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A molecular phylogenetic approach to Longidoridae (Nematoda: Dorylaimida)

Yu HE [1], Sergei A. SUBBOTIN [2], Tatiana V. RUBTSOVA [2], Franco LAMBERTI [3,†], Derek J.F. BROWN [4] and Maurice MOENS [1,5,*]

[1] *Agricultural Research Centre, Burg. Van Gansberghelaan 96, 9820 Merelbeke, Belgium*
[2] *Institute of Parasitology, RAS, Leninskii Prospekt 33, Moscow 177071, Russia*
[3] *Istituto per la Protezione delle Piante, Sezione di Bari, CNR, Bari, Italy*
[4] *Central Laboratory of General Ecology, Gagarin Street 2, 1113 Sofia, Bulgaria*
[5] *Laboratory for Agrozoology, Ghent University, Coupure 555, 9000 Ghent, Belgium*

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Summary – The Longidoridae are a group of ectoparasitic nematodes including two subfamilies and six genera with hundreds of species. Sequences of the D2 and D3 expansion region of the large subunit (LSU) rRNA nuclear gene were amplified and used to reconstruct the phylogeny of longidorids. Phylogenetic analyses with maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) were performed with one outgroup taxon and 62 longidorid sequences. Confidence of inferred clades was assessed by non-parametric bootstrapping for MP and Bayesian posterior probability for ML. All analyses placed *Paralongidorus* species as an inner group within the otherwise monophyletic genus *Longidorus*. The genus *Xiphinema*, except for *X. americanum*-group species, was placed as the sister group of *Longidorus* with strong support from the ML and BI analyses. The *X. americanum*-group was strongly supported as an exclusive clade to other genus *Xiphinema* species. The position of the *Xiphidorus* clade was not well resolved and the phylogenetic analyses did not support it as a sister group to *Longidorus* as previously inferred from morphology. Secondary structure models were constructed for the D2/D3 region of LSU rRNA for all studied species. It was found that sequence-based and structural morphometric rRNA phylogenies were incongruent.

Keywords – amphid structure, evolution, large subunit, *Longidorus*, *Paralongidorus*, phylogeny, rRNA, secondary structure, *Xiphidorus*, *Xiphinema*, *Xiphinema americanum*-group.

The family Longidoridae Thorne, 1935 belongs to the Dorylaimida Pearse, 1942 and is subdivided into two subfamilies: Longidorinae Thorne, 1935 and Xiphinematinae Dalmasso, 1969. Within the Longidorinae, the genera *Longidorus* Micoletzky, 1922 (107 valid species), *Paralongidorus* Siddiqi, Hooper & Khan, 1963 (42 valid species), *Longidoroides* Khan, Chawla & Saha, 1978 (19 valid species), *Xiphidorus* Monteiro, 1976 (eight valid species), *Paraxiphidorus* Coomans & Chaves, 1995 (three valid species) and *Australodorus* Coomans, Olmos, Casella & Chaves, 2004 (monotypic) are classified into two tribes: Xiphidorini Coomans, 1985 with the genera *Australodorus*, *Xiphidorus* and *Paraxiphidorus*, and Longidorini Coomans, 1985 for the remaining three genera (Coomans, 1985). One genus, *Xiphinema* Cobb, 1913, is classified in the Xiphinematinae with 296 nominal taxa corresponding to 234 valid taxa, 49 junior synonyms and 13 *species inquirendae* (Coomans *et al.*, 2001). All species live ectoparasitically. Some can vector plant viruses (nepoviruses) and are classified as quarantine pests (Taylor & Brown, 1997).

Within the longidorids, the *Xiphinema americanum*-group attracts special attention. According to Loof and Luc (1990) the common morphological characters for this group are: spiral or C-shaped small body, two well developed genital branches, no uterine differentiation, short conical to broadly convex-conoid tail, and vulva positioned at 40-60% of the body length from the head. As more populations of *X. americanum* were sampled and investigated from different geographical localities, taxono-

† Franco Lamberti died in 2004.
* Corresponding author, e-mail: m.moens@clo.fgov.be

mists encountered many morphological varieties among populations studied. Lima (1965) and Tarjan (1969) suggested that *X. americanum* is a complex of several species. Since then, 51 nominal species have been placed in this group (Lamberti *et al.*, 2000). The traditional identification of these species by morphology and morphometrics is very difficult because many of the characters used overlap. Moreover, questions concerning the true phylogeny behind such a complicated group and the position of the species within this group have been raised (Lamberti *et al*., 2002). So far, phylogenetic analyses of Longidoridae are few. The phylogeny, based on selected morphological characters, of the genus *Xiphinema* was analysed in great detail by Coomans *et al*. (2001). Rubtsova *et al*. (2001) used a molecular approach to investigate the phylogenetic position of a few species from the genera *Longidorus* and *Paralongidorus*. Recently, the phylogeny of longidorids was inferred from ITS1 analyses (Ye *et al*., 2004) and 18S rDNA sequences (Oliveira *et al.*, 2004).

Molecular systematic approaches are useful for producing phylogenies, especially in cases where morphological characters lead to ambiguous interpretation. In recent years, ribosomal RNA genes have attracted the attention of many systematists and evolutionists because of their functional importance, their ease of amplification and because they are assumed to record the evolutionary history of the organism relatively faithfully (Lydeard *et al.*, 2000). In nematodes, rRNA gene sequences were used to infer the phylogeny of many groups, including some phytoparasitic taxa (*e.g*., Al-Banna *et al*., 1997; Blaxter *et al*., 1998; Kaplan *et al*., 2000; Subbotin *et al*., 2001).

When assembled into the ribosome together with other proteins, ribosomal RNA is usually folded into a complicated secondary and tertiary structure. Although some of these structures have been detected by X-ray crystallisation (Cate *et al*., 1999) or by cryo-electron microscopic reconstruction (Mueller *et al*., 2000), most of the structures deposited in the public domain, such as the Antwerp database of large (De Rijk *et al*., 1999) and small rRNA sequences (Van de Peer *et al*., 1996) (http://www.psb.ugent.be/rRNA/index.htm), and the comparative RNA website (http://www.rna.icmb.utexas.edu/) (Cannone *et al*., 2002), are derived from comparative analysis, which generates the folding from the common compensatory substitutions and pairing patterns on many sequences. The secondary structure of rRNA provides a very useful template for improved construction of sequence alignments, and critical for phylogenetic construction (Kier, 1995; Hickson *et al*., 1996). Researchers have achieved some success by using alignments refined with the aid of the secondary structure and an optimised computer algorithm (Titus & Frost, 1996). Additionally, secondary structure can provide useful information for assessing the sequence in weighted parsimony or other weighted methods. Structural motifs may themselves contain information useful for phylogeny inference (Lydeard *et al.*, 2000).

In this paper we report on the first phylogenetic analyses of the family Longidoridae using molecular data collected from the LSU rRNA gene. We also tried to provide systematists with persuasive molecular information to aid in the reconstruction of those taxa. Species were sampled and used from all of the longidorid genera, with the exception of *Longidoroides*, which was, however, synonymised with the genus *Paralongidorus* (Siddiqi *et al.*, 1993), a status that is still questioned (Coomans, 1996), *Paraxiphidorus* and the monotypic *Australodorus*.

Materials and methods

Taxon sampling

Nematode samples collected for this study, together with their authorities, are listed in Table 1 and include 23 species from the genus *Longidorus*, two species from the genus *Paralongidorus*, two species from the genus *Xiphidorus*, and 35 species from the genus *Xiphinema*. The majority of the populations were identified on the basis of both morphometrics and morphology; only for some populations was the identification made on the basis of general morphology. These latter populations are marked with an asterisk in Table 1.

Total DNA extraction

One juvenile or adult nematode was transferred into 13 μl ddH_2O and cut into two to five pieces with a sterilised scalpel. Ten μl 2 $\times$ worm lysis buffer (20 mM Tris-HCl (pH 8.0), 100 mM KCl, 3.0 mM Mg_2Cl, 2.0 mM DTT, 0.9% Tween 20) and 0.1 μl proteinase K stock solution (20 mg/ml) was added to a 200 or 500 μl microcentrifuge tube. The nematode fragments were pipetted up in 9.9 μl ddH_2O and added to the tube, which was then briefly centrifuged and stored at -70°C for at least 10 min. Subsequently, each tube was incubated at 65°C for 1-2 h and the proteinase K was denatured at 95°C for 10 min. Finally, the DNA suspensions were cooled to 4°C and stored at -20°C

Table 1. *Taxon sampling for longidorids and outgroup used in this study.*

Nematode species	Code	Locality of sample	GenBank accession	Source (ID)
Aporcelaimellus obtusicaudatus (Bastian, 1865) Altherr, 1968	AOFD	Merelbeke, Belgium	AY601632	A. Coomans, S.A. Subbotin
Longidorus africanus Merny, 1966	CA46	California, USA	AY601583	F. Lamberti
L. apulus Lamberti & Bleve-Zacheo, 1977	CAN23	Mola di Bari, Italy	AY601571	T.C. Vrain
L. arthensis Brown, Grunder, Hooper, Klingler & Kunz, 1994	CAN115	Suter, Switzerland	AY601570	T.C. Vrain
L. athesinus Lamberti, Coiro & Agostinelli, 1992	EU105	Italy	AY601574	D.J.F. Brown
L. attenuatus Hooper, 1961	CAN17	Germany	AY601572	T.C. Vrain
L. breviannulatus Norton & Hoffmann, 1975	CAN268	Nebraska, USA	AY601576	T.C. Vrain
L. caespiticola Hooper, 1961	EU20	Scotland, UK	AY601567	D.J.F. Brown
L. camelliae Zheng & Brown, 2000	EU130	Hangzhou, China	AY601585	J. Zheng
L. carpathicus Lišková, Robbins & Brown, 1997	Carpa	Kirchbichel, Germany	AF480072	T. Rubtsova
L. diadecturus Eveleigh & Allen, 1982	CAN31	Elkins, White river, USA	AY601584	T.C. Vrain
L. edmundsi Hunt & Siddiqi, 1977	VE275	Caribbean sea beach, Cuba	AY601575	F. Lamberti
L. elongatus (de Man, 1876) Micoletzky, 1922	EU1	Ingraston, Scotland, UK	AY601578	D.J.F. Brown
L. euonymus Mali & Hooper, 1974	EU124	Zabagr, Hungary	AY601573	D.J.F. Brown
L. goodeyi Hooper, 1961	EU26	Peebles, Scotland, UK	AY601581	D.J.F. Brown
L. helveticus Lamberti, Kunz, Grunder, Molinari, De Luca, Agostinelli & Radicci, 2001	SV46	Camenzuid, Switzerland	AY601566	F. Lamberti
L. intermedius Kozlowska & Seinhorst, 1979	Inter	Planegg, Germany	AF480074	T. Rubtsova
L. juvenilis Dalmasso, 1969	CAN196	Moca, Slovakia	AY601579	T.C. Vrain
L. latocephalus Lamberti, Choleva & Agostinelli, 1983	BLUE	Nylstrom, South Africa	AY601568	F. Lamberti
L. latocephalus	CAN114	Greece	AY601569	T.C. Vrain
L. leptocephalus Hooper, 1961	EU8	Scotland, UK	AY601580	D.J.F. Brown
L. macrosoma Hooper, 1961	LM1	Switzerland	AY601565	J. Grunder
L. piceicola Lišková, Robbins & Brown, 1997	EU112	Branisko, Slovakia	AY601577	D.J.F. Brown
L. profundorum Hooper, 1966	Prof	Gandesbergen, Germany	AF480073	T. Rubtsova
L. sturhani Rubtsova, Subbotin, Brown & Moens, 2001	Vise348	Augen, Germany	AF480071	T. Rubtsova
Paralongidorus maximus (Bütschli, 1874) Siddiqi, 1964	Max592	Harrier Sand, Germany	AF480083	T. Rubtsova
Paralongidorus sp.	CAN201	Bosaka H1 SL, Czech Republic	AY601582	T.C. Vrain
Xiphidorus minor Rashid, Coomans & Sharma, 1986	VE269	Amazon forest, Venezuela	AY601612	F. Lamberti
Xiphidorus sp.	CAN248	Argentina	AY601611	T.C. Vrain
Xiphinema abrantinum Roca & Pereira, 1991	CAN223	Portugal	AY601625	T.C. Vrain
X. americanum Cobb, 1913	PE24	Pennsylvania, USA	AY601599	F. Lamberti
X. americanum	XA1	USA	AY601591	D.J.F. Brown
X. bakeri Williams, 1961	CAN27	Fayetteville, USA	AY601623	T.C. Vrain
*X. basiri** group sp.	EU125	San Jose, Cuba	AY601629	D.J.F. Brown
*X. basiri** group sp.	EU126	San Jose, Cuba	AY601630	D.J.F. Brown
*X. brasiliense** Lordello, 1951	EU41	Para State, Brazil	AY601616	D.J.F. Brown
X. brevicollum Lordello & Da Costa, 1961	Xb1	South Africa	AY601601	A. Coomans
X. brevicollum	EU132	Beijing, China	AY601604	J. Zheng
X. brevicollum	EU29	Brazil	AY601605	D.J.F. Brown
X. brevisicum Lamberti, Bravo, Agostinelli & Lemos, 1994	EU5	Braga, Portugal	AY601610	L. Poiras
X. bricolense Ebsary, Vrain & Graham, 1989	CAN39	Winfield, BC, Canada	AY601594	T.C. Vrain
X. bricolense	PE18	Pennsylvania, USA	AY601596	F. Lamberti
X. californicum Lamberti & Bleve-Zacheo, 1979	CA54	Kearney, CA, USA	AY601592	F. Lamberti

Table 1. *(Continued).*

Nematode species	Code	Locality of sample	GenBank accession	Source (ID)
X. chambersi Thorne, 1939	AB3	Lee county, AL, USA	AY601617	F. Lamberti
X. coxi Tarjan, 1964	GG10	Jenkil Tsloud, GG, USA	AY601631	F. Lamberti
X. dentatum[*] Sturhan, 1978	EU111	Branisko, Slovakia	AY601627	D.J.F. Brown
X. diversicaudatum[*] (Micoletzky, 1927) Thorne, 1939	EU7	Forest, Braga, Portugal	AY601624	D.J.F. Brown
X. diffusum Lamberti & Bleve-Zacheo, 1979	CAN162	South Africa	AY601600	T.C. Vrain
X. elongatum Schuurmans Stekhoven & Teunissen, 1938	CAN24	Israel	AY601618	T.C. Vrain
X. georgianum Lamberti & Bleve-Zacheo, 1979	GG14	Jenkil Tsloud, GG, USA	AY601586	F. Lamberti
X. incognitum Lamberti & Bleve-Zacheo, 1979	PE42	Pennsylvania, USA	AY601597	F. Lamberti
X. index Thorne & Allen, 1950	EU25	Argentina	AY601628	D.J.F. Brown
X. insigne Loos, 1949	EU131	Hangzhou, China	AY601619	J. Zheng
X. italiae Meyl, 1953	BAR1	Italy	AY601613	F. Lamberti
X. pachtaicum (Tulaganov, 1938) Kirjanova, 1951	T48	Castelnuovo Berarjengo, Italy	AY601606	F. Lamberti
X. pachtaicum	M21	Albata, Moldova	AY601607	L. Poiras
X. pacificum Ebsary, Vrain & Graham, 1989	GG15	Pike, GG, USA	AY601590	F. Lamberti
X. pachydermum Sturhan, 1983	EU109	Portugal, type locality	AY601608	D.J.F. Brown
X. pyrenaicum[*] Dalmasso, 1969	EU121	Cyprus	AY601626	D.J.F. Brown
X. radicicola Goodey, 1936	V1273	Chu'momray, Vietnam	AY601622	C.N. Nguyen
X. rivesi Dalmasso, 1969	PE20	Pennsylvania, USA	AY601588	F. Lamberti
X. rivesi	PE1	Pennsylvania, USA	AY601589	F. Lamberti
X. santos Lamberti, Lemos, Agostinelli & d'Addabo, 1993	CAN224	Portugal	AY601587	T.C. Vrain
X. savanicola Luc & Southey, 1980	CAN72	Dakar, Senegal	AY601620	T.C. Vrain
X. setariae Luc, 1958	EU27	Brazil	AY601621	D.J.F. Brown
X. simile Lamberti, Choleva & Agostinelli, 1983	M5	Anenii Nou, Moldova	AY601609	L. Poiras
X. taylori Lamberti, Ciancio, Agostinelli & Coiro, 1992	EU117	Spa, Slovakia	AY601602	D.J.F. Brown
X. taylori	TN1	Treuna, Italy	AY601603	F. Lamberti
X. thornei Lamberti & Golden, 1986	CO3	Colorado, USA	AY601595	F. Lamberti
X. thornei	OR4	Molella, Oregon, USA	AY601593	F. Lamberti
X. utahense Lamberti & Bleve-Zacheo, 1979	CO5	Colorado, USA	AY601598	F. Lamberti
X. vuittenezi Luc, Lima, Weischer & Flegg, 1964	EU123	Zabagr, Hungary	AY601614	D.J.F. Brown
Xiphinema sp.	EU110	Portugal	AY601615	D.J.F. Brown

[*] Populations identified on the basis of general morphology.

until use. No additional purification was required for subsequent PCR procedure.

PCR Amplification

The D2 and D3 expansion regions of the large subunit rDNA were amplified using the primers D2A (5′-ACAAGTACCGTGAGGGAAAGTTG-3′) and D3B (5′-TCGGAAGGAACCAGCTACTA-3′). The cycling profile of the PCR was 94°C for 3 min, 35 cycles of 94°C for 30 s, 54°C for 40 s, and 72°C for 1 min followed by an extension at 72°C for 10 min. PCR products were visualised under UV after separation in a 1% agarose gel and staining with ethidium bromide. The fragments were recovered from the gel by excision and purified with Gel purification kit (Qiagen-Westburg, Leusden, The Netherlands).

Sequencing and sequence analyses

A direct sequencing strategy was used for the amplified product. DNA fragments were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions (PE Applied Biosystems, Foster City, CA, USA). The final se-

quences were determined by an ABI prism 377 genetic analyser (Applied Biosystems). The GenBank accession numbers for sequences are given in Table 1. Sequences were assembled and edited with BioEdit (Hall, 1999).

Secondary structure construction and analyses

The secondary structure model of the D2 region was inferred with the aid of Mfold (Zuker *et al*., 1999) and the Vienna RNA package (Hofacker *et al.*, 2003). The Vienna RNA package was also used to aid in the construction of the secondary structure template of the D3 region by imposing constraints based on the predicted secondary structures of *Caenorhabditis elegans* (Maupas, 1900) Osche, 1952 (Ellis *et al*., 1986), *Drosophila melanogaster* Meigen, 1930 (Tautz *et al*., 1988) and *Xenopus laevis* Daudin, 1803 (Clark *et al*., 1984). The variability of the secondary structure templates was analysed by tree edit distance comparisons as implemented in the Vienna RNA package.

Phylogeny inference

Alignment

A sequence alignment was made with ClustalX 1.8 (Thompson *et al*., 1997) using default parameters (gap opening penalty = 15.55 and gap extension penalty = 6.66), followed by manual editing in BioEdit (Hall, 1999) based on the secondary structure templates. The analyses were based on this manually optimised alignment.

Phylogenetic analyses

The datasets of the D2 and D3 sequences were analysed with PAUP*4b10 (Swofford, 2002). As D2 and D3 regions may evolve at different rates and with different historical records of evolution, the homogeneity test (Farris *et al*., 1994) was used to measure the incongruence between the two regions so as to decide whether to perform analyses on the combined dataset. Homogeneity of nucleotide compositions was given by χ^2 statistics implemented in PAUP.

Different phylogenetic methods have different strengths and weaknesses; the use of multiple methods increases the confidence of the inferred phylogeny. Trees were therefore constructed using the algorithms of maximum parsimony (MP) and maximum likelihood (ML) methods as implemented in PAUP. Weighted parsimony was also performed on the combined D2 and D3 dataset (a weight of 1 was assigned to nucleotides in ambiguously aligned regions and a weight of 3 to those in robustly aligned regions). The weight strategies referred to the secondary structure model of D2 and D3 regions. A weight of 1 was assigned to nucleotides in regions for which the homologous nucleotides are difficult to be defined and a weight of 3 to those in homologous regions.

The MP method was used with heuristic search, tree bisection and reconnection (TBR) swapping algorithm and ten random additions of sequences. One hundred non-parametric bootstrap replicates (BS) were analysed with heuristic search algorithm. Decay indices (DI) (Bremer, 1994) were calculated by Autodecay (Ericsson, 2001).

The appropriate ML model was selected by the Log Likelihood ratio test (LRT) implemented in the software Modeltest (Posada & Crandall, 1998). Nested models were evaluated by LRT and the best model was selected for the ML method. The starting tree for LRT was obtained by the neighbour joining (NJ) method or the best MP tree. The heuristic search with the TBR swapping algorithm and random sequence addition. The TBR was limited to 10 000 trees due to the extended computation time. Eleven searches were performed. Eleven ML trees were compared using the Shimodaira-Hasegawa (SH) test with resampling estimated log-likelihood (RELL) approximation (Shimodaira & Hasegawa, 1999). The selected best tree topology (with the highest log likelihood value) was tested by the Swofford-Hillis (SOWH) test (Goldman *et al*., 2000) using parametric bootstrap methods. One hundred parametric bootstrap replicates were produced by SeqGen implementing Monte Carlo simulation (Rambaut & Grassly, 1997) based on the ML estimated parameters of the given topology.

A tree based on the RNA secondary structure was constructed from tree edit distances. The secondary structure comparison was reduced to a comparison of the ordered labelled trees that were used to represent the secondary structures, as proposed by Shapiro and Zhang (1990) using the NJ method. The tree edit distances matrix was computed using Vienna RNA package. Confidence assessments for the MP analyses, branch and topology supports were performed by non-parametric bootstrap analysis. Decay indices were calculated for each branch. Because of the computational limitations encountered with ML, we could not perform extensive non-parametric bootstrap analyses on the big data sets. We chose Bayesian inference (BI) analyses to estimate posterior probabilities (BPP) for the phylogenetic relationships inferred by ML analysis. MrBayes 2.0 (Huelsenbeck & Ronquist, 2001)

was used for the analysis in combination with the model previously identified by Modeltest. Bayesian analysis was implemented with random starting trees, four incrementally heated Markov chains and 1.0×10^6 generations and sampling Markov chains at 100 generation intervals. Trees obtained before the stationary point were discarded as 'burn-in' samples. Analyses were performed three times to avoid local optima traps (Huelsenbeck & Ronquist, 2001).

An alternative phylogenetic hypothesis test was carried out by imposing constraints on the MP analyses. For these analyses, species were forced into groups according to the phylogeny of the family as inferred from morphological data (Coomans, 1985, 1996). The trees obtained with these constraints were tested by implementing SH-test (Shimodaira & Hasegawa, 1999) with likelihood setting previously generated from the Modeltest. The tested hypothetical monophyletic groups included the subfamilies Longidorinae and Xiphinematinae, and the genera *Xiphidorus*, *Xiphinema* and *Paralongidorus*.

Results and discussion

Secondary structure of D2 and D3 expansion region

Secondary structures of the expansion region were inferred for each species. The general secondary structure of the D2 region of longidorids was composed of three long stem and loop structures (Fig. 1A); some variations were observed (data not shown). The D3 structure was rather conserved across the species studied (Fig. 1B). Variation was found in the D4_1 stem and loop region of D3 structure, which were absent in several *Longidorus* species.

Phylogeny

The base composition of the D2 and D3 expansion regions did not reveal high heterogeneity between the species. No significant differences in base composition were observed in the D2 region ($\chi^2 = 209.49$, d.f. $= 219$, $P = 0.67$) or the D3 region ($\chi^2 = 53.05$, d.f. $= 219$, $P = 1.0$). Partition homogeneity analyses (Farris *et al*., 1994) resulted in $P = 0.22$, which supported the analyses of the combined D2 and D3 regions. The g1 statistic for the D2 and D3 datasets was -0.35 and -0.29, respectively, indicating that the data sets contain good phylogenetic signal (Hillis & Huelsenbeck, 1992). Statistic g1 was calculated by evaluating the tree length distribution of 10 000 random trees; the g1 for the combined dataset equalled -0.37.

The ML and MP analyses of the D2 dataset and the combined dataset produced similar tree topologies. Analyses of the D3 dataset did not resolve all lineages although they also recovered the strongly supported clades inferred from D2 alone and from the combined dataset (data not shown).

MP analysis of the combined dataset resulted in 5154 maximum parsimonious trees with a tree length of 2732. A consensus tree of the 5154 equally scored trees is presented in Figure 2. Bootstrap values and decay indices calculated for the consensus trees are added to the corresponding nodes. The tree topology obtained from the weighted MP analysis was the same as the one obtained from unweighted MP analysis (data not shown).

Maximum likelihood analyses were performed on the combined datasets. The tree topology obtained from the combined dataset is similar to that of the MP trees. The selected model was GTR + Γ + I (general time reversible plus gamma rates and proportion of invariable sites). Eleven heuristic searches resulted in 11 ML trees that were compared by the Kishino-Hasegawa tests (KH-test). The 11 topologies were not significantly different. The tree with the highest likelihood score (lnL $= -12998.65$) was selected as the default best ML tree (Fig. 3A).

The NJ tree inferred from the tree edit distance of the secondary structures maintained clades statistically strongly supported in the ML and MP analyses (Fig. 3B).

Position of genera

Both the ML tree (Fig. 3A) and MP tree (Fig. 2) showed the same phylogenetic clades. The monophyly of the genus *Longidorus* was strongly supported with 1.0 BPP for the tree inferred by ML analysis and 92% BS in the MP analysis.

Non-*X. americanum*-group species and *X. americanum*-group species (both classified in the genus *Xiphinema*) were strongly supported as two isolated clades (1.0 BPP for non-*X. americanum*-group and 0.92 for *X. americanum*-group in the ML analysis, and 100% BS for the non-*X. americanum*-group and 53% for the *X. americanum*-group in the MP analysis). The analysis of the ITS1-rRNA by Ye *et al.* (2004) also yielded trees with two separate clades within *Xiphinema*. The non-*X. americanum*-group was supported as a sister clade to *Longidorus* (0.98 BPP in ML analysis and 69% BS in MP analysis).

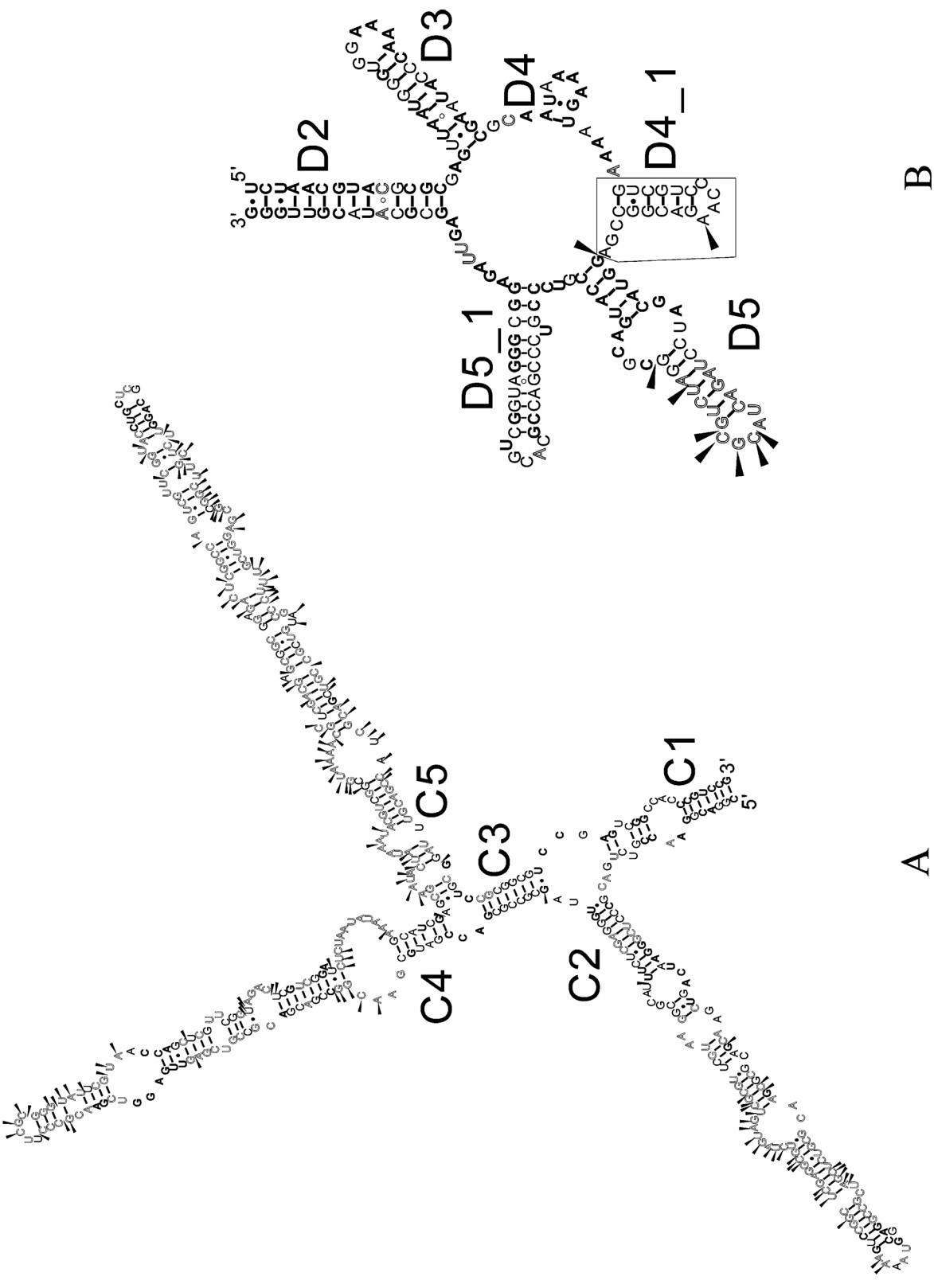

Fig. 1. *Secondary structure models of the D2 and D3 expansion regions of the LSU rRNA for species of the Longidoridae. Both model sequences came from* Xiphinema brevicollum *with code XB1. In the D2 model (A), constant sites, polymorphic sites with only one variant and polymorphic sites with more than one variant are shown by bold faced letters, normal letters and hollow letters, respectively. In the D3 model (B), stem-loop D4_1 is absent in the secondary structures of some species. The deletion region is circled. Arrows point at deletion/insertion positions.*

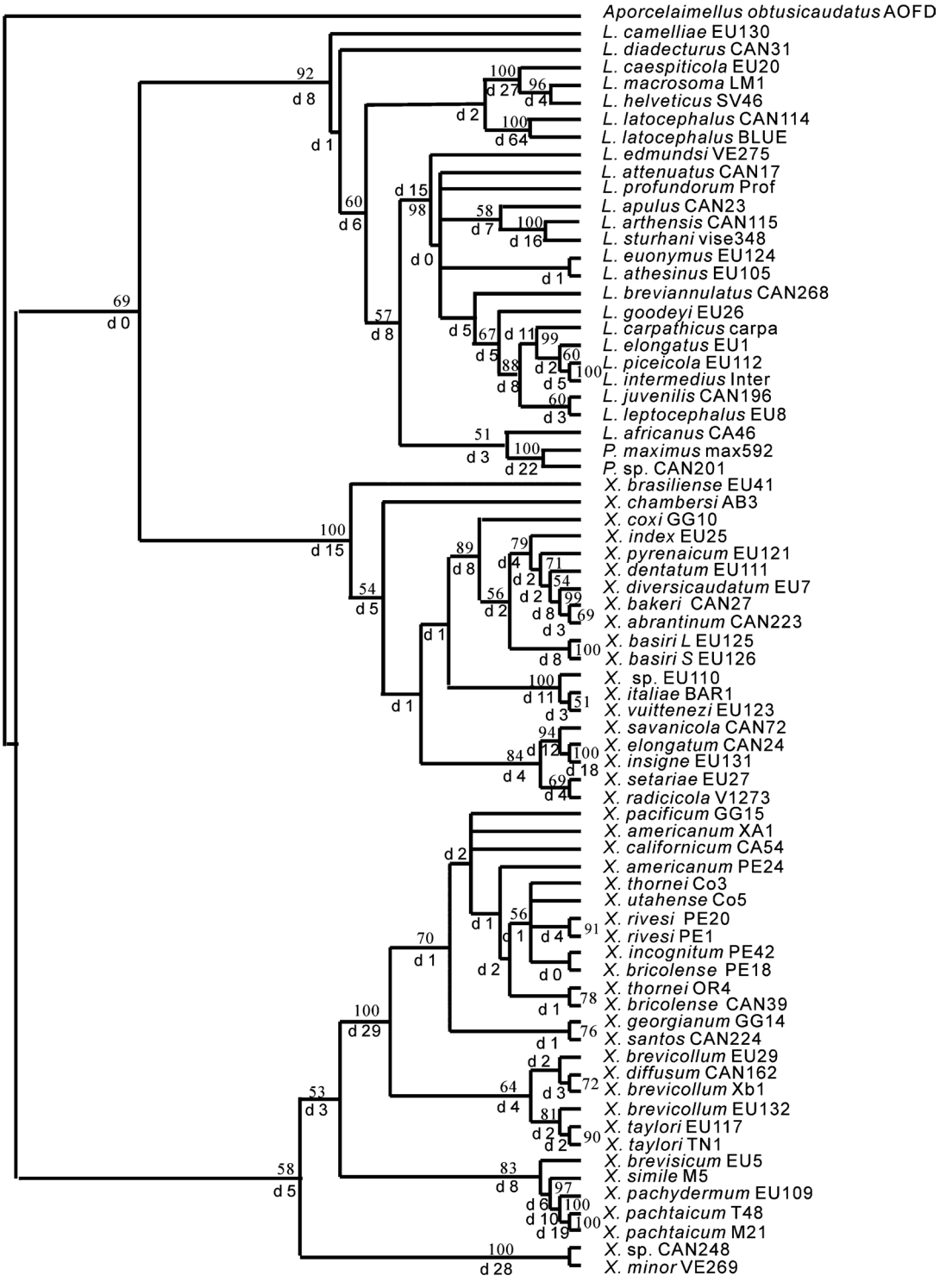

Fig. 2. *Phylogeny of the Longidoridae inferred from maximum parsimony analysis of the sequences of the D2 and D3 expansion regions of the LSU rRNA gene. Bootstrap support (more than 50%) and decay index (starting with d) are shown in the MP consensus tree.*

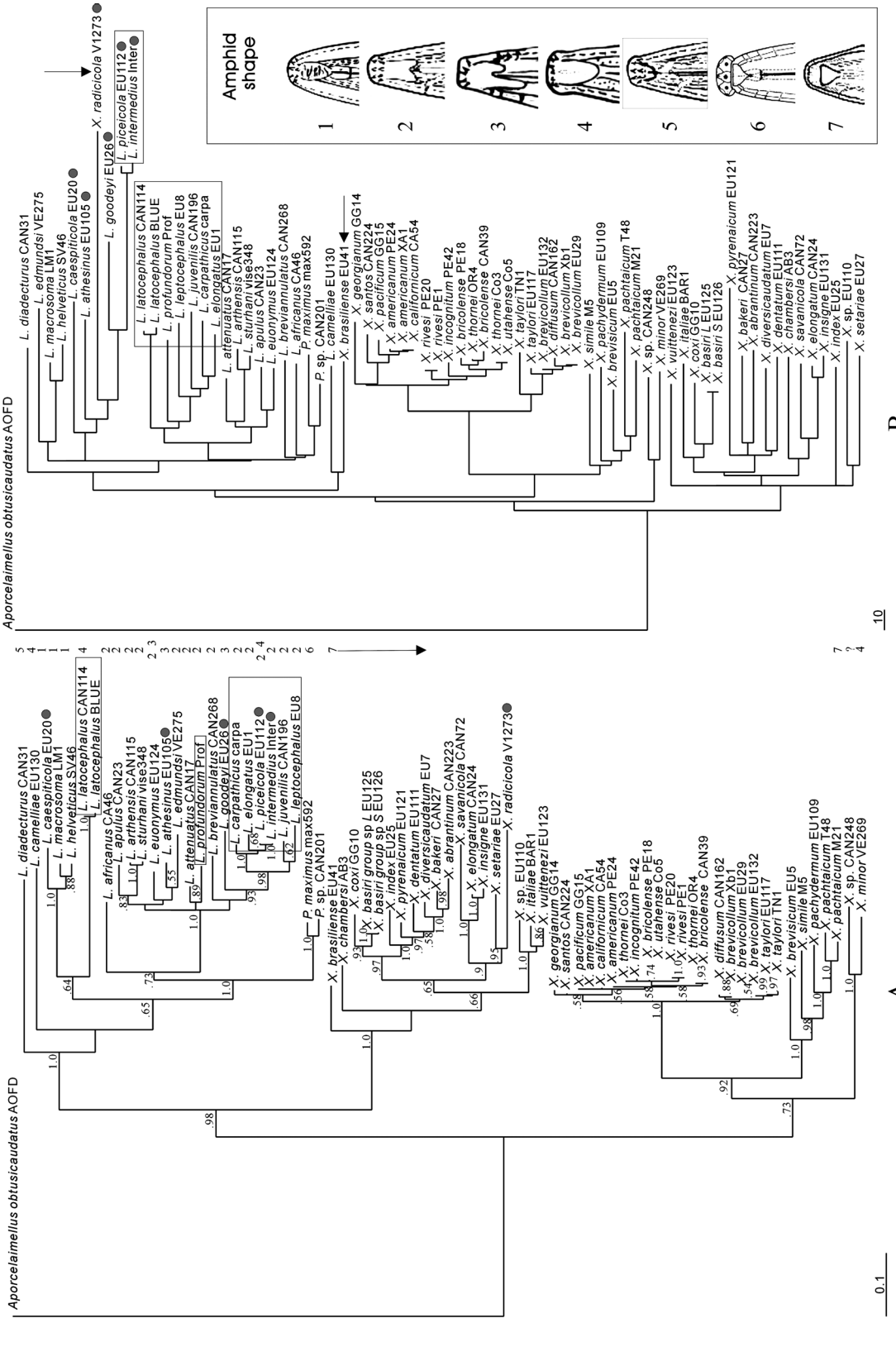

Fig. 3. *Phylogenetic relationships within the Longidoridae as inferred from (A) ML analysis of nucleotide sequence data and (B) NJ analysis of structural rRNA morphometric matrix. The contoured species have the D4_1 stem-loop structure deleted in D3 region; the bullets indicate species with variant structures in D2. Arrows point at the species placed erroneously to other taxon. Bayesian posterior probabilities are added to the corresponding nodes in the ML tree. The amphid shape of* Longidorus *(shown in the rightmost contoured area) is mapped to the ML tree. The correspondence between shape of amphids and the terminal nodes of the tree is indicated by corresponding numbers. Amphid Type 1: elongated funnel shape not lobed; 2: pouch shape with symmetrical lobes; 3: pouch shape with asymmetrical lobes; 4: cup shape without lobes; 5: funnel shape not lobed; 6: stirrup-shape; 7: wide stirrup-shape (after Chen* et al., *1997).*

Table 2. *Results of the SH-tests for different topologies and alternative hypotheses.*

Topologies and hypothesis tested	−*Ln*L	Difference of −*Ln*L	*P* value
ML tree	12998.65	best	–
MP tree	13022.52	23.88	0.49
Distance tree based on secondary structure[a]	14003.06	1004.41	0.00*
Bayesian consensus tree[b]	13048.84	50.20	0.29
All *Xiphinema* species constrained into one group	13064.22	65.58	0.109
Paralongidorus constrained to be a group outside of *Longidorus*	13184.11	185.46	0.002*
Xiphidorus constrained to be a group outside of all *Xiphinema* species	13198.79	200.14	0.001*

* $P < 0.05$ indicates the significant differences between the two inferred tree topology.
[a] Distance tree inferred from the tree edit distance of secondary structure.
[a] The 50% majority consensus tree obtained in the result of BI analysis.

The two *Xiphidorus* species were grouped together with 1.0 BPP in the ML analysis and 100% BS in the MP analysis. Instead of a closer position to *Longidorus*, the *Xiphidorus* species were grouped with the *X. americanum*-group species (0.73 in ML analysis and 58% BS in MP analysis).

The clade composed of the two *Paralongidorus* species was strongly supported (1.0 BPP in ML analysis and 100% BS in MP analysis). It clustered as an internal clade of the genus *Longidorus* confirming the results of the phylogenetic analysis made by Rubtsova *et al*. (2001).

Our analyses of both the sequence and secondary structure based phylogenies support four major clades within the Longidoridae: *i*) the *Longidorus* clade (including *Paralongidorus*); *ii*) the clade composed of the non-*X. americanum*-group species of the genus *Xiphinema*; *iii*) the *X. americanum*-group clade; and *iv*) the *Xiphidorus* clade.

Recently published analyses of ITS1-rRNA (Ye *et al*., 2004) and 18S rRNA (Oliveira *et al.*, 2004) also revealed two distinct major groups within the genus *Xiphinema* and are fully congruent with the results of our analysis. The phylogenetic testing of our D2/D3 tree (Table 2) did not refute the monophyly of the genus *Xiphinema* even though it was split into two major clades ($P = 0.109$). The genus *Paralongidorus* was rejected as a valid taxon ($P = 0.002$). The genus *Xiphidorus* was rejected as a group outside of genus *Xiphinema* ($P = 0.001$).

Molecular evolution of the secondary structure

Although the tree generated from the tree edit distances of the secondary structure shared the major clades with trees obtained from ML and MP analyses, it nevertheless differed significantly ($P = 0.00$) from the ML tree inferred from the sequence data (Table 2). Remarkable differences were observed for the positions of two species (*X. radicicola* – V1273, *X. brasiliense* – EU41) belonging to the non-*X. americanum*-group (Figs 2, 3A), which in the secondary structure tree were grouped with *Longidorus* species, because their derived secondary structures in stem loop C5 of the D2 region were similar to those in several *Longidorus* species (Fig. 3B). These derived structures presumably represent convergence. The notable derived feature of the D3 region was the loss of the D4_1 stem-loop structure in several *Longidorus* species. Eight species that had lost the D4_1 structure were distributed in three clades obtained with sequence analyses: six species forming the strongly supported clade including *L. carpathicus*, *L. elongatus*, *L. piceicola*, *L. intermedius*, *L. juvenilis* and *L. leptocephalus* (0.98 BPP in ML analysis), one species, *L. profundorum*, was positioned with *L. attenuatus* in one clade supported with 0.89 BPP in ML analysis, and one species, *L. latocephalus*, in the clade containing *L. caespiticola* (Fig. 3A). We also noted that six species from the above mentioned three clades were clustered into one clade in the NJ tree inferred from the secondary structure distance matrix (Fig. 3B) while two species (*L. piceicola* and *L. intermedius*) from the clade including *L. carpathicus* and *L. leptocephalus* were positioned with *L. goodeyi* in another clade because of their derived C5 stem-loop structure in the D2 expansion region (Fig. 3B). Additionally, some minor differences of structural evolution resulted in changes of the positions of several species inside the major clades (Fig. 3). All discrepancies described above may reflect differences of evolutionary rates between the nucleotide sequences and their secondary structures.

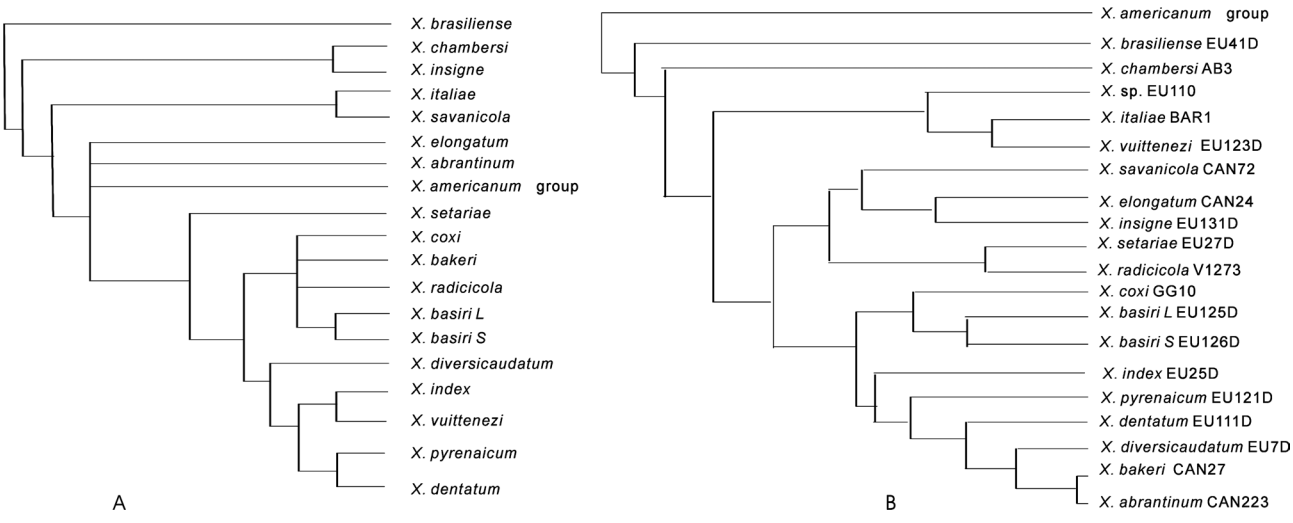

Fig. 4. *A: Tree derived from the phylogeny of the genus* Xiphinema *proposed by Coomans* et al. *(2001); B: Tree containing* Xiphinema *species (except for* X. americanum*-group) derived from the molecular ML tree.*

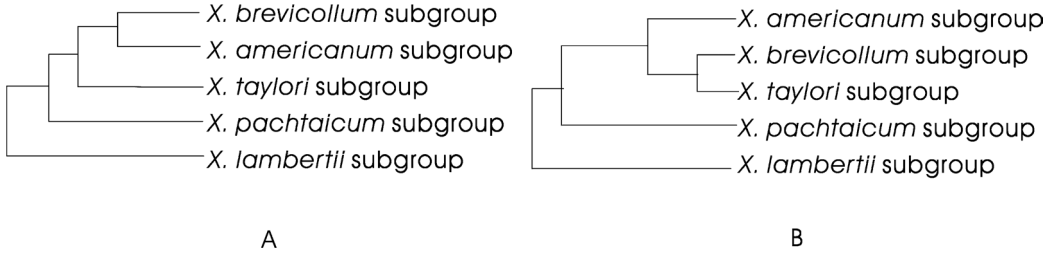

Fig. 5. *A: Tree derived from the phylogeny of* Xiphinema americanum*-group proposed by Lamberti and Ciancio (1993); B: Tree containing the* Xiphinema americanum*-group species derived from the molecular ML tree.*

Correlation with morphological characters and groups

Longidorus *and* Paralongidorus

The only interesting correspondence between morphological characters and phylogenetic trees inferred from molecular data is the grouping of *Longidorus* species coincident with similarity in the amphid structure, as previously noticed by Rubtsova *et al*. (2001) (Fig. 3A). Two groups were observed in the tree. One group included *L. caespiticola*, *L. helveticus* and *L. macrosoma* with funnel shaped amphid pouches (Type 1, see Fig. 3A); the other group included *L. africanus*, *L. apulus*, *L. arthensis*, *L. athesinus*, *L. attenuatus*, *L. breviannulatus*, *L. carpathicus*, *L. edmundsi*, *L. elongatus*, *L. euonymus*, *L. intermedius*, *L. juvenilis*, *L. leptocephalus*, *L. piceicola*, *L. profundorum*, and *L. sturhani* with symmetrically (Type 2), or *L. euonymus* and *L. goodeyi* with asymmetrically (Type 3) lobed amphids. The remaining species, *L. camelliae*, *L. adecturus* (Type 5) and *L. latocephalus*, did not form one group although *L. camelliae* and *L. latocephalus* share the same Type 4 amphid pouch. The amphids with a stirrup-shaped pouch were only found in species of the genus *Paralongidorus*, which formed a separate group within *Longidorus*. Examining more species of *Paralongidorus* should reveal the distribution of the other amphid shapes reported for the genus.

Mapping of amphid types on the tree suggests that Type 3 and Type 4 appeared several times during evolution of the genus *Longidorus*. The correspondence implies that evolution of some molecules may be synchronous with the evolution of some morphological characters, even when there is no obvious morphogenetic link between both. This synchronous evolution facilitates the recovery of correct phylogeny based on both molecular and morphological analyses, especially for extant taxa lacking informative fossil records, such as nematodes.

Xiphinema *and* Xiphidorus

The phylogeny of the genus *Xiphinema* was constructed by Coomans *et al*. (2001) based on 44 morphological characters. To facilitate the analyses, the authors subdivided the sampled species into several groups according to tail shape. The tree topology obtained from our analyses is very close to the topology obtained by Coomans *et al*. (2001) as redrawn here in Figure 4A. Congruent groupings included those of *X. dentatum* and *X. pyrenaicum*, the group *X. dentatum*, *X. pyrenaicum*, *X. index* and *X. diversicaudatum*, the group including *X. coxi* and *X. basiri*, and the large group including all species cited above as well as *X. bakeri*, *X. setariae* and *X. radicicola*. The positions for other species also corresponded fairly well between both analyses. However, the two topologies differed distinctly in the position of the *X. americanum*-group. A reasonable explanation is that the evolutionary rates of the D2 and D3 expansion regions of these species are not synchronised with the evolution of the selected morphological character (tail shape).

Giving consideration to all *Xiphinema* species, and excluding those of the *X. americanum*-group, we conducted a KH-test (with RELL approximation) between two derived trees. One tree was derived from the ML tree based on the combined D2 and D3 dataset (Fig. 3A); the other was derived from the tree shown in Figure 4. The result does not show significant differences between the two topologies ($P < 0.05$, $P = 0.001$).

Examining more species of the genus *Xiphidorus* may reveal the distribution of the three reported amphid shapes within the genus, besides the cup-shaped amphid pouch (Type 4) observed in *Xiphidorus minor*.

The Xiphinema americanum *lineage*

In our analyses, the *X. americanum* lineage appears as a clade close to *Xiphidorus*, albeit with low support from bootstrap analyses (Fig. 2). Within the lineage, we observed two groups well supported in our analyses. The first group (*X. americanum* subgroup) included *X. americanum*, *X. brevicollum* and several virus vector species; the second group (*X. pachtaicum* subgroup) included *X. pachtaicum*, *X. pachydermum* and *X. brevisicum*, an amphimictic species (Lamberti *et al*., 2000). In comparison with cluster analysis based on morphological characters (Lamberti & Ciancio, 1993), an analysis that resulted in the subdivision of the *X. americanum*-group into the *X. brevicollum*, *X. americanum*, *X. taylori*, *X. pachtaicum*, and *X. lamberti* subgroups, our results merged the *X. taylori* subgroup into the *X. brevicollum* subgroup, itself part of the *X. americanum* subgroup (Fig. 5). As we did not have a species from the *X. lamberti* subgroup at our disposal, we could not infer position and consequently the relationships of this subgroup remain unclear.

Conclusion

This is the first extensive study using large subunit rDNA molecular data to infer the phylogeny of the family Longidoridae. Our analysis revealed four major groups within Longidoridae: *Longidorus*, the *X. americanum*-group, other *Xiphinema* species and *Xiphidorus*. The genus *Paralongidorus* was clustered as an internal group of the genus *Longidorus*, and ML testing rejected the validity of this genus. Although the result of the alternative phylogenetic hypotheses testing (Table 2) did not refute the monophyly of the genus *Xiphinema*, the species of this genus were split into two distinct clades in all trees. The genus *Xiphinema* was originally described by Cobb in 1913 (Lamberti *et al.*, 2000), the type species being *X. americanum*, itself a member, of course, of the *X. americanum*-group. If additional analyses of other genes reveal the same phylogenetic pattern in the distribution of *Xiphinema s.l.* species, then it may become necessary to restrict *Xiphinema* to the species of the *X. americanum*-group and establish one or more additional genera, along the lines hypothesised by Lamberti and Bleve Zacheo (1979), for the remainder of the species. Comparative analysis revealed that sequence-based *vs* structural phylogenies can lead to different results and are not always congruent.

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