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Phylogenetic relationships of Longidoridae species (Nematoda: Dorylaimida) from North America inferred from 18S rDNA sequence data

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Summary

A phylogenetic analysis of 18S rDNA sequences was conducted to construct evolutionary trees and evaluate taxonomic relationships among *Longidorus* and *Xiphinema* nematode species from North America. A multiple sequence alignment, including *Californidorus cylindricaudatus* as an outgroup, comprised of 1747 characters of which 1479 were constant, and 146 (8.4 %) were parsimony informative. All sequences had a higher A + T (52.8 %) than G + C composition (47.2 %). Interspecies sequence variation (0.1 % – 9.1 %) was greater than intraspecies sequence variation (0.1 % – 0.6 %). Phylogenetic analysis, using both maximum parsimony and maximum likelihood analysis, revealed three phylogenetically distinct clades representing extant taxonomy, namely, *Longidorus*, *Xiphinema* and the *X. americanum*-group. Within the *Longidorus* clade, four sub-clades were evident in the maximum likelihood tree. It is concluded that 18S rDNA sequences are appropriate for inferring phylogenetic relationships among longidorid species.

Key words: 18S rDNA; *Longidorus*; phylogeny; *Xiphinema*; *Xiphinema americanum*-group

Introduction

Longidorids are migratory ectoparasitic nematodes that feed on an extensive range of crop, weed, and tree species. They are long and slender, ranging from 1.5 mm (e.g. *Xiphinema* species) to over 12 mm (e.g. *Paralongidorus* species) in length and are readily distinguished from most other plant-parasitic nematodes by having a posterior enlargement of the oesophagus and greatly attenuated odontostyle. Nineteen species in *Longidorus*, *Paralongidorus* and *Xiphinema* have been proven to be vectors of plant viruses causing reductions in yield and quality of a range of horticultural and agricultural crops (Brown *et al*., 1993, 1994; Taylor and Brown, 1997).

Fifty-seven species in the three genera *Longidorus*, *Paralongidorus* and *Xiphinema* occur in North America, with many being widely distributed (Norton *et al*., 1984; Robbins and Brown, 1991; Robbins and Brown, 1996; Coomans *et al*., 2001; Ye, 2002). Species identification is based primarily on morphological and morphometric criteria, and as the number of valid longidorid species increases (Taylor and Brown, 1997), their identification is becoming increasingly difficult as many of the taxonomic characters overlap. With the loss of skilled expertise, identification to putative species level, especially in the *Xiphinema americanum*-group and *Longidorus,* is becoming problematical.

Molecular sequences, particularly of 18S rDNA, have proven useful in resolving phylogenetic relationships among living taxa including nematode species at different taxonomic levels (Fitch *et al*., 1995; Liu *et al*., 1997; Aleshin *et al*., 1998; Blaxter *et al*., 1998; Kampfer *et al*., 1998; de Ley *et al*., 2002; Dorris *et al*., 2002; Floyd *et al*., 2002; Kanzaki and Futai, 2002). However, with the exception of Oliveira *et al*. (2004), 18S rDNA sequences for almost all longidorids are still unknown. The region is among the slowest evolving in living organisms but is long enough to yield statistically valid information which allows the inference of phylogenetic history across a very broad taxonomic range (Nadler, 1992; Fitch *et al*., 1995; Nadler and Hudspeth, 1998). Also, the presence of many copies of 18S rDNA per genome, and its homogenization through concerted evolution, greatly reduces intra-specific variation (Hillis and Dixon, 1991).

In a study of molecular relationships among longidorids, we have determined the nucleotide sequence of 18S rDNA from 15 species of longidorids mainly from North America, and evaluated its potential usefulness for reconstructing the evolutionary relationships of species within this nematode group.

Materials and Methods

Nematode specimens were collected in the period 1999 to 2001 from several different habitats, but mostly from soil around hardwood trees growing on sandy stream bank soil in Arkansas, USA. Specimens were extracted using a combined decanting, sieving (sieve size: 75 µm, 850 µm) and sugar centrifugal flotation (specific gravity = 1.167) technique (Jenkins, 1964). Fresh nematode specimens were hand picked and transferred to 1 M NaCl in microtubes and preserved at – 80°C prior to DNA extraction. Sixteen populations belonging to 12 species of *Longidorus*, 3 species of *Xiphinema,* and *Californidorus cylindricaudatus*

Table 1. *Longidorus* and *Xiphinema* populations and species used for 18S rDNA sequencing with *Californidorus cylindricaudatus* Robbins, 1985 used as an outgroup

Species	Population code	Host	Locality	GenBank accession number
L. africanus Merny, 1966	Long-162	Grape (*Vitis* sp.)	San Diego County, California	AY283164
L. biformis Ye & Robbins, 2004	Long-4	Elm (*Ulmus americana* L.)	Middle Fork of the White River, near Elkins, Washington Co., AR	AY283171
L. biformis Ye & Robbins, 2004	Long-149	Osage orange (*Maclura pomifera* L.)	Osage Creek, ½ mile North of Hwy. 412, Carroll Co., AR	AY283162
L. breviannulatus Norton & Hoffman, 1975	Long-140	Cottonwood (*Populus deltoides* Marsh)	Toad Suck Park, Perry Co., AR	AY283161
L. crassus Thorne, 1974	Long-115	Oak (*Quercus* sp. L.)	Illinois River, County Road 62 Bridge, Washington Co., AR	AY283158
L. diadecturus Eveleigh & Allen, 1982[a]	Long-23A	Elm (*Ulmus americana* L.)	Middle Fork of the White River, near Elkins, Washington Co., AR	AY283167
L. diadecturus Eveleigh & Allen, 1982[a]	Long-23B	Elm (*Ulmus americana* L.)	Middle Fork of the White River, near Elkins, Washington Co., AR	AY283166
L. fragilis Thorne, 1974	Long-97	Willow (*Salix* sp. L.)	Haroldton Access, Arkansas River, near Van Buren, Crawford Co., AR	AY283172
L. grandis Ye & Robbins, 2003	Long-201	River cane (*Arundinaria gigantea* (Walt.) Chapm.)	Big Piney Creek Access Area, Highway 164, Pope Co., AR	AY283165
L. paralongicaudatus Ye & Robbins, 2003	Long-137	Hickory (*Carya* sp. Nutt.)	Illinois River, County Road 62 Bridge, Washington Co., AR	AY283160
L. paravineacola Ye & Robbins, 2003[b]	Long-108A	Osage orange (*Maclura pomifera* L.)	Osage Creek, ½ mile North of Hwy. 412, Carroll Co., AR	AY283157
L. paravineacola Ye & Robbins, 2003[b]	Long-108B	Osage orange (*Maclura pomifera* L.)	Osage Creek, ½ mile North of Hwy. 412, Carroll Co., AR	AY283156
L. paravineacola Ye & Robbins, 2003	Long-123	Box elder (*Acer negundo* L.)	Illinois River, County Road 62 Bridge, Washington Co., AR	AY283159
L. vineacola Sturhan & Weischer, 1964	Long-235	Grass (Gramineae)	Island of Coll, UK	AY283169
L. sp. California	Long-161	Citrus (*Poncirus trifoliata* (L.) Raf.)	Oakville, Napa County, CA	AY283163
L. sp. Georgia	Long-234	Pine (*Pinus* sp. L.)	Forest service nursery, USDA, GA	AY283168
X. americanum sensu lato Cobb, 1913	Xiph-4	Crabapple (*Pyrus* (*Malus*) sp. L.)	Univ. of Ark. Main Exp. Sta., Fayetteville, Washington Co., AR	AY283170
X. bakeri Williams, 1961	Xiph-23	Red bud (*Cercis canadensis* L.)	Middle Fork of the White River, Near Elkins, Washington Co., AR	AY283173
X. chambersi Thorne, 1939	Xiph-41	Maple (*Acer sp*. L.)	Shirey Bay - Rainey Brake Wildlife Management Area, Lawrence Co., AR	AY283174
Californidorus cylindricaudatus	Cali-1	Elm (*Ulmus americana* L.)	Middle Fork of the White River, near Elkins, Washington Co., AR	AY283155

a – Long-23A had longer odontostyle (114 µm) than Long-23B (102 µm); b – Only two individual nematodes in this population were available to be sequenced, Long-108A and Long-108B

were used in the study (Table 1). *Longidorus vineacola* from Scotland was included because of its similar morphological features with *L. paravineacola* described from Arkansas, USA. The sequence of *C. cylindricaudatus* was used as an outgroup as this species is a distantly related member of the Dorylaimida (Jairajpuri, 1982; Robbins, 1985) located within clade I of the phylogeny proposed by Blaxter *et al*. (1998). Two published sequences from Gen Bank, *X. rivesi* Dalmasso, 1969 (Gene Bank accession number: AF036610) and *L. elongatus* (AF036594), were also included for comparative purposes.

DNA from a minimum of two individual adult females of each species was extracted using a modified lysis method described by Stanton *et al*. (1998). Nematodes were placed into a 0.5 ml micro-centrifuge tube containing 20 µl 0.25 M NaOH and incubated at 25°C overnight. Thereafter, the samples were incubated at 99°C for 3 minutes and 10 µl 0.25 M HCl, 5 µl 0.5 M Tris-HCl, (pH 8.0) and 5 µl 2 % Triton X-100 were added. Samples were incubated at 99°C for a further 3 minutes.

Two Ready-to-Go PCR beads (Amersham International, Little Chalfont, UK) were placed into a 0.2 ml micro-centrifuge tube and 47.5 µl distilled water, 0.5 µl template DNA and 1 µl of each 10 µM primer pair were added. Combinations of primers SSU_F_02, SSU_F_04, SSU_F_07, SSU_F_22, SSU_R_09, SSU_R_13, and SSU_R_18 (http://nema.cap.ed.ac.uk/biodiversity/sourhope/nemoprimers.html) and XIPHF and XIPHR (Oliveira *et al*., 2004) were used to determine the sequence of 18S rDNA. PCR conditions were as follows: 94°C for 2 min 45 s, then 40 cycles of 94°C for 1 min, 57°C for 45 s, and 72°C for 2 min. The extension phase was 72°C for 10 min. PCR products were separated on 1 % agarose gel and visualised by staining with ethidium bromide. PCR products were purified for sequencing using the protocol listed by the manufacturer (QiAquick PCR Purification Kit, QIAGEN Inc., Crawley, West Sussex, UK).

Purified DNA fragments were sequenced directly in both directions using each PCR primer pair, one forward and one reverse using a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Warrington, UK). For each sequencing reaction the following reagents were added to a 0.5 ml micro-centrifuge tube: 4 µl terminator Ready Reaction Mix, 1.0 µl primer (3.4 µM) and 5 µl template purified DNA, according to the instructions listed by the manufacturer. DNA was sequenced using an ABI 377 DNA sequencer. GenBank accession numbers for the nematodes used in this study are listed in Table 1.

Sequences were aligned using GCG (Genetics Computer Group) PILEUP program (with a gap weight of 5 and a gap length weight of 1). The distance matrix option of PAUP* 4.0b10 (Swofford, 2002) was used to calculate genetic distances according to the Tajima-Nei model (Tajima and Nei, 1984). Maximum likelihood and unweighted parsimony analysis on the alignments were conducted using PAUP* 4.0b10. Gaps were treated as missing characters for all analyses. The reliability of the phylogenetic trees produced was determined using a bootstrap test (Felsenstein, 1985). Parsimony bootstrap analysis included 1000 re-samplings using the Branch and Bound algorithm of PAUP*. For maximum likelihood analysis (Yang, 1994), the default likelihood parameter settings of PAUP* were used (HKY85 6-parameter model of nucleotide substitution, empirical base frequencies, and transition/transversion ratio set to 1.259418:1). These parameters were used to carry out a heuristic search using PAUP*, utilising either the single most parsimonious tree as the starting tree, or step-wise addition, with 1000 bootstrap replications.

Results

A multiple sequence alignment, including *C. cylindricaudatus* as an outgroup, comprised of 1747 characters of which 1479 were constant and 146 that were parsimony informative. The aligned DNA data matrix, including the outgroup taxon, is available at TreeBASE (http://www.treebase.org, submission number SN1448). Average composition of nucleotides amongst the longidorids was 27.4 % (A), 25.4 % (T), 21.2 % (C) and 26.0 % (G). Interspecies sequence variation (0.1 % – 9.1 %; Table 2) was greater than intraspecies sequence variation (0.1 % – 0.6 %). Individuals of *L. diadecturus* from the same population but with different mean odontostyle lengths (114 µm *vs*. 102 µm) exhibited sequence variation (10 bp). Additionally,

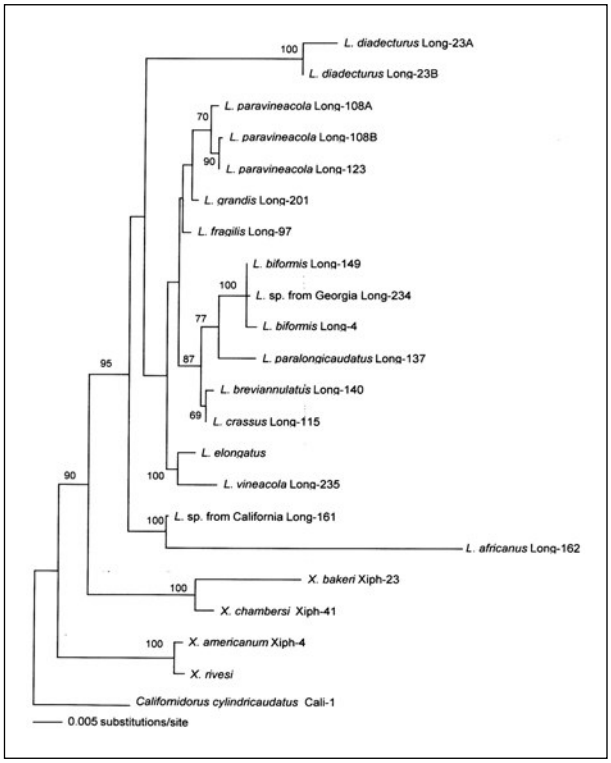

Fig. 1. Best maximum likelihood tree for *Longidorus* and *Xiphinema* inferred from 18S rDNA sequences. Branch lengths are proportional to the number of inferred changes. Numbers at the branch points represent the percentage of bootstrap replicates supporting the indicated clades and subclades. (Ln likelihood = – 4919.48352)

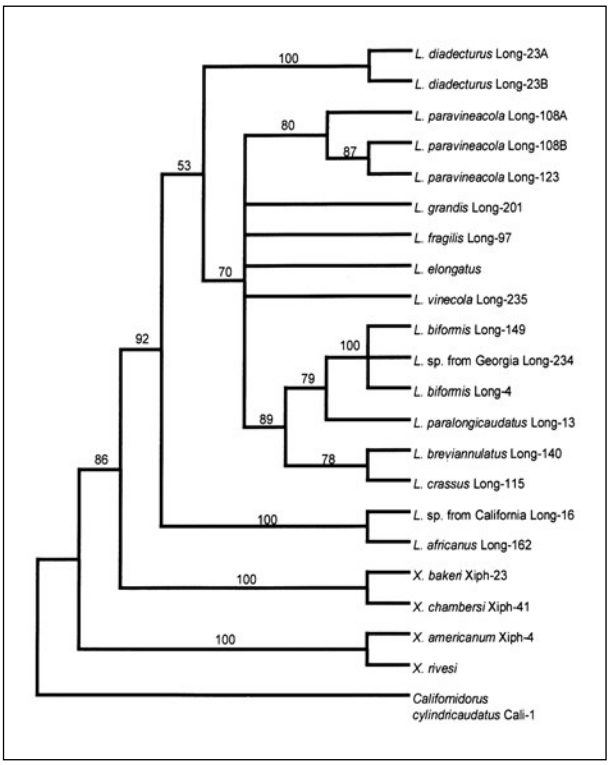

Fig. 2. Consensus tree from the maximum parsimony analysis for *Longidorus* and *Xiphinema* inferred from 18S rDNA sequences. Numbers at the branch points represent the percentage of bootstrap replicates supporting the indicated clades and subclades. (tree length (TL) = 398; consistency index (CI) = 0.7789; re-tention index (RI) = 0.7766)

intraspecific pairwise sequence variation (0.1 % – 0.3 %) occurred in each of two populations of *L. biformis* and *L. paravineacola*, however as the level of variation was small this could be accounted by PCR artifacts.

Maximum likelihood analysis resulted in a tree (Fig. 1) with three distinct clades representing extant taxonomy, namely, *Longidorus*, *Xiphinema* and the *X. americanum*-group species. *Xiphinema bakeri* and *X. chambersi* comprised a distinct clade, as did *X. americanum* and *X. rivesi* (a member of the *X. americanum*-group). Within the *Longidorus* clade, some statistically supported sub-clades were revealed (Fig 1): i) *L. africanus* and an undescribed species from California; ii) *L. diadecturus*, iii) species from Arkansas and Georgia that were further sub-divided: one comprising *L. biformis*; *L. paralongicaudatus*; *L. breviannulatus*; *L. crassus*; and an undescribed species from Georgia and another comprising *L. paravineacola* and iv) the two European species *L. vineacola*; and *L. elongatus*. Maximum parsimony analysis resulted in 3 equally most parsimonious trees with a branch length of 398, a consistency index value of 0.7789 and a retention index of 0.7766 using the branch and bound search algorithm of PAUP*. The consensus tree topology of the maximum parsimony analysis (Fig. 2) was similar to that of the maximum likelihood method.

Discussion

Phylogenies based at the species level have been calculated for a few nematode groups using the ITS and D2/D3 regions (Al-Banna *et al*., 1997; Adams *et al*., 1998;

Table 2. Tajima-Nei pairwise distance matrix of 18S rDNA nucleotide sequence for species and populations of *Longidorus* and *Xiphinema*

	Species (population code)	1	2	3	4	5	6	7
1	*L. diadecturus* (Long-23A)							
2	*L. diadecturus* (Long-23B)	0.006						
3	*X. bakeri* (Xiph-23)	0.060	0.058					
4	*X. chambersi* (Xiph-41)	0.052	0.051	0.021				
5	*X. americanum* (Xiph-4)	0.054	0.051	0.055	0.045			
6	*X. rivesi*	0.056	0.054	0.055	0.044	0.003		
7	*L. paravineacola* (Long-108A)	0.040	0.034	0.048	0.031	0.038	0.041	
8	*L. paravineacola* (Long-108B)	0.043	0.036	0.051	0.033	0.039	0.042	0.003
9	*L. paravineacola* (Long-123)	0.042	0.036	0.050	0.033	0.038	0.041	0.002
10	*L. grandis* (Long-201)	0.039	0.033	0.047	0.029	0.037	0.040	0.005
11	*L. fragilis* (Long-97)	0.037	0.031	0.048	0.029	0.037	0.040	0.006
12	*L. elongatus*	0.038	0.031	0.048	0.035	0.039	0.042	0.007
13	*L. vineacola* (Long-235)	0.038	0.032	0.047	0.034	0.041	0.043	0.012
14	*L. biformis* (Long-149)	0.044	0.039	0.051	0.036	0.038	0.041	0.014
15	*L.* sp. (Long-234)	0.044	0.039	0.051	0.037	0.039	0.042	0.015
16	*L. biformis* (Long-4)	0.046	0.041	0.052	0.037	0.039	0.043	0.015
17	*L. breviannulatus* (Long-140)	0.041	0.036	0.049	0.033	0.037	0.040	0.010
18	*L. crassus* (Long-115)	0.039	0.034	0.049	0.032	0.036	0.039	0.009
19	*L. paralongicaudatus* (Long-137)	0.043	0.039	0.053	0.036	0.041	0.044	0.015
20	*L.* sp. (Long-161)	0.037	0.034	0.044	0.033	0.034	0.036	0.018
21	*Californidorus cylindricaudatus* (Cali-1)	0.056	0.054	0.059	0.042	0.041	0.041	0.036
22	*L. africanus* (Long-162)	0.078	0.076	0.091	0.083	0.086	0.089	0.070

Continued

	Species (population code)	8	9	10	11	12	13	14
1	*L. diadecturus* (Long-23A)							
2	*L. diadecturus* (Long-23B)							
3	*X. bakeri* (Xiph-23)							
4	*X. chambersi* (Xiph-41)							
5	*X. americanum* (Xiph-4)							
6	*X. rivesi*							
7	*L. paravineacola* (Long-108A)							
8	*L. paravineacola* (Long-108B)							
9	*L. paravineacola* (Long-123)	0.001						
10	*L. grandis* (Long-201)	0.005	0.005					
11	*L. fragilis* (Long-97)	0.008	0.007	0.004				
12	*L. elongatus*	0.009	0.009	0.009	0.007			
13	*L. vineacola* (Long-235)	0.015	0.014	0.012	0.011	0.009		
14	*L. biformis* (Long-149)	0.015	0.015	0.014	0.012	0.015	0.016	
15	*L.* sp. (Long-234)	0.016	0.015	0.014	0.013	0.015	0.015	0.001
16	*L. biformis* (Long-4)	0.017	0.016	0.015	0.012	0.015	0.017	0.002
17	*L. breviannulatus* (Long-140)	0.011	0.011	0.009	0.008	0.012	0.015	0.009
18	*L. crassus* (Long-115)	0.011	0.010	0.008	0.006	0.011	0.014	0.008
19	*L. paralongicaudatus* (Long-137)	0.016	0.015	0.014	0.013	0.016	0.019	0.011
20	*L.* sp. (Long-161)	0.020	0.019	0.017	0.017	0.016	0.016	0.022
21	*Californidorus cylindricaudatus* (Cali-1)	0.037	0.036	0.035	0.035	0.037	0.038	0.039
22	*L. africanus* (Long-162)	0.070	0.070	0.068	0.068	0.067	0.066	0.072

Continued

	Species (population code)	15	16	17	18	19	20	21
1	*L. diadecturus* (Long-23A)							
2	*L. diadecturus* (Long-23B)							
3	*X. bakeri* (Xiph-23)							
4	*X. chambersi* (Xiph-41)							
5	*X. americanum* (Xiph-4)							
6	*X. rivesi*							
7	*L. paravineacola* (Long-108A)							
8	*L. paravineacola* (Long-108B)							
9	*L. paravineacola* (Long-123)							
10	*L. grandis* (Long-201)							
11	*L. fragilis* (Long-97)							
12	*L. elongatus*							
13	*L. vineacola* (Long-235)							
14	*L. biformis* (Long-149)							
15	*L.* sp. (Long-234)							
16	*L. biformis* (Long-4)	0.002						
17	*L. breviannulatus* (Long-140)	0.009	0.011					
18	*L. crassus* (Long-115)	0.009	0.010	0.001				
19	*L. paralongicaudatus* (Long-137)	0.012	0.013	0.011	0.010			
20	*L.* sp. (Long-161)	0.023	0.024	0.020	0.018	0.025		
21	*Californidorus cylindricaudatus* (Cali-1)	0.040	0.040	0.038	0.036	0.036	0.035	
22	*L. africanus* (Long-162)	0.072	0.074	0.069	0.068	0.075	0.052	0.086

Subbotin *et al*., 2001; Ye *et al*., 2004) and increasingly 18S rDNA (Fitch *et al*., 1995; de Ley *et al*., 2002; Kanzaki and Futai, 2002; Oliveira *et al*., 2004). Maximum parsimony and maximum likelihood analyses of the 18S rDNA sequences yielded similar supporting tree topologies. Longidorid species were separated into three phylogenetically distinct clades representing currently accepted taxonomic groups, namely, *Longidorus*, *Xiphinema* and the *X. americanum*-group. For the latter two this concurs with Oliveira *et al*. (2004) who reported that species belonging to the *X. americanum*-group originating from Brazil clustered separately from indigenous *Xiphinema* species. Most of the inferred relationships were well resolved. Congruent results from different models indicated that the topology was probably the result of phylogenetic signal and not an artifact of the particular methods chosen, since both methods make different assumptions concerning the evolutionary process. In contrast to Oliveira *et al*. (2004) who reported that 18S rDNA sequences did not discriminate *Xiphinema* at the species level, here, 18S rDNA sequences contained sufficiently useful phylogenetic information for inferring relationships within longidorids at the genus and with few exceptions at species level for *Longidorus*.

Interspecific sequence variation ranged from 0.1 – 9.1 %, with those species with the greatest morphological and morphometric disparity generally representing the largest sequence variation, e.g. *X. bakeri* vs. *L. africanus*. However, some putative species that were morphologically disparate (e.g. *L. biformis* and an undescribed species from Georgia) had nearly identical 18S rDNA sequences, differing by only 4 bp. Conversely, *L. paravineacola* (Arkansas) and *L. vineacola* (Europe) parthenogenetically and sexually reproducing species, respectively, whilst being morphologically very similar had non-homologous 18S rDNA sequences. Similarly, *L. breviannulatus* is morphologically similar to the European species *L. elongatus* (Ye, 2002), but their 18S rDNA sequences varied by 1.2 %.

Our results suggest that 18S sequences show variation among longidorid taxa. The phylogenetic trees, inferred from rDNA sequences, serve as an independent evaluation of currently used morphology-based systematics and provide support for the current morphological groupings of longidorid species (Ye, 2002).

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