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Phylogeny of the Trichostrongylina (Nematoda) Inferred from 28S rDNA Sequences

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We produced a molecular phylogeny of species within the order Strongylida (bursate nematodes) using the D1 and D2 domains of 28S rDNA, with 23 new sequences for each domain. A first analysis using *Caenorhabditis elegans* as an outgroup produced a tree with low resolution in which three taxa (*Dictyocaulus filaria, Dictyocaulus noerneri,* and *Metastrongylus pudendotectus*) showed highly divergent sequences. In a second analysis, these three species and *C. elegans* were removed and an Ancylostomatina, *Bunostomum trigonocephalum,* was chosen (on the basis of previous morphological analyses) as the outgroup for an analysis of the phylogenetic relationships between and within the Strongylina (strongyles) and Trichostrongylina (trichostrongyles). A very robust tree was obtained. The Trichostrongylina were monophyletic, but the Strongylina were paraphyletic, though this requires confirmation. Within the Trichostrongylina, the three superfamilies defined from morphological characters are confirmed, with the Trichostrongyloidea sister group to a clade including the Molineoidea and Heligmosomoidea. Within the Trichostrongyloidea, the Cooperiidae, Trichostrongylidae, and Haemonchidae were polytomous, the Haemonchinae were monophyletic, but the Ostertagiinae were paraphyletic. The sister-group relationships between Molineoidea and Heligmosomoidea were unsuspected from previous morphological analysis. No unequivocal morphological synapomorphy could be found for the grouping Molineoidea + Heligmosomoidea, but none was found which contradicted it.

Key Words: **ribosomal DNA; 28S; Nematoda; Strongylida; Trichostrongylina.**

INTRODUCTION

Classification of the Nematoda, one of the most species-rich phyla, has been hampered by the lack of reliable morphological characters. Molecular phylogenies for this phylum, based on 18S rDNA, have recently been proposed (Aleshin *et al.,* 1998a,b; Blaxter *et al.,* 1998; Kampfer *et al.,* 1998). One of the results of these studies was the paraphyly of the Rhabditida and recognition of sister-group relationships between the Strongylida (bursate nematodes) and *Caenorhabditis elegans* (Aleshin *et al.,* 1998a,b; Blaxter *et al.,* 1998).

The order Strongylida is one of the most important orders of parasitic Nematoda in number of species and includes many species of medical or veterinary importance. Four suborders (Durette-Desset and Chabaud, 1993) are distinguished on the basis of morphological characters, such as the mouth and the caudal bursa: the Ancylostomatina (hookworms), Strongylina (strongyles), Trichostrongylina (trichostrongyles), and Metastrongylina (lungworms). Traditional classifications, based on morphology and without a cladistic approach, are available for the Strongylida (Chabaud, 1974; Durette-Desset *et al.,* 1994), and paleogeographical reconstructions have been proposed with great degrees of detail for the Trichostrongylina (Durette-Desset, 1985). Attempts to construct a phylogenetic classification of the group using cladistic methods have been, until now, limited to the subfamily Pudicinae (Durette-Desset and Justine, 1991), the family Trichostrongylidae (Hoberg and Lichtenfels, 1994), and the superfamily Trichostrongylidea (Durette-Desset *et al.,* 1999). Molecular studies are numerous in this group (see review by Gasser and Newton, 2000), but have used mostly the ITS1 and ITS2 regions of rDNA, for diagnostic purposes (Audebert *et al.,* 2000; Chilton *et al.,* 1995, 1998; Conole *et al.,* 1999; Dallas *et al.,* 2000; Hoste *et al.,* 1993, 1995; Hung *et al.,* 1997; Leignel *et al.,* 1997; Newton *et al.,* 1998a; Stevenson *et al.,* 1995, 1996; Zarlenga *et al.,* 1998), or the mtDNA for characterization of species (Hoberg *et al.,* 1999), sometimes with phyloge-

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TABLE 1

Species Used in the Analysis

Species	Accession No. D1	Accession No. D2	Accession No. D8	Host	Locality	Method
Ancylostomatina						
Bunostomum trigonocephalum (Rudolphi, 1808)	AF210008	AF210028	AF210045	*Capreolus capreolus*	France	A, B
Strongylina						
Chabertia ovina (Gmelin, 1790)	AF210009	AF210031		*C. capreolus*	France	A, B
Oesophagostomum venulosum (Rudolphi, 1809)	AF210010	AF210034		*C. capreolus*	France	A, B
Oesophagostomum dentatum (Rudolphi, 1803)	AF210000	AF210018	AF210041	*Sus scrofa*	Denmark	B
Triodontophorus serratus (Looss, 1900)	AF210011	AF210035	AF210047	*Equus caballus*	France	A, B
Trichostrongylina						
Ashworthius sidemi Schulz, 1933	AF210012	AF210027	AF210043	*C. capreolus*	France	A, B
Mecistocirrus digitatus Linstow, 1906	AF209997	F210022		*Bos taurus*	Guadeloupe	B
Haemonchus contortus (Rudolphi, 1803)	AF209999	AF210024		*Ovis aries*	Zaire[a]	A, B[b]
Ostertagia leptospicularis Assadov, 1953	AF210005	AF210029		*C. capreolus*	France	A, B
Graphidium strigosum (Dujardin, 1845)	AF209994	AF210017	AF210039	*Oryctolagus cuniculus*	France	A, B[b]
Teladorsagia circumcincta (Stadelmann, 1894)	AF209996	AF210021		*O. aries*	France	A, B[b]
Spiculopteragia spiculoptera (Gushanskaya, 1934)	AF210004	AF210026	AF210044	*C. capreolus*	France	A, B
Cooperia oncophora (Railliet, 1898)	AF210001	AF210019		*B. taurus*	England[a]	B
Trichostrongylus capricola Ramson, 1907	AF210006	AF210030		*C. capreolus*	France	A, B
Trichostrongylus retortaeformis (Zeder, 1800)	AF209992	AF210015	AF210042	*Or. cuniculus*	Australia[a]	B
Nematodirus europaeus Jansen, 1972	AF210013	AF210033		*C. capreolus*	France	A, B
Nematodirus spathiger (Railliet, 1896)	AF209995	AF210020		*O. aries*	France	B
Nematodiroides zembrae (Bernard, 1965)	AF209993	AF210016	AF210037	*Or. cuniculus*	Spain	B
Dictyocaulus noerneri Railliet and Henry, 1907	AF210007	AF210032		*C. capreolus*	France	A, B
Dictyocaulus filaria (Rudolphi, 1809)	AF210002	AF210036		*O. aries*	France	B
Heligmosomoides polygyrus polygyrus (Dujardin, 1845)	AF209998	AF210023	AF210040	*Apodemus flavicollis*	France	B
Ohbayashinema erbaevae Durette-Desset, Ganzorig, Audebert, and Masao, 2000	AF209991	AF210014	AF210038	*Ochotona daurica*	Bouriatia	B
Metastrongylina						
Metastrongylus pudendotectus Vostokov, 1905	AF210003	AF210025	AF210046	*S. scrofa*	France	A, B[b]

Note. Methods (A and B) are detailed in the text.

[a] Laboratory strain of the Institut National de la Recherche Agronomique, France.

[b] Domain D2 sequenced by both Methods A and B.

netic reconstructions (Chilton and Gasser, 1999; Hoste *et al.,* 1998; Hung *et al.,* 1996) limited to the intrageneric level. Some recent studies, however, investigated phylogenetic relationships within the equine strongyles (Hung *et al.,* 1999, 2000) and the hookworms (Blaxter, 2000).

The validity and relationships of high rank categories (suborders) within the Strongylida have not been tested with cladistic methods from morphological characters, and an attempt using 5.8S rDNA concluded that this molecule was not phylogenetically informative at this phylogenetic level (Chilton *et al.,* 1997b). The only available high-rank-level molecular phylogenetic study concerning the Strongylida, based on ITS2 sequences, was restricted to the order Strongylina (Chilton *et al.,* 1997a). A subsequent study updated this work with additional sequences of Ancylostomatina (Dorris *et al.,* 1999).

The present paper proposes a phylogeny of the Strongylida, with emphasis on the Trichostrongylina and Strongylina, based on the domains D1 and D2 of 28S rDNA (Hassouna *et al.,* 1984), including 23 new sequences for each domain.

MATERIAL AND METHODS

Material

The specimens used were collected from their hosts (Table 1), carefully rinsed in salt water (NaCl 0.9%), and conserved in 70% ethanol or frozen at −20°C or in liquid nitrogen prior to further manipulations. Sequences were produced by two teams using different protocols. The team in Reims (Method A in Table 1) sequenced only domain D2; the team in Paris (Method B in Table 1) sequenced domain D1 + D2, or domain D1 for the species treated for D2 by the other team, and sometimes domain D8. For a few species, domain D2 was sequenced by both teams and sequences were exactly the same (Methods A, B[b] in Table 1).

Extraction

Method A. A single male worm was used for each species. The posterior part (and/or anterior part) of the worm was cut for mounting in Amman lactophenol,

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                                  1 1111111112 2222222223 3333333334 4444444445 5555555556 6666666667
                       1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

T. circumcincta        TCAAGAGAAC GTGAAATCGC TGGAGTGGAA CCGGAGAGAG TTGACGTAGC CGGGTAACTA AGTTTCCTAA
H. polygyrus           ---------- ---------- ---------- ---------- ---------- ---------- ---C-A----
Ohbayashinema          ---------- ---------- ---------- ---------- ---------- ---------- ---C-A----
T. retortaeformis      ---------- ---------- ---------- ---------- ---------- ---------- ----------
N. zembrae             ---------- ---------- ---------- ---------- ---------- ---------- -----A-CG-
G. strigosum           ---------- ---------- ---------- ---------- ---------- ---------- ----------
C. elegans             ---------- ---------- ---------- -------C-- ----T--T-- TT--AG--*- --C--GG-G-
O. dentatum            ---------- ---------- ---------- ---------- ---------T -A-------- -----G-C--
C. oncophora           ---------- ---------- ---------- ---------- ---------- ---------- ----------
N. spathiger           ---------- ---------- ---------- ---------- ---------- ---------- ---C-A-CG-
M. digitatus           ---------- ---------- ---------- ---------- --------AT T--------- ------T-G-
H. contortus           ---------- ---------- ---------- ---------- --------A- ---------- ----------
M. pudendotectus       ---------- ---------T --A------- T--------- ---------T TT---GGA-- ---A-TA-G-
S. spiculoptera        ---------- ---------- ---------- ---------- ---------- ---------- ----------
A. sidemi              ---------- ---------- ---------- T--------- --------A- T--------- ----------
B. trigonocephalum     ---------- ---------- ---------- ---------- ---------T T--------- -------C--
O. leptospicularis     ---------- ---------- ---------- ---------- --------A- ---------- ----------
T. capricola           ---------- ---------T ---------- ---------- ---------- ---------- ----------
C. ovina               ---------- ---------- ---------- ---------- ---------T -A-------- -----G-C--
D. noerneri            ---------- ---------T --A------- A--------- --A-TA---T -TA--G-A-- -A-G-TA-TT
N. europaeus           ---------- ---------- ---------- ---------- ---------- ---------- -----G-CG-
O. venulosum           ---------- ---------- ---------- ---------- ---------T -A-------- -----G-C--
T. serratus            ---------- ---------- ---------- ---------- ---------T ---------- -----G-C--
D. filaria             ---------- ---------T --A------- A--------A --A-TA---T -TA--G-A-- -A-GCTAC--


                                                  1 1111111111 1111111111 1111111111 1111111111 1111111111
                       7777777778 8888888889 9999999990 0000000001 1111111112 2222222223 3333333334 4444444445
                       1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

Tc                     **TGTCGC*T TAATT***** ****GTGGCT GCAGGTGATG TATGTTGCCT ATGGCTGCGC TGACAGCACT GGCTGTGCGT
Hp                     **------*- -----***** ****---A-- --G--A---- ---------- ---------- -------G-- ---C------
Oh                     **------*- -----***** ****---A-- --G--A---- ---------- ---------- -------G-- ---C------
Tr                     **G-----*- -----***** ****------ ---------- ---------- -C-------- ---------- ----------
Nz                     **-C----*- ---C-***** ****---AG- TAG-TGA--- ---------- ---------- -------G-- ----------
Gs                     **------*- -----***** ****------ A--------- ---------- ---------- ---------- ----------
Ce                     CTG-----*- --G--***** ****---ATC -TT-CC-GGT GTC---T--- --*---A--- C---G--GT- -----CT---
Od                     **A-----*- -----***** ****---A-G --G--A---- ------SS-- -C-------- ---------- ----------
Co                     **------*- -----***** ****------ ---------- ---------- ---------- -------G-- ----A-----
Ns                     **------*- ---C-***** ****---A-C TAG-TGA-C- ---------- ---------- -------GT- ----------
Md                     *T------*- -----***** ****------ A--------- ---------- ----T---AT --------G- ----A-----
Hc                     **------*- -----***** ****------ A--------- ---------- ----T----- ---------- ----------
Mp                     *GAA---TA- -C---AATTT GAATA--ATC AAGTACT--- ----CCAT-A --AA---T-- --------TC -A--ACA---
Ss                     **------*- -----***** ****------ ---------- ---------- ---------- ---------- ----------
As                     **--C---*- -----***** ****------ A--------- ---------- --A-T----- ---------- ----A-----
Bt                     **A-----*- -----***** ****---A-- AAG--A---- ---------- ---A------ ---------- ----A-----
Ol                     **------*- -----***** ****---A-- A-----A--- ---------- ----T----- ---------- ----------
Ta                     **G-C---*- -----***** ****---A-- -T-------- ---------- -A-------- ---------- ----------
Co                     **A-----*- -----***** ****---A-G --G--A---- ---------- -C-------- ---------- ----------
Dn                     AT-A-TATA- AT--ATATAT ATAT-ATATA TA-TAAC--- -----CA-TA -C----A--T -A---A--T- AATA-CA-AA
Ne                     **------*- -----***** ****---A-C TAG-TG--C- ---------- ---------- -------GT- -----C----
Ov                     **A-----*- -----***** ****---A-G --G--A---- ---------- ---------- ---------- ----------
Ts                     **AA----*- -----***** ****---ATA --G-CGA--- ---------- -G-A------ ---------- ----------
Df                     AA-CG-ATAC G--CG***** *TAT---ATC TAGTAAC--- -----CA-TA -C-A--A--- -----AT-T- -ATA-CA-A-
```

FIG. 1. Alignment of the sequences of the D2 domain. *Sites eliminated from the analysis. Three species show high A-T content: *D. filaria, D. noerneri,* and *M. pudendotectus.*

allowing morphological examination and identification of species. Extraction of genomic DNA was performed on the other parts of the worm, which were dried, then crushed, and incubated at 60°C in a solution containing CTAB (Winnepenninckx *et al.,* 1993). Proteins were eliminated by the addition of chloroform. RNase (0.5 units) was added to the aqueous phase, which was then incubated at 37°C for 30 min to remove RNA. Total

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          1111111111 1111111111 1111111111 1111111111 1111111112 2222222222 2222222222 2222222222
          5555555556 6666666667 7777777778 8888888889 9999999990 0000000001 1111111112 2222222223
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

Tc        TCTGACCCGT TTGT****** ***GTTGCCC ACTTC***** ******GGTG GTGTCTTAC* TTACGGTT** ***AGGTTTG
Hp        --C--A---- --AGC***** ***------- --CGTTTCGG C*****---- -------G-* -GG---A-** ***-------
Oh        --C--A---- --AGC***** ***------- --CG-TTCGG C*****---- -------G-* -GG---A-** ***-------
Tr        ---------- ---C****** ***------- -----***** ******---- -------G-* C-------** ***-------
Nz        --C--A---A -CTC****** ***------- --CG-TCCGG C*****---- -------GT* C-T---A-** ***-------
Gs        ---------- ----****** ***------- -----***** ******---- --------* --------** ***-------
Ce        ---AG----A CA--****** ***------- -TC--GCAAG A*****-AA- -------G-* -GG----AGT ***G----C-
Od        --C--A---- C--C****** ***------- --C--***** ******---- -------G-* --T---G-** ***-------
Co        ---------- ---C****** ***------- -----***** ******---- -------G-* C-------** ***-------
Ns        --C--A---- --AC****** ***------- --CGTTCGC* ******---- -------GT* ------G-** ***-------
Md        ------A-A- ----****** ***------- ----TTTATC ******---- -------G-* ---T-T--** ***-A--C--
Hc        ---------- ----****** ***------- --CGTCACC* ******---- -------G-* --------** ***----C--
Mp        --C--A-TA- G-TGACAT** ***------- -T-CATATTC ACATTGAA-- -------GTG -----T--TG *TG-------
Ss        ---------- -C-C****** ***------- -----***** ******---- -------G-* --------** ***-------
As        ------ATA- ----****** ***------- ----TTAAT* ******---- ----------* A--T-T--** ***----C--
Bt        --C--A---- --TC****** ***------- --C-TTGT** ******---- -------GT* ------G-** ***-------
Ol        ---------- ----****** ***------- -----***** ******---- ----------* --------** ***-A-----
Ta        -----A---- ---C****** ***------- -----***** ******---- -------G-* C-------** ***-------
Co        --C--A---- ---C****** ***------- -----***** ******---- -------G-* --T---G-** ***-------
Dn        -----GTTAA ACA-****** ***------- -T-ATTATTA TTATTATTAT TATAA-AGTG G-G-CTAATG *TTTAACAGT
Ne        -----A---- --AC****** ***------- --CG-TTGC* ******---- -------GT* ------G-** ***-------
Ov        --C--A---- ---C****** ***------- --CCATCACT G*****---- -------G-* --T---G-** ***-------
Ts        --C--A---C ---C****** ***------- -----***** ******---- -------G-* --G--AA-** ***-------
Df        --C-TA-T-- GATAAATGAA CAT------- -T-ATTATTA TAATA**--- -----A--T* G-TTTAACAG TTCGA-----


          2222222222 2222222222 2222222222 2222222222 2222222222 2222222222 2222222223 333
          3333333334 4444444445 5555555556 6666666667 7777777778 8888888889 9999999990 000
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890 123

Tc        TCATTGCTAG TGTTTTATCC TGC******T TGCAGGGTGT ********** ********** ******CAGT GTG
Hp        --G-G----- C-----TG-- CTACTTCA*- --T------- ********** ********** ******---- ---
Oh        --G-G----- C-----TG-- CTACTTCA*C --T------- ********** ********** ******---- ---
Tr        ----G----- ---------- ---******A ---------- ********** ********** ******---- ---
Nz        --G-G----- C--------- ---******- ---------- ********** ********** ******---- ---
Gs        ----G----- ---------- ---******- ---------- ********** ********** ******---- ---
Ce        -GGCG----- C----AG-TA C--******* -AGT-T---- GACGT***** ********** ******-G-- ---
Od        --G-G----- ---------- ---******- C--------- ********** ********** ******---- ---
Co        ----G----- C-------T- ---******A -----A---- ********** ********** ******---- ---
Ns        --GCG----A C--------- ---******- ---------- ********** ********** ******---- ---
Md        -T--G----- --------T- ---******A ------A--- ********** ********** ******---- --A
Hc        ----G----- ---------- ---******A ---------- ********** ********** ******---- ---
Mp        -TG-ATG-T- ATG--A--AA ATTATATAA- ATA-TTA--- ********** ********** ******---A C-A
Ss        ----G----- ---------- ---******- ---------- ********** ********** ******---- ---
As        ----G----- --------T- ---******A -----AA--- ********** ********** ******---- ---
Bt        --G-G----A ---------- ---******- ---------- ********** ********** ******---- ---
Ol        ----G----- ---------- ---******- ---------- ********** ********** ******---- ---
Ta        ----G----- ---------- ---******A ---------- ********** ********** ******---- ---
Co        --G-G----- ---------- ---******- C--------- ********** ********** ******---- ---
Dn        AA-G-TTGTT GCG---TATT AATGTTTATC AT-GATCGA- CGATCGATCG ATCGAATCGA TAGTGTT-AC ---
Ne        --GCG----A C--------- ---******- ---------- ********** ********** ******---- ---
Ov        --G-G----- ---------- ---******- ---------- ********** ********** ******---- ---
Ts        ----G----- ---------- ---*****TG ---------- ********** ********** ******---- ---
Df        -TGCAAGAT- --A-A-TAAT ATTTCAATAG ----AAA-T- GAATTCGTTT CATTTTTTGC AATGATG
```

FIG. 1—*Continued*

genomic DNA was precipitated by the addition of two-thirds of the total volume of isopropanol and stored at 4°C overnight. After centrifugation, the pellet was washed, dried, and resuspended in 50 μl of ultrapure water.

Method B. Worms were identified at the species level, or hosts experimentally infested with well-known laboratory strains were used. Extraction of genomic DNA was performed from one to five animals according to their size, preferably from females, which

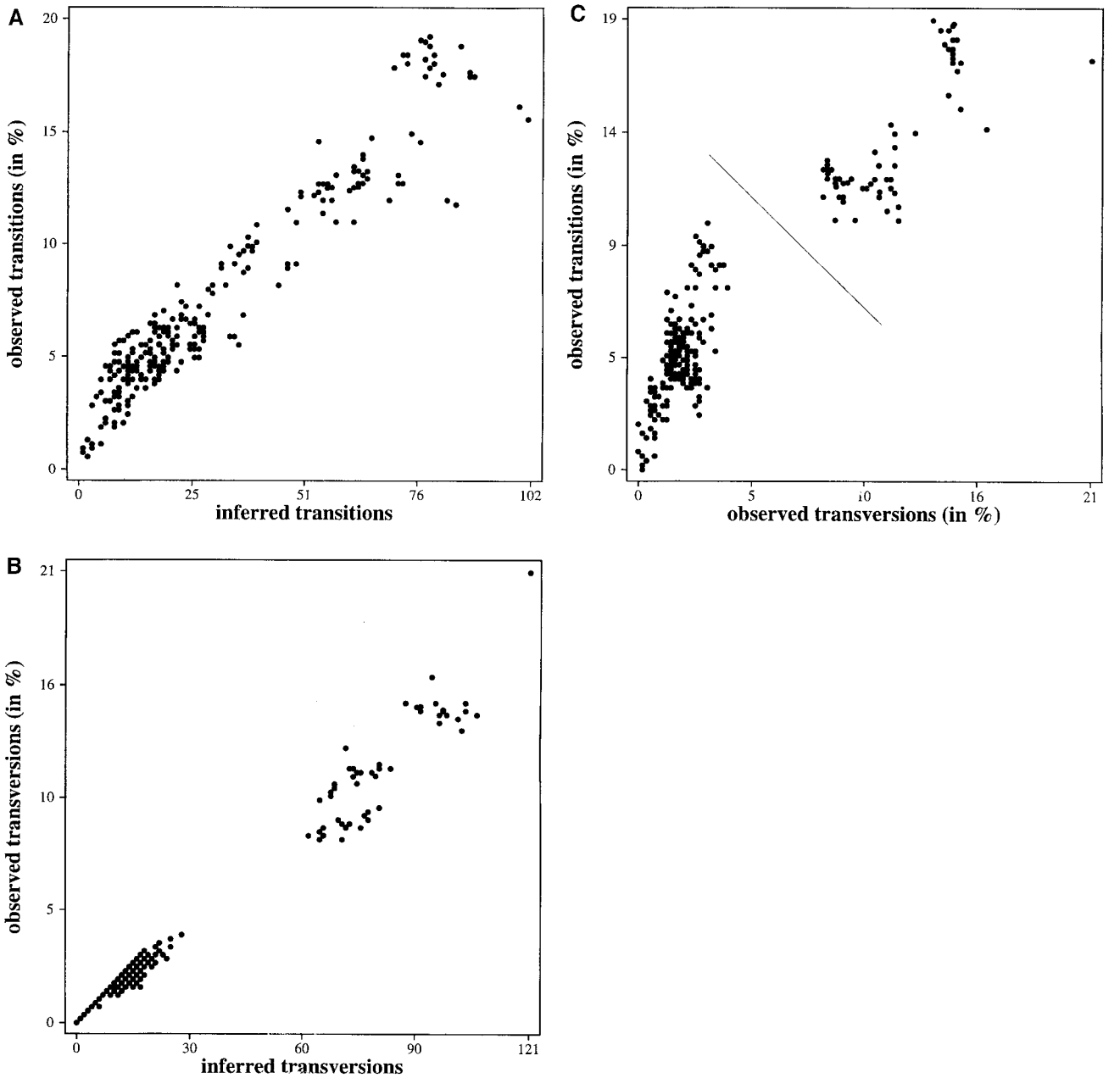

FIG. 2. Saturation curves. (A) Observed transitions against inferred transitions; (B) observed transversions against inferred transversions; (C) observed transitions against observed transversions. Plots above the line correspond to *Caenorhabditis elegans, Metastrongylus pudendotectus,* and *Dictyocaulus noerneri.*

are larger. Specimens were crushed in 700 μl of a solution containing CTAB supplemented with 1 μl of proteinase K and incubated overnight according to the method of Winnepenninckx *et al.* (1993), modified. Proteins were eliminated by the addition of chloroform: isoamyl alcohol 96:4. Total genomic DNA was precipitated by the addition of two-thirds of the total volume of isopropanol and stored 4 h at −20°C. After centrifugation, the pellet was washed, dried, and resuspended in 20 μl of ultrapure water.

PCR

Method A. Polymerase chain reactions (PCR) of the D2 domain were performed in a 50-μl volume using 5 μl of extraction DNA solution and 50 pmol of each of the two primers. Primer sequences were C2′, 5′-GAAAAGAACTTTGRARAGAGA-3′, and D2, 5′-TCCGTGTTTCAAGACGGG-3′. The PCR mix usually contained (final concentrations) 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 200

TABLE 2

Percentages of A, T, G, and C Compared between *Metastrongylus pudendotectus, Dictyocaulus noerneri,* and *Dictyocaulus filaria* and Mean of All Other Taxa

	D1 + D2					D2 alone				
Species	A	C	G	T	A + T	A	C	G	T	A + T
Metastrongylus pudendotectus	30.4	15.3	23.5	30.7	61.1	30.3	14.1	21	34.5	64.8
Dictyocaulus noerneri	33.6	13.6	20.8	31.9	65.5	34.4	12.7	16.9	36	70.4
Dictyocaulus filaria	32.5	14.7	22.6	30.3	62.8	34	13.2	19.5	33.3	67.3
Mean of all other taxa ± confidence interval ($P < 0.05$)	24.9 ± 2.1	19.6 ± 2.0	29.1 ± 2.6	26.3 ± 2.5	51.2 ± 4.3	19.4 ± 2.5	21.4 ± 3.1	30.1 ± 3.1	29.1 ± 4.0	48.5 ± 5.9

μM each dNTP, and 0.5 μl (2.5 units) of *Taq* polymerase (Eurobio). Temperature cycling was performed using a Eppendorf personal thermocycler. Thermal cycling was denaturation at 94°C for 5 min, then 35 cycles of denaturation at 94°C for 1 min, annealing at 57 to 61°C (depending of the studied species) for 1 min, and extension at 72°C for 1 min. An elongation at 72°C for 10 min completed the DNA amplification. The quality of PCR was checked by electrophoresis in 1.5% agarose gel containing ethidium bromide (Sambrook *et al.,* 1989). The length of each DNA gene was measured by comparison with a 100 bp ladder molecular weight marker (Pharmacia Biotech). PCR products were cloned into a pUC 18 vector using the Sure Clone ligation kit (Pharmacia Biotech) following the manufacturer's instructions.

Method B. PCR of domains D1 and D2 (including partial domain C1 and complete domains D1, C2, and D2) was performed in a 50-μl volume using 1 μl of extraction DNA solution and 30 pmol of each of the two primers C1′, ACCCGCTGAATTTAAGCAT, and D2, TCCGTGTTTCAAGACGG. The PCR mix usually contained 2.5 μl DMSO, 5 μl *Taq* buffer 10× (Bioprobe), 1.5 U *Taq* polymerase (Bioprobe), 165 μM each dNTP. Thermal cycling was denaturation at 94°C for 3 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The quality of PCR was checked by electrophoresis of 5 μl in 1.5% agarose gel containing ethidium bromide (Sambrook *et al.,* 1989). The length of each gene was measured by comparison with the molecular weight marker XIV (Boehringer Mannheim). PCR of the D1 domain was performed with the primers C1′ and C2, TGAACTCTCTCTTCAAAGTTCTTTTC, with a similar protocol. Primers for the D8 domain were C8′, CCGTAACTTCGGGAAAAGGATTGGC, and NEMD8, TTAGACAGTCGGATTCCCTGA.

Sequencing

Method A. Sequencing of the insert was done by the Society ESGS (Cybergene Group, Évry, France). Different clones were sequenced until three identical sequences were obtained.

Method B. Sequencing was performed with the Thermo Sequenase Cycle Sequencing Kit (Amersham). Universal primers used were C1′ and C2 for domain D1 (and a part of domains C1 and C2); universal primers C2′, GAAAAGAACTTTGRARAGAGAGT, and D2 for domain D2; and NEMD8 and C8′ for domain D8.

Data Processing and Phylogenetic Analysis

Sequences were aligned by eye with MUST (Philippe, 1993). A preliminary study of domain D8 in 11 species revealed that it was almost invariable, with the exception of *Metastrongylus pudendotectus,* which showed some differences. Thus, this domain was useless for this level of phylogenetic analysis. Domains D1 (including partial C1, complete D1, and partial C2) and D2 were concatenated with AFAS, a subset program of MUST. Absolute saturation tests (Hassanin *et al.,* 1998; Lavoué *et al.,* 2000; Philippe *et al.,* 1994) were performed using MUST (COMP-MAT and AF-PAUP) by plotting the pairwise number of observed nucleotide differences against the pairwise number of substitutions in the MP tree from PAUP 3.1.1 (Swofford, 1993). Phylogenetic analyses were performed with PAUP* version 4.0b4 (Swofford, 1998) using the maximum parsimony criterion and heuristic and branch and bound analyses; distances were computed using PAUP* with the neighbor-joining method; bootstraps were computed with 1000 replicates; Bremer indices (BI) (Bremer, 1994) were computed with Autodecay (Eriksson, 1997), a Hypercard stack using PAUP*; trees were edited with Treeview 1.5.3 (Page, 1996), all using a G3 Power Macintosh. Alignments are available

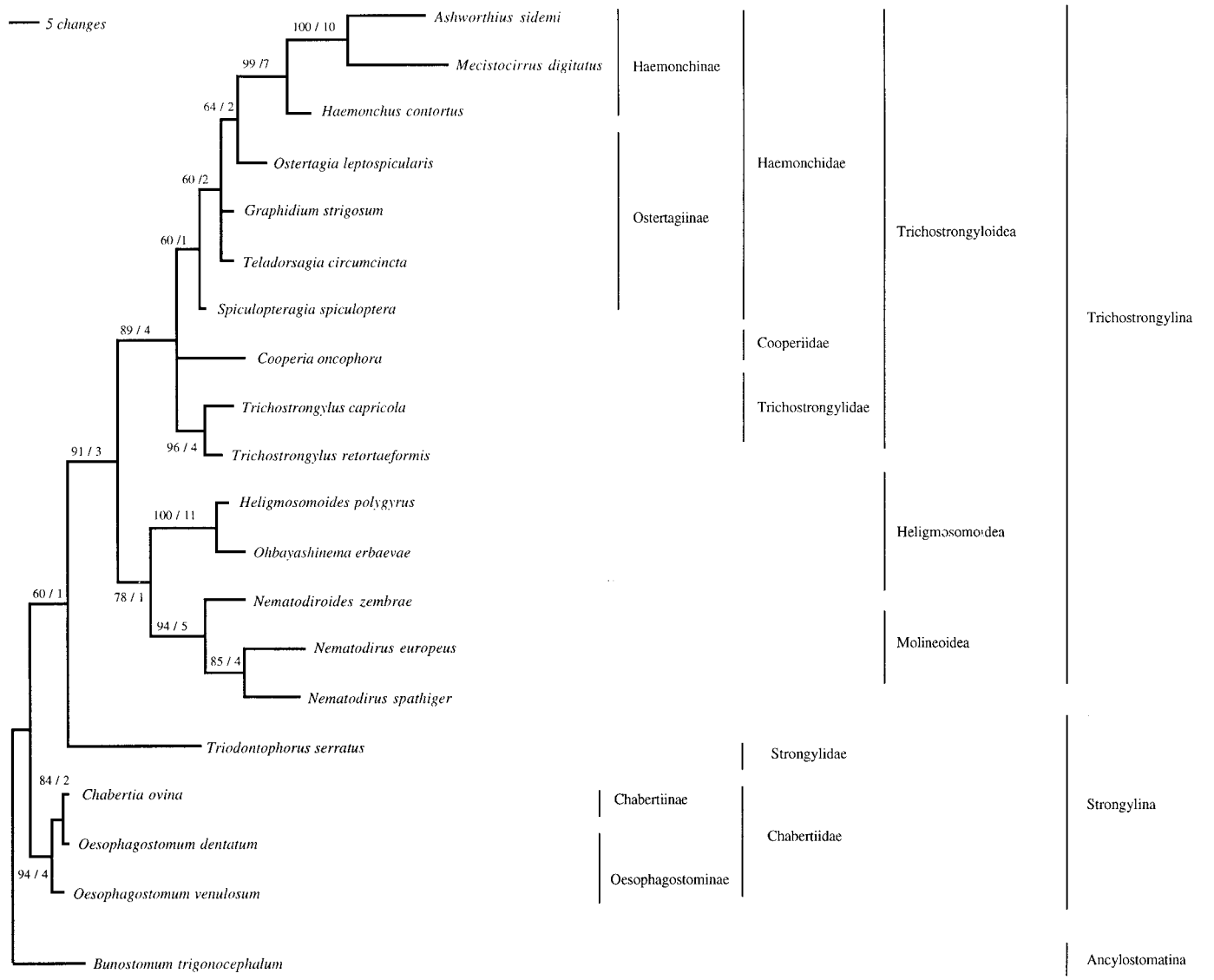

FIG. 3. Molecular phylogeny of the Strongylina. Phylogram of a consensus tree computed from four equiparsimonious trees obtained by a parsimony analysis. CI = 0.6545, RI = 0.7240, length is 246 steps; bootstrap values and Bremer indices are indicated above each branch.

on request to the authors or from http://www.mnhn.fr/mnhn/bpph/Data/IndexData.html.

Morphological Data

Morphological characters that are stable within the suprafamilial taxa considered were listed from classical morphological works and keys (Chabaud, 1965, 1974; Durette-Desset, 1983, 1985; Durette-Desset and Chabaud, 1993; Lichtenfels, 1980a,b) and checked by examination of selected specimens.

RESULTS

The phylogenetic analysis was conducted in two steps. In the first step, we tried to infer the relationships between the major groups (Ancylostomatina, Strongylina, Trichostrongylina, and Metastrongylina) within the Strongylida. In the second step, we restricted our analysis to the relationships within the Strongylina and Trichostrongylina, with emphasis on the latter.

First Step of Phylogenetic Analysis

The outgroup chosen for the analysis was *C. elegans,* according to a molecular phylogeny of the Nematoda based on 18S rDNA in which this species was found closely related to the Strongylida, within "Group V" of Blaxter *et al.* (1998).

After alignment of the sequences (Fig. 1), we found that three species, namely *Dictyocaulus filaria, Dictyocaulus noerneri,* and *M. pudendotectus,* presented highly divergent parts in their sequences (e.g., bases

86–94 and 182–196 in Fig. 1). The two *Dictyocaulus* species presented divergences at bases 271–296, and, furthermore, *D. filaria* had insertions at bases 165–173. Therefore, we deleted *D. filaria* from the analysis and provisionally retained the two other species. No absolute saturation was found for transitions, absolute saturation for transversions was low, but saturation of transitions against transversions was attributable to the three taxa *C. elegans, D. noerneri,* and *M. pudendotectus* (Fig. 2). The nucleotidic frequencies in the two species of *Dictyocaulus* and in *M. pudendotectus* were different from those of the other species, with high levels of A-T (Table 2), particularly in the D2 region, with several A-T-rich regions inserted within the sequence (Fig. 1).

The phylogenetic analysis using all species except *D. filaria,* with *C. elegans* as outgroup, produced four equiparsimonious trees, 577 steps in length, with consistency index (CI) excluding uninformative characters of 0.6690 and RI of 0.6094. Among 502 characters, 271 sites were variable and 151 were parsimony informative. A bootstrap analysis yielded very low bootstrap values. We concluded that *C. elegans* was an outgroup too distant from the ingroup and that the divergent sequences of *D. noerneri* and *M. pudendotectus* produced artifacts, particularly long branch attraction toward the outgroup, in the analysis. We therefore removed the sequences of *D. noerneri* and *M. pudendotectus* and restricted the analysis to the members of the Strongylina and Trichostrongylina with a closer outgroup.

Second Step of Phylogenetic Analysis

The ingroup was restricted to the Strongylina and Trichostrongylina. The Metastrongylina could not be included in this analysis because *M. pudendotectus* had a divergent sequence (see above). The choice of the outgroup for this analysis could not be based on a previous general molecular analysis at a high level or a cladistic study of interordinal relationships of the Strongylida as both are lacking. The Ancylostomatina, which are characterized by a primitive buccal capsule lacking a corona radiata (Chabaud, 1974), were tentatively chosen as an outgroup. A molecular analysis of the Strongylina and Trichostrongylina of marsupials based on 18S rDNA and published with minimum details (Dorris *et al.,* 1999) also used the Ancylostomatina as outgroup.

The level of saturation within this set of taxa was low (not shown). A parsimony analysis produced four equiparsimonious trees, 246 steps in length, with a CI of 0.6545, CI excluding uninformative characters of 0.5707, RI of 0.7240. Among 502 characters, 134 were variable and 90 were parsimony informative. The neighbor-joining analysis produced a similar tree for all major nodes, but some minor differences were found within the Trichostrongyloidea. In the consensus tree (Fig. 3) produced from the parsimony analysis, the main features were the Strongylina appeared paraphyletic; a clade which included the Heligmosomoidea and Molineoidea was relatively well supported (bootstrap value 78, BI 1), and within it, the Heligmosomoidea (bootstrap 100, BI 11) and Molineoidea (bootstrap 94, BI 5) were very robust; and the Trichostrongyloidea, a robust clade (bootstrap 89, BI 4), was the sister group of the Heligmosomoidea + Molineoidea clade. Paraphyly of the Strongylina was caused by the taxon *Triodontophorus serratus* but the corresponding grouping was weakly supported (bootstrap 60, BI 1). Within the Strongylina, the subfamily Oesophagostominae appeared paraphyletic because *Chabertia ovina,* a Chabertiinae, grouped with *Oesophagostomum dentatum* with strong support (bootstrap 84, BI 2). Within the Trichostrongyloidea, a polytomy was observed with three branches, corresponding to the Cooperiidae, Trichostrongylidae, and Haemonchidae. Monophyly of the Haemonchidae, the family for which we had the highest number of species, was supported by relatively low bootstrap (60) and BI (1) values. Within the Haemonchidae, the Ostertagiinae appeared paraphyletic (with, however, low values for the grouping producing paraphyly) but the Haemonchinae were strongly monophyletic with high bootstrap (99) and BI (7) values.

Morphological Assessment of the Molecular Results

A clade grouping the Molineoidea and the Heligmosomoidea was never suggested in previous morphological analyses (Chabaud, 1974; Durette-Desset *et al.,* 1994) of the Trichostrongylina. To test this new hypothesis of phylogenetic relationships, we tried to find morphological characters (Table 3, Fig. 4), stable at the suprafamilial level, that could falsify or confirm this phylogeny. Characters of the male bursa appeared too variable to be used at this level. Morphological characters, mapped onto the phylogenetic scheme inferred from our molecular analysis, are presented in Fig. 5. A buccal capsule (Figs. 4A and 4B) is found only in the Ancylostomatina and Strongylina; consequently, the absence of buccal capsule (Figs. 4C–4F) was considered a synapomorphy for the Trichostrongylina. The corona radiata (Fig. 4B) is a buccal structure found only in the Strongylina and therefore is interpreted as an autapomorphy for this group (but a few Trichostrongylina also have a simple corona radiata). A thorough examination of the ovejector in the female (Figs. 4N–4R) revealed a previously neglected character: the sphincters are long in the Ancylostomatina and Strongylina, but annular in the other groups; we propose that an annular sphincter is a synapomorphy for the Trichostrongylina. This is the only synapomorphy we found for the Trichostrongylina. The cephalic vesicle (Figs. 4A–4F) and the synlophe (Figs. 4G–4M) are two characters that present the same pattern: they are

TABLE 3

Morphological Characters That Could Provide Synapomorphies for the Major Groups

Character	Ancylostomatina	Strongylina	Trichostrongylina		
			Trichostrongyloidea	Molineoidea	Heligmosomoidea
Buccal capsule	Present	Present	Absent	Absent	Absent
Corona radiata	Absent	Present	Absent	Absent	Absent
Cephalic vesicle	Absent	Absent	Absent/present	Present	Present
Synlophe	Absent	Absent	Absent/present	Present	Present[a]
Ovejector sphincter	Long	Long	Annular	Annular	Annular
Female apparatus	Didelphic	Didelphic	Didelphic (except *Impalaia* and *Minutostrongylus*)	Didelphic (except *Ortleppstrongylus*)	Didelphic or monodelphic
Caudal spine	Absent	Absent	Absent	Present	Absent/present

[a] In addition, the synlophe is asymmetrical in the Heligmosomoidea.

absent in the Ancylostomatina and Strongylina and present in the Molineoidea and Heligmosomoidea, but the two character states (absent and present) are found in different families of the Trichostrongyloidea. The synlophe, in addition, is asymmetrical (i.e., the orientation axis of the cuticular ridges is oblique in relation to the sagittal axis (Durette-Desset, 1985)) only in the Heligmosomoidea (Fig. 4M). The monodelphic female tract (Fig. 4R) could be proposed as an autapomorphy for the Heligmosomoidea, in contrast to all other groups in which the female apparatus is didelphic (Figs. 4N–4Q); however, the Heligmosomoid *Suncinema* is didelphic. The tail of the female (Figs. 4R and 4S) has no caudal spine in the Ancylostomatina, Strongylina, and Trichostrongyloidea, but a caudal spine is present in the Molineoidea; however, the character is equivocal in the Heligmosomoidea and thus does not provide a synapomorphy for the grouping Molineoidea + Heligmosomoidea. Finally (Fig. 5), no unambiguous morphological character could be found which could be considered a synapomorphy for the grouping Molineoidea + Heligmosomoidea, a grouping suggested by our molecular analysis. But no morphological character unambiguously contradicted the sister-group relationships found in our molecular analysis.

DISCUSSION

In the first analysis, a low resolution was produced by the use of an outgroup probably too phylogenetically distant, i.e., *C. elegans*. Highly divergent sequences were also found in three taxa, *D. filaria* (removed from the analysis) and *D. noerneri* and *M. pudendotectus,* which contained A-T-rich regions that rendered sequence alignment questionable (Table 2). It seems that these A-T rich regions are also present in the D2 domain of the 28S rDNA of other nematodes, such as *Strongyloides ratti* (A + T = 0.72, GenBank U39490) and *Strongyloides stercoralis* (A + T = 0.74, GenBank U39489 and U38855). Preliminary evaluation of the A-T richness is needed before any phylogenetic analyses within the Nematoda. Our results suggest that the D1 and D2 regions of 28S rDNA are not suitable for a study of the relationships between the major taxa of the relatively large group ("Group V" in Blaxter *et al.,* 1998) which includes *C. elegans*.

In the second analysis, with a reduced data set of 20 species, a robust tree of phylogenetic relationships between and within the Strongylina and the Trichostrongylina was obtained.

The Strongylina were found paraphyletic because *T. serratus* did not group with the other Strongylina but instead with the clade Trichostrongylina. This node, however, was weakly supported. It may be remarked that the genus *Triodontophorus* includes parasites of horses, in contrast to the other Strongylina used in the analysis. However, no member of the third family of the Strongylina, the Cloacinidae, was included in our analysis. For these reasons, we consider that the possible paraphyly of the Strongylina requires confirmation by additional species sampling.

The Trichostrongylina were found to be monophyletic. Within them, the Heligmosomoidea and the Molineoidea formed a clade. This result cannot be compared to a cladistic, morphology-based analysis of the internal higher group relationships within the Strongylida, which is lacking. The Trichostrongyloidea and the Molineoidea share the following characters: the same type of simple synlophe with bilateral symmetry (or synlophe absent), didelphy of the female genital apparatus (with a few exceptions, see Table 3), and a male caudal bursa with various types including 2-1-2 (according to the terminology of Durette-Desset, 1971) in the Molineoidea. In contrast, the Heligmosomoidea have an asymmetrical synlophe, a monodelphic (rarely didelphic) female genital apparatus, and several types of male caudal bursa except the type 2-1-2. If the grouping Heligmosomoidea + Molineoidea, found in the present molecular analysis, is valid, this suggests that a synlophe with bilateral sym-

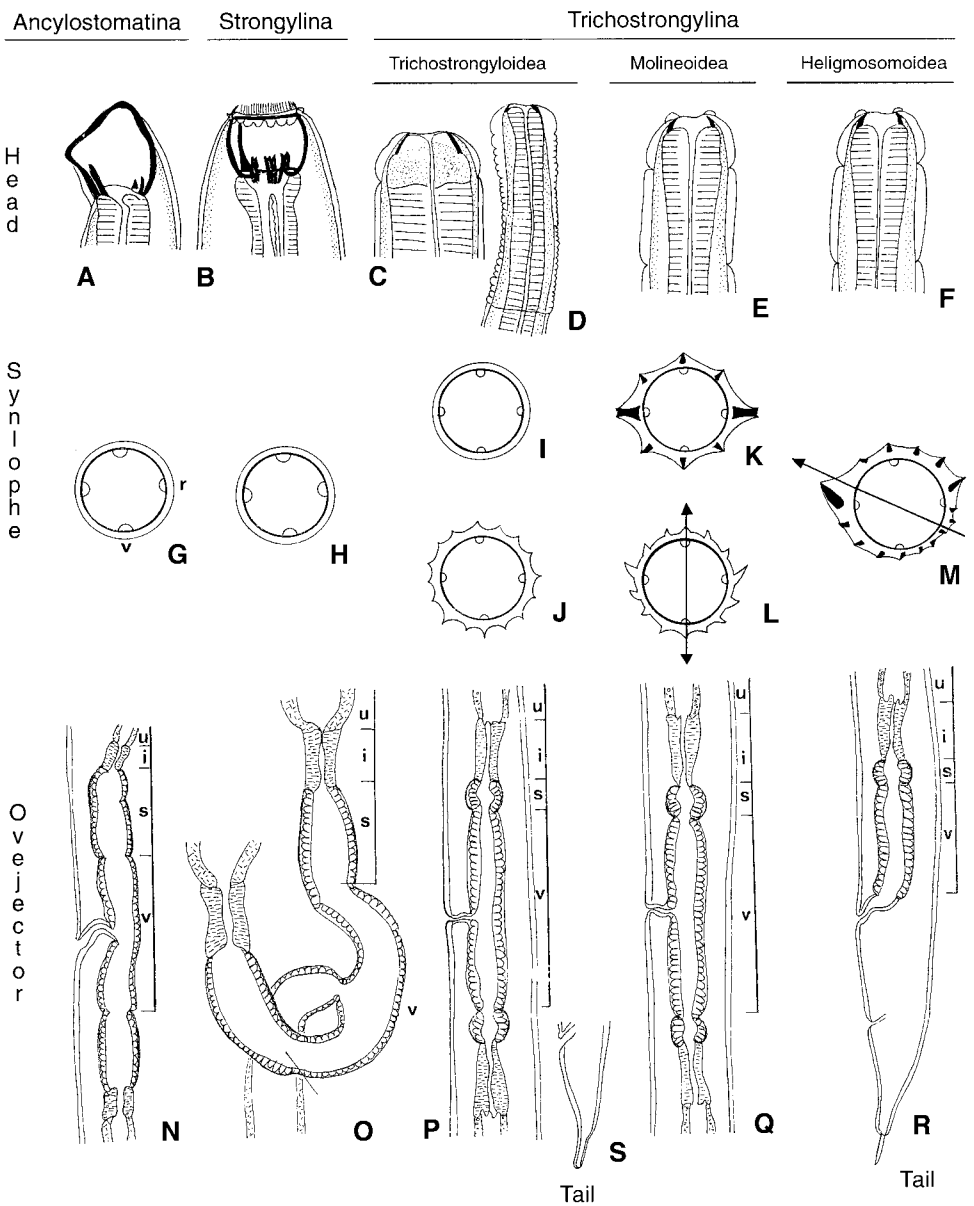

FIG. 4. Morphological characters within the Strongylida (bursate nematodes). Only characters stable at the suprafamilial level were selected. Head: (A) *Bunostomum* (Ancylostomina), (B) *Triodontophorus* (Strongylina), (C) general Trichostrongyloidea (except Cooperinae), (D) Cooperinae (Trichostrongyloidea), (E) general Molineoidea, (F) general Heligmosomoidea. Synlophe: (G) general Ancylostomina, (H) general Strongylina, (I) general Trichostrongyloidea (except Cooperinae), (J) Cooperinae (Trichostrongyloidea), (K) Molineoidae (Molineoidea), (L) Anoplostrongylinae and Nematodirinae (Molineoidea), (M) general Heligmosomoidea. v, ventral; r, right. Ovejector: (N) general Ancylostomina, (O) general Strongylina, (P) general Trichostrongyloidea, (Q) general Molineoidea, (R) general Heligmosomoidea. u, uterus; i, infundibulum; s, sphincter; v, vestibule. Tail: (S) without caudal spine, in a Trichostrongyloidea; (R) with caudal spine, in an Heligmosomoidea.

metry and female didelphy are plesiomorphous characters and that the presence of these characters in both the Trichostrongyloidea and the Molineoidea does not demonstrate close phylogenetic relationships between them. Independently, Audebert (1999), using ITS2 sequences, obtained similar results, i.e., a monophylum Heligmosomoidea + Molineoidea sister group of the Trichostrongyloidea. A formal taxon grouping the Heligmosomoidea + Molineoidea has never been proposed from morphological analyses.

Within the Strongylina, the Oesophagostominae were found paraphyletic. This was already found from a molecular analysis of the ITS2 region (Newton *et al.,* 1998b). The classical interpretation of the Chabertiidae, composed of the Chabertiinae and Oesophagostominae, should probably be reevaluated from these congruent molecular results.

Within the Trichostrongyloidea, we found a polytomy of the three families Haemonchidae, Trichostrongylidae, and Cooperiidae. In a recent cladistic

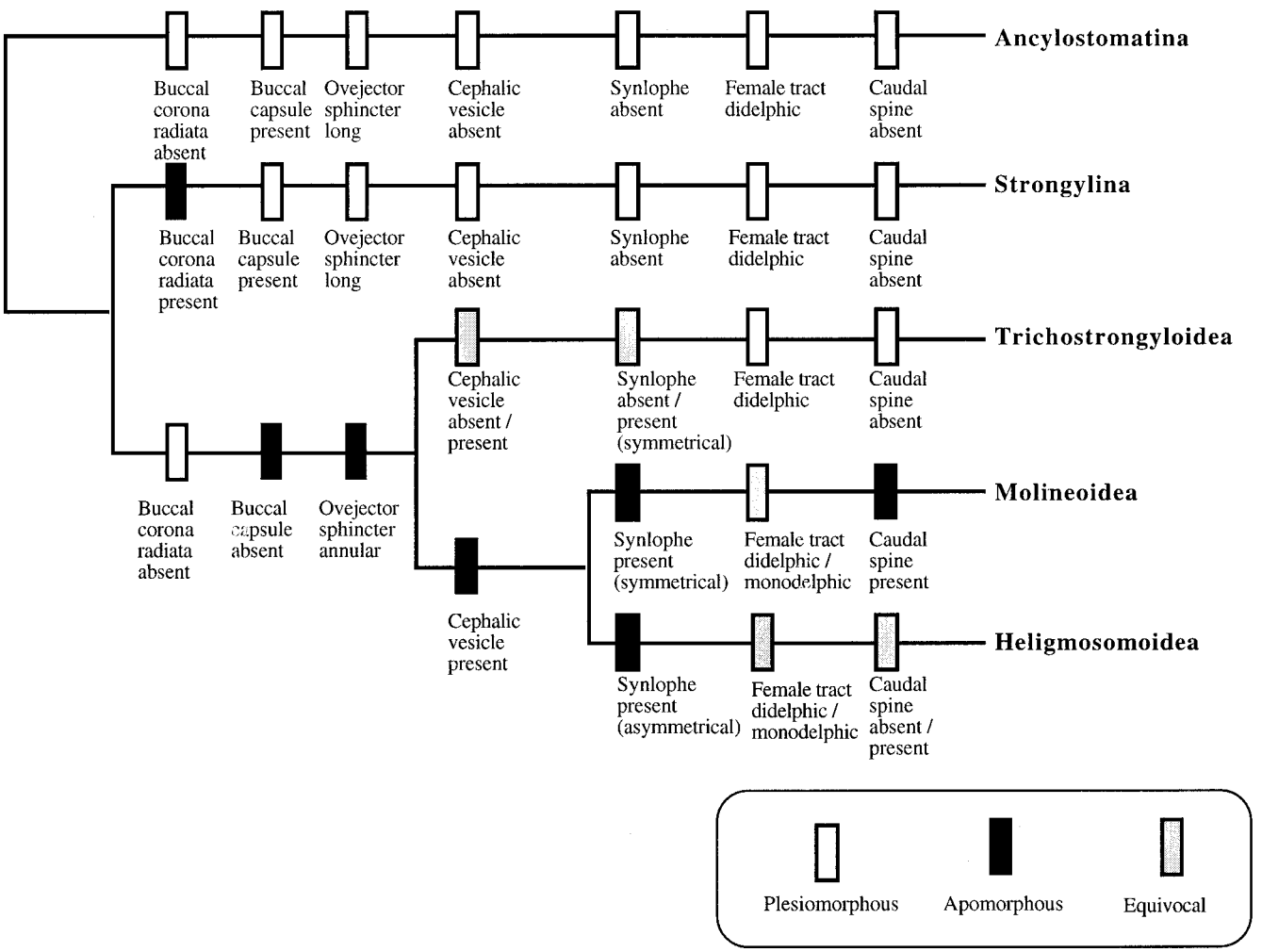

FIG. 5. Morphological characters mapped on a scheme of phylogenetic relationships inferred from our molecular study. The Metastrongylina, for which no pertinent molecular data were available, are not considered. The presence of an annular sphincter in the ovejector could be a synapomorphy for the Trichostrongylina. No unequivocal morphological character could be found that supports the new finding, sister-group relationships between the Molineoidea and the Heligmosomoidea, but no morphological character falsified it.

morphological analysis, dichotomic sister-group relationships between these three clades were proposed from shared derived characters, with the Trichostrongylidae sister group to the Haemonchidae + Cooperiidae (Durette-Desset *et al.,* 1999). The Ostertagiinae were found paraphyletic in our molecular analysis, but their monophyly was supported by two characters of the male caudal bursa in the morphological analysis (Durette-Desset *et al.,* 1999).

In conclusion, our molecular analysis confirmed monophyly of the Trichostrongylina and, within them, of the Trichostrongyloidea, Heligmosomoidea, and the Molineoidea. Sister-group relationships between the Heligmosomoidea and the Molineoidea, previously never proposed on the basis of morphology, were strongly supported. These new relationships were seminal and prompted us to seek morphological characters that could provide robust synapomorphies, supporting the tree suggested by the molecular analysis. No such character was found for the grouping Molineoidea + Heligmosomoidea, but no unambiguous character was found to falsify it.

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