2000

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J. Parasitol., 86(2), 2000 p. 380–393

PHYLOGENY OF THE ASCARIDOIDEA (NEMATODA: ASCARIDIDA) BASED ON THREE GENES AND MORPHOLOGY: HYPOTHESES OF STRUCTURAL AND SEQUENCE EVOLUTION

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ABSTRACT: Ascaridoid nematodes parasitize the gastrointestinal tract of vertebrate definitive hosts and are represented by more than 50 described genera. We used 582 nucleotides (83% of the coding sequence) of the mitochondrial gene cytochrome oxidase subunit 2, in combination with published small- and large-subunit nuclear rDNA sequences (2,557 characters) and morphological data (20 characters), to produce a phylogenetic hypothesis for representatives of this superfamily. This combined evidence phylogeny strongly supported clades that, with 1 exception, were consistent with Fagerholm's 1991 classification. Parsimony mapping of character states on the combined evidence tree was used to develop hypotheses for the evolution of morphological, life history, and amino acid characters. This analysis of character evolution revealed that certain key features that have been used by previous workers for developing taxonomic and evolutionary hypotheses represent plesiomorphic states. Cytochrome oxidase subunit 2 nucleotides show a strong compositional bias to A+T and a substitution bias to thymine. These biases are most apparent at third positions of codons and 4-fold degenerate sites, which is consistent with the nonrandom substitution pattern of A+T pressure. Despite nucleotide bias, cytochrome oxidase amino acid sequences show conservation and retention of critical functional residues, as inferred from comparisons to other organisms.

Perhaps due to their importance as disease agents, large size, and availability, e.g., *Ascaris suum*, species of ascaridoids are among the most thoroughly studied nematode parasites of vertebrates. For example, members of this superfamily have been used for studies of respiratory biochemistry (Saz and Weil, 1960; Komuniecki et al., 1993), molecular genetics (Neuhaus et al., 1987; Kageyama, 1998), immunology (Jones et al., 1994), reproductive biology (Von Beneden, 1883; Foor, 1970), comparative morphology (Fagerholm, 1989, 1991; Hugot et al., 1991), development (Boveri, 1899; Pilitt et al., 1981), life cycles (Huizinga, 1967; Klöser et al., 1992; Køie and Fagerholm, 1995), population genetics (Anderson et al., 1993; Nascetti et al., 1993; Nadler, 1996), and pathology (Beaver, 1956; Overstreet and Meyer, 1981; Kazacos, 1986). In contrast, much less is known about the evolutionary history of taxa in this superfamily, although certain organisms, e.g., *Ascaris, Anisakis,* have been studied in detail at the microevolutionary level (Paggi et al., 1991; Anderson et al., 1993, 1995; Anderson and Jaenike, 1997).

Structural features of ascaridoids, particularly external ones, show considerable variation among genera and, in some cases, species. Likewise, their life cycles also show a diversity of patterns. However, although many general features of ascaridoid life cycles have been established, detailed studies have been limited to relatively few species, and in some cases, recent investigations have yielded new findings for such thoroughly studied species as *A. suum* (Murrell et al., 1997). Evolutionary scenarios to explain observed patterns of life-cycle variation have frequently been proposed. For example, it has been debated whether the monoxenous (1-host) or heteroxenous life cycle is ancestral for the group (Fülleborn, 1927; Chitwood and Chitwood, 1950; Mozgovoi, 1953; Sprent, 1954; Chabaud, 1955; Anderson, 1988). Testing such competing evolutionary scenarios requires a phylogenetic framework for comparison.

Most evolutionary hypotheses for ascaridoids were developed prior to the widespread use of cladistic analysis, and these hypotheses were typically based on interpretation of variation in 1 or a few key structures or life history features (Gibson, 1983; Sprent, 1983). More rarely, the assumption of host–parasite coevolution (Osche, 1963; Gibson, 1983) was used to develop evolutionary hypotheses. Emphasis on different key features (or differences in their interpretation) by different investigators led to different hypotheses for transformation and polarity of character states, and conflicting hypotheses of relationships (Mozgovoi, 1953; Hartwich, 1954, 1957, 1974; Osche, 1958, 1963; Gibson, 1983; Sprent, 1983; Fagerholm, 1991). Predictably, differences in evolutionary hypotheses have also led to instability of ascaridoid classification. For example, Hsu's (1933) classification emphasized the presence or absence of the ventriculus in forming 2 major groups (genera in terrestrial mammals [excepting *Toxocara*], and genera in fish, birds, aquatic reptiles, and aquatic mammals), whereas Hartwich (1974) emphasized the structure of the secretory–excretory system in combination with the concepts of Chabaud (1965) in organizing ascaridoids into 5 families and 8 subfamilies. More recently, nucleotide (nt) sequences have provided data for developing phylogenetic hypotheses for some ascaridoids (Nadler, 1992, 1995; Nadler and Hudspeth, 1998; Zhu et al., 1998), and trees based on ribosomal DNA sequences are mostly consistent with a more recent classification of the group (Fagerholm, 1991). However, rDNA gene trees have failed to provide entirely satisfactory resolution for certain questions (Nadler and Hudspeth, 1998; Zhu et al., 1998) and represent inferences based on a single locus.

In the present study, phylogenetic relationships among 20 ascaridoid taxa, comprising 14 genera, were inferred using a combined analysis of previously published nuclear-encoded rDNA sequences (Nadler and Hudspeth, 1998), mitochondrially encoded cytochrome oxidase subunit 2 (*cox2*) sequences, and morphological data. This combined evidence approach (Eernisse and Kluge, 1993; Kluge, 1998) is interpreted to represent the best phylogenetic estimate, given the available data. The *cox2* (nucleotide sequences abbreviated *cox2*, amino acid sequences as COII) sequence data were also analyzed separately

Received 7 May 1999; revised 25 August 1999; accepted 25 August 1999.

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to infer a tree for this gene. Due to the maternal inheritance of mitochondrial DNA (mtDNA) in most organisms, including *Ascaris* (Anderson, Komuniecki et al., 1995), *cox2* represents a genetically independent locus from genes in the nucleus. Hypotheses for the evolution of morphological, life history, and COII data were developed relative to the tree inferred from the combined analysis. The pattern of *cox2* substitution was also estimated using the combined analysis tree and a parsimony-based approximation method to produce a rate matrix of change.

MATERIALS AND METHODS

Taxa for molecular systematics

Studied taxa and their classification (sensu Fagerholm, 1991) included 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., *Contracaecum multipapillatum, Pseudoterranova decipiens, Terranova caballeroi,* Ascarididae (Baird, 1853), *Ascaris lumbricoides, A. suum, Baylisascaris procyonis, Baylisascaris transfuga, Parascaris equorum, Porrocaecum depressum, Toxocara canis, Toxascaris leonina,* and *Iheringascaris inquies,* a genus not included by Fagerholm (1991). Based on small subunit (SSU) ribosomal DNA (rDNA) gene trees (Nadler and Hudspeth, 1998) and hypotheses of relationship based on morphological similarity (Inglis, 1965), *Cruzia americana,* a member of the Cosmocercoidea (Ascaridida), was included as an outgroup for the Ascaridoidea. Collection site and host data for these taxa have been reported (Nadler and Hudspeth, 1998).

DNA amplification and sequencing

Nucleic acids were extracted from frozen (−70 C) tissue samples of body wall muscle (larger species) or from individual or pooled samples of whole adults or juveniles, as previously described (Nadler and Hudspeth, 1998). Methods for polymerase chain reaction (PCR) amplification and sequencing of nuclear rDNA were reported in Nadler and Hudspeth (1998), and these sequences deposited in GenBank (SSU sequences U94365–94383, large subunit [LSU] sequences U94749–94769). The *cox2* locus was amplified by PCR using several different strategies. Amplification primers were designed based on the sequences (Okimoto et al., 1992) and mtDNA gene order of *A. suum* (X54253) and *Caenorhabditis elegans* (X54252). In an attempt to obtain complete *cox2* sequences, amplification primers were designed to anneal to the transfer RNAs (tRNA-Gly, tRNA-His) flanking *cox2* (in *A. suum, C. elegans,* and *Onchocerca volvulus,* AF015193), or to genes (cytochrome oxidase subunit 1 [*cox1*], large ribosomal-RNA, [L-rRNA]) adjacent to these tRNAs in *A. suum* and *C. elegans.* The laboratory designations for these PCR primers, their corresponding positions in complete *A. suum* mtDNA (accession X54253; positions in parentheses), their orientation (>forward, <reverse), and their sequences are: no. 56 (L-rRNA, 12,064–12,046) <TGACTTTCTGTTAACTCCG, no. 57 (*cox1,* 10,295–10,315) >GGTCATAGTTACCAGTCTGAG, no. 103 (t-RNA-His, 11,319–11,300) <ACAAGCATTTTCACATAAAC, no. 104 (t-RNA-Gly, 10,549–10,568) >TTTTTGTACATTTGACTTCC. Most attempts to amplify the complete gene using primers flanking *cox2* were successful for the Ascaridinae (subfamily of *A. suum*) but not for most species from other families. Taxa that were successfully amplified by flanking primers and confirmed by sequencing included *B. procyonis* (primers 56/57), *B. transfuga* (primers 56/57), *P. decipiens* (primers 103/104), and *T. leonina* (primers 104/56). For all Ascaridida, 2 internal *cox2* primers were successfully used to amplify 629 bp (90%) of coding sequence: no. 210 (11,267–11,248) <CACCAACTCTTAAAATTATC, and no. 211 (10,639–10,665) >TTTTCTAGTTATATAGATTGRTTTYAT. For the no. 210/no. 211 PCR, an initial DNA denaturation at 94 C for 4 min was followed by 25 cycles of PCR using *Taq* or $Vent_R$ (New England Biolabs, Beverly, Massachusetts) polymerase; 94 C for 30 sec, 45 C for 30 sec, and 72 C for 50 sec, followed by a postamplification extension for 5 min at 72 C. Although PCR conditions were empirically optimized for individual templates, typical conditions for 50-μl reactions included 300 ng of DNA template, 4 mM $MgCl_2$, and a 2-fold concentration of the degenerate primer no. 211 (final concentration 1 μM) relative to primer no. 210. For direct sequencing, 1 of 2 methods of PCR template preparation was used. PCR products were either purified by agarose gel electrophoresis in TAE buffer, pH 8.0 (Ausubel et al., 1989) and product recovered from gel slices using a QIAEX (Qiagen, Valencia, California) kit, or products were washed 3 times by spin filtration (Millipore Ultrafree-MC 30,000 NMWL, Bedford, Massachusetts) using TE buffer (pH 8.0). Prior to sequencing, PCR templates were quantified by fluorometry using Hoechst 33258 dye and a Hoefer TKO 100 minifluorometer with TKO 130 capillary cuvette; 200–400 fmol of PCR product was used per sequencing reaction. Direct sequencing of *cox2* PCR products yielded reliable sequence data for primer no. 210, but typically not for the degenerate primer no. 211. To obtain full-length double-stranded sequence of the amplified DNA, PCR products were cloned into pGEM-T vector (Promega, Madison, Wisconsin). For cloning, PCR products were washed 3 times with TE (pH 8.0) by spin filtration, ligated into pGEM-T vector, and used to transform DH5α *Escherichia coli.* With the exception of the outgroup, *C. americana,* a minimum of 2 clones were sequenced for each taxon (1 for each DNA strand). Typically, clones displayed absolute complementarity, although in some cases a third clone was needed to fully verify (double-strand) the sequence. Sequencing reactions were performed manually using the Delta-Taq cycle sequencing kit (Amersham, Piscataway, New Jersey) and α^{32}P-dATP (specific activity >3,000 Ci/mmol), with annealing temperatures for the labeling and termination steps adjusted empirically for each sequencing primer. 7-Deaza-dGTP was used in cycle and termination mixes to reduce potential gel compression artifacts. Cesium chloride-purified plasmid DNA was used as template. Double-stranded *cox2* sequence was obtained using 2 pGEM-T vector primers of our own design (pGEM-T 2974–2997 >GGCCAGTGAATTGTAATACGACTC; 110–135 <GACACTATAGAATACTCAAGCTATGC) and 3 *cox2* primers for each taxon. The *cox2* primers included the PCR primer no. 210, and 2 internal priming sites with opposite orientations. The internal priming sites were found to be variable among species, therefore, 2 different sequencing primers were used for these regions: no. 20 <TCCCTAAACTCATAACTTC, no. 21 <TCCCTAAACTCATAACTCC, no. 332 >CACCAGTGATATTGAAGTTACG, and no. 388 >CATCAGTGGTATTGGAGTTATG. A sequence obtained from noncloned PCR product template using primer no. 210 was included in each contig and used to confirm that clones were consistent with the products obtained directly from PCR.

Sequence analysis

The invariant 47 nt corresponding to the 210/211 *cox2* PCR primers were excluded from sequence analysis, because potential sequence variation within PCR priming regions is masked by incorporation of synthetic primers during amplification. The nematode mtDNA genetic code (Wolstenholme et al., 1987; Okimoto et al., 1992) was used to predict amino acid sequences from the 582 nt obtained for all taxa. Predicted polypeptide sequences were aligned using CLUSTAL V (Higgins et al., 1992); alignment of the coding nucleotide sequences was straightforward. Nuclear rDNA sequences were aligned based on secondary structure (SSU) and similarity (LSU), as described in Nadler and Hudspeth (1998). For parsimony analyses of the rDNA data and the combined datasets, inferred gaps in rDNA were recoded conservatively such that each unambiguous contiguous gap was represented as 1 character, with ''nucleotide present'' or ''nucleotide absent'' as the character states (Swofford, 1993; Crandall and Fitzpatrick, 1996). This recoding procedure yielded 44 characters for the rDNA alignment; the unrecoded gap states were treated as missing data in maximum parsimony (MP) analyses.

Trees were inferred from the mitochondrial and nuclear sequence data sets using unweighted MP (PAUP version 3.1.1). Parsimony analysis of *cox2* data was also performed with transversion substitutions weighted 4 times transitions. The transversion weighting stepmatrix was selected by using the topologies of the 2 most-parsimonious *cox2* trees (unweighted analysis) and a tree from neighbor-joining (log/det paralinear distances) as user trees to estimate parameters of a likelihood model using PAUP* (version 4d64). The transition : transversion ratio estimated from these 3 trees ranged from 3.41 to 3.65 (mean 3.53), which was rounded to 4 for the stepmatrix. Trees were rooted by *C. americana*;

this choice was supported by analysis of the SSU rDNA data with additional outgroups (Nadler and Hudspeth, 1998). Combined analyses of rDNA, *cox2*, and morphological data were performed using unweighted MP. Alternative tree topologies were evaluated by parsimony criteria using the winning sites test (Prager and Wilson, 1988) and Templeton's modified test (Templeton, 1983) using PAUP*. Tree space was searched using heuristic methods (tree-bisection reconnection branch-swapping, MULPARS), with 100 replicates of random stepwise addition. Reported consistency indices do not include uninformative characters. Bootstrap MP trees (2,000 replicates) were produced using heuristic searches with MULPARS option, TBR branch-swapping, and simple stepwise addition.

Substitution rate patterns for *cox2* were inferred using the combined analysis tree and the parsimony-based approximation method of Yang and Kumar (1996) as calculated in the PAMP program of the PAML package (version 1.3C; Yang, 1997). Numbers of unambiguous substitutions, as determined by parsimony, were assessed relative to trees and codon positions using MacClade (v 3.05; Maddison and Maddison, 1992). Base compositional bias was calculated according to the formula given in Thomas and Wilson (1991).

Morphological data and mapping

Morphological data (Fagerholm, 1991; Gibson, 1983) were coded as unordered states (for characters and state assignments, see Nadler and Hudspeth [1998]). MacClade was used to examine the most parsimonious distribution of character states on the tree inferred from the combined data. Developing hypotheses for morphological character evolution were limited to the tree topology inferred for the ingroup (Ascaridoidea) because many structural characters were not comparable for the outgroup, *C. americana*.

RESULTS

Nucleotide sequences for *cox2* were deposited in GenBank (accession numbers AF179905–179923); alignments and tree files were deposited in TreeBase (Sanderson et al., 1994). Regions of COII protein sequence that are conserved among other organisms were also primarily conserved in these Ascaridida (Fig. 1). Twenty-nine of 37 highly conserved amino acid sites were unchanged (Fig. 1), and of the remaining 8 sites with replacements, 2 showed lack of conservation in comparisons of nematodes (Ascaridida, *C. elegans, O. volvulus*—amino acid sequences of the latter 2 species not shown) to other organisms (site 179 and 181; the latter also had 2 autapomorphic changes among ascaridoids). Four sites showed single autapomorphic changes (sites 103, 104, 110, 208), 1 had a synapomorphic change (site 212), and 1 (site 175, replacement in the outgroup) was an equivocal change with respect to the combined analysis tree. The region on the N-terminal side of the aromatic sequence (Fig. 1) has an abundance of nonpolar amino acids including leucine, isoleucine, proline, valine, phenylalanine, and methionine, a characteristic of transmembrane helices in COII of other organisms.

For *cox2* codons, 69% of first positions, 84.5% of second positions, and 7.7% of third positions were constant among these Ascaridida. Frequencies of nucleotides over all 3 codon positions (Table I) showed an overrepresentation of T in the coding strand of species; *H. pelagicum* and *H. reliquens* were the only taxa with frequencies of T (averaged over all positions) less than 40%. For most taxa, A+T content was higher at third codon positions than first or second (Table I). For mean nucleotide frequencies over all taxa, values of base-compositional bias were 0.078 (all positions), 0.031 (first positions), 0.045 (second positions), 0.231 (third positions), and 0.485 (4-fold degenerate sites). Substitution rate matrices (*Q* matrices) for *cox2* revealed a bias to T, with the greatest bias for the transition substitution C to T (Table II). For all 3 codon positions and 4-fold degenerate sites, rates of substitution from nucleotides C, A, or G to T were always greater than the corresponding reverse substitution. This pattern was most pronounced for 4-fold degenerate sites. For the combined evidence tree, parsimony changes by codon position or type of site included (unambiguous changes/total length): first positions (96/138), second positions (32/49), third positions (456/719), 4-fold degenerate sites (121/180). Thus, ratios of substitution at first : second :third positions of codons were 3:1:14.3 (unambiguous substitutions) and 2.8:1:14.7 (total tree length).

Previously published results (Nadler and Hudspeth, 1998) revealed that parsimony analysis of the rDNA data (2,557 nt characters, 44 recoded gap characters, 173 parsimony informative sites) yielded 3 trees of equal length with consistency indices of 0.54 that are summarized by strict consensus (Fig. 2). The Anisakinae, Ascaridinae, Contracaecinae, and Raphidascarididae (sensu Fagerholm, 1991) were supported strongly (≥99%) in bootstrap parsimony analysis of these rDNA data (Fig. 2).

Equal-weighted parsimony analysis of the *cox2* nucleotide data (582 nt characters) yielded 2 trees (length 886, consistency index [CI] 0.40, 201 parsimony informative sites). The strict consensus of these trees (Fig. 3) contains polytomies representing conflict between the trees, as each was fully dichotomous. This *cox2* strict consensus tree has considerably less structure than the strict consensus of rDNA trees, and bootstrap MP analysis of the *cox2* data set indicated poor support for many groups (Fig. 3). Transversion-weighted parsimony analysis of the *cox2* nucleotide data yielded 1 fully dichotomous tree (Fig. 4) with much greater resolution than the consensus tree for equal-weighting; however, transversion-weighted bootstrap MP analysis also revealed poor support for many clades (data not shown).

The combined analysis of all molecular plus morphological characters (3,159 characters, 517 parsimony informative characters) yielded 1 most parsimonious tree (Fig. 5, length 1,866, CI 0.47). Except for the relationship of *T. canis*, the topology of this tree was consistent with Fagerholm's (1991) classification. Bootstrap resampling and MP analysis showed strong support for some clades and moderate support for other groups (Fig. 5).

The evolution of morphological, life history, and COII amino acid characters were explored relative to the combined evidence tree (Figs. 1, 5). The range of consistency indices for structural and life history characters (Table III) showed that some of these characters were explained by the minimum number of required changes, whereas others were inferred to be highly homoplastic.

Alternative tree topologies depicting certain traditional expectations were compared to the combined evidence tree using statistical tests. These alternative topologies (Table IV) included restoring monophyly to the Ascarididae and Toxocarinae, restoring a monophyletic *Hysterothylacium* with an *Iheringascaris* sister group, depicting *G. pelagia* as a member of the Anisakidae, and the most parsimonious alternative topology for *Parascaris* within the Ascaridinae, i.e., not depicting *Parascaris* as the sister-taxon of *Ascaris*.

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                          26          36          46          56          66
Anisakis cl 1             NFYCSLLFGV LSFVSVMFGY LLFSNFYFKS KKIEYQFGEL LCSIFPTLIL
Anisakis cl 4             NFNCSLLFGV LSFVSVMFGY LLFSNFYFKS KKIEYQFGEL LCSIFPTLIL
Ps. decipiens             NFNCSLLFGV LSFVSVMFVY LLFSSFYFKS KKIEYQLGEL LCSIFPTSIL
Te. caballeroi            NFNCSLLFGV LSFVSAMFFY LLFSKFYFKS KKVEYQLGEL LCSIFPTLIL
Co. multipapillatum       NFNCSLLFGV LSFVTVMFFY LLLSRFYFKS KKIEYQFGEL LCSVFPTLIL
To. canis                 NFNCSLLFGV LSFVSTSFVY LLLSKFYFKS KKIEYQFGEL LRSVFPTLIL
As. lumbricoides          NFNCSLLFGV LSFVSTMFVY LLLSSFYFKS KKIEYQFGEL LCSVFPTLIL
As. suum                  NFNCSLLFGV LSFVSTMFVY LLLSSFYFKS KKIEYQFGEL LCSVFPTLIL
Pa. equorum               NFNCSLLFGV LSFVSTMFVY LLLSNFYFKS KKIEYQFGEL LCSVFPTLIL
Ba. procyonis             NFNCSLLFGV LSFVSTMFIY LLLSRFYFKS KKVEYQFGEL LCSVFPTLIL
Ba. transfuga             NFNCSLLFGV LSFVSTMFIY LLLSRFYFKS KKVEYQFGEL LCSVFPTLIL
Tx. leonina               NFNCSLLFGV LPFVSTMFVY LLLSCFYFKS KKVEYQFGEL LCSVFPTLIL
Po. depressum             NFNCSLLFGI LAFVCTMFIY LLLNSFYFKS KKIEYQFGEL LCSVFPTLIL
Go. pelagia               NFNCSLLFGV LSFVSAVFAY LLLSHFYFKS KKIEYQFGKL LCSVFPTLIL
Hy. pelagicum             NFNCKLLFGV LSFVSTVFAY LMLSHFYFKS KKIEYQFGEL LCSVFPTLIL
Hy. reliquens             NFNCSLLFGV LSFVSTVFAY LMLSHFYFKS KKIEYQFGEL LCSVFPTLIL
Ih. inquies               NFNCSLLFGV LSFVSTVFAY LLLSHFYFKS KKIEYQFGEL LCSVFPTLIL
Hy. fortalezae            NFNCSLLFGV LSFVCTLFVY LLLSHFYFKS KKIEYQFGEL LCSVFPTLIL
He. tunicatus             NFNCSLLFGV LVFVSTMFVY LLLSSYYFKS KKIEYQLGEL LCSVFPTLIL
Cr. americana             NFNCSLLFGV LAFVSTMFIF LIFNRFYFKS KKIEYQFGEL LCSVFPTLIL
                          ** * ****. * **   * . *... .**** **.***.* * * *.*** **
                                    0                               0

                          76          86          96          106         116
Anisakis cl 1             VAQMVPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Anisakis cl 4             VAQMVPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFGD IPGLEFDSYM
Ps. decipiens             VAQMVPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Te. caballeroi            VAQMIPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Co. multipapillatum       VMQMIPSLSL LYYYGLMNLD SSLTIKVTGH QWYWSYEFSD IPGLEFDSYM
To. canis                 VMQMVPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFSD IPGLEFDSYM
As. lumbricoides          VMQMVPSLSL LYYYGLMNLD SSLTVKVTGH QWYWSYEFSD IPGLEFDSYM
As. suum                  VMQMVPSLSL LYYYGLMNLD SSLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Pa. equorum               VMQMVPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Ba. procyonis             VMQMVPSLSL LYYYGLMNLD SSLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Ba. transfuga             VMQMVPSLSL LYYYGLMNLD SSLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Tx. leonina               VMQMVPSLSL LYYYGLMNLD SSLTIKVTGH QWYWSYEFSD VPGLEFDSYM
Po. depressum             VIQMVPSLSL LYYYGLMNLD SSLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Go. pelagia               VMQMIPSLSL LYYYGLMNLD SNLTIKVTGH QWYWTYEFSD IPGLEFDSYM
Hy. pelagicum             VMQMIPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Hy. reliquens             VMQMIPSLSL LYYYGLMNLD SNLTIKVTGH QWYWSYEFSD IPGLEFDSYM
Ih. inquies               VMQMIPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFSD IPGLEFDSYM
Hy. fortalezae            VIQMVPSLSL LYYYGLMNLD SSLTIKVTVH QWYWSYEFSD IPGLVFDSYM
He. tunicatus             VMQMVPSLSL LYYYGLMNLD SSLTVKVIGH QWYWSYEFSD IPGLEFDSYM
Cr. americana             VMQTIPSLSL LYYYGLMNLD SNLTVKVTGH QWYWSYEFSD IPGLEFDSYM
                          * * .***** ********** *.**.** * ****.***.* .*** *****
                                    0                   1
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FIGURE 1. Alignment of COII sequences. Amino acids are numbered according to the complete protein of *Ascaris suum*. Invariant sites among these taxa are marked with an asterisk; sites with chemically conserved amino acid replacements are marked with a period. Sequence regions with high levels of amino acid conservation, as inferred from comparisons of other organisms, are shaded. The combined evidence tree (Fig. 5) was used to infer unambiguously autapomorphic (underlined) and shared-derived (bold) amino acid replacements. Conserved elements of COII include the membrane-spanning domains (boxes marked 0), aromatic sequence (box 1), negatively charged cytochrome *c* binding residue (box 2), Cu_A binding histidine residue (box 3), Cu_A binding cysteine residue (box 4), negatively charged cytochrome *c* binding residue (box 5), and Cu_A binding histidine residue (box 6).

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                          126         136         146         156         166
Anisakis cl 1             KSVDQLELGE PRLLEVDNRC VVPCDINVRF CITSGDVIHS WALPSMSIKL
Anisakis cl 4             KSVDQLELGE PRLLEVDNRC VVPCDINVRF CITSGDVIHS WALPSMSIKL
Ps. decipiens             KSVDQLELGE PRLLEVDNRC VVPCDTNIRF CITSGDVIHS WALPSMSIKL
Te. caballeroi            KSIDQLLPGE PRLLEVDNRC VVPCDINVRF CITSGDVIHS WALPSMSIKL
Co. multipapillatum       KSLDQLELGE PRLLEVDNRC VIPCDTNIRF CITSGDVIHS WALPSLSIKL
To. canis                 KSLDQLELGE PRLLEVDNRC VVPCDTNIRF CITSGDVIHS WALPSMAIKL
As. lumbricoides          KSLDQLELGE PRLLEVDNRC VVPCDVNIRF CITSGDVIHS WALPSMSIKL
As. suum                  KSLDQLELGE PRLLEVDNRC VVPCDVNIRF CITSGDVIHS WALPSMSIKL
Pa. equorum               KSLDQLELGE PRLLEVDNRC VVPCDVNIRF CITSGDVIHS WALPSMSIKL
Ba. procyonis             KSLDQLELGE PRLLEVDNRC VVPCDVNIRF CITSGDVIHS WALPSMSIKL
Ba. transfuga             KSLDQLELGE PRLLEVDNRC VVPCDVNVRF CITSGDVIHS WALPSMSIKL
Tx. leonina               KSLDQLELGE PRLLEVDNRC VVPCDVNVRF CITSGDVIHS WALPSMSIKL
Po. depressum             KSLDQLELGE PRLLEVDNRC VVPCDTNIRF CITSSDVIHS WALPSMSIKL
Go. pelagia               KSVDQLALGE PRLLEVDNRC VVPCDTNIRF CITSGDVIHS WALPSMSIKL
Hy. pelagicum             KSVDQLELGE PRLLEVDNRC VVPCDTNIRF CITSGDVIHS WALPSMAIKL
Hy. reliquens             KSVDQLELGE PRLLEVDNRC VIPVDANIRF CITSGDVIHS WALPSMAIKL
Ih. inquies               KSLDQLELGE PRLLEVDNRC VIPCDTNIRF CITSGDVIHS WALPSMAIKL
Hy. fortalezae            KSLDQLELGE PRLLEVDNRC VVPCDINVRF CITSGDVIHS WALPSMSIKL
He. tunicatus             KSLDQLNLGE PRLLEVDNRC VVPCNTNIRF CITSADVIHS WALPSMAIKL
Cr. americana             KSLDQLNLGE PRLLEVDNRC VVPCETNIRF CVTSADVIHS WALPTMSIKV
                          **.***  ** ********** *.* . *.** *.**.[*]**[*]* ****...**.
                                                              2  3

                          176         186         196         206
Anisakis cl 1             DAMSGILSTV SYSFPTVGVF YGQCSEICGA NHSFMPIALEVTLL
Anisakis cl 4             DAMSGILSTV SYSFPTVGVF YGQCSEICGA NHSFMPIALEVTLL
Ps. decipiens             DAMSGILSTL SYSFPTVGVF YGQCSEICGA NHSFMPIALEVTLL
Te. caballeroi            DAMSGILSTL SYSFPVLGVF YGQCSEICGA NHSFMPISLEVILL
Co. multipapillatum       DAMSGILSTL SYSFPVVGVF YGQCSEICGA NHSFMPIALEVTLM
To. canis                 DAMSGILTTL SYSFPVVGVF YGQCSEICGA NHSFMPIVLEVTLL
As. lumbricoides          DAMSGILSTL SYSFPVVGVF YGQCSEICGA NHSFMPVALEVTLL
As. suum                  DAMSGILSTL SYSFPVVGVF YGQCSEICGA NHSFMPVALEVTLL
Pa. equorum               DAMSGILSTL SYSFPVVGVF YGQCSEICGA NHSFMPVALEVTLL
Ba. procyonis             DAMSGILSTL SYSFPVVGVF YGQCSEICGA NHSFMPVALEVTLL
Ba. transfuga             DAMSGILSTL SYSFPVVGVF YGQCSEICGA NHSFMPVALEVTLL
Tx. leonina               DAMSGILSTL SYSFPVIGVF YGQCSEICGA NHSFMPIALEVTLL
Po. depressum             DAMSGILTTL SYSFPLVGVF YGQCSEICGA NHSFMPIALEVTLL
Go. pelagia               DAMSGILSTL SYSFPVVGVF YGQCSEICGA NHSFMPIALEVTLL
Hy. pelagicum             DAMSGMLSTL SYSFPVVGVF YGQCSEICGA NHSFMPIALEVTLL
Hy. reliquens             DAMSGILSTL SYSFPVIGVF YGQCSEICGA NHSFMPIALEVTLL
Ih. inquies               DAMSGILSTL SYSFPVVGVF YGQCSEICGA NHSFMPIALEVTLL
Hy. fortalezae            DAMSGILSTL SYSFPVIGVF YGQCSEICGA NHNFMPIALEVTLL
He. tunicatus             DAMSGVLTTL SYNFPVVGVF YGQCSEICGA NHSFMPIALEVTLL
Cr. americana             DAMSGILNTL SYSFPVIGVF YGQCSEICGA NHSFMPIALEVTLL
                          *****.* *. **.** .*** ***[*][*]**** *[*].***. *** *.
                                                  4  5       6
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FIGURE 1. Continued.

DISCUSSION

Sequence comparisons

The amino terminus of these COII proteins contains many hydrophobic amino acids, consistent with the location of transmembrane helix anchors in COII proteins of other organisms (Bisson et al., 1982), including nematodes (Thomas and Wilson, 1991). The transmembrane helices show the highest amount of amino acid variation among these ascaridoids (Fig. 1); however, this observation is expected because many conservative amino acid replacements in the transmembrane region of COII appear to be selectively neutral as inferred from comparative analysis of other organisms (Templeton, 1996). The aromatic sequence region, GxQWYWxYEY (where x's designate sites without ap-

TABLE I. Nucleotide frequencies of *cox2* sequences by taxon and position.

Taxon	Nucleotide				Position A+T		
	T	C	A	G	First	Second	Third
Anisakis cl 1	0.4519	0.1168	0.2079	0.2234	0.5876	0.6495	0.7422
Anisakis cl 4	0.4553	0.1117	0.2045	0.2285	0.5876	0.6495	0.7422
Ascaris lumbricoides	0.4656	0.0928	0.2131	0.2285	0.6237	0.6495	0.7629
Ascaris suum	0.4656	0.0928	0.2096	0.2320	0.6237	0.6495	0.7526
Baylisascaris procyonis	0.4605	0.0928	0.1890	0.2577	0.6185	0.6495	0.6805
Baylisascaris transfuga	0.4416	0.0962	0.1821	0.2801	0.6186	0.6495	0.6031
Contracaecuram multipapillatum	0.4502	0.1065	0.2165	0.2268	0.6443	0.6495	0.7062
Cruzia americana	0.4381	0.1100	0.2268	0.2251	0.6495	0.6546	0.6907
Goezia pelagia	0.4416	0.1271	0.2337	0.1976	0.6031	0.6444	0.7784
Heterocheilus tunicatus	0.4433	0.0928	0.2302	0.2337	0.6392	0.6598	0.7216
Hysterothylacium fortalezae	0.4485	0.1117	0.2027	0.2371	0.6391	0.6650	0.6495
Hysterothylacium pelagicum	0.3780	0.1838	0.2302	0.2079	0.5412	0.6547	0.6288
Hysterothylacium reliquens	0.3952	0.1718	0.2165	0.2165	0.5670	0.6546	0.6134
Iheringascaris inquies	0.4210	0.1357	0.2234	0.2199	0.5928	0.6495	0.6907
Parascaris equorum	0.4485	0.0962	0.2027	0.2526	0.6237	0.6598	0.6701
Porrocaecum depressum	0.4794	0.0893	0.1942	0.2371	0.6598	0.6495	0.7113
Pseudoterranova decipiens	0.4502	0.1134	0.2285	0.2079	0.6185	0.6392	0.7783
Terranova caballeroi	0.4433	0.1203	0.2268	0.2096	0.6083	0.6547	0.7474
Toxocara canis	0.4399	0.1065	0.1993	0.2543	0.6340	0.6546	0.6289
Toxascaris leonina	0.4708	0.0979	0.2010	0.2302	0.6185	0.6495	0.7474
Average	0.4444	0.1133	0.2119	0.2303	0.6149	0.6518	0.7023

TABLE II. Rate matrix estimate of the pattern of nucleotide substitution for *cox2* based on parsimony and the combined evidence tree.

From	To			
	T	C	A	G
All positions				
T	−0.667416	0.293187	0.126929	0.247300
C	1.319390	−1.472471	0.063034	0.090047
A	0.294809	0.032533	−1.203737	0.876395
G	0.489936	0.039642	0.747536	−1.277113
First positions				
T	−0.892312	0.642053	0.083447	0.166811
C	1.792079	−1.960081	0.142793	0.025209
A	0.109765	0.067294	−0.787650	0.610591
G	0.233341	0.012634	0.649327	−0.895302
Second positions				
T	−0.615646	0.411544	0.102190	0.101912
C	1.042744	−1.693887	0.128755	0.522388
A	0.160663	0.079893	−0.847132	0.606576
G	0.209621	0.424073	0.793576	−1.427270
Third positions				
T	−0.476605	0.208100	0.098435	0.170070
C	5.178309	−5.541286	0.155168	0.207809
A	0.811710	0.051421	−3.252610	2.389479
G	0.424680	0.020854	0.723578	−1.169112
4-fold degenerate sites				
T	−0.503096	0.147581	0.123087	0.232429
C	6.291296	−7.302287	0.448629	0.562362
A	2.968347	0.253793	−4.405633	1.183493
G	2.676191	0.151891	0.565054	−3.393135

parent amino acid conservation), is normally highly conserved, presumably because it mediates electron transfer from subunit II to heme *a* of subunit I (Müller et al., 1988; Overholtzer et al., 1996). In these ascaridoids, the aromatic region is represented by the consensus sequence GHQWYWSYEF (positions 104–113), where each of the underlined amino acids had a replacement in 1 ascaridoid. The position 104 replacement (valine) in *H. fortalezae* was unexpected based on conservation in other organisms; however, functional studies in yeast show that replacements of the conserved glycine with other small uncharged residues (such as valine) does not compromise subunit II function (Overholtzer et al., 1996). The conservative replacement at position 113 in the aromatic sequence (phenylalanine for tyrosine) was found in all Ascaridida; in *C. elegans* and *O. volvulus* the tyrosine is conserved (Okimoto et al., 1992; Keddie et al., 1998). Another highly conserved region in other organisms is the DV(hydrophobic amino acid)H sequence (Müller et al., 1988); ascaridoids have retained the aspartic acid (D) at position 161 (providing a negative charge for cytochrome *c* binding), followed by the invariant valine, an isoleucine, and the invariant histidine, which is 1 of 2 involved in copper A (Cu_A) binding. Subunit II sequences also typically have a conserved PGR sequence at positions 179–181. Nematodes sequenced to date have the glycine, but the other 2 residues are characterized by nonconservative replacements. The Ascaridida and *C. elegans* (Okimoto et al., 1992) have the sequence SG (hydrophobic amino acid), whereas *O. volvulus* has NGI (Keddie et al., 1998). A region near the C-terminus of COII proteins contains the other critical ligands to Cu_A and a second charged amino acid involved in cytochrome *c* binding. The consensus sequence for this region, CSE(hydrophobic amino acid) CGxxHxxxPI, is found in the Ascaridida (positions 199–212) with the exception of the final isoleucine, which in *Ascaris*,

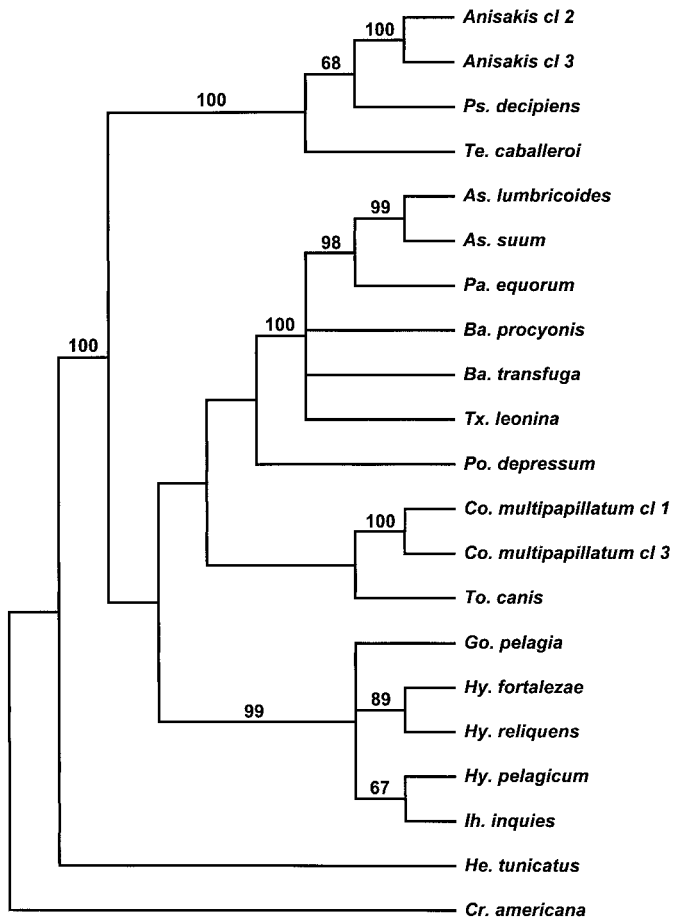

FIGURE 2. Strict consensus of 3 equally parsimonious trees inferred from unweighted analysis of rDNA sequence data (2,557 characters, 173 parsimony-informative sites, 1,047 steps, CI 0.538). Results from bootstrap resampling and MP inference are also shown relative to the strict consensus tree for clades receiving >65% bootstrap support. The bootstrap 50% majority-rule consensus tree differed from the topology shown in that *T. leonina* was resolved as the ''basal'' lineage within the Ascaridinae, and (*C. multipapillatum* cl 1, *C. multipapillatum* cl 2) and *T. canis* were each depicted as polytomies within the clade that includes taxa representing the Ascarididae and Raphidascarididae.

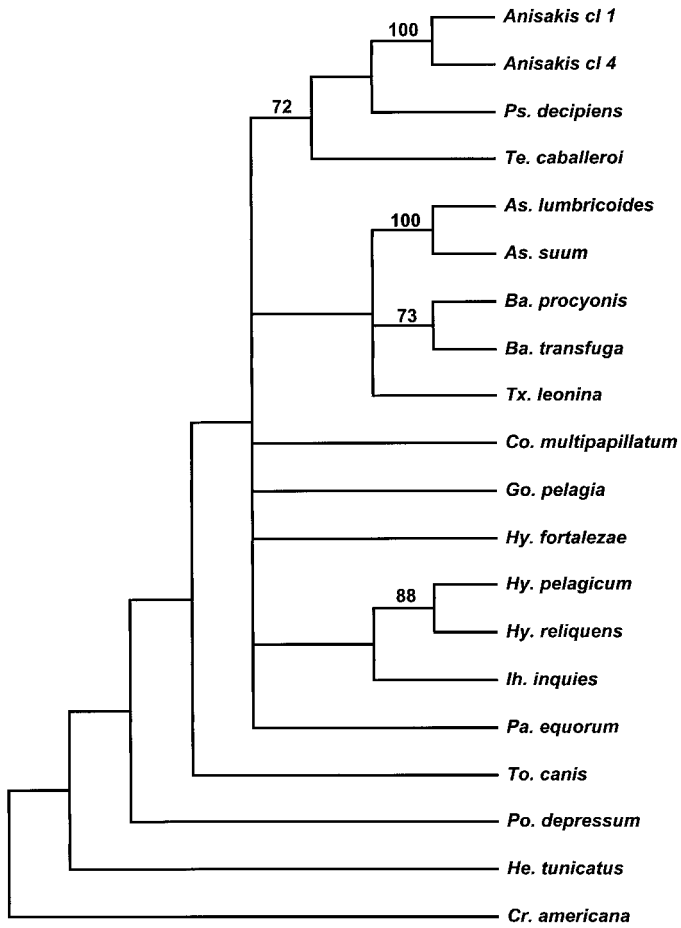

FIGURE 3. Strict consensus of 2 equally parsimonious trees inferred from unweighted analysis of *cox2* nucleotide data (582 characters, 201 parsimony-informative sites, 886 steps, CI 0.40). Results from bootstrap resampling and MP inference are also shown relative to the strict consensus tree; percentages of clades (>65%) are shown above internal nodes. The bootstrap 50% majority-rule consensus tree had less resolution than the strict consensus shown and is defined by the topology: (*C. americana,* (*H. tunicatus,* (*P. depressum,* (*T. canis,* ((((*Anisakis* cl 1, *Anisakis* cl 4), *P. decipiens*), *T. caballeroi*), ((*H. pelagicum, H. reliquens*), *I. inquies*), *H. fortalezae, G. pelagia, P. equorum, C. multipapillatum,* ((*B. procyonis, B. transfuga*), *T. leonina,* (*A. lumbricoides, A. suum*)))))))).

Parascaris, and *Baylisascaris* is replaced by another hydrophobic amino acid (valine).

These amino acid sequences for ascaridoids show conservation of the most critical functional residues characteristic of COII in other organisms (Holm et al., 1987). Subunit II of cytochrome oxidase is a key component of electron transfer during aerobic respiration, and given the observed conservation among ascaridoids, it seems possible that the aerobic larval component of the aerobic/anaerobic metabolism of *A. suum* (Komuniecki and Komuniecki, 1995) may also be present in other members of the superfamily, including species developing in aquatic environments.

Like other nematode mtDNA sequences (Thomas and Wilson, 1991; Okimoto et al., 1992; Hyman and Azevedo, 1996; Hugall et al., 1997; Blouin et al., 1998; Keddie et al., 1998), *cox2* is extremely A+T rich in ascaridoids. The value for third-position base compositional bias of ascaridoid *cox2* is the same as for *Caenorhabditis* species (Thomas and Wilson, 1991), which is only slightly less biased than *Drosophila yakuba* (Clary and Wolstenholme, 1985). Averaged over all taxa, base-compositional bias of ascaridoid *cox2* was 10-fold greater at 4-fold degenerate sites than for first or second codon positions. Rate matrix analysis (Table II) reveals a strong pattern of substitutional bias to thymine at all codon positions; this bias was most pronounced at 4-fold degenerate sites, which represent sites most likely to be selectively neutral within protein coding genes (Wolfe et al., 1989). This pattern and the observed A+T bias is consistent with A+T pressure (Jukes and Bhushan, 1986; Jermiin et al., 1995), a pattern of nonrandom substitution resulting in the accumulation of A+T nucleotides in protein-coding genes, particularly at synonymous sites. For ascaridoid *cox2,* differential patterns of compositional and substitutional bias by codon position are consistent with an underlying mutational bias (Sueoka, 1992) that has been previously suggested for nematode mtDNA (Thomas and Wilson, 1991; Okimoto et

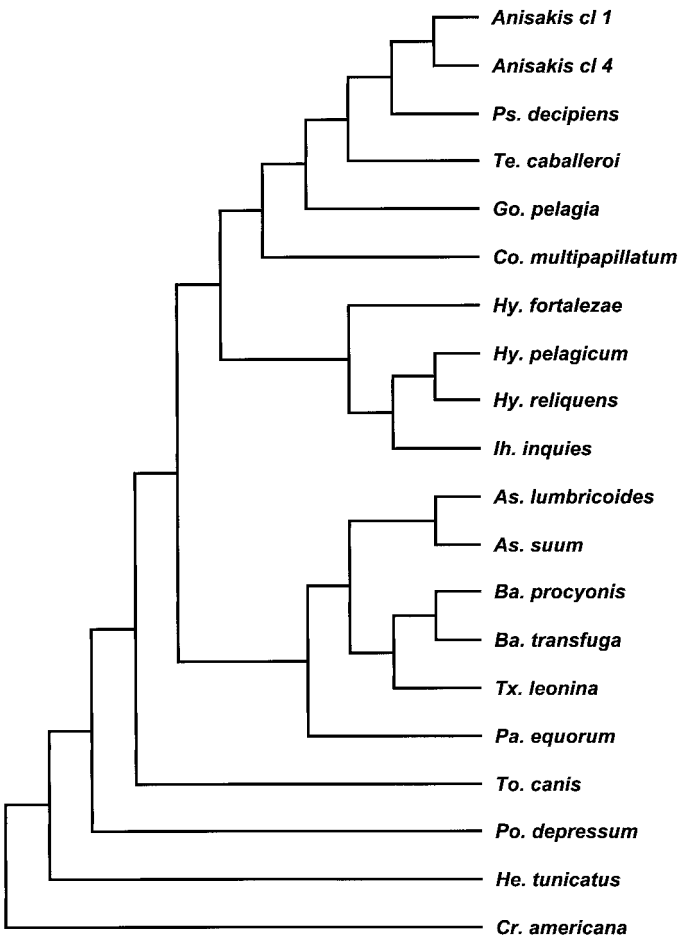

FIGURE 4. Parsimony tree for *cox2* nucleotide data (1,859 steps), inferred using a transversion-weighted stepmatrix.

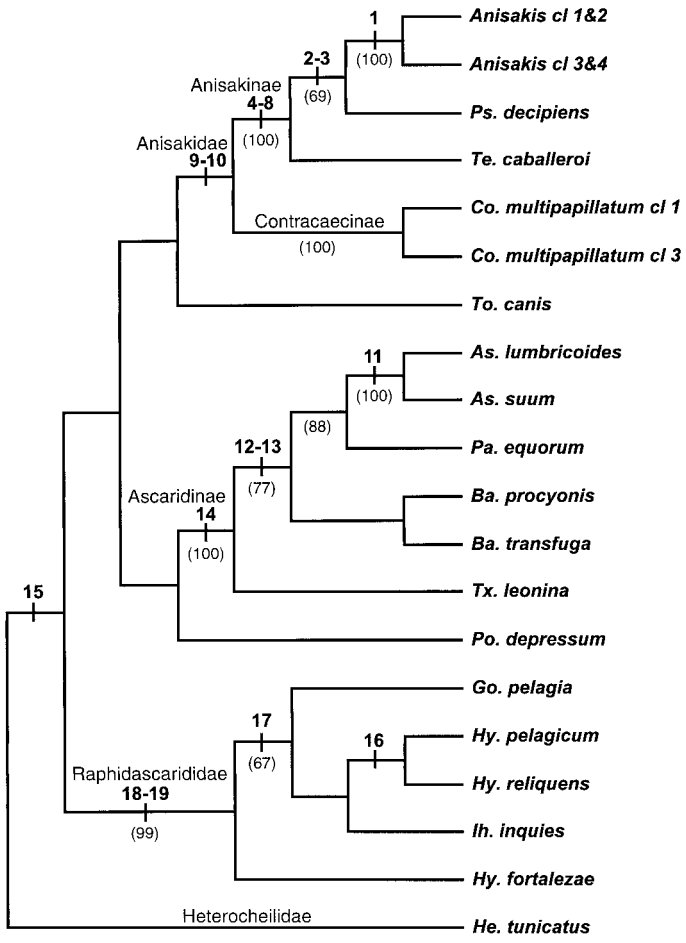

FIGURE 5. Maximum parsimony tree inferred from combined analysis of all molecular and morphological data (3,159 characters, 517 parsimony informative, 1,866 steps, CI 0.47). Inferred synapomorphic changes in morphological and amino acid characters with respect to the combined evidence tree are labeled: 1, AA 185 Val; 2, AA 191 Thr; 3, distal papillae proximal; 4, AA 69 Ile; 5, AA 77 Ala; 6, caudal plates present; 7, ventriculus long; 8, lips reduced; 9, left-glandular excretory system; 10, excretory pore between lips; 11, proximal papillae grouped posterior; 12, AA 212 Val; 13, distal papillae united; 14, ventriculus absent; 15, gubernaculum absent; 16, AA 47 Met; 17, AA 44 Ala; 18, AA 50 His; 19, left-filamental excretory system. Results from bootstrap resampling and MP inference are shown relative to the single most parsimonious tree; percentages of clades (>60%) are shown below internal nodes. The topology of the bootstrap 50% majority-rule consensus tree showed less resolution and is defined by the topology: (*H. tunicatus*, ((((*Anisakis* cl 2, *Anisakis* cl 3), *P. decipiens*), *T. caballeroi*), ((((*H. pelagicum*, *H. reliquens*), *I. inquies*, *G. pelagia*), *H. fortalezae*), (*C. multipapillatum* cl 1, *C. multipapillatum* cl 3), *P. depressum*, *T. canis*, (*T. leonina*, ((*B. procyonis*, *B. transfuga*), (*P. equorum*, (*A. lumbricoides*, *A. suum*)))))))).

al., 1992; Hyman and Azevedo, 1996; Hugall et al., 1997; Blouin et al., 1998). Due to a relatively rapid rate of change, compositional bias, and tendency for multiple hits (Thomas and Wilson, 1991; Blouin et al., 1998), it has been suggested that nematode mtDNA is likely to be of less phylogenetic utility than many nuclear genes (Blouin et al., 1998). The comparatively poor resolution and higher homoplasy of the *cox2* gene tree in comparison to rDNA (Nadler and Hudspeth, 1998) for the same set of taxa is consistent with this suggestion, and it is noteworthy that *cox2* is among the more conservatively evolving mtDNA genes.

Phylogenetic hypotheses

Previous analyses of SSU and LSU rDNA sequences (Nadler, 1992; Nadler and Hudspeth, 1998) showed that rDNA is informative for inferring ascaridoid phylogeny. Yet, statistical comparison of alternative trees and results from bootstrap resampling also revealed that these rDNA sequences did not provide an entirely robust hypothesis. In an attempt to improve upon this phylogenetic estimate, sequence data were obtained from another locus (*cox2*), and all available nucleotide sequence and morphological data were combined for parsimony analysis. This approach to phylogenetic analysis, typically referred to as combined or total evidence (Eernisse and Kluge, 1993; Kluge, 1998), has been advocated as the method of choice to provide the best estimate of evolutionary history by maximizing the explanatory power of the available data. A practical advantage of this approach is that it is efficient for large data sets. In addition, parsimony is the only method conducive to combining disparate types of data, such as nucleotide sequences and morphology, in a single analysis.

Parsimony analysis of the combined data yielded a single tree (Fig. 5) that has greater congruence with Fagerholm's (1991) classification than the individual gene trees inferred from rDNA

TABLE III. Mapping of structural and life history characters on the combined evidence tree.

Character	States*	Steps	CI
1. Median papilla	2	3	0.33
2. Paracloacal papillae	2	4	0.25
3. Distal papillae number	2	1	1.00
4. Distal papillae type	4	3	1.00
5. Proximal papillae type	3	2	1.00
6. Spicules	2	2	0.50
7. Gubernaculum	2	1	1.00
8. Caudal plates	2	1	1.00
9. Caudal alae	2	2	0.50
10. Ventriculus	3	2	1.00
11. Intestinal cecum	2	3	0.33
12. Lips	2	1	1.00
13. Lip denticles	2	2	0.50
14. Interlabia	2	6	0.17
15. Excretory pore	3	2	1.00
16. Excretory system	3	2	1.00
17. Cervical alae	3	6	0.33
18. Eggshell	2	2	0.50
19. Ventricular appendix	2	2	0.50
20. Proximal papillae pairs	3	3	0.67
21. Definitive host	4	4	0.75
22. Life history	2	2	0.50

* 1, Single/joined; 2, separate/joined double; 3, 2 pair/4 pair; 4, distal united/distal unjoined/proximal/subventral rows; 5, straight rows/grouped anterior/grouped posterior; 6, alate/rod; 7, present/absent; 8, present/absent; 9, present/absent; 10, short/long/absent; 11, present/absent; 12, prominent/reduced; 13, present/absent; 14, present/absent; 15, between nerve ring and lips/near nerve ring/between lips; 16, left–right filamental/left glandular/left-filamental; 17, present/absent; 18, thick-walled/thin-walled; 19, present/absent; 20, <6 pairs/6–50 pairs/>50 pairs; 21, mammal/fish/bird/reptile; 22, terrestrial/aquatic.

or *cox2* (Figs. 2–4). Nevertheless, certain clades in this tree remain weakly supported as assessed by bootstrap resampling, and statistical comparisons show that some alternative topologies representing traditional expectations are not worse. Although the combined evidence tree does not encompass the full diversity of the Ascaridoidea (>50 described genera), this phylogenetic hypothesis suggests that Fagerholm's (1991) taxonomic summary represents a reasonable working classification that can be examined in greater detail as more taxa are included in phylogenetic analysis. One disagreement between the combined evidence tree and all modern classifications concerns *T. canis* that was depicted as the most basal branch of the anisakids. Classically, *Toxocara* and *Porrocaecum* have been retained as a distinct subfamily (Toxocarinae) within the Ascarididae because they possess a ventriculus and have a terrestrial life cycle (Gibson, 1983; Fagerholm, 1991). Clearly, the former character cannot be considered informative for inferring group membership because the presence of a ventriculus is the plesiomorphic condition as inferred from the combined evidence. Nevertheless, restoring monophyly to the Ascarididae and Toxocarinae such that the latter subfamily is the sister group to the Ascaridinae requires only 6 additional steps by parsimony, which was not significantly worse. Thus, from a statistical perspective, these data are insufficient to discriminate between the representation in Figure 5 and the traditional alternative hypothesis for the Toxocarinae.

In the combined evidence tree, species of *Hysterothylacium* were not monophyletic. This finding was also obtained for analyses of rDNA and combined analysis of rDNA and morphology (Nadler and Hudspeth, 1998) and herein for transversion-weighted analysis of *cox2*. Analysis of alternative trees for rDNA showed that a monophyletic *Hysterothylacium* with an *Iheringascaris* sister group was not significantly worse (Nadler and Hudspeth, 1998). However, with the combined evidence, restoring a monophyletic *Hysterothylacium* (((*Hysterothylacium* spp.), *Iheringascaris*), *Goezia*) required a minimum of 23 additional steps, and was significantly worse. In contrast, the topology ((((*H. pelagicum, H. reliquens*), *I. inquies*), *H. fortalezae*), *G. pelagia*) required 6 additional steps and was not significantly worse. Deardorff and Overstreet (1980) proposed resurrecting *Iheringascaris* (formerly *Thynnascaris inquies*, a genus previously used for several other species of *Hysterothylacium*) because *I. inquies* has conspicuous annulations and additional caudal papillar rows when compared to *Hysterothylacium* species. The combined evidence tree (Fig. 5) does not provide a topological basis for this generic distinction, and comparison of alternative topologies also suggests that *Iheringascaris* is best represented as more closely related to some *Hysterothylacium*, than as a sister taxon to a monophyletic *Hysterothylacium.*

The Anisakidae as typically constituted (Anisakinae plus Contracaecinae) was recovered by maximum likelihood analy-

TABLE IV. Alternative trees compared using statistical tests and the combined data.

Tree topology*	Length	$P^{\dagger}$ Templeton's test	$P^{\dagger}$ winning sites
MP tree (Fig. 5): (20,(((((11,12),13),9),10),((((((1,2),16),17),(7,8)),18),(15,(19,((5,6),(14,(3,4))))))))	1,866		
Alt tree 1: (20,(((((11,12),13),9),10),(((((1,2),16),17),(7,8)),((15,18),(19,((5,6),(14,(3,4))))))))	1,872	0.38	0.40
Alt tree 2: (20,(((((11,12),10),13),9),((((((1,2),16),17),(7,8)),18),(15,(19,((5,6),(14,(3,4))))))))	1,889	<0.005	0.001
Alt tree 3: (20,(((((11,12),13),10),9),((((((1,2),16),17),(7,8)),18),(15,(19,((5,6),(14,(3,4))))))))	1,872	0.30	0.33
Alt tree 4: (20,((((11,12),13),10),(((((((1,2),16),17),9),(7,8)),18),(15,(19,((5,6),(14,(3,4))))))))	1,911	<0.001	<0.001
Alt tree 5: (20,(((((11,12),13),10),9),((((((1,2),16),17),(7,8)),18),(15,(19,(14,((5,6),(3,4))))))))	1,882	0.03	0.03

* Alt tree 1, monophyletic Toxocarinae as sister group to Ascaridinae; Alt tree 2, monophyletic *Hysterothylacium* with *Iheringascaris* sister group; Alt tree 3, most parsimonious *Hysterothylacium* + *Iheringascaris* clade with *Goezia* sister group; Alt tree 4, most parsimonious topology with *Goezia* within the Anisakidae; Alt tree 5, most parsimonious topology excluding a *Parascaris–Ascaris* clade. Parenthetical tree descriptions written as rooted, although tests do not require rooted trees. Key to taxa: 1, *Anisakis* cl 2; 2, *Anisakis* cl 3; 3, *A. lumbricoides*; 4, *A. suum*; 5, *B. procyonis*; 6, *B. transfuga*; 7, *C. multipapillatum* cl 1; 8, *C. multipapillatum* cl 3; 9, *G. pelagia*; 10, *H. fortalezae*; 11, *H. pelagicum*; 12, *H. reliquens*; 13, *I. inquies*; 14, *P. equorum*; 15, *P. depressum*; 16, *P. decipiens*; 17, *T. caballeroi*; 18, *T. canis*; 19, *T. leonina*; 20, *H. tunicatus.*

† Probability of obtaining a more extreme test statistic under the null hypothesis of no difference between the two trees (2-tailed).

sis of rDNA (Nadler and Hudspeth, 1998) and in the combined evidence tree. However, for the combined evidence, this clade was not recovered in bootstrap MP analysis. In some other estimates of gene trees (MP rDNA, equal-weighted *cox2* MP), *Contracaecum* taxa did not form a clade with the other Anisakidae. For the rDNA gene tree, the relationship of *Contracaecum* was equivocal as assessed by statistical comparisons of alternative topologies (Nadler and Hudspeth, 1998). Likewise, in equal- and transversion-weighted bootstrap MP analysis of *cox2, Contracaecum* was unresolved among the ingroup taxa. So although the best single estimate of phylogeny (the combined evidence tree) yielded a monophyletic Anisakidae, support for this clade appears to be weak given these data.

In the transversion-weighted *cox2* gene tree, a clade was recovered that included Anisakinae, Contracaecinae, and *Goezia,* consistent with representation of *Goezia* as a subfamily within the Anisakidae (Hartwich, 1974; Gibson, 1983). In contrast, this grouping of *Goezia* with the anisakids was not recovered in the transversion-weighted bootstrap tree, and *Goezia* was included among the Raphidascarididae with high (99%) bootstrap support in the combined evidence tree. Furthermore, for the combined evidence, the shortest alternative tree depicting *G. pelagia* within the Anisakidae was significantly worse (requiring 45 additional steps), indicating that both the most parsimonious explanation of the combined data and statistical evaluation refutes inclusion of *Goezia* with the anisakids.

Like previous analyses of rDNA data, or the combined analysis of rDNA and morphology (Nadler, 1992; Nadler and Hudspeth, 1998), the combined evidence tree did not yield robust support for relationships among more distantly related ascaridoid clades as inferred from bootstrap parsimony analysis. Thus, although the single tree inferred from combined evidence is the best explanation of these data, some of the deeper nodes in this tree should be interpreted cautiously. Given this caveat, the combined evidence tree does not support the hypothesis that ascaridoids of marine mammal definitive hosts are most closely related to species maturing in marine fish (Osche, 1963). However, unlike results for rDNA alone (Nadler and Hudspeth, 1998), the combined evidence tree is consistent with the hypothesis that parasites of piscivorous aquatic mammals and birds are more closely related to those from terrestrial mammalian hosts, than those maturing in bony fish (Anderson, 1984). With respect to definitive host, neither taxa maturing in mammals nor those from all aquatic hosts are monophyletic; only species maturing in fish form a clade. The combined evidence tree does not appear consistent with the hypothesis of a broad pattern of ascaridoid host–parasite coevolution (Osche, 1958, 1963). It seems more likely that these host–parasite associations reflect a variety of complex patterns of definitive host acquisition with subsequent speciation, including reaching novel hosts via transfer from prey hosts to predator hosts through the food chain (Sprent, 1982, 1992), or, conversely, transfers from predator hosts to prey hosts via precocity (Anderson and Bartlett, 1993).

Only recently have investigators provided explicit phylogenetic proposals for relationships among ascaridoid genera (Nadler, 1992, 1995; Nadler and Hudspeth, 1998). In the combined evidence tree, moderate (69%) bootstrap support was observed for a clade consisting of *Anisakis* and *Pseudoterranova,* species that use marine mammals as definitive hosts. The clade consisting of *Ascaris* and *Parascaris* was reliably supported (88%) by bootstrap resampling, and the shortest alternative topology (16 more steps) without the *Ascaris–Parascaris* clade was significantly worse by statistical tests. Topologies that did not preserve this clade were also significantly worse for the rDNA data alone (Nadler and Hudspeth, 1998). The *Ascaris–Parascaris* clade is unexpected based on morphological dissimilarities of gross labial features. It appears likely that the labial differences of *Parascaris* and *Ascaris,* which occur late during larval development (Pilitt et al., 1979, 1981), are an example of heterochrony.

In the combined evidence tree, extremely strong bootstrap support (100%) was observed for the Ascaridinae, and within this clade, monophyly of *Ascaris, Parascaris,* and *Baylisascaris* received moderate support (77%). The *Baylisascaris* species were also monophyletic with moderate (77%) bootstrap support. Some trees based on rDNA have provided evidence for *Baylisascaris* monophyly (Nadler, 1992), whereas trees based on other regions of rDNA sequence have not (Nadler and Hudspeth, 1998). Inferred substitutions from analysis of *cox2* support *Baylisascaris* monophyly in all most parsimonious trees (73% of bootstrap MP trees in unweighted analysis; 62% in transversion-weighted analysis).

Several cryptic species of *Anisakis* have been identified by allozyme electrophoresis (Nascetti et al., 1986). Clones of 28S rDNA obtained from pooled juvenile samples also revealed unexpected sequence divergence consistent with multiple species (Nadler and Hudspeth, 1998). The 2 *cox2* clones representing *Anisakis* also appeared to represent separate species. With respect to the combined tree, clone 1 had 6 autapomorphies, whereas clone 4 had 5 autapomorphies, and there were a total of 15 nt differences between these clones. These clones were treated separately for inferring the *cox2* gene tree, and as expected, they were monophyletic (with 100% bootstrap support). *Anisakis* juveniles of different species are morphologically quite similar, and sequence data (such as rDNA or mtDNA) or sequence-specific tests (e.g., PCR-restriction fragment length polymorphism, SSCP) correlated with morphologically or biochemically diagnosed adults may provide the only practical method for identification of juveniles.

The validity of human and pig *Ascaris* as separate species has remained controversial (Gibson, 1983; Anderson et al., 1993; Anderson and Jaenike, 1997). Studies of human and pig *Ascaris* using restriction site data (Anderson et al., 1993; Anderson, Romero-Abal et al., 1995; Anderson and Jaenike, 1997) have not revealed a single genetic marker that distinguishes host-associated *Ascaris,* yet interpretation of data representing multilocus genotypes is consistent with the inference of restricted gene flow between these putative species. Addressing the species status of host-associated *Ascaris* taxa with sequence data requires population-level sampling that was not done in the current study. However, it is noteworthy that for the combined evidence tree, *A. lumbricoides* had 7 unambiguous autapomorphies (6 rDNA characters, 1 *cox2* character) and *A. suum* had 1 such autapomorphy (a rDNA character). This suggests that rDNA sequences may provide useful data for evaluating species status of *Ascaris* in a phylogenetic context (Adams, 1998), although regions of rDNA with more rapid rates of change are likely to provide greater phylogenetic resolution for this question. In a nonphylogenetic context, the pig- and hu-

man-derived specimens had 2 nucleotide differences for *cox2*, both transitions (A–G) at third positions of codons that were silent at the amino acid level. The rDNA sequences had a total of 7 nt differences for the pig- and human-derived worms (Nadler and Hudspeth, 1998). For both LSU rDNA and *cox2* sequences, *A. suum* and *A. lumbricoides* had the smallest pairwise distance among taxa in the comparisons. These results are consistent with other observations of low genetic differentiation between these host-associated taxa (Nascetti et al., 1979; Nadler, 1987; Anderson et al., 1993).

Unlike analysis of rDNA (for which *H. fortalezae* and *H. reliquens* received strong bootstrap support), or *cox2* (for which *H. pelagicum* and *H. reliquens* received strong bootstrap support), no sister taxa within the Raphidascarididae received even moderate bootstrap support in the combined evidence tree, presumably due to character conflicts resulting from combining the molecular data. The conflict between inferred *Hysterothylacium* sister taxa from separate analyses of rDNA (Fig. 2) and *cox2* (Figs. 3, 4) represents the only instance where these different loci strongly support conflicting clades. One potential explanation is that this difference reflects discordance between the histories of rDNA and the mitochondrial genome. An alternative is that elements of sequence evolution, such as the comparatively long branches in the mitochondrial gene tree may be misleading the parsimony analysis. An argument against the latter explanation is that *H. pelagicum* and *H. reliquens* are also sister taxa in maximum-likelihood analysis of the *cox2* data (data not shown), and likelihood inference is less sensitive to long-branch attraction (Felsenstein, 1981).

Character evolution

Patterns of character evolution, as explored using the combined evidence tree, suggest that certain features that have been used to develop evolutionary scenarios and classifications represent either plesiomorphic states or highly homoplastic characters. As examples of the former, the left–right filamental (H-shaped) excretory system and the presence of a ventriculus, features emphasized by several investigators as indicators of relationship (Gibson, 1983; Sprent, 1983), are both plesiomorphic character states. Likewise, presence or absence of the intestinal cecum has been emphasized as a key feature by certain workers (Hartwich, 1954), yet mapping indicates that this character is highly homoplasious.

Parsimony analysis revealed putative morphological synapomorphies with respect to the combined evidence tree (Fig. 5). For example, states of 2 characters (left-glandular excretory system, excretory pore between lips) are synapomorphic for the Anisakidae. The Anisakinae are defined by 3 morphological synapomorphies (presence of caudal plates, ventriculus long, reduced lips), the Ascaridinae by 1 (ventriculus absent), and the Raphidascarididae by 1 (excretory system left-filamental). Excretory system structure, which is complex and variable among taxa, has been considered an important character for developing evolutionary hypotheses (Hartwich, 1954, 1957; Gibson, 1983; Sprent, 1983). Similarly, Fagerholm (1991) proposed that distribution patterns of cloacal papillae may also be useful for interpreting relationships. Although coding of cloacal papillae patterns may prove difficult, the combined tree supports Fagerholm's (1991) idea, in that the state of united distal papillae is apomorphic for *Baylisascaris, Ascaris,* and *Parascaris,* whereas the configuration of distal papillae in a more proximal position is apomorphic for *Anisakis* and *Pseudoterranova.* Inferences about synapomorphic morphological states based on trees obtained from analysis of combined evidence are likely to be particularly valuable for developing additional testable evolutionary hypotheses, because for certain rare taxa only morphological data are readily available.

Although COII amino acid sequences are not independent from their underlying nucleotide sequences (codons), parsimony mapping of deduced amino acids can reveal apomorphic patterns that are not evident when individual nucleotide sites are considered without reference to the genetic code. These amino acid replacements may also be viewed in light of functional constraints on COII evolution and previous comparative studies of COII sequences (Müller et al., 1988; Thomas and Wilson, 1991). Parsimony mapping of amino acid characters on the combined evidence tree revealed shared-derived patterns for clades (Figs. 1, 5). For example, the Raphidascarididae share a derived histidine for amino acid 50 of the alignment. Within this family, the clade composed of *H. pelagicum, H. reliquens, I. inquies,* and *G. pelagia* have a derived alanine at position 44, and the sister taxa *H. pelagicum* and *H. reliquens* share a methionine at position 47. The Anisakinae share 2 derived amino acid states, isoleucine at position 69 and alanine at position 77. Within this subfamily, the clade consisting of *Anisakis* and *Pseudoterranova* share a threonine at position 191, and the *Anisakis* taxa share a derived valine at position 185. The Ascaridinae have a shared-derived valine at position 151, and within this subfamily, *Baylisascaris* spp., *P. equorum,* and *Ascaris* spp. have a synapomorphic valine at position 212. The amino acid replacement at position 212 is chemically conservative (valine for isoleucine) but occurs at a site that appears to be virtually invariant based on comparison of sequences from diverse organisms (Müller et al., 1988). This is the only shared-derived replacement that occurs within the most highly conserved regions of COII (Fig. 1, shaded boxes). Thirty-five autapomorphic amino acid replacements occur relative to the combined tree; 5 of these occur within highly conserved regions, and all of these are chemically conservative replacements.

Although the life cycles of many ascaridoids are not known in detail, available information indicates substantial variation in potential transmission patterns, ranging from monoxenous life cycles requiring no intermediate host, e.g., *Ascaris, Parascaris,* and certain *Baylisascaris,* to heteroxenous patterns requiring intermediate hosts and involving paratenesis, e.g., *Anisakis.* Certain authorities have regarded the monoxenous pattern (and typically nematodes with such life cycles) as ancestral (Chitwood and Chitwood, 1950; Mozgovoi, 1953). Other investigators (Fülleborn, 1927; Sprent, 1954; Chabaud, 1955; Anderson, 1988) have proposed that heteroxeny is ancestral for ascaridoids, with the direct life-cycle pattern of *Ascaris,* which involves somatic and tracheal migrations, representing loss of intermediate hosts, i.e., secondary monoxeny (Fülleborn, 1927; Chabaud, 1955; Anderson, 1988). In the combined evidence tree, ascaridoid species with classical secondary monoxeny (*Ascaris, Parascaris*) are a clade within the Ascaridinae, some with facultative monoxenous patterns, i.e., where somatic/tracheal migrations can yield patent infections (even though most infections are acquired through other routes) are sister to this clade,

and others with modified facultative monoxeny, i.e., *T. leonina*, branch earlier, consistent with Fülleborn's (1927) hypothesis. Some species of *Toxocara* are also capable of facultative monoxeny, and strict interpretation of the combined evidence tree suggests that monoxeny evolved more than once within the Ascaridoidea. However, if the true evolutionary history is represented by a monophyletic Toxocarinae as the sister group to the Ascaridinae, a result not significantly worse than the combined evidence tree, then it is equivocal if the monoxenous pattern evolved more than once within the superfamily.

ACKNOWLEDGMENTS

We thank J. Brattey, B. Font, K. Gasser, R. Overstreet, R. Palmer, and J. Sakanari for help collecting specimens. We are grateful to M. Hudspeth for help with troubleshooting sequencing reactions. H.-P. Fagerholm and E. Hoberg provided information on morphological characters. This work was supported in part by National Science Foundation grant DEB-9208024.

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ANNOUNCEMENT . . .

The 4th International Symposium on Monogenea will be held 9–13 July 2001 in Brisbane, Queensland, Australia. Further details will be forthcoming. Contact persons are: Dr. Ian D. Whittington and Dr. Leslie A. Chisholm, Department of Microbiology and Parasitology, The University of Queensland, Brisbane, Qld. 4072, Australia.

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