

# Ribosomal DNA and Phylogeny of the Ascaridoidea (Nemata: Secernentea): Implications for Morphological Evolution and Classification

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**Nematodes of the superfamily Ascaridoidea are parasites of the alimentary tract of vertebrates and include species that are of medical and economic importance. Existing evolutionary hypotheses for these organisms have frequently been based on interpretation of one or few "key" structural or life history features. We used nuclear-encoded small (1764 characters) and large sub-unit (757 characters) ribosomal DNA sequences to estimate the phylogeny of representative taxa from this superfamily. Trees inferred by maximum parsimony and maximum likelihood methods strongly support clades that are primarily consistent with one recent classification of the group. In contrast, most previously proposed phylogenetic hypotheses were significantly worse when compared to the maximum likelihood tree by a statistical method. Hypotheses for the evolution of morphological and life history characters were explored by parsimony mapping these features on several tree topologies, including optimal molecular trees and alternative topologies reflecting traditional expectations deemed not worse in statistical tests. The results identify some consistent putative shared-derived morphological features, but also strongly suggest that some key features emphasized by previous workers represent ancestral states or highly homoplastic characters.** © 1998 Academic Press

## INTRODUCTION

Nematodes of the superfamily Ascaridoidea (Secernentea: Ascaridida) have been studied extensively with respect to alpha-taxonomy (e.g., Davey, 1971; Sprent, 1977, 1978, 1979; Deardorff and Overstreet, 1979, 1981; Fagerholm and Gibson, 1987; Bruce and Cannon, 1990; Petter *et al.*, 1991; Bruce *et al.*, 1994), morphology (Snyder, 1985, 1989; Fagerholm, 1989, 1991; Hugot *et al.*, 1991; De and Dey, 1992), life cycles (Huizinga, 1967;

Klöser *et al.*, 1992; Køie and Fagerholm, 1995), geographic and host distribution (Fagerholm, 1988; Bratney and Ni, 1992; Bristow and Berland, 1992; Jensen *et al.*, 1994), population genetic structure (Paggi *et al.*, 1991; Anderson *et al.*, 1993; Nascetti *et al.*, 1993; Nadler, 1996), and host pathogenesis (Deardorff and Overstreet, 1980; Overstreet and Meyer, 1981). Many species, as juveniles or adults, are potential causative agents of disease in humans, domesticated animals, and wildlife (Beaver, 1956; Norris and Overstreet, 1976; Kazacos, 1986). Evolutionary hypotheses for the Ascaridoidea have frequently been based on consideration of one or few "key" structures or life history features (Gibson, 1983; Sprent, 1983). In some cases (Osche, 1963; Gibson, 1983), the assumption of host-parasite cophylogeny has been used as additional evidence to infer higher-level relationships among taxa. Most published evolutionary hypotheses for the Ascaridoidea have not been developed by phylogenetic methods, and conflicting proposals for transformation and polarity of morphological character-states are common among systematic treatments (Gibson, 1983; Sprent, 1983; Fagerholm, 1991). In part, phylogenetic analysis of these nematodes has been hindered by a paucity of morphological data, with less than 25 variable structural features currently defined for a superfamily with more than 50 genera (Fagerholm, 1991). In contrast, patterns of nuclear ribosomal-RNA variation seem promising for estimating relationships in this group (Nadler, 1992, 1995). However, the only detailed analysis to date (Nadler, 1992) included limited taxonomic diversity and few nucleotides sites, and was insufficient to discriminate statistically among certain alternative hypotheses of relationship.

Ascaridoid classifications and inferred patterns of character evolution have differed markedly among investigators (Mozgovoi, 1953; Hartwich, 1954, 1957, 1974; Osche, 1958, 1963; Yamaguti, 1961; Chabaud, 1965; Gibson, 1983; Sprent, 1983; Fagerholm, 1991). For example, two common points of contention are whether monoxenous (one-host) or heteroxenous trans-

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mission (life cycle) patterns are ancestral, and which vertebrates (i.e., aquatic versus terrestrial taxa) were the first definitive hosts. Classifications have varied according to the features emphasized by individual investigators. For example, Hsu (1933) emphasized the presence or absence of the ventriculus in forming two major groups [genera in terrestrial mammals (excepting *Toxocara*) versus genera in fish, birds, aquatic reptiles, and aquatic mammals], whereas Hartwich (1957) primarily used the structure of the secretory-excretory system in combination with the systematic framework of Chabaud (1965) to summarize higher-level categories of the superfamily in key form (Hartwich, 1974). Differences in the organization and ranking of groups above the genus level have also been common. For example, two of the four family groupings recognized by Hartwich (1974) and Gibson (1983) were considered to be either of questionable affinity or nonnatural by Sprent (1983, 1992) and were excluded from the classification of Fagerholm (1991). Instability of ascaridoid classifications is one consequence of the absence of a robust phylogenetic hypothesis.

In the present study, phylogenetic relationships among 20 ascaridoid taxa representing 14 genera were estimated using nuclear-encoded rDNA sequences, including near-complete SSU (1756 nt per taxon) and partial LSU (757 nt) sequences. Statistical methods were used to compare the gene tree inferred from maximum likelihood estimation to results obtained by other approaches, including maximum parsimony and hypotheses based on traditional expectations. Hypotheses of character evolution were explored by mapping structural and life history character states on estimates of organismal phylogeny inferred from both the rDNA analysis and topological alternatives to the rDNA trees representing "traditional" relationships that were not significantly worse in statistical tests.

## MATERIALS AND METHODS

### Taxa

Nineteen ascaridoid taxa (Nemata: Ascaridida, Ascaridoidea) representing (classification *sensu* Fagerholm, 1991) the Heterocheilidae (Railliet and Henry, 1912; *Heterocheilus tunicatus*), Raphidascarididae (Hartwich, 1954, rank *sensu* Fagerholm, 1991; *Goezia pelagia*, *Hysterothylacium fortalezae*, *Hysterothylacium pelagicum*, *Hysterothylacium reliquens*), Anisakidae (Railliet and Henry, 1912; *Anisakis sp.*, *Contraecaecum multipapillatum*, *Pseudoterranova decipiens*, *Terranova caballeroi*), Ascarididae [Baird, 1853; *Ascaris lumbricoides* (i.e., human source), *Ascaris suum* (i.e., pig source), *Baylisascaris procyonis*, *Baylisascaris transfuga*, *Parascaris equorum*, *Porrocaecum depressum*, *Toxocara canis*, *Toxascaris leonina*], and *Itheringascaris iniquus* (genus not included by Fagerholm, 1991) were studied. In addition to the published sequence data for the

trichostrongylid nematode *Haemonchus similis* (Zarlenga *et al.*, 1994a; L04152) and the Adenophorean *Plectus sp.* (Baldwin *et al.*, 1997; U61761), *Cruzia americana* (Acaridida: Cosmocercoidea) was sequenced as a more closely related outgroup for the Ascaridoidea (Inglis, 1965). Host (definitive or other = intermediate/paratenic) and collection sites for these taxa included: *H. tunicatus*, *d = Trichechus manatus*, Citrus County, FL; *G. pelagia*, *d = Chaetodipterus faber*, MS Gulf Coast, East Ship Island; *H. fortalezae*, *d = Lutjanus campechanus*, MS Gulf Coast, 25 mi S Horn Island; *H. pelagicum*, *d = Coryphaena hippurus*, MS Gulf Coast, off barrier islands; *H. reliquens*, *o = Micropogonias undulatus*, Davis Bayou, Ocean Springs, MS; *Anisakis sp.*, *o = rockfish*, Northern CA coast; *C. multipapillatum*, *o = Mugil curema*, Grand Lagoon, Horn Island, MS; *P. decipiens*, *o = Myoxocephalus scorpius*, Dantzic Point, Burin Peninsula, Newfoundland; *T. caballeroi*, *d = Nerodia cyclopion*, Hammond, LA; *A. lumbricoides*, *d = Homo sapiens*, Covington, LA; *A. suum*, *d = Sus scrofa*, Cassopolis, MI; *B. procyonis*, *d = Procyon lotor*, River Ridge, LA; *B. transfuga*, *d = Ursus americana*, Pocahontus Co., West Virginia; *P. equorum*, *d = Equus caballus*, Baton Rouge, LA; *P. depressum*, *d = Strix varia*, Baton Rouge, LA; *T. canis*, *d = Canis familiaris*, DeKalb, IL; *T. leonina*, *d = Vulpes vulpes*, Brookings, SD; *I. iniquus*, *d = Rachycentron canadum*, near Petit Bois oil rig, MS; and *C. americana*, *d = Didelphis virginiana*, Hammond, LA.

### DNA Amplification and Sequencing

Total nucleic acids were extracted from frozen ( $-70^{\circ}\text{C}$ ) tissue samples of dissected body wall muscle (larger species), or from individual or pooled samples of whole adults or juveniles (as indicated). When feasible, nematode intestines were removed prior to tissue digestion. Tissues were homogenized in TE buffer (pH 8.0) and digested using Proteinase K (0.8  $\mu\text{g}/\mu\text{l}$  final concentration) at  $50^{\circ}\text{C}$  until only the cuticle remained. Total nucleic acids were obtained from the supernatant using a standard phenol-chloroform enrichment, ethanol/ammonium acetate precipitation procedure (Ausubel *et al.*, 1989). The resulting pellet was resuspended and treated with 50  $\mu\text{g}$  of RNase A (1 h at  $37^{\circ}\text{C}$ ), and total DNA was recovered by reprecipitation.

Small subunit (SSU) rDNA was amplified and sequenced using several different methods. Internal (numbering according to *C. elegans*; Ellis *et al.*, 1986, X03680) PCR primers (937–955 >CCCGATTGATTCTGTCCGC; 2667–2690 <TGATCCTTCTGCAGGTTACCTAC) were designed to amplify the 18S region based on an alignment of published near-complete or partial sequences for *C. elegans* (X03680), *Nematodirus battus* (Zarlenga *et al.*, 1994a, U01230), *Haemonchus contortus* (Zarlenga *et al.*, 1994b, L04153), *Strongyloides stercoralis* (Putland *et al.*, 1993, M84229), and *A. suum* (Neuhaus *et al.*, 1987, X06225). For amplification of the SSU rDNA, an

initial DNA denaturation at 94°C for 4 min was followed by 25 cycles of the polymerase chain reaction (PCR with Taq polymerase; 94°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min), followed by a postamplification extension for 5 min at 72°C. PCR conditions (annealing temperature, number of cycles, amount of starting DNA template) were adjusted empirically to improve the specificity of the reaction and quality of the sequencing results. For some taxa, the SSU was amplified in two overlapping fragments; this frequently reduced the number of ambiguities obtained from direct sequencing reactions of PCR products. In these cases, the 5' end was amplified using 937–955 >CCCGATTGATTCTGTCGGC; 2003–2020 <CAACCATACTTCCCCCGG (25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min 10 s), and the 3' fragment was amplified using 1301–1324 >CGGAGAGGGAGCCTGAGAAACGGC; 2667–2690 <TGATCCTTCTGCAGGTTACCTAC, with PCR conditions as described for the amplification of the entire SSU. PCR products were purified by agarose gel electrophoresis in TAE buffer, pH 8.0 (Ausubel *et al.*, 1989) and product of the appropriate size was recovered from gel slices using GeneClean II (Bio 101) or QIAEX (Qiagen) extractions. Purified PCR templates were quantified by fluorometry using Hoechst 33258 dye and a Hoefer TKO 100 minifluorometer with TKO 130 capillary cuvette. Fifty to 400 fmol of PCR product was used per sequencing reaction; higher amounts of template usually resulted in fewer ambiguous nucleotides per reaction. For most taxa, direct sequencing of SSU PCR products yielded unambiguous sequence when the template:primer ratio was optimized. This suggests that multiple copies of the PCR-amplified SSU rDNA genes are highly similar or identical in sequence within individuals; this result was supported by sequences obtained from cloned SSU PCR product. For example, for taxa where direct sequencing did not work reliably, full-length SSU PCR product was washed by spin filtration (Millipore Ultrafree-MC 30,000 NMWL), ligated into pGEM-T vector (Promega), and cloned into DH5 $\alpha$  *Escherichia coli*. A minimum of two clones were sequenced (one for each strand), with additional clones used to resolve sequencing ambiguities as required. Typically, clones representing different strands displayed absolute complementarity. Sequencing reactions were performed using the Delta-Taq cycle sequencing kit (Amersham) and [ $\alpha$ -<sup>32</sup>P]ATP (sp act >3000 Ci/mmol), with annealing temperatures for the labeling and termination steps adjusted empirically for each sequencing primer. Seven-deaza-dGTP was used in cycle and termination mixes to reduce gel compression artifacts resulting from template secondary structure. A total of 13 primers were used to obtain complete double-stranded sequence for the SSU region; all primer sequences are available from the authors. SSU sequences are deposited in GenBank (U94365–U94383).

Large subunit (LSU) rDNA sequences were obtained

by TA-cloning of PCR products as described previously. Internal LSU PCR primers (numbering by *C. elegans* rDNA, 3745–3764 >AGCGGAGGAAAAGAACTAA; 4359–4377 <ATCCGTGTTTCAAGACGGG) were designed based on an alignment of complete or partial sequences for *C. elegans* (X03680), *Onchocerca gibsoni* (Qu *et al.*, 1986, M15308), *Brugia pahangi* (Qu *et al.*, 1986, M15409), and *Nematospiroides dubius* (Qu *et al.*, 1986, M15310). These primers yielded a PCR product of approximately 782 bp among the Ascaridida. Double-stranded sequences were obtained using CsCl-purified plasmid DNA, custom-designed pGEM-T vector sequencing primers (pGEM-T 2974–2997 >GGCCAGTGAATTGTAATACGACTC; 110–135 <GACACTATA-GAATACTCAAGCTATGC), and two internal primers (>AGAGAGTTCAAGAGGGCGT; <AAGCTCTCAGGC-CATACC) that were designed based on sequence data obtained from the clones. A minimum of two clones were sequenced for each taxon (one for each strand); with two exceptions (below), these clones displayed absolute complementarity. For *Anisakis* sp. and *Contra-caecum multipapillatum*, which were amplified from extractions of pooled juvenile specimens, unexpected levels of LSU sequence differences were observed between clones during contig assembly. For these taxa, double-stranded sequence was obtained for multiple clones, and the clones were treated separately in subsequent analyses. LSU sequences are deposited in GenBank (U94749–U94769).

#### Sequence Analysis

SSU sequences were aligned to the secondary structure model of *Haemonchus similis* using the Ribosomal Data Base Project ALIGN SEQUENCE program (Maidak *et al.*, 1994). LSU sequences were aligned initially using CLUSTAL V (Higgins *et al.*, 1992), and the resulting output was adjusted manually to increase sequence similarity. The effect of uncertainties in these multiple alignments on tree topology was explored by producing a second data matrix that excluded regions of potential ambiguity (six SSU sites and 13 LSU sites of 2513 sites in the combined SSU and LSU data). Invariant regions corresponding to the flanking PCR primers were excluded from all analyses. Aligned sequences and inferred trees have been deposited in the TreeBase (<http://herbaria.harvard.edu/treebase/>) database (Sanderson *et al.*, 1994).

The aligned data were analyzed by two character-state methods: maximum parsimony (MP) using PAUP (Version 3.1.1; Swofford, 1993) and maximum likelihood (ML) using PAUPSTAR (Test Version 4d.54). Although SSU sequences were analyzed separately due to the availability of additional outgroups, LSU and SSU data were ultimately combined since the nuclear rDNA array is inherited as a composite transcription unit. For parsimony analyses, inferred gaps were recoded conservatively and added to the end of the matrix

**TABLE 1**  
**Morphological and Life History Characters and States for Ascaridoid Taxa Studied**

	<i>H. tunicatus</i>	<i>P. equorum</i>	<i>A. lumbricoides</i>	<i>A. suum</i>
Median papilla	Single	2-Joined	Single	Single
Paracloacal papillae	Separate	Joined double	Joined double	Joined double
Distal papillae	2 Pair	4 Pair	4 Pair	4 Pair
Distal papillae	?	Distal united	Distal united	Distal united
Proximal papillae	Straight rows	Grouped anterior	Grouped posterior	Grouped posterior
Spicules	Alate	Rod	Rod	Rod
Gubernaculum	Present	Absent	Absent	Absent
Caudal plates	Absent	Absent	Absent	Absent
Caudal alae	Absent	Absent	Absent	Absent
Definitive host	Mammal	Mammal	Mammal	Mammal
Ventriculus	Short	Absent	Absent	Absent
Intestinal caecum	Present	Present	Absent	Absent
Lips	Prominent	Prominent	Prominent	Prominent
Lip denticles	Absent	Present	Present	Present
Interlabia	Absent	Present	Absent	Absent
Excretory pore	Between nr <sup>a</sup> and lips	Near nr	Near nr	Near nr
Excretory system	Left-right filamental	Left-right filamental	Left-right filamental	Left-right filamental
Cervical alae	Absent	Absent	Absent	Absent
Life history	Aquatic	Terrestrial	Terrestrial	Terrestrial
Monoxenous cycle	?	Present	Present	Present
Eggshell	?	Thick-walled	Thick-walled	Thick-walled
Ventricular appendix	Absent	Absent	Absent	Absent
Proximal papillae	<6 Pairs	>50 Pairs	>50 Pairs	>50 Pairs
	<i>B. procyonis</i>	<i>B. transfuga</i>	<i>T. leonina</i>	<i>T. canis</i>
Median papilla	2-Joined	2-Joined	Single	Single
Paracloacal papillae	Joined double	Joined double	Joined double	Joined double
Distal papillae	4 Pair	4 Pair	4 Pair	4 Pair
Distal papillae	Distal united	Distal united	Distal unjoined	Distal unjoined
Proximal papillae	Grouped anterior	Grouped anterior	Straight rows	Straight rows
Spicules	Rod	Rod	Rod	Alate
Gubernaculum	Absent	Absent	Absent	Absent
Caudal plates	Absent	Absent	Absent	Absent
Caudal alae	Absent	Absent	Absent	Absent
Definitive host	Mammal	Mammal	Mammal	Mammal
Ventriculus	Absent	Absent	Absent	Short
Intestinal caecum	Absent	Absent	Absent	Present
Lips	Prominent	Prominent	Prominent	Prominent
Lip denticles	Present	Present	Present	Present
Interlabia	Present	Present	Absent	Absent
Excretory pore	Near nr	Near nr	Near nr	Near nr
Excretory system	Left-right filamental	Left-right filamental	Left-right filamental	Left-right filamental
Cervical alae	Narrow	Narrow	Wide	Wide
Life history	Terrestrial	Terrestrial	Terrestrial	Terrestrial
Monoxenous cycle	Present	Present	Present	Present
Eggshell	Thick-walled	Thick-walled	Thick-walled	Thick-walled
Ventricular appendix	Absent	Absent	Absent	Absent
Proximal papillae	>50 Pairs	>50 Pairs	6-50 Pairs	>50 Pairs
	<i>P. depressum</i>	<i>Anisakis sp.</i>	<i>P. decipiens</i>	<i>C. multipapillatum</i>
Median papilla	Single	Single	?	Single
Paracloacal papillae	Joined double	Joined double	Joined double	Separate
Distal papillae	4 Pair	4 Pair	4 Pair	4 Pair
Distal papillae	Distal unjoined	Proximal	Proximal	Distal unjoined
Proximal papillae	Straight rows	Straight rows	Straight rows	Straight rows
Spicules	Alate	Rod	Alate	Alate
Gubernaculum	Absent	Absent	Absent	Absent
Caudal plates	Absent	Present	Present	Absent
Caudal alae	Present	Present	Present	Absent
Definitive host	Bird	Mammal	Mammal	Bird
Ventriculus	Short	Long	Long	Short
Intestinal caecum	Present	Absent	Present	Present
Lips	Prominent	Reduced	Reduced	Prominent

TABLE 1—Continued

	<i>P. depressum</i>	<i>Anisakis sp.</i>	<i>P. decipiens</i>	<i>C. multipapillatum</i>
Lip denticles	Present	Present	Present	Absent
Interlabia	Present	Absent	Absent	Present
Excretory pore	Near nr	Between lips	Between lips	Between lips
Excretory system	Left-right filamental	Left-glandular	Left-glandular	Left-glandular
Cervical alae	Wide	Absent	Absent	Absent
Life history	Terrestrial	Aquatic	Aquatic	Aquatic
Monoxenous cycle	Absent	Absent	Absent	Absent
Eggshell	Thick-walled	Thin-walled	Thin-walled	Thin-walled
Ventricular appendix	Absent	Absent	Absent	Present
Proximal papillae	6–50 Pairs	>50 Pairs	?	>50 Pairs
	<i>G. pelagia</i>	<i>H. fortalezae</i>	<i>H. reliquens</i>	<i>H. pelagicum</i>
Median papilla	Single	Single	Single	Single
Paracloacal papillae	Joined double	Separate	Joined double	Separate
Distal papillae	4 Pair	4 Pair	4 Pair	4 Pair
Distal papillae	Subventral rows	Subventral rows	Subventral rows	Subventral rows
Proximal papillae	Straight rows	Straight rows	Straight rows	Straight rows
Spicules	Alate	Alate	Alate	Alate
Gubernaculum	Absent	Absent	Absent	Absent
Caudal plates	Absent	Absent	Absent	Absent
Caudal alae	Absent	Absent	Absent	Absent
Definitive host	Fish	Fish	Fish	Fish
Ventriculus	Short	Short	Short	Short
Intestinal caecum	Present	Present	Present	Present
Lips	Prominent	Prominent	Prominent	Prominent
Lip denticles	Absent	Absent	Absent	Absent
Interlabia	Absent	Present	Present	Present
Excretory pore	Near nr	Near nr	Near nr	Near nr
Excretory system	Left-filamental	Left-filamental	Left-filamental	Left-filamental
Cervical alae	Absent	Wide	Wide	Wide
Life history	Aquatic	Aquatic	Aquatic	Aquatic
Monoxenous cycle	Absent	Absent	Absent	Absent
Eggshell	Thin-walled	?	?	?
Ventricular appendix	Present	Present	Present	Present
Proximal papillae	6–50 Pairs	6–50 Pairs	6–50 Pairs	6–50 Pairs
	<i>I. iniquis</i>		<i>T. caballeroi</i>	
Median papilla	Single		2-Joined	
Paracloacal papillae	Joined double		Joined double	
Distal papillae	4 Pair		4 Pair	
Distal papillae	Subventral rows		?	
Proximal papillae	Straight rows		Straight rows	
Spicules	Alate		Alate	
Gubernaculum	Absent		Absent	
Caudal plates	Absent		Present	
Caudal alae	Absent		Absent	
Definitive host	Fish		Reptile	
Ventriculus	Short		Long	
Intestinal caecum	Present		Present	
Lips	Prominent		Reduced	
Lip denticles	Absent		Present	
Interlabia	Present		Absent	
Excretory pore	Near nr		Between lips	
Excretory system	Left-filamental		Left-glandular	
Cervical alae	Narrow		Absent	
Life history	Aquatic		Aquatic	
Monoxenous cycle	Absent		?	
Eggshell	?		?	
Ventricular appendix	Present		Absent	
Proximal papillae	6–50 Pairs		>50 Pairs	

Note. Missing data are indicated with a question mark. Most states were obtained from Fagerholm (1991).

<sup>a</sup> Nerve ring.

such that each unambiguous contiguous gap was represented as one character and "nucleotide present" was coded as the alternative character state (Swofford, 1993; Crandall and Fitzpatrick, 1996). All unrecoded gaps (the original inferred indels) in the data matrix were treated as missing data in the MP analyses. Maximum-parsimony searches were performed using heuristic methods (TBR, tree-bisection reconnection). Reported consistency indices do not include uninformative characters. Bootstrap MP trees (2000 replicates) were produced using heuristic searches and TBR branch-swapping with the MULPARS option. Due to computational limitations, a subset of taxa (12 or more) was used to estimate ti:tv and the shape of the gamma distribution ( $\alpha$ ) for the subsequent ML analysis of each complete data set. The shape of the gamma distribution ( $\alpha$ ), the proportion of variable sites, ti:tv, and the topology for 12 or more taxa (representing all clades) were estimated simultaneously by PAUPSTAR. For example, for the combined SSU plus LSU dataset, the taxa excluded from analysis were: *Anisakis* clone 2, *A. lumbricoides*, *B. procyonis*, *C. multipapillatum* clone 3, *H. fortalezae*, *H. pelagicum*, *H. reliquens*, *P. depressum*, and *T. caballeri*. Subsequent ML searches using all taxa were performed using these estimates of ti:tv and  $\alpha$  (formula of Yang and Kumar, 1996) whereas the program was allowed to estimate the proportion of invariable sites, employing the Hasegawa-Kishino-Yano ML model with rate heterogeneity. Starting trees for ML searches were obtained by neighbor-joining, and heuristic searches were performed with TBR branch-swapping. The statistical significance of alternative tree topologies was assessed using the test of Kishino and Hasegawa (1989) as implemented by PAUPSTAR. Parameters of the likelihood model estimated previously (ti:tv,  $\alpha$ ) were employed for the tests, whereas the proportion of invariable sites was a parameter estimated by the program.

Morphological and life history data (Fagerholm, 1991; Gibson, 1983) were coded as unordered states; 15 of these characters were binary and 8 multistate (Table 1). When characters included in the analysis were polymorphic within a genus, states specific for the species sequenced were used. Hypotheses of character evolution were explored by mapping (MacClade Version 3.05; Maddison and Maddison, 1992) character states on trees estimated from the sequence data or on certain "traditional" alternative topologies that were not significantly worse in ML tests. The influence of the morphological characters (considered unordered) on the topology of the parsimony tree was explored by a combined MP analysis of molecular plus morphological characters. This analysis was limited to the Ascaridoidea because too many structural characters were not comparable for the most closely related outgroup taxon (*C. americana*). Rooting of the combined rDNA tree (SSU and LSU data) was based on results obtained from analyses of the SSU data with additional outgroups.

## RESULTS

The SSU dataset with the trichostrongylid outgroup consisted of 1795 characters (including 31 recoded gap characters), of which 1383 were invariant and 76 phylogenetically informative by parsimony criteria. Heuristic MP searches recovered six trees (length 526, C.I. = 0.58); the topology of the strict consensus of these trees (Fig. 1) does not conflict with the bootstrap MP tree for these data (Fig. 2). Estimates of ti:tv and the shape of the gamma distribution for this SSU dataset yielded values of 1.6468 and 0.823261, respectively. These estimated parameters were used in a heuristic ML search, which recovered three trees; the strict consensus of these ML trees (Fig. 3) is very similar to, but slightly more resolved than, the MP tree topologies. In all trees (six MP and one ML), the cosmoceroid (*C. americana*) is basal among the Ascaridida, and *H. tunicatus* (Heterocheilidae) is basal among the Ascaridoidea. Clades recovered in all MP, bootstrap

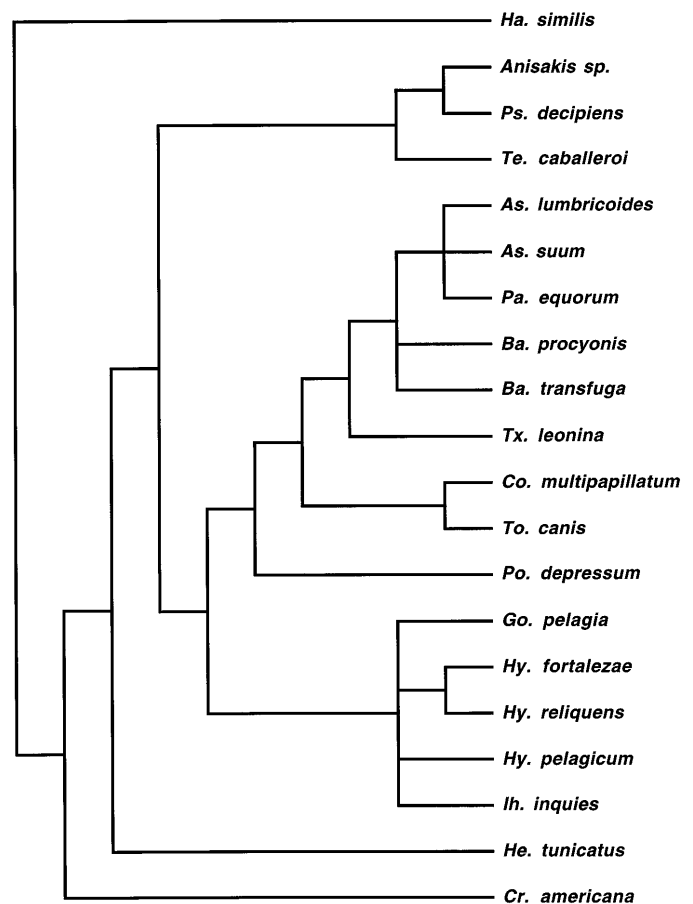
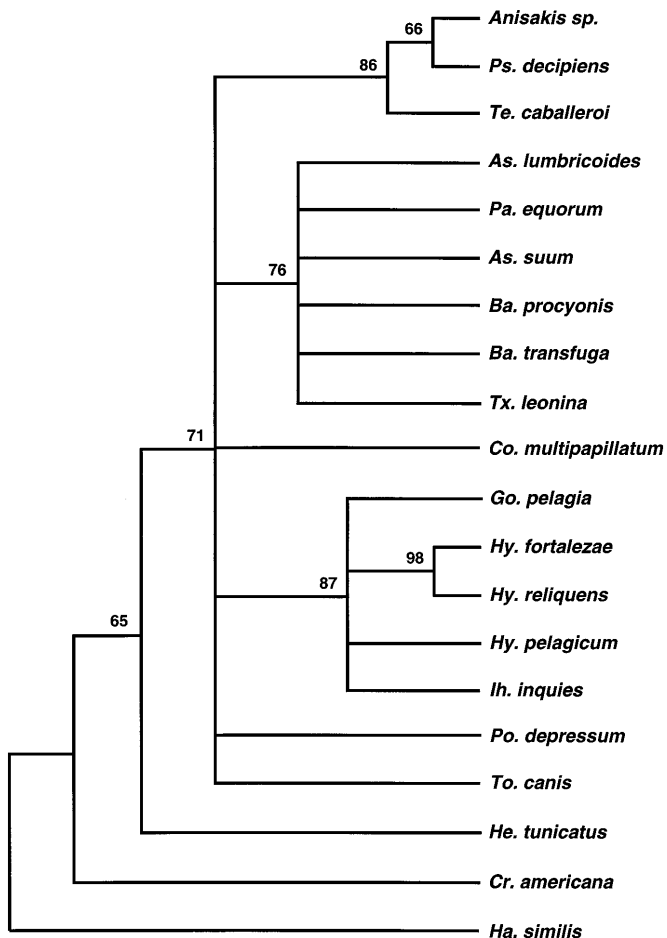


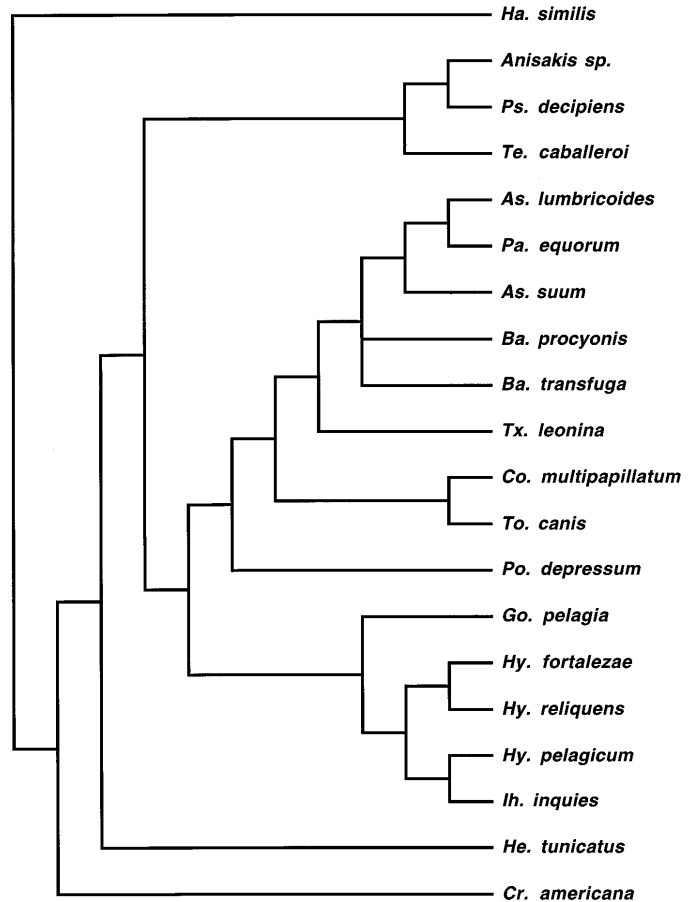
FIG. 1. Strict consensus MP tree obtained from analysis of SSU rDNA sequence data with unambiguous contiguous gaps recoded as a fifth character state (1795 total characters; 76 parsimony informative). Six equally parsimonious trees were recovered (526 steps, C.I. 0.581 excluding uninformative characters). The trees were rooted by the *H. similis* outgroup (Strongyloidea).

parsimony, and ML trees include species representing genera of the Anisakinae (*Anisakis*, *Pseudoterranova*, *Terranova*), Ascaridinae (*Ascaris*, *Parascaris*, *Baylisascaris*, *Toxascaris*), and Raphidascarididae (*Goezia*, *Hysterothylacium*, *Iheringascaris*), as principally defined by Fagerholm (1991). These clades also received reasonable support (>75%) in the bootstrap parsimony tree.

A separate parsimony analysis of SSU data was performed using an alignment that included the Adenophorean *Plectus* as an outgroup in addition to the trichostrongylid *H. similis*. The published *Plectus* sequence is incomplete (total 1676 bp), requiring the exclusion of some sites in both the 5' and 3' ends of the sequences. For the reported result, unambiguous gaps were recoded as separate characters, but the same topology was recovered when gaps were treated as missing data. This aligned SSU dataset consisted of 1739 characters (including 41 recoded gap characters), of which 1279 were invariant and 108 phylogenetically informative by parsimony criteria. Heuristic MP searches recovered six trees (length 596, C.I. = 0.64);



**FIG. 2.** MP 50% majority-rule bootstrap consensus tree obtained from analysis of SSU rDNA data with contiguous gaps recoded as a fifth character state. Bootstrap percentages of clades (2000 iterations) are shown above internal nodes.



**FIG. 3.** Strict consensus of three trees of equal likelihood (-ln likelihood 4814.36920) obtained from analysis (heuristic search with tree-bisection-reconnection) of SSU rDNA (1764 characters) using PAUP\*. The transition/transversion ratio and the shape of the gamma distribution were estimated prior to this search (see Materials and Methods).

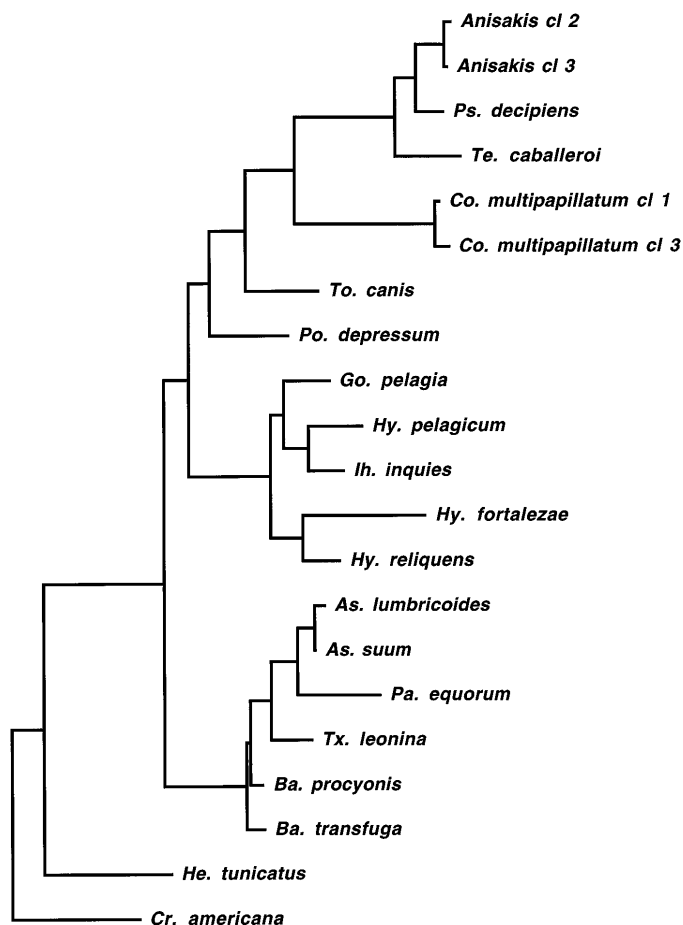
the topology of these trees with respect to the ingroup taxa was identical to that recovered using more complete SSU sequences with the trichostrongylid outgroup (Fig. 1). The bootstrap MP consensus tree for the dataset that included *Plectus* was very similar in topology to Fig. 2. Levels of bootstrap support for family and subfamily groups in this tree were similar to those recovered using the trichostrongylid outgroup: Anisakinae (84%), Ascaridinae (71%), and Raphidascarididae (93%). Sixty-one percent of the trees supported a monophyletic Ascaridoidea, and the representative Ascaridida were recovered in 100% of the replicates.

Unlike the SSU analyses, the combined analysis of LSU plus SSU sequences did not include an outgroup from an order other than the Ascaridida. Although the LSU sequence of *H. similis* is currently unavailable, given the observed LSU sequence divergence between ascaridoids and the cosmocercoid outgroup (*C. americana*), it is likely that alignment of the 26S sequences would be problematic even if representatives of other

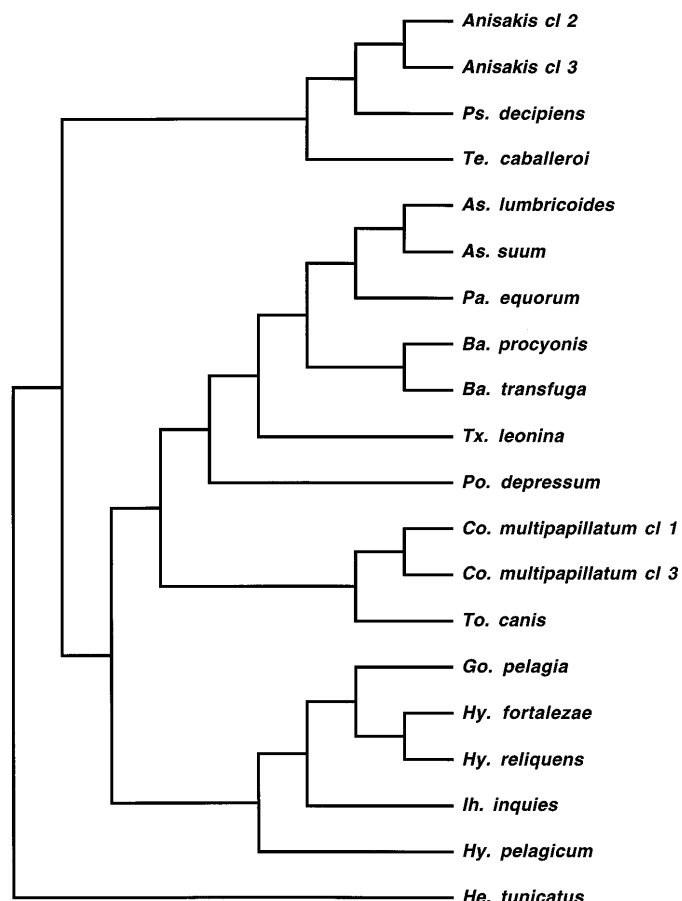


ambiguity. This dataset consisted of 2523 characters (including 29 recoded gap characters), of which 2047 were constant and 165 phylogenetically informative by parsimony criteria. Heuristic MP analysis recovered three trees (length 997, C.I. 0.54). The topology of the strict consensus of these three trees was identical to the strict consensus tree for the combined rDNA data without exclusion of sites (Fig. 4). Furthermore, of the nine clades receiving  $\geq 70\%$  bootstrap support in the analysis of the combined data (without exclusion), only one had reduced bootstrap support in analysis of the dataset with excluded sites (i.e., *H. fortalezae*, *H. reliquens*) was recovered in 87% of the replicates.

The combined analysis of all molecular plus (unordered) morphological characters yielded a single most parsimonious tree (Fig. 7, length 1004, C.I. 0.54), which was similar in topology to the combined SSU plus LSU result (Figs. 4, 5). Bootstrap parsimony analysis yielded



**FIG. 6.** ML tree (-ln likelihood 8622.10411) obtained from analysis (heuristic search with tree-bisection-reconnection) of SSU plus LSU rDNA sequence data (2513 characters) using PAUP\*. Branch lengths are scaled to the expected number of substitutions per site. The transition/transversion ratio and the shape of the gamma distribution were estimated prior to this search (see Materials and Methods). This tree was rooted by the *C. americana* outgroup (Cosmocercoidea), as supported from the SSU analyses.



**FIG. 7.** MP tree based on combined analysis of all rDNA sequence data (2557 characters) plus 21 unordered morphological characters for the ingroup taxa. One most parsimonious tree was recovered by heuristic search (1004 steps, C.I. 0.543 excluding uninformative characters). This tree was rooted by the basal ingroup taxon, *H. tunicatus*.

a topology like that of Fig. 5 (no bootstrap values were markedly increased) except this analysis weakly supported a monophyletic *Baylisascaris* (57% of replicates).

Thirty-four alternative tree topologies were selected for statistical testing against the optimal maximum likelihood tree (Table 2) using the combined SSU plus LSU dataset. Some of these topologies represented previously published evolutionary hypotheses, whereas others were selected to determine if certain traditional alternatives to unexpected outcomes of the nucleotide-based trees were worse in a statistical framework, given these rDNA data. In addition, clades in the rDNA trees defining families and subfamilies (sensu Fagerholm, 1991) were tested to evaluate if arrangements that violated these groupings were worse.

Many of the alternative topologies were significantly worse than the ML tree. For example, the "non-traditional" ML and MP inference of an (*Ascaris* spp., *Parascaris*) clade was compared to alternative topolo-

TABLE 2

Alternative Tree Topologies Tested Using the Maximum Likelihood Method of Kishino and Hasegawa (1989) and the Combined rDNA Data

Tree topology	-ln Likelihood	s.d. difference	$P^a$
ML tree, Fig. 6 (9, (11, ((6, (5, (21, ((4, 3), 16))))), ((((((1, 2), 18), 19), (8, 7)), 20), 17), ((14, 12), (10, (15, 13))))));	8622.10411		
Tree 2 (1, (2, (((((((((3, 4), 16), (5, 6)), 21), 17), ((7, 8), 20)), (10, ((12, 14), (13, 15))))), (9, 11), 19), 18));	8625.45728	10.13488	0.7408
Tree 3 (1, (2, (((((((((3, 4), 16), 21), 5), 6), 17), ((7, 8), 20)), (10, ((12, 14), (13, 15))))), (9, 11), 19), 18));	8623.73237	7.77281	0.8341
Tree 4 (1, (2, (((((((((3, 4), 16), (5, 6)), 21), 17), ((7, 8), 20)), (10, (13, 15)), (12, 14))), (9, 11), 19), 18));	8624.64180	9.62457	0.7921
Tree 5 (9, (11, ((6, (5, (21, ((4, 3), 16))))), ((((((1, 2), 18), 19), (8, 7)), 20), 17), ((14, 12), (13), 15), 10));	8630.07556	6.56634	0.2249
Tree 6 (9, (11, ((6, (5, (21, (4, 3))), 16), ((((((1, 2), 18), 19), (8, 7)), 20), 17), ((14, 12), (10, (15, 13))))));	8656.32631	9.72176	0.0004*
Tree 7 (9, (11, ((21, (16, (6, (5, (4, 3))))), ((((((1, 2), 18), 19), (8, 7)), 20), 17), ((14, 12), (10, (15, 13))))));	8653.83869	13.00040	0.0147*
Tree 8 (9, (11, ((6, 5), (21, ((4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 20), 17), ((14, 12), (10, (15, 13))))));	8622.62272	1.16762	0.6570
Tree 9 (9, (((1, 2), 18), 19), (8, 7)), (((14, 12), (15, 13)), 10), (11, ((17, 20), (6, (5, (21, ((4, 3), 16))))));	8680.28589	14.23053	<0.0001*
Tree 10 (9, (11, (((14, 12), (15, 13)), 10), ((8, 7), (((1, 2), 18), 19), (17, 20), (6, (5, (21, (16, (4, 3))))))));	8629.42169	6.88038	0.2876
Tree 11 (9, (11, (10, (((14, 12), (15, 13)), (8, 7), (((1, 2), 18), 19)), (17, (20, (6, (5, (21, (16, (4, 3))))))));	8713.97119	19.40731	<0.0001*
Tree 12 (9, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), 11), (8, 7)), 20), 17), ((14, 12), (10, (15, 13))))));	8680.34529	13.70082	<0.0001*
Tree 13 (9, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), (8, 7)), 20), 17), (19, ((14, 12), (10, (15, 13))))));	8740.49750	18.38610	<0.0001*
Tree 14 (9, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 19), (8, 7)), 20), 17), (18, ((14, 12), (10, (15, 13))))));	8793.76867	22.08030	<0.0001*
Tree 15 (9, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), 10), (8, 7)), 20), 17), ((14, 12), (15, 13))))));	8717.88531	18.97309	<0.0001*
Tree 16 (9, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), 15), (8, 7)), 20), 17), ((14, 12), (10, 13))))));	8753.64722	20.88567	<0.0001*
Tree 17 (9, (11, ((6, (5, (21, (4, 3), 16))), 15), ((((((1, 2), 18), 19), (8, 7)), 20), 17), ((14, 12), (10, 13))))));	8746.35202	21.08608	<0.0001*
Tree 18 (9, (11, ((5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 20), 17), (6, ((14, 12), (10, (15, 13))))));	8722.95154	19.86861	<0.0001*
Tree 19 (9, (11, ((5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 20), 17), 6), ((14, 12), (10, (15, 13))))));	8722.95154	19.86861	<0.0001*
Tree 20 (9, (11, ((6, (20, (5, (21, (4, 3), 16))))), ((((((1, 2), 18), 19), (8, 7)), 17), ((14, 12), (10, (15, 13))))));	8697.31808	15.91639	<0.0001*
Tree 21 (9, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 17), (20, ((14, 12), (10, (15, 13))))));	8624.15245	2.61183	0.4330
Tree 22 (9, (11, ((6, (17, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 20), ((14, 12), (10, (15, 13))))));	8690.73567	16.27983	<0.0001*
Tree 23 (9, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 20), 17), ((14, 12), (10, (15, 13))))));	8622.20556	0.63732	0.8735
Tree 24 (9, (11, ((6, (5, (21, (4, 3), 16))), (17, (((1, 2), 18), 19), (8, 7)), 20), ((14, 12), (10, (15, 13))))));	8622.23973	0.55578	0.8072
Tree 25 (9, (11, (17, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 20), ((14, 12), (10, (15, 13))))));	8624.61882	3.60883	0.4860
Tree 26 (9, (11, ((6, (5, (21, (4, 3), 16))), (20, (((1, 2), 18), 19), (8, 7)), 17), ((14, 12), (10, (15, 13))))));	8624.15246	2.61184	0.4330
Tree 27 (9, (11, (20, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 17), ((14, 12), (10, (15, 13))))));	8627.41440	4.10993	0.1965
Tree 28 (9, (20, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), (8, 7)), 17), ((14, 12), (10, (15, 13))))));	8682.11896	13.41882	<0.0001*
Tree 29 (9, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), (8, 7)), 19), 20), 17), ((14, 12), (10, (15, 13))))));	8671.28082	11.99751	<0.0001*
Tree 30 (9, (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), 20), 17), ((8, 7), ((14, 12), (10, (15, 13))))));	8626.65955	3.77512	0.2277
Tree 31 (9, (11, ((6, (5, (21, (4, 3), 16))), (8, 7)), ((((((1, 2), 18), 19), 20), 17), ((14, 12), (10, (15, 13))))));	8626.89420	5.25656	0.3622
Tree 32 (9, (11, ((6, (8, 7), (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), 20), 17), ((14, 12), (10, (15, 13))))));	8685.01062	13.29706	<0.0001*
Tree 33 (9, (11, ((8, 7), (6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), 20), 17), ((14, 12), (10, (15, 13))))));	8625.26012	5.81924	0.5876
Tree 34 (9, ((8, 7), (11, ((6, (5, (21, (4, 3), 16))), ((((((1, 2), 18), 19), 20), 17), ((14, 12), (10, (15, 13))))));	8670.99937	14.02239	0.0005*
Tree 35 (9, (11, ((21, (6, 5), (4, 3), 16)), (20, 17)), (((1, 2), 18), 19), (8, 7)), (((14, 12), (13), 15), 10));	8634.16811	10.64783	0.2573

Note. Trees 2–4 are the 3 equally parsimonious trees recovered from analysis of the combined rDNA data. Other tested topologies are discussed in the text. Key to taxa: 1 (*Anisakis* clone 1), 2 (*Anisakis* clone 3), 3 (*A. lumbricoides*), 4 (*A. suum*), 5 (*B. procyonis*), 6 (*B. transfuga*), 7 (*C. multipapillatum* clone 1), 8 (*C. multipapillatum* clone 3), 9 (*C. americana*), 10 (*G. pelagia*), 11 (*H. tunicatus*), 12 (*H. fortalezae*), 13 (*H. pelagicum*), 14 (*H. reliquens*), 15 (*I. iniquus*), 16 (*P. equorum*), 17 (*P. depressum*), 18 (*P. decipiens*), 19 (*T. caballeri*), 20 (*T. canis*), 21 (*T. leonina*).

<sup>a</sup> Probability of getting a more extreme  $T$  value under the null hypothesis of no difference between the two trees (two-tailed test).

\* Significant at  $P < 0.05$ .

gies that included ((*A. suum*, *A. lumbricoides*), *T. leonina*, *B. procyonis*, *B. transfuga*, *P. equorum*) and ((*A. suum*, *A. lumbricoides*), *B. procyonis*, *B. transfuga*, *P. equorum*, *T. leonina*). Both of these more “traditional” interpretations of relationships, which are based on overall morphological similarity and previous taxonomic organizations, were significantly worse (Table 2, trees 6, 7) than the tree depicting an *Ascaris-Parascaris* clade. With respect to phylogenetic hypotheses for the entire superfamily, the evolutionary hypotheses of Osche (1963) and Gibson (1983) were significantly worse than the ML hypothesis (Table 2, trees 11 and 9, respectively). Of tests that involved placing a taxon

with a clade composed of species representing a different family, 8 out of 12 tested arrangements (Table 2, trees 12–19, 21, 23, 30, 31) were significantly worse. The nonsignificant results involved placing a member of the (non-monophyletic) *Toxocarinae* with the *Raphidascarididae* (Table 2, trees 21, 23), or the *Contracaecum* taxa with the *Raphidascarididae* or *Ascaridinae*. Poor resolution with respect to the position of the *Toxocarinae* was reinforced by the observation that trees with *Toxocara* or *Porrocaecum* basal to the (*Anisakidae*, *Raphidascarididae*), or ((*Anisakidae*, *Raphidascarididae*), *Ascaridinae*) were also not significantly worse (Table 2, trees 24–27). By contrast, topologies

placing *Toxocara* or *Porrocaecum* within the Ascaridinae or basal among the Ascaridoidea were significantly worse (Table 2, trees 20, 22, 28, respectively).

Some of the alternative topologies tested were not significantly worse than the ML tree. Trees not worse included the three most parsimonious trees (Table 2, trees 2–4), and a tree consistent with the evolutionary hypotheses described by Sprent (1983) (Table 2, tree 10). Other alternative trees that were not worse included relationships expected based on beta taxonomy and rearrangements of taxa within certain clades (of the rDNA gene trees) based on overall morphological similarity. For example, an alternative topology (Table 2, tree 5) with a monophyletic *Hysterothylacium* and an *Iheringascaris* sister group (*H. fortalezae*, *H. reliquens*), *H. pelagicum*, *I. inquires*) was not significantly worse than the (*H. pelagicum*, *I. inquires*) clade recovered in the ML and MP trees. Likewise, a monophyletic *Baylisascaris* (Table 2, tree 8) was not significantly worse than the paraphyletic arrangement of species recovered in the ML tree and one of the MP trees. The most unexpected inference from MP analysis of SSU plus LSU data (Fig. 4) was the sister-taxon relationship between *Contraecum* and *Toxocara* (which represent different families in all modern classifications), and the relationship of *Contraecum* to other ascaridoids. However, statistical topology tests indicate that this MP result should be cautiously interpreted since many alternative topologies involving *Contraecum* were not worse. These included representing *Contraecum* as the sister group to the Raphidascaididae (Table 2, tree 30), sister group to the Ascaridinae (Table 2, tree 31), and basal to all ingroup taxa except *Heterocheilus* (Table 2, tree 33). In contrast, topologies depicting *Contraecum* nested within the Anisakinae or Ascaridinae, or basal to all Ascaridoidea, were significantly worse (Table 2, trees 29, 32, 34, respectively). Finally, a topology that combined four traditional expectations with the remaining framework of the ML tree (i.e., traditional placements of *Toxocara* and *Porrocaecum*, *Toxascaris*, monophyletic *Baylisascaris*, and monophyletic *Hysterothylacium*) was also not significantly worse (Table 2, tree 35).

Given the inability to reject statistically certain traditional groupings using the framework of the ML tree, patterns of morphological and life history evolution were explored on six different topologies including the ML tree, the three equally parsimonious trees, the combined rDNA plus morphology tree, and the ML tree as modified to represent traditional expectations as described previously (Table 2, tree 35). The range of consistency indices for individual characters on these trees (Table 3) showed that some characters were explained in all these trees by the minimum number of changes, whereas others always required extra steps. Of the five characters that required no extra steps on all trees, two (gubernaculum and distal papillae num-

TABLE 3

**Consistency Indices for Characters Obtained by Parsimony Mapping on Six Inferred Trees, Including the ML Tree as Modified To Represent Certain Traditional Expectations<sup>a</sup> ("Traditional Tree," Table 2, Tree 35)**

Character	C.I., ML tree	C.I., "traditional" tree	C.I., combined molecular and morpho- logy tree	C.I. Range for three MP trees
Median papilla type	0.25	0.33	0.33	0.25–0.33
Paracloacal papillae	0.25	0.25	0.25	0.25
Distal papillae number	1.0	1.0	1.0	1.0
Distal papillae type	0.75	1.0	1.0	0.75–1.0
Proximal papillae type	0.67	1.0	1.0	0.67–1.0
Spicule type	0.50	0.50	0.50	0.50
+/- Gubernaculum	1.0	1.0	1.0	1.0
+/- Caudal plates	1.0	1.0	1.0	1.0
+/- Caudal alae	0.50	0.50	0.50	0.50
Definitive host type	0.75	0.75	0.75	0.75
Ventriculus (+/-, type)	1.0	1.0	1.0	1.0
+/- Intestinal caecum	0.33	0.33	0.33	0.33
Lip type	1.0	1.0	1.0	1.0
+/- Lip denticles	0.33	0.50	0.33	0.33
+/- Interlabia	0.17	0.20	0.20	0.20
Excretory pore position	1.0	1.0	0.67	0.67
Excretory system type	1.0	1.0	0.67	0.67
+/- Cervical alae	0.29	0.40	0.33	0.29–0.33
Life history type	0.33	1.0	0.50	0.50
+/- Monoxenous cycle	0.50	0.50	0.50	0.50
Eggshell type	0.50	1.0	0.50	0.50
+/- Ventricular appendix	0.50	0.50	0.50	0.50
Proximal papillae type	0.50	0.50	0.50	0.50

<sup>a</sup>This topology was not significantly worse by statistical comparison. See Table 1 for character matrix and additional descriptions.

ber) had inferred changes of state between *H. tunicatus* and the remaining ingroup taxa in all six trees. For the other three characters with consistency indices of one for all six trees, four character states appear to be apomorphic for groups of taxa. Three of these presumptive synapomorphies define the Anisakinae: the presence of caudal plates, reduced lip size, and a long ventriculus. Likewise, the absence of a ventriculus is inferred to be a shared derived state for the Ascaridinae. Six additional characters that showed some homoplasy when mapped on certain molecular trees (ML or MP) required none when mapped on the ML tree modified to include "traditional expectations." These characters/states (and the groups they define if this traditional tree is used for exploring character evolution) include: distal papillae type (Anisakinae-proximal pattern; Raphidascaididae-subventral rows; [*Baylisascaris*, *Parascaris*, *Ascaris*]-distal and united), proximal papillae type (*Ascaris* spp.-grouped posterior), excretory system type (Anisakidae-left glandular; Raphidas-

carididae; left filamental), life history (Ascarididae–terrestrial), eggshell thickness (Ascarididae–thick), and excretory pore position (Anisakidae–between subventral lips). The inferred patterns of change in two features, eggshell thickness and life history (aquatic versus terrestrial), are identical on the trees. Mappings of 11 other characters (9 binary) required extra steps on all six trees (Table 3, C.I.  $\leq$  0.50 in all cases); these characters included surface structures of cuticular origin (e.g., presence or absence of labial denticles, presence or absence of caudal/cervical alae) and seemingly more complex internal structures such as appendices of the alimentary tract.

Levels of uncorrected pairwise nucleotide distance ( $p$  distances) for the LSU rDNA data revealed obvious differentiation between clones of two species where DNA was extracted from pooled juvenile specimens. Clones of *Anisakis* sp. had a  $p$  distance of 0.0081 whereas clones of *Contraecum* had a  $p$  distance of 0.0150. In comparison, the  $p$  distance between *B. procyonis* and *B. transfuga* was 0.0229, and the average  $p$  distance among four genera of the Ascaridinae was 0.0579. Human and swine-source *Ascaris* (classically considered the cryptic species *A. lumbricoidea* and *A. suum*, respectively) had a  $p$  distance of 0.0014.

## DISCUSSION

A previous investigation of partial SSU and LSU sequences established that ribosomal DNA is informative for inferring relationships among ascaridoid species (Nadler, 1992); however, the limited sampling of taxa and nucleotides in that study precluded detailed conclusions regarding evolutionary pattern. For example, the 395 nt sites analyzed were insufficient to discriminate statistically between most alternative tree topologies of interest. Nevertheless, optimal trees in that study were consistent with certain traditional expectations. One goal of this study was to determine if adding more taxa and nucleotides would extend the previous results and allow for a greater level of statistical resolution in evaluating alternative hypotheses for this rDNA gene tree. The larger data set was more effective in discriminating statistically between alternative trees, although these data were not uniformly effective for resolving relationships among all studied taxa.

Trees estimated in this study strongly supported several of the higher taxonomic groups defined by Fagerholm (1991). In the rDNA trees (ML and MP), taxa representing the Ascaridinae, Contraecinae, Anisakinae, and Raphidascarididae (sensu Fagerholm, 1991) were each monophyletic and reliably supported by bootstrap resampling. Alternative topologies that separated members of these family or subfamily groups were significantly worse in statistical tests based on maximum likelihood. Concepts of ascaridoid phylogeny

(and classification) as proposed by Osche (1963) and Gibson (1983), which were developed based on interpretations of few “key” characteristics, were significantly worse than the ML tree, given these rDNA data. In contrast, the hypothesis of Sprent (1983), which is primarily consistent with the classification of Fagerholm (1991), was not significantly worse. Although this study does not fully encompass the tremendous diversity of the Ascaridoidea (>50 genera), the rDNA-based phylogenetic hypotheses suggest that the taxonomic summary of Fagerholm (1991) may represent the best working hypothesis for classification of the group.

There is no indication of monophyly in the rDNA trees for the two genera that normally constitute the Toxocarinae (Hartwich, 1974; Fagerholm, 1991; Gibson, 1983). Classically, the two genera in this subfamily (sensu Gibson, 1983), *Toxocara* and *Porrocaecum*, have been considered closely related because they possess a ventriculus (a plesiomorphic trait as inferred by mapping in this study) and are parasites of hosts that acquire infection through a terrestrial life cycle. Bootstrap resampling and statistical testing of alternative topologies shows that these rDNA data are not robust for assessing the relationships of *Toxocara* or *Porrocaecum* among the ascaridoids, with the exception of rejecting the hypothesis that they belong within the Ascaridinae, or are basal among the Ascaridoidea. However, it is noteworthy that the Ascarididae as recognized traditionally in virtually all modern classifications (Ascaridinae, Angusticaecinae, Toxocarinae) was not recovered in analyses of these data. Although this and previously published molecular studies (Nadler, 1992, 1995) are equivocal regarding monophyly of the Ascarididae, it is notable that other systematists have also questioned this taxonomic organization (Fagerholm, 1991). From a molecular perspective, sequences from additional loci and taxa are necessary to resolve the issue of monophyly of this family.

The Anisakidae as typically constituted (Anisakinae plus Contraecinae) was recovered in the ML tree using all rDNA data, but not the MP trees. In the MP trees, the *Contraecum* taxa were part of a clade with *Toxocara*, although this relationship was not recovered in the bootstrap MP consensus tree. Poor resolution with respect to the phylogenetic relationship of *Contraecum* was also supported by tests of alternative topologies using ML. Because the relationship of *Contraecum* is poorly resolved, and the traditional representation was recovered by ML, this representation was one of several trees used to explore patterns of change in morphological characters.

Not unexpectedly, monophyly of the sampled Ascaridoidea was recovered in MP analysis of the SSU data with the Adenophorean *Plectus* as the root, and for ML and MP analyses of the more complete SSU data using the Strongylid (*Haemonchus*) outgroup. Among the Ascaridoidea, trees inferred from the SSU or combined

SSU plus LSU data uniformly depicted the heterocheilid as basal among the Ascaridoidea. This observation is consistent with previous results (Nadler, 1992, 1995) and the hypothesis of Sprent (1982, 1983, 1992), who considered heterocheilids as the basal ascaridoid lineage based on structural features he interpreted as plesiomorphic (features of the excretory system, labia, and arrangement of esophageal gland nuclei). Neither the SSU tree or combined SSU plus LSU trees yielded robust support for relationships among families and subfamilies as revealed by bootstrap resampling. However, trees inferred from combined analysis of the SSU plus LSU data do not support previous hypotheses suggesting that ascaridoids of marine mammal definitive hosts are most closely related to those maturing in marine fish (Osche, 1963), or that parasites of aquatic mammals are most closely related to those from terrestrial mammalian hosts (Anderson, 1984). Neither species using mammals as definitive hosts or those from all aquatic environments are monophyletic. This suggests that the complex patterns of definitive host acquisition as envisioned by Sprent (1982, 1992), which involve ascaridoids exploiting food chains in acquiring new hosts, may be a more accurate representation of ascaridoid evolution than a general pattern of host-parasite cophylogeny (Osche, 1958, 1963).

Few investigators have provided explicit proposals of how genera within ascaridoid families are related. With these rDNA data, levels of bootstrap support for clades within families and subfamilies varied substantially; thus, weakly supported clades should be viewed as provisional hypotheses requiring further testing. It is notable that strong bootstrap support was observed for a clade consisting of *Anisakis* and *Pseudoterranova*, species that use marine mammals as their definitive hosts. The genus *Pseudoterranova* has a particularly convoluted history of nomenclature (summarized by Gibson, 1983); however, because *P. decipiens* is not the sister taxon of *Terranova caballeroi* in the rDNA trees, this topological result is consistent with the generic distinction of *P. decipiens* [formerly *Terranova decipiens* (Karokhin, 1946)], from other *Terranova* species (Myers, 1959; Gibson, 1983).

The clade consisting of *Ascaris* and *Parascaris* was also strongly supported by bootstrap resampling and statistical testing. This grouping, which is unexpected based on structural dissimilarities of the lips and reflected by the taxonomic history of these genera (Sprent, 1968), was recovered in both a previous study of rDNA (Nadler, 1992) and a preliminary analysis of SSU and mitochondrial (Cytochrome oxidase II) sequences (Nadler, 1995). However, unlike the previous studies, statistical testing of alternative topologies using these rDNA data showed that the traditional *Ascaris* plus *Baylisascaris* clade was significantly worse. Thus, it appears that structural dissimilarities of *Parascaris*, which are most pronounced for labial features,

may be misleading with respect to evolutionary relationships. Although detailed comparative studies of development among ascaridoid nematodes are rare, it is interesting that in *Parascaris*, much of this marked labial differentiation occurs relatively late during ontogeny (Pilitt *et al.*, 1979), whereas during the same period in *Ascaris*, comparatively few changes in lip structure occur (Pilitt *et al.*, 1981).

*Goezia pelagia*, which represents a genus sometimes listed as a subfamily within the Anisakidae (Hartwich, 1974; Gibson, 1983), was included among the Raphidascarididae. Strong bootstrap support was noted for *H. fortalezae* and *H. reliquens* as sister taxa, whereas a clade consisting of *H. pelagicum* and *I. inquires* received less support. Although the optimal ML and MP tree topologies did not support a monophyletic *Hysterothylacium* (formerly *Thynnascaris*), an alternative topology with a monophyletic *Hysterothylacium* and an *Iheringascaris* (also formerly *Thynnascaris*) sister group was not significantly worse by ML comparison. *Iheringascaris* (Pereira), which is currently a monotypic genus, was resurrected by Deardorff and Overstreet (1980). The main features used to differentiate *Iheringascaris* from *Hysterothylacium* are conspicuous plicated cuticular annulations and an additional lateral pair of caudal papillar rows in the former genus; biological differences include the observation that *I. inquires* may embed in the definitive fish host's mucosal tissue whereas species of *Hysterothylacium* do not. The optimal ML and MP tree topologies are not consistent with genus-level distinction for *Iheringascaris*, although tests of alternative topologies indicate that additional data are needed to assess if taxa now reassigned to *Hysterothylacium* (originally described as *Contraecaeum*, *Ascaris*, or *Thynnascaris*) are monophyletic.

Patterns of character evolution, as explored using multiple trees, suggested that certain key features that have been used to group ascaridoids for classifications are either plesiomorphic states or highly homoplastic features. The "H-shaped" (left-right filamental) excretory system and the presence of a ventriculus, two features that have been emphasized by many investigators, are plesiomorphic states as inferred from parsimony mapping. The presence, absence, and form of appendices of the alimentary tract (i.e., intestinal caecum) have been emphasized in certain classifications and evolutionary hypotheses (Hartwich, 1954); however, mapping suggests that presence or absence of appendices is highly homoplasous. The presence or absence of cuticular alae (caudal and cervical), which are readily visible and variable features of ascaridoids, was also inferred to be highly homoplasous by mapping. Perhaps the expression of certain cuticular features (e.g., alae, interlabia, labial denticulation) is "switched" on or off relatively easily through minor regulatory or developmental changes. This would explain why the presence or absence of certain cuticular

features can occasionally vary substantially between species in the same genus (Sprent, 1983).

Character mapping also suggested that certain states may be synapomorphic for taxonomic groups defined both classically and by analysis of rDNA. For example, the absence of a ventriculus appears to be a shared-derived condition defining the Ascaridinae. By extension, the Angusticaecinae (which also lack a ventriculus) are hypothesized to be the sister group to the Ascaridinae. When characters are mapped on the ML tree or a traditional tree (i.e., as modified to represent traditional placements for *Toxocara*, *Porrocaecum*, *Toxascaris*, *Baylisascaris*, and *Hysterothylacium*), additional character-states are inferred to be synapomorphic for groups of taxa. Obviously these inferences are tentative and rest on the assumption that the ML tree may be more robustly supported by inclusion of additional characters. Some of these features have previously been depicted as informative for revealing evolutionary pattern. For example, excretory system structure has been considered important for inferring relatedness (Hartwich, 1954, 1957; Gibson, 1983; Sprent, 1983), and parsimony mapping (on ML and traditional trees) supports the hypothesis that left glandular (defining Anisakidae) and left filamental (defining Raphidascarididae) conditions represent apomorphic states. Likewise, Fagerholm (1991) proposed that patterns of cloacal papillae may be useful in interpreting evolutionary relationships. Although not all of Fagerholm's (1991) hypotheses of papillae character evolution were examined, this study suggests that the type and pattern of distal cloacal papillae may be useful for defining certain clades.

Species within the Ascaridoidea show substantial variation in patterns of transmission, ranging from monoxenous life cycles requiring no intermediate host (e.g., *Ascaris* and *Parascaris*), to obligate heteroxenous patterns involving one or more intermediate hosts in addition to the definitive host. Scenarios for the evolution of these life cycle patterns, and by extension the nematodes, have received considerable attention. For example, certain authorities regarded the monoxenous pattern as ancestral (Chitwood and Chitwood, 1950; Mozgovoi, 1953), whereas others (e.g., Fülleborn, 1927; Sprent, 1954; Chabaud, 1955; Anderson, 1988) believed that heteroxeny is plesiomorphic for ascaridoids, with the direct life cycle pattern of *Ascaris* representing a loss of intermediate hosts (i.e., secondary monoxeny, Fülleborn, 1927; Chabaud, 1955; Anderson, 1988). In the rDNA trees, ascaridoid species with a classical monoxenous transmission pattern (*Ascaris*, *Parascaris*) are a crown group within the Ascaridinae; those with facultative monoxenous patterns (i.e., known for certain *Baylisascaris* spp., *Tx. leonina*) are closely related and basal to *Parascaris* and *Ascaris*, supporting Fülleborn's (1927) hypothesis that the direct life cycle pattern is a derived feature involving loss of intermediate hosts. Some species of *Toxocara* are also known to

be capable of classical monoxeny, and this suggests that the direct life cycle may have evolved more than once within the Ascaridoidea. Confirmation of this hypothesis requires better resolution of relationships with respect to *Toxocara*.

Patterns of LSU sequence differentiation between clones of *Anisakis* sp. and *Contracaecum* are consistent with previous reports of sibling species complexes for these groups (Nascetti *et al.*, 1986, 1993) based on allozyme data. For these genera, the juvenile nematodes used for DNA isolation were collected from single intermediate hosts, and for *Contracaecum*, morphological features characteristic of *multipapillatum* were observed by microscopy. For *Anisakis*, cryptic species identified by allozyme electrophoresis (Nascetti *et al.*, 1986) have yet to be described formally, but given the morphological similarity among *Anisakis* juveniles, correlation of molecular markers such as rDNA for diagnosed adult specimens may provide the only practical method of identifying single juveniles. The species status of human and pig *Ascaris* has long been controversial (for review see Gibson, 1983; Anderson *et al.*, 1993), and has not been fully resolved despite intensive molecular studies of human- and pig-associated worms at sites where they are sympatric and cross-infection is likely (Anderson *et al.*, 1993). Thus, sequence data from one individual of each putative species from North America cannot resolve this systematic problem. However, it is noteworthy that both SSU (*p* distance of 0.00342) and LSU sequences (*p* distance of 0.0014) showed sequence differentiation for the *Ascaris* taxa. For the LSU sequences, *A. suum* and *A. lumbricoides* had the smallest pairwise distance among taxa in the comparisons; for the SSU sequences, three pairwise comparisons of other species (*P. decipiens* vs *Anisakis* sp.; *T. leonina* vs *B. procyonis*; *B. procyonis* vs *B. transfuga*) had somewhat lower *p* distances. These results suggest that in addition to internal transcribed spacer variation between pig and human source *Ascaris* (Anderson, 1995), regions of the small and large rDNA subunits may be useful for investigating the host fidelity and specific status of *Ascaris*.

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