafossate clade was consistently found a clade consisting of the cyclophyllidean, nippotaeniidean, and tetrabothriidean taxa (Table V). Members of the other tetrafossate orders, Lecanicephalidea, Proteocephalidea, and Tetraphyllidea, typically formed either a sister clade (e.g., Fig. 3B) or formed the basal lineages of a larger tetrafossate clade (e.g., Figs. 2A, B, 3A).

The taxonomic affinities of the tetrabothriideans have been controversial (see Hoberg et al., 1997, 1999a, 1999b). Commonly, this group has been considered to be either closely related to, or actual members of, the order Cyclophyllidea (Fuhrmann, 1931; Wardle and McLeod, 1952; Yamaguti, 1959; Dubinina, 1980; Schmidt, 1986). This affiliation is based in part on their shared possession of a compact vitellarium (shared also by nippotaeniids) and because both groups parasitize tetrapod definitive hosts. Others, however, have argued that the tetrabothriideans are most closely allied to the Tetraphyllidea. This affiliation is based in part on the presumed homology between the "bothridia" and accessory suckers found on the scolices of members of both groups (Euzet, 1959). In addition, Hoberg (1987, 1994) and Brooks et al. (1991) have suggested that members of both groups share a homologous pattern of scolex morphogenesis in their final larval stage. Support for a sister group relationship between the tetrabothriideans and the cyclophyllideans, however, was argued subsequently by Hoberg et al. (1997, 1999a, 1999b; Fig. 1A). This was also supported by 50% consensus of the EPTs in the analysis of Mariaux (1998), but not by strict consensus (Fig. 1B). In the present study, all data partitions and methods of analysis strongly supported a clade consisting of the cyclophyllidean, nippotaeniidean, and tetrabothriidean taxa. Although the exact branching order of the 3 taxa was not universally supported, it nonetheless seems clear from these results that the tetrabothriideans are closer to the order Cyclophyllidea than to the Tetraphyllidea. Among the eucestodes, only cyclophyllideans and tetrabothriideans utilize tetrapods as their primary host group; the remaining eucestodes and their relatives are predominantly parasites of fishes. The derived position of these 2 orders within the Eucestoda is consistent from a phylogenetic perspective with the more recent origins of the major tetrapod groups relative to the origins of the fishes.

In the past, the nippotaeniideans have generally been thought to occupy a basal position within the Eucestoda owing largely to their "primitive" scolex, which consists of a single terminal sucker. Freeman (1973), for example, considered them to have evolved directly from the protocestode stalk. Both Yamaguti (1959) and Brooks et al. (1991) postulated a position of the nippotaeniideans intermediate between the more basal Pseudophyllidea and the higher tetrafossate orders. Analysis by Hoberg et al. (1997) supported the monophyly of a derived clade including the nippotaeniideans together with the cyclophyllideans and tetrabothriideans. The results presented here corroborate the Nippotaeniidea as a derived group of tapeworms. In this case, the simple scolex of the adult nippotaeniidean is likely to represent either a reversal to a plesiomorphic condition or a larval condition retained in the adult form resulting from paedomorphic development.

The phylogenetic position of the proteocephalideans has been controversial because of their morphological similarity to both cyclophyllideans and tetraphyllideans. Like the cyclophyllideans, they possess a scolex with 4 suckers, but they have a proglottid morphology so similar in the arrangement of the male and female organs to that of tetraphyllideans that they are often

indistinguishable. Unlike either the cyclophyllideans (parasites of tetrapods) or tetraphyllideans (parasites of elasmobranchs), the proteocephalideans are primarily parasites of freshwater fishes, although some members of the family Proteocephalidae occur in reptiles and amphibians (Rego, 1994). Thus, their host associations do not support a close affinity to either cyclophyllidean or tetraphyllidean tapeworms. Understandably, there has been disagreement as to the phylogenetic position of group. Fuhrmann (1931) considered the proteocephalideans to be closest to the Tetraphyllidea, as did Euzet et al. (1981). However, Euzet (1959), Freeman (1973), Jarecka (1975), and Dubinina (1980) postulated a proteocephalidean origin of the Cyclophyllidea, thus making the proteocephalideans paraphyletic. Brooks et al. (1991) found the proteocephalideans to be the sister group of the cyclophyllideans. Hoberg et al. (1997) showed them to be the sister group to the clade including the Cyclophyllidea, Nippotaeniidea, and Tetrabothriidea, with the orders Tetraphyllidea and Lecanicephalidea forming the basal lineages of a larger tetrafossate clade (Fig. 1A). The analysis of Mariaux (1998) showed weak support for a sister group relationship between the orders Proteocephalidea and Diphyllidea (Fig. 1B).

All data partitions and methods of analysis in the present study showed strong support for the position of the proteocephalidean *Proteocephalus perplexus* closest to the tetraphyllidean species *Anthobothrium laciniatum, Calliobothrium* sp., and *Platybothrium auriculatum.* Furthermore, the majority of analyses showed these 4 taxa to form a monophyletic group (Table V). No analysis supported a close relationship between the proteocephalidean and the Cyclophyllidea. The placement of the proteocephalidean among the 6 tetraphyllidean taxa suggests that the ancestral hosts of the group may have been elasmobranchs, in which case, the group's present association with teleost definitive hosts represents a secondary colonization.

It has been suggested that the large and diverse order Tetraphyllidea represented the progenitor of the other tetrafossate orders (Euzet, 1959; Freeman, 1973). Taxonomically, the order has been, and remains, poorly defined. Paraphyly of the group has been suggested previously by Euzet et al. (1981) and was shown by the cladistic analyses of Brooks et al. (1991), Hoberg et al. (1997), and Caira et al. (1999) based on morphological data and by Mariaux (1998) based on partial sequences of 18S rDNA. Two representatives each of 3 of the 7 families of the Tetraphyllidea as treated by Euzet (1994b) were included in the analyses herein, including representatives of the 2 major families, the Onchobothriidae and Phyllobothriidae, and the minor family, Litobothriidae. Monophyly of the Tetraphyllidea was not supported by any of the analyses (Table V). This was due not only to the inconsistent placement of *Rhinebothrium maccallumi* outside of the group, but also to the placement of the proteocephalidean *Proteocephalus perplexus* within a clade including the tetraphyllidean species *Anthobothrium laciniatum, Calliobothrium* sp., and *Platybothrium auriculatum.* Analyses of the 18S rDNA data partition supported a basal position of *R. maccallumi,* with respect to the other phyllobothriid and onchobothriid taxa, but still placed the taxon within the tetrafossate clade. Its position outside of a tetrafossate clade and its grouping with the diphyllidean taxon *Macrobothridium* sp. based on the *Ef-1α* data partitions (Figs. 3A, B, 4A) is highly questionable and may have been influenced by its relatively divergent *Ef-1α* sequence (Fig. 3B). The taxonomically problematic genus *Rhinebothrium* deserves further consideration using molecular data because it may well represent one of the early lineages of the Tetraphyllidea. Although the cladistic analysis of Caira et al. (1999) showed the Tetraphyllidea to be paraphyletic, monophyly of the family Onchobothriidae was supported. In the present study, however, monophyly of the 2 onchobothriid species *Calliobothrium* sp. and *P. auriculatum* was supported only by ME analyses of 18S rDNA and by parsimony analysis of Ef-1α amino acid sequences (Table V). Other analyses showed the family to be paraphyletic. Monophyly of the 2 phyllobothriid species *A. laciniatum* and *R. maccallumi* was not supported by any of the analyses in the present study (Table V).

The tetraphyllidean family Litobothriidae was erected originally as a distinct order by Dailey (1969). Its ordinal status was recognized subsequently by Wardle et al. (1974) and Schmidt (1986). However, Brooks et al. (1991) considered this group to be tetraphyllidean, and Euzet (1994b) formally moved the litobothriideans to the order Tetraphyllidea, erecting the new family Litobothriidae to house them. Following the classification of Euzet (1994b), the ordinal-level analyses of Hoberg et al. (1997) and Mariaux (1998) also considered the litobothriideans to be members of the Tetraphyllidea. Thus, the phylogenetic position of the litobothriideans has not been demonstrated previously. Monophyly of the litobothriidean species *Litobothrium alopias* and *Renyxa amplifica* was supported by all analyses in the present study (Table V). Together, these taxa generally formed the basal lineage of a clade including the lecanicephalidean, proteocephalidean, and tetraphyllidean taxa, minus *R. maccallumi.* However, in a few analyses they were separated from the other tetraphyllidean taxa by the lecanicephalideans. From both a morphological and genetic perspective, the litobothriideans appear to be as distinct from the more traditional members of the Tetraphyllidea as do the lecanicephalideans, and ordinal status of the Litobothriidea may be warranted at least until a monophyletic assemblage of "tetraphyllideans" can be better circumscribed.

A close relationship between the Lecanicephalidea and Tetraphyllidea has long been recognized. Nonetheless, most workers have considered the lecanicephalideans to represent a distinct lineage (Wardle and McLeod, 1952; Yamaguti, 1959; Freeman, 1973; Dubinina, 1980; Schmidt, 1986; Khalil et al., 1994). Present analyses support the lecanicephalideans as members of the clade including the Tetraphyllidea and Proteocephalidea, although they were occasionally found to form the basal lineages of a clade including the cyclophyllidean, nippotaeniidean, and tetrabothriidean taxa (Figs. 3A, 4A). In the analyses herein, the lecanecephalideans, like the litobothriideans, are positioned close to, but outside of the Tetraphyllidea, the position of *Rhinebothrium maccallumi* notwithstanding. These results also confirm the taxonomic position of *Eniochobothrium gracile,* listed as incerte sedis by Euzet (1994a), as being within the order Lecanicephalidea.

Utility of 18S rDNA and *Ef-1α* data for cestode systematics

Analysis of both the 18S rDNA and *Ef-1α* sequence data showed strong support for relationships among species closely related taxonomically (e.g., *Diphyllobothrium stemmacephalum*

and *Schistocephalus solidus*) and for nodes uniting the more recently diverged major lineages (e.g., Cyclophyllidea, Nippotaeniidea, and Tetrabothriidea). Nodes separating basal lineages were typically weak, and this lack of character support was predominately responsible for the differences in results found among the 3 methods of analysis (Table V). Neither the complete 18S rDNA sequences nor the partial *Ef-1α* sequences appeared to be significantly more informative, and separate analyses resulted in similar patterns of nodal support, both weak and strong. Perhaps the primary difference in the phylogenetic content of the genes is related to the distribution of variable sites. Among-site variation in *Ef-1α* was largely constant, with only 2 short coding regions that showed higher levels of variation, including insertions and deletions, and 1 intron region (Appendix B). Typical of the 18S rDNA gene, however, variation was highly skewed with a majority of sites strongly conserved and, interspersed among them, distinct regions showing variation at all taxonomic levels. By and large, both genes appeared to do well at the level of order; that is, nodal support grouping representatives of the same order was generally strong. Conversely, implications of paraphyly were strongly supported as well. This was also evident from the analysis of 18S rDNA by Mariaux (1998). Indeed, it may be in the circumscription of natural groups at the ordinal level that data from these genes will be most valuable in cestode systematics. This will require a considerably broader sampling of taxa than presently available for molecular analysis (however, see Mariaux (1998) with regard to the Cyclophyllidea).

Within orders, present data are insufficient for most groups to evaluate their utility at this taxonomic level, although it is likely that it will depend greatly on the order in question. Using the same conserved regions of the 18S rDNA gene, Mariaux (1998) was able to achieve significant resolution among species of cyclophyllideans, whereas no resolution was obtained among species of proteocephalideans. Moreover, comparison of partial 18S rDNA sequences of a large number of tetraphyllidean taxa (Olson et al., in press) shows the differences in the level of variation among genera to be similar to that among eucestode orders.

There is no doubt that great disparity exists in the ages and degrees of divergence both within and among the major lineages of tapeworms, and such extremes are unlikely to be encompassed by the phylogenetic content of any single gene locus. Still, current results indicate that the early radiation of the basal lineages may have evolved in a relatively short period of time, insufficient to have left behind a large number of phylogenetically informative characters, either molecular or morphological. It is necessary, then, that specific gene loci be targeted for recovering more restricted branching patterns, such as that of the basal lineages and of more problematic taxa, such as Diphyllidea, Trypanorhyncha, and the larger Tetraphyllidea, including Lecanicephalidea, and Proteocephalidea. Knowledge from a combination of gene loci may eventually enable the construction of a "super tree" (Wilkinson and Thorley, 1998), in which compatible components are linked to form a complete phylogeny for the Cestoidea, well supported across basal and distal nodes alike.

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APPENDIX A—18S RIBOSOMAL DNA ALIGNMENT

Shading indicates stem regions consistent with the secondary structural hypothesis and numbering system of Neefs et al. (1990). In cases where stems are interspersed by nonpairing sites, continuous shading of the first sequence indicates continuity of the stem. In cases where adjacent stem regions are contiguous, vertical lines separate the boundaries of the adjacent stems. Variable regions (V1–V9) are marked by bars (note that the V6 region is absent from eukaryotic small-subunit rDNA). Dashes (-) indicate alignment gaps; question marks (?) indicate undetermined or ambiguous character states; dots (.) indicate character states identical to those of the first sequence. Asterisks (*) below the last sequence indicate sites removed from the analyses. Sequences are numbered as follows:

1. *Pseudomurraytrema* sp. (Monogenea: Monopisthocotylidea)
2. *Polystomoides malayi* (Monogenea: Polypisthocotylidea)
3. *Schizochoerus liguloideus* (Cestoidea: Amphilinidea)
4. *Gyrocotyle rugosa* (Cestoidea: Gyrocotylidea)
5. *Spathebothrium simplex* (Cestoidea: Spathebothriidea)
6. *Hunterella nodulosa* (Cestoidea: Caryophyllidea)
7. *Diphyllobothrium stemmacephalum* (Cestoidea: Pseudophyllidea)
8. *Schistocephalus solidus* (Cestoidea: Pseudophyllidea)
9. *Haplobothrium globuliforme* (Cestoidea: Haplobothriidea)
10. *Tentacularia* sp. (Cestoidea: Trypanorhyncha)
11. *Hepatoxylon* sp. (Cestoidea: Trypanorhyncha)
12. *Macrobothridium* sp. (Cestoidea: Diphyllidea)
13. *Echinobothrium fautleyae* (Cestoidea: Diphyllidea)
14. *Renyxa amplifica* (Cestoidea: Tetraphyllidea)
15. *Litobothrium alopias* (Cestoidea: Tetraphyllidea)
16. *Eniochobothrium gracile* (Cestoidea: Lecanicephalidea)
17. *Cephalobothrium* cf. *aetobatidis* (Cestoidea: Lecanicephalidea)
18. *Rhinebothrium maccallumi* (Cestoidea: Tetraphyllidea)
19. *Platybothrium auriculatum* (Cestoidea: Tetraphyllidea)
20. *Calliobothrium* sp. (Cestoidea: Tetraphyllidea)
21. *Anthobothrium laciniatum* (Cestoidea: Tetraphyllidea)
22. *Proteocephalus prolixus* (Cestoidea: Proteocephalidea)
23. *Tetrabothrius forsteri* (Cestoidea: Tetrabothriidea)
24. *Hymenolepis diminuta* (Cestoidea: Cyclophyllidea)
25. *Amurotaenia decidua* (Cestoidea: Nippotaeniidea)

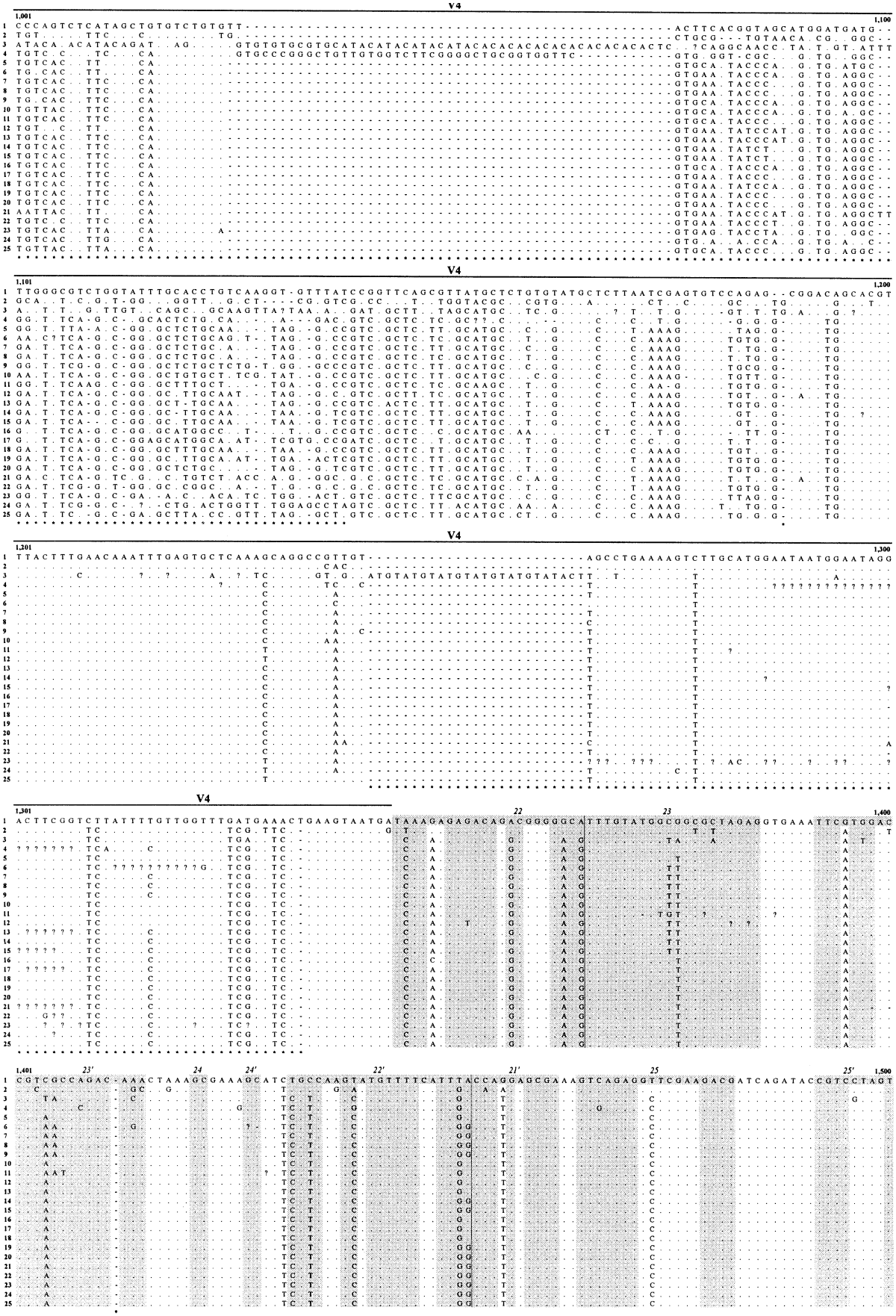
APPENDIX B—*ELONGATION FACTOR 1-α* ALIGNMENT

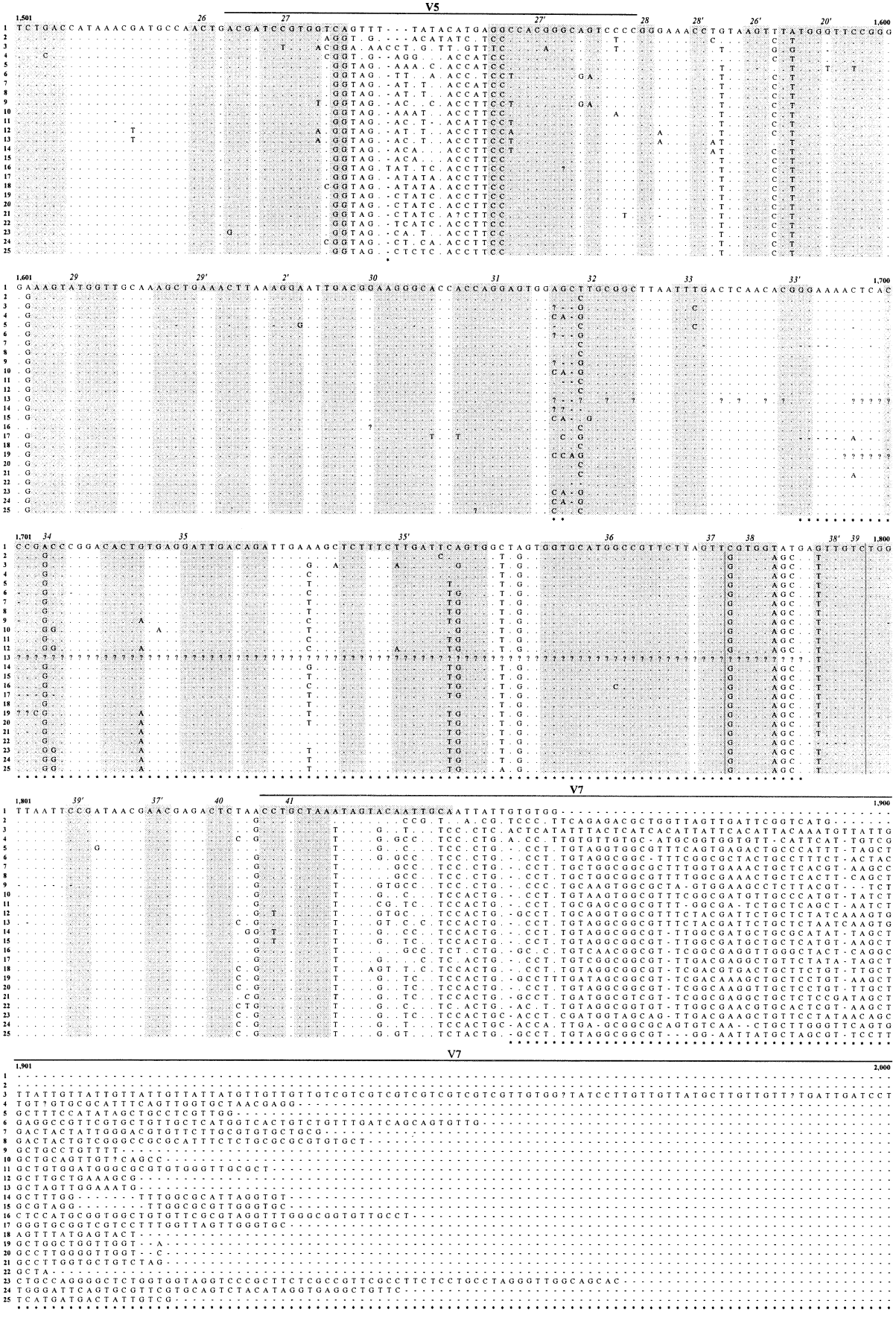
Shaded regions indicate positions excluded from analyses. Dashes (-) indicate alignment gaps; question marks (?) indicate undetermined or ambiguous character states; dots (.) indicate character states identical to those of the first sequence. Noncoding intron region indicated by a bar. Sequences are numbered as follows:

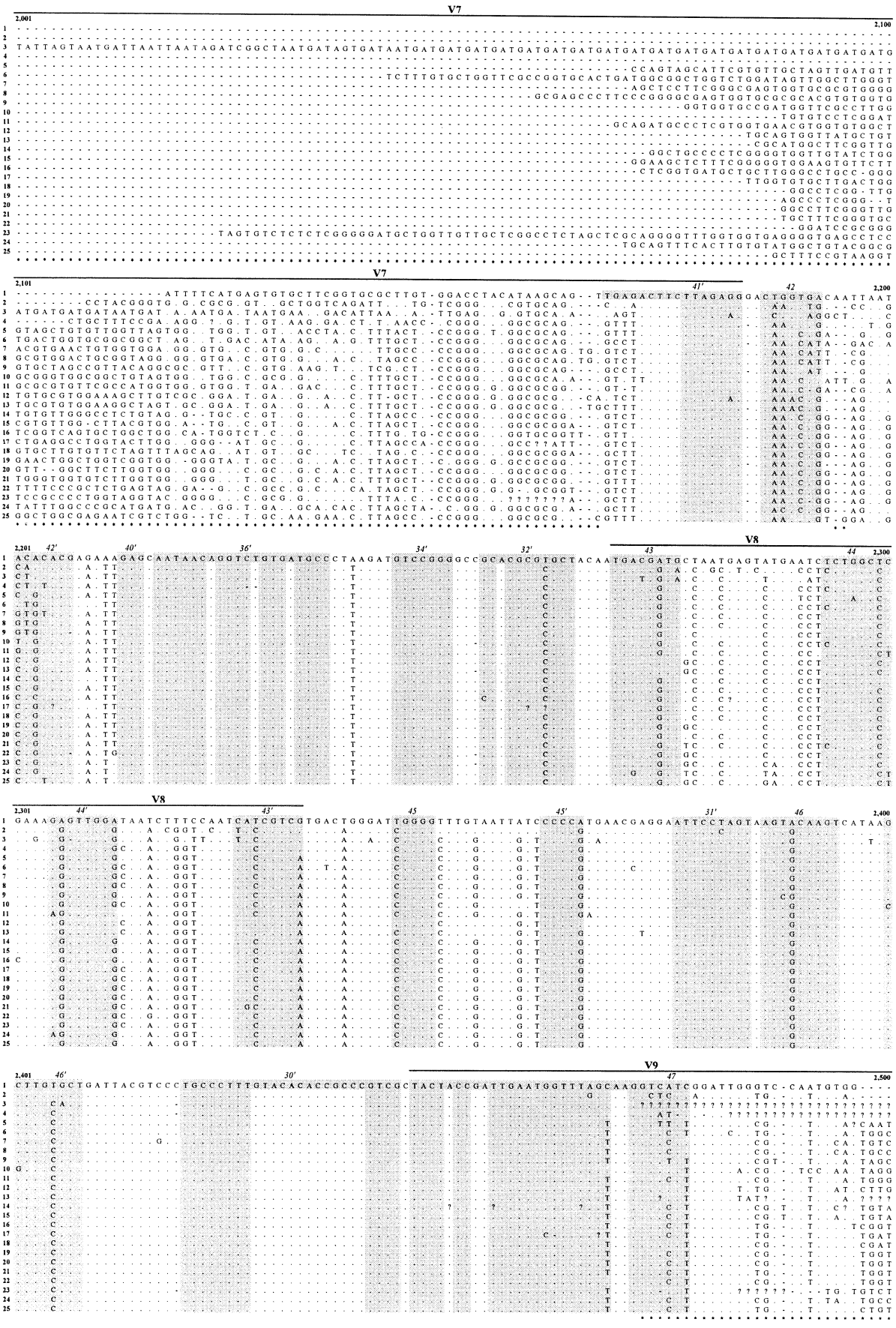
1. *Dugesia japonica* (Turbellaria: Tricladida)
2. *Schistosoma mansoni* (Digenea: Schistosomatidae)
3. *Neomicrocotyle pacifica* (Monogenea: Polypisthocotylidea)
4. *Schizochoerus liguloideus* (Cestoidea: Amphilinidea)
5. *Gyrocotyle rugosa* (Cestoidea: Gyrocotylidea)
6. *Spathebothrium simplex* (Cestoidea: Spathebothriidea)
7. *Hunterella nodulosa* (Cestoidea: Caryophyllidea)
8. *Diphyllobothrium stemmacephalum* (Cestoidea: Pseudophyllidea)
9. *Schistocephalus solidus* (Cestoidea: Pseudophyllidea)
10. *Haplobothrium globuliforme* (Cestoidea: Haplobothriidea)
11. *Tentacularia* sp. (Cestoidea: Trypanorhyncha)
12. *Hepatoxylon* sp. (Cestoidea: Trypanorhyncha)
13. *Macrobothridium* sp. (Cestoidea: Diphyllidea)
14. *Renyxa amplifica* (Cestoidea: Tetraphyllidea)
15. *Litobothrium alopias* (Cestoidea: Tetraphyllidea)
16. *Eniochobothrium gracile* (Cestoidea: Lecanicephalidea)
17. *Cephalobothrium* cf. *aetobatidis* (Cestoidea: Lecanicephalidea)
18. *Rhinebothrium maccallumi* (Cestoidea: Tetraphyllidea)
19. *Platybothrium auriculatum* (Cestoidea: Tetraphyllidea)
20. *Calliobothrium* sp. (Cestoidea: Tetraphyllidea)
21. *Anthobothrium laciniatum* (Cestoidea: Tetraphyllidea)
22. *Proteocephalus prolixus* (Cestoidea: Proteocephalidea)
23. *Tetrabothrius forsteri* (Cestoidea: Tetrabothriidea)
24. *Hymenolepis diminuta* (Cestoidea: Cyclophyllidea)
25. *Amurotaenia decidua* (Cestoidea: Nippotaeniidea)

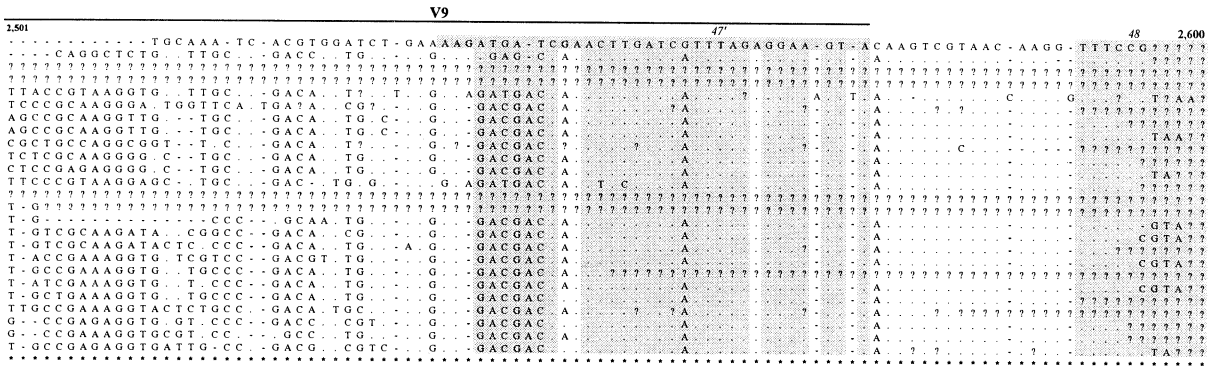
Appendix A: 18S ribosomal DNA alignment


V3
V4

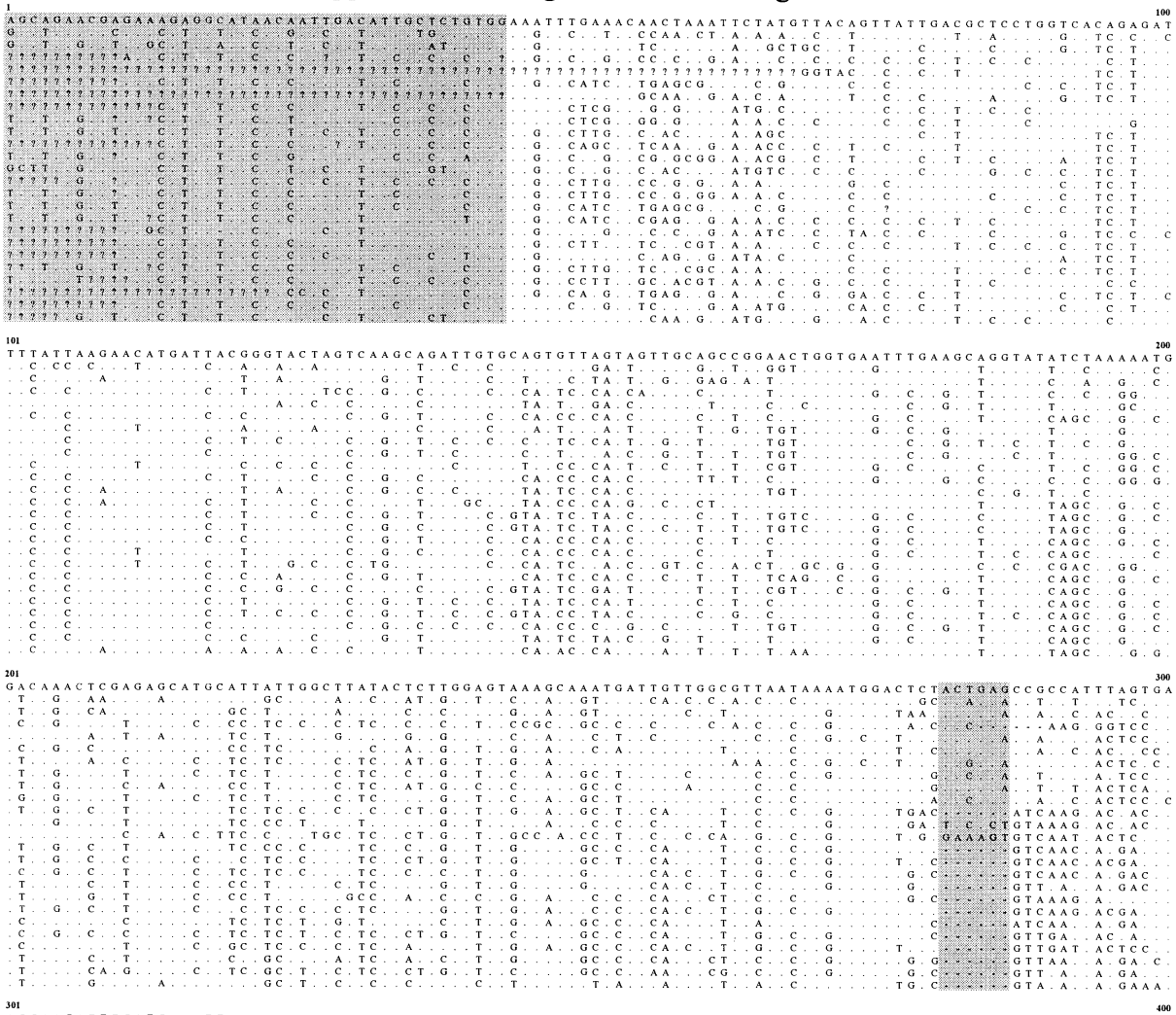

V4
V4
V4
V4

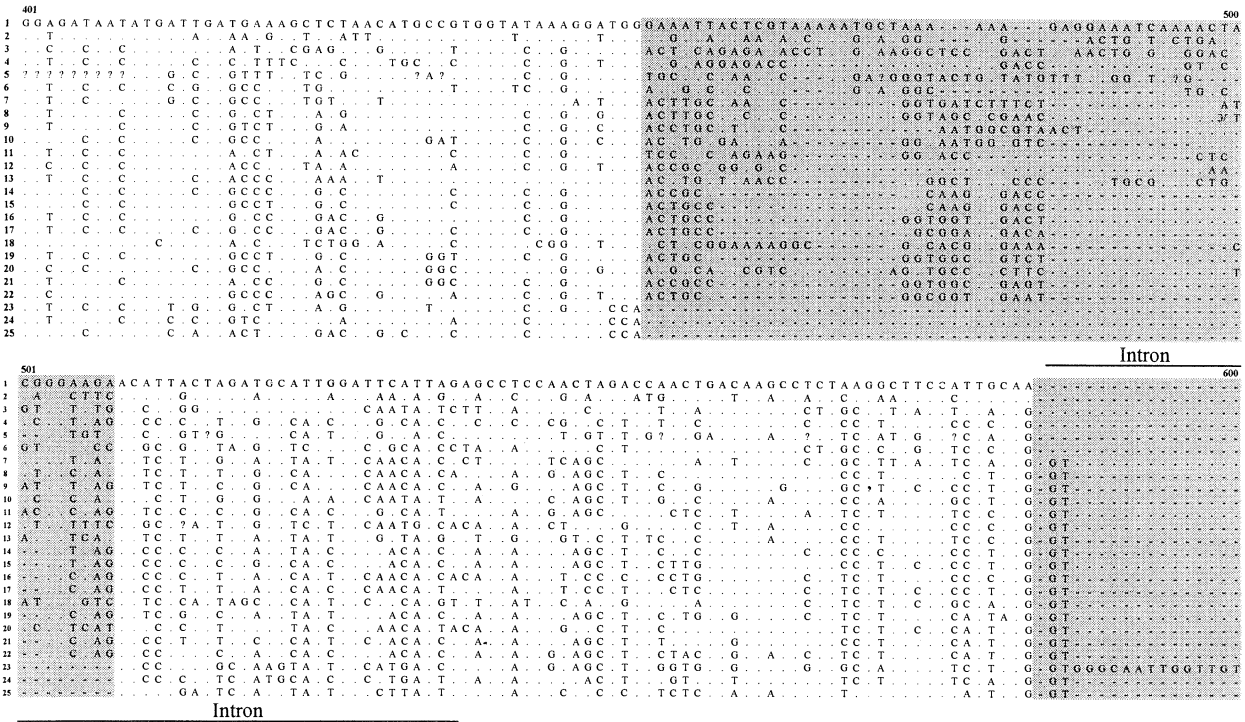

V5
V7
V7

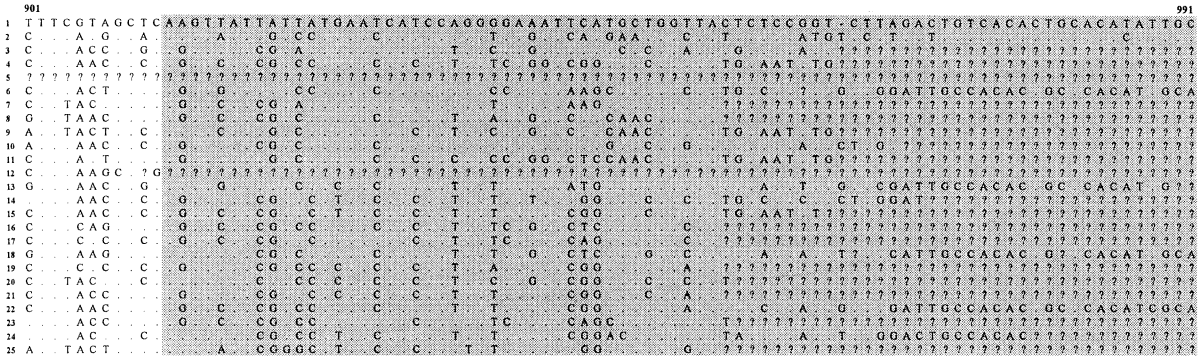

V7
V8
V9



Appendix B: Elongation factor-1a alignment




Intron
Intron


901
991