Journal of Nematology 36(1):14–19. 2004.

Phylogenetic Relationships and Genetic Variation in *Longidorus* and *Xiphinema* Species (Nematoda: Longidoridae) Using ITS1 Sequences of Nuclear Ribosomal DNA[1]

Weimin Ye,[2] Allen L. Szalanski,[3] and R. T. Robbins[2]

Abstract: Genetic analyses using DNA sequences of nuclear ribosomal DNA ITS1 were conducted to determine the extent of genetic variation within and among *Longidorus* and *Xiphinema* species. DNA sequences were obtained from samples collected from Arkansas, California and Australia as well as 4 *Xiphinema* DNA sequences from GenBank. The sequences of the ITS1 region including the 3′ end of the 18S rDNA gene and the 5′ end of the 5.8S rDNA gene ranged from 1020 bp to 1244 bp for the 9 *Longidorus* species, and from 870 bp to 1354 bp for the 7 *Xiphinema* species. Nucleotide frequencies were: A = 25.5%, C = 21.0%, G = 26.4%, and T = 27.1%. Genetic variation between the two genera had a maximum divergence of 38.6% between *X. chambersi* and *L. crassus*. Genetic variation among *Xiphinema* species ranged from 3.8% between *X. diversicaudatum* and *X. bakeri* to 29.9% between *X. chambersi* and *X. italiae*. Within *Longidorus,* genetic variation ranged from 8.9% between *L. crassus* and *L. grandis* to 32.4% between *L. fragilis* and *L. diadecturus*. Intraspecific genetic variation in *X. americanum sensu lato* ranged from 0.3% to 1.9%, while genetic variation in *L. diadecturus* had 0.8% and *L. biformis* ranged from 0.6% to 10.9%. Identical sequences were obtained between the two populations of *L. grandis,* and between the two populations of *X. bakeri*. Phylogenetic analyses based on the ITS1 DNA sequence data were conducted on each genus separately using both maximum parsimony and maximum likelihood analysis. Among the *Longidorus* taxa, 4 subgroups are supported: *L. grandis, L. crassus,* and *L. elongatus* are in one cluster; *L. biformis* and *L. paralongicaudatus* are in a second cluster; *L. fragilis* and *L. breviannulatus* are in a third cluster; and *L. diadecturus* is in a fourth cluster. Among the *Xiphinema* taxa, 3 subgroups are supported: *X. americanum* with *X. chambersi, X. bakeri* with *X. diversicaudatum,* and *X. italiae* and *X. vuittenezi* forming a sister group with *X. index*. The relationships observed in this study correspond to previous genera and species defined by morphology.

Key words: DNA sequencing, ITS1, *Longidorus,* molecular phylogenetics, *Xiphinema.*

Longidorids (Nematoda: Longidoridae) are economically important plant-parasitic nematodes as well as virus vectors, especially of woody plants. Because of their economic importance, the identification of species in this group is essential for their management. The taxonomic differentiation of longidorid species is difficult because they exhibit little morphological diversity. Taxonomic difficulties often arise from under estimation of intraspecific variability of certain morphological characters currently used for distinguishing species. Because of this uncertainty, a molecular phylogeny of this group should be developed that is independent of morphology. To date, no extensive molecular study has examined the evolutionary relationships of longidorids as a whole.

The first ribosomal transcribed spacer (ITS1) is part of the eukaryotic cistron of ribosomal DNA, located between the genes coding for 18S and 5.8S rRNA. This spacer has become a popular marker for phylogenetic studies of closely related species of animals, plants, and fungi at the population and species level because it shows a comparatively high rate of evolution due to its noncoding structure and can be easily isolated via PCR from almost any taxon, using conserved primers in the adjacent rRNA coding regions (Cherry et al., 1997; Ferris et al., 1994; Joyce et al., 1994; Nasmith et al., 1996; Nguyen et al., 2001; Szalanski et al., 1997, 2000; Wendt et al., 1993).

In recent years, methods of identification of plant-parasitic nematodes based on molecular approaches have been developed that are complementary to traditional methods. Previous molecular studies have focused on several species within the *Xiphinema americanum*-group. Vrain et al. (1992), using ribosomal DNA ITS probes designed from *X. bricolensis* Ebsary, Vrain & Graham, 1989, sequences, demonstrated genetic discontinuities between several North American populations belonging to the *X. americanum*-group. Populations studied segregated into only four discrete groups, which supported the morphological description of *X. americanum sensu stricto, X. bricolensis* Ebsary, Vrain & Graham, 1989, *X. californicum* Lamberti & Bleve-Zacheo, 1979, and *X. rivesi* Dalmasso, 1969. Molinari et al. (1997) distinguished *X. index* Thorne & Allen, 1950, *X. santos* Lamberti, Lemos, Agostinelli & D'Addabo, 1993, *X. vuittenezi* Luc, Lima, Weischer & Flegg, 1964 and *Longidorus latocephalus* Lamberti, Choleva & Agostinelli, 1983 based on the enzyme superoxide dismutase and ITS PCR. Recently, Lamberti et al. (2001) characterized six species of *Longidorus* from Switzerland and described a new species *L. helveticus* Lamberti, Kunz, Grunder, Molinari, De Luca, Agostinelli & Radicci, 2001 by using superoxide dismutase, esterase isozyme analysis, and PCR-RFLP of amplified ITS. However, the ribosomal DNA sequences and their variations for almost all the longidorids are still unknown.

Received for publication 5 June 2002.

[1] Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

[2] Former Graduate Research Assistant and Professor, Department of Plant Pathology, Nematology Laboratory, 2601 N. Young Ave., University of Arkansas, Fayetteville, AR 72704. Current address of senior author: Hubbard Center for Genome Studies, University of New Hampshire, Environmental Technology Bldg., 4th Floor, 35 Colovos Rd., Durham, NH 03824.

[3] Assistant Professor, Department of Entomology, University of Arkansas, Fayetteville, AR 72701.

E-mail: rrobbin@uark.edu

This paper was edited by B. C. Hyman.

The authors thank D. J. F. Brown from the Scottish Crop Research Institute for providing *Longidorus elongatus* specimens for DNA sequencing.

In this study, we present a genetic analysis of ITS1 rDNA sequences for longidorids primarily from Arkansas and analyzed the extent of ITS1 rDNA sequence variation within and among species to determine the suitability of the marker for population genetic and molecular phylogenetic analysis.

Materials and Methods

Materials: Twenty populations belonging to 11 species of *Longidorus* and *Xiphinema* were used in ITS1 DNA sequencing (Table 1). Fresh nematode specimens were handpicked and transferred to 1M NaCl in microcentrifuge tubes and preserved at −80 °C prior to further manipulations.

DNA extraction: Individual nematodes from each population were transferred with a pick to 1.5 ml microcentrifuge tubes containing 20 µl nematode lysis buffer (1% SDS, 0.1M Tris pH 8.0, 0.2M NaCl, 5mM EDTA). The nematode was crushed using a pipet tip. The extract was centrifuged at 13,000 rpm for 5 minutes at 4 °C to force the solution to the bottom of the tube and then frozen at −20 °C for 20 minutes. The samples were then boiled in water for 5 minutes. Two µl of a 20-mg/ml Proteinase K solution was added and incubated at 58 °C for 3 hours. DNAs were purified using a Bio101 Geneclean Kit III (Bio101, Carlsbad, CA).

Table 1. *Longidorus* and *Xiphinema* populations and species used in ITSI DNA sequencing.

Species	Population number	Host	Locality
L. biformis Ye & Robbins, 2003[a]	Long-4	Elm (*Ulmus* americana L.)	Middle Fork of the White River, near Elkins, Washington Co., AR
L. biformis Ye & Robbins, 2003[a]	Long-105	Hackberry (*Celtis occidentalis* L.)	Crooked Creek, Yellville, Marion Co., AR
L. biformis Ye & Robbins, 2003[a]	Long-149	Osage orange (*Maclura pomifera* Nutt.)	Osage Creek, Highway 412, Carrol Co., AR
L. biformis Ye & Robbins, 2003[a]	Long-264	Hickory (*Carya* sp.)	Frog Bayou, Highway 162, south of Alma, Crawford Co., AR
L. breviannulatus Norton & Hoffman, 1975	Long-124	Willow (*Salix* sp.)	Haroldton Access, Arkansas River, Van Buren, Crawford Co., AR
L. crassus Thorne, 1974	Long-115	Oak (*Quercus* sp.)	Illinois River Bridge, County Road 62, Washington Co., AR
L. diadecturus Eveleigh & Allen, 1982	Long-87	*Box elder (Acer negundo L.)*	War Eagle Mill, near Rogers, Benton Co., AR
L. diadecturus Eveleigh & Allen, 1982	Long-146	Red bud (*Cercis Canadensis* L.)	Crooked Creek, Yellville, Marion Co., AR
L. elongatus (de Man) Thorne & Swanger, 1936	Long-198	Greenhouse culture	Scottish Crop Research Institute, Scotland, UK
L. fragilis Thorne, 1974	Long-224	Willow (*Salix* sp.)	Haroldton Access, Arkansas River, Van Buren, Crawford Co., AR
L. grandis Ye & Robbins, 2003[a]	Long-148	Osage orange (*Maclura pomifera* Nutt.)	Osage Creek, Highway 412, Carrol Co., AR
L. grandis Ye & Robbins, 2003[a]	Long-201	River cane (*Arundinaria gigantea* (Walt) Muhl	(Walt) Big Piney Creek Access Area, Highway 164, Pope Co., AR
L. paralongicaudatus Ye & Robbins, 2003[a]	Long-143	Elm (*Ulmus* Americana L.)	Frog Bayou, Highway 162, south of Alma, Crawford Co., AR
X. americanum sensu lato	Xiph-4	Crabapple	University of Arkansas Farm, Fayetteville, Washington Co., AR
X. americanum sensu lato	Xiph-7	Grape (*Vitis* sp.)	San Diego Co., CA
X. americanum sensu lato	Xiph-10	Unknown	Australia
X. americanum sensu lato	Xiph-59	Elm (*Ulmus* americana L.)	Illinois River Bridge, County Road 62, Washington Co., AR
X. bakeri Williams, 1961	Xiph-23	Red bud (*Cercis Canadensis* L.)	Middle Fork of the White River, near Elkins, Washington Co., AR
X. bakeri Williams, 1961	Xiph-47	Osage orange (*Maclura pomifera* Nutt.)	Osage Creek, Highway 412, Carrol Co., AR
X. chambersi Thorne, 1939	Xiph-2	Maple (*Acer* sp.)	Frog Bayou, Highway 162, south of Alma, Crawford Co., AR
X. diversicaudatum (Micoletzki, 1927) Thorne, 1939	GenBank AJ437027		Dordogne, France
X. index Thorne & Allen, 1950	GenBank AJ437026		Frejus, France
X. italiae Meyl, 1953	GenBank AJ437029		Apulia, Italy
X. vuittenezi Luc, Lima Weischer & Flegg, 1964	GenBank AJ437028		Rhein Valley, Germany

[a] New species described in separate papers (Ye and Robbins, 2003, 2004).

PCR: Polymerase chain reactions (PCR) of the ITS1 were performed in a 50-µl volume using 2 µl of DNA solution. Primers used were rDNA2 (5′ TTGATTACGTCCCTGCCCTTT 3′) (Vrain et al., 1992) and rDNA5.8S (5′ ACGAGCCGAGTGATCCACCG 3′) (Cherry et al., 1997). These two primers amplify a 3′ portion of the rDNA 18S gene, the entire ITS1 region, and a 5′ portion of the 5.8S gene. The PCR mixture contained 5 µl 10X hot start *Taq* DNA polymerase incubation buffer (Qiagen, Valencia, CA), 2 µl $MgCl_2$ (2.5mM), 4 µl dNTP-mixture (0.2mM each), 1 µl each primer (0.4 µM), and 0.4 µl (2 units) hot start *Taq* DNA polymerase (Qiagen, Valencia, CA). This mixture was placed into a Hybaid Express thermal cycler. The thermal cycling profile was denaturation at 95 °C for 15 minutes, then 40 cycles of denaturation at 94 °C for 45 seconds, annealing at 53 °C for 45 seconds, and extension at 72 °C for 90 seconds. A final extension was performed at 72 °C for 10 minutes. The quality of PCR was checked by electrophoresis of 4 µl of the PCR reaction in 1% agarose gel containing ethidium bromide. Products were visualized and photographed under UV light. The length and concentration of each PCR product was measured by comparison with a low DNA mass ladder (Invitrogen, Carlsbad, CA).

Cloning: PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into pGemT easy vector System I (Promega, Madison, WI). The plasmids were transformed into *Escherichia coli* strain JM109 competent cells (Promega, Madison, WI) according to the manufacturer's protocols. Clones containing putative rRNA inserts were identified through blue/white color selection and further identified either by *Eco*R I restriction digestion at 37 °C for 2 hours or by PCR using vector primers T7 and SP6. The plasmid preparations were extracted using the QIAquick Spin Miniprep Kit (Qiagen, Valencia, CA).

Sequencing: Plasmid inserts from at least five colonies of the same individual were sequenced in both directions with two vector primers T7 and SP6 by dideoxynucleotide chain termination using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) in an Applied Biosystems 377 automated sequencer in the DNA Core Laboratory, University of Arkansas. GenBank accession numbers for the sequences obtained in this study are AF511409 to AF511428. In addition, DNA sequences for the following four *Xiphinema* species were obtained from GenBank: *X. italiae* Meyl, 1953 (AJ437029); *X. diversicaudatum* (Micoletzky, 1927) Thorne, 1939 (AJ437027); *X. vuittenezi* Luc, Lima, Weischer, & Flegg, 1964 (AJ437028); and *X. index* Thorne & Allen, 1950 (AJ437026).

Alignment and phylogenetic analysis: Sequences were aligned using GCG (Genetics Computer Group) PILEUP program (with a gap weight of 1 and a gap length weight of 0). The distance matrix option of PAUP* 4.0b8 (Swofford, 2001) was used to calculate genetic distances according to the Kimura 2-parameter model (Kimura, 1980) of sequence evolution. *Longidorus* DNA sequences were aligned using *X. americanum* Cobb, 1913 and *X. bakeri* Williams, 1961 as the outgroup taxa, and the *Xiphinema* DNA sequences were aligned using *L. grandis* Ye & Robbins, 2003 and *L. biformis* Ye & Robbins, 2003 as the outgroup. Maximum likelihood and unweighted parsimony analysis on the alignments were conducted using PAUP* 4.0b8 (Swofford, 2001). Gaps were treated as missing characters for all analyses. The reliability of trees was tested with a bootstrap test (Felsenstein, 1985). Parsimony bootstrap analysis included 1,000 resamplings 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 2: 1). These parameters were employed to carry out a heuristic search using PAUP*, using either the single most parsimonious tree as the starting tree, or step-wise addition.

Results

The PCR product ranged from 1020 bp to 1244 bp for all the *Longidorus* species and from 870 bp to 1354 bp for all the *Xiphinema* species. Multiple sequence alignment generated by PILEUP had 1906 characters, of which 762 are constant (40%) and 859 are parsimony-informative (45%). Average nucleotide composition among longidorids is 25.5% (A), 27.1% (T), 21.0% (C), 26.4% (G), 52.6% (A+T), and 47.4% (G+C). Genetic variation between the two genera had a maximum divergence of 38.6% between *X. chambersi* Thorne, 1939 and *L. crassus* Thorne, 1974. Within *Longidorus,* genetic variation ranged from 8.9% between *L. crassus* and *L. grandis* to 32.4% between *L. fragilis* Thorne, 1974 and *L. diadecturus* Eveleigh & Allen, 1982. Genetic variation among *Xiphinema* species ranged from 3.8% between *X. diversicaudatum* and *X. bakeri* to 29.9% between *X. chambersi* and *X. italiae.* Intraspecific genetic variation in *X. americanum sensu lato* ranged from 0.3% to 1.9% whereas genetic variation in *L. diedecturus* was 0.8% and *L. biformis* ranged from 0.6% to 10.9%. Identical sequences were obtained between the two populations of *L. grandis,* and between the two populations of *X. bakeri.*

The *Longidorus* alignment had a total of 1906 characters including the two *Xiphinema* outgroup taxa. Of these characters, 939 (49%) were variable and 601 (32%) were informative. The aligned *Xiphinema* data matrix including the outgroup taxa resulted in a total of 1906 characters. Of these characters, 916 (48%) were variable and 495 (26%) were informative. The *Longidorus* dataset had only one most parsimonious tree

(Fig. 1) (Length = 1729, C.I = 0.79), as documented using the Branch and Bound algorithm of PAUP*. Inside the Glade of *Longidorus* species, 3 subgroups were formed. *Longidorus grandis, L. crassus,* and *L. elongatus* (de Man, 1876) Micoletzky, 1922 are in one cluster; *L. biformis* and *L. paralongicaudatus* Ye & Robbins, 2003 are in a second cluster; and *L. fragilis* and *L. breviannulatus* are in a third cluster. The maximum likelihood tree (–Ln likelihood = 9704.66018) was identical to the parsimony tree. Parsimony analysis of the *Xiphinema* dataset found one most parsimonious tree (Fig. 2) (length = 1532, CI = 0.84), as documented using the Branch and Bound algorithm of PAUP*. Three distinct clades were formed: *X. americanum* with *X. chambersi, X. bakeri* with *X. diversicaudatum,* and *X. italiae* and *X. vuittenezi* forming a sister group with *X. index*. The *Xiphinema* maximum likelihood tree (Fig. 3) (–Ln likelihood = 8716.26041) was similar to the parsimony tree with the exception that *X. chambersi* did not form a sister group with *X. americanum* and *X. index* formed a common clade with the *X. bakeri/X. diversicaudatum* group.

Discussion

The level of intraspecific genetic variation in the nuclear ribosomal ITS1 region from *Longidorus* in this study was high compared to other studies involving the nuclear ribosomal internal transcribed spacer region.

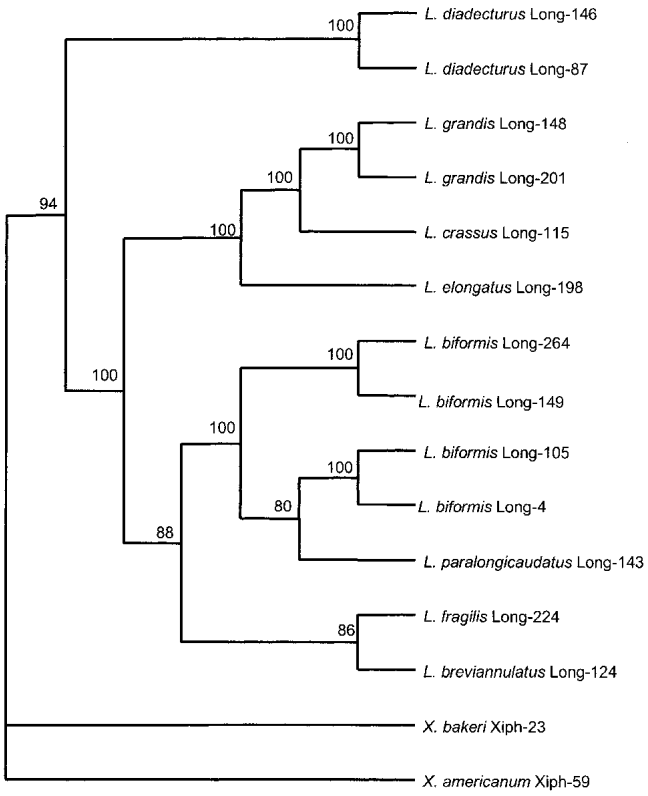

Fig. 1. Consensus tree from the maximum parsimony bootstrap analysis for ITS1 of *Longidorus*. The percentages of bootstrap replicates supporting the clades are indicated at the branch points.

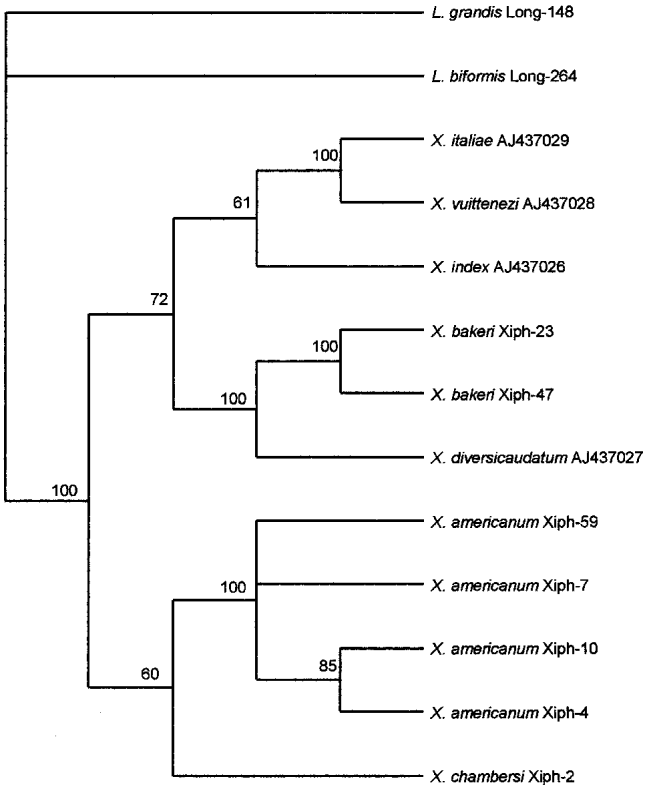

Fig. 2. Consensus tree from the maximum parsimony bootstrap analysis for ITS1 of *Xiphinema*. The percentages of bootstrap replicates supporting the clades are indicated at the branch points.

Among seven species of another Adenophorean, *Trichostrongylus,* little or no intraspecific variation was detected in ITS1, while sequence differences among species ranged from 1.3% to 5.7% (Hoste et al., 1998). Another study by Hoste et al. (1993) found no intraspecific variation in the ITS2 region of three populations of *Trichostrongylus colubriformis* Giles, 1892. Several studies have observed intraspecific variation for ITS1 and ITS2 in nematodes. Intraspecific genetic variation of the ITS1 region in *Globodera* cyst nematodes can range from 0 to 0.7% for *G. tabacum* Lownsbery and Lownsbery, 1954; 0 to 2.5% for *G. pallida* Stone, 1973; and 0.1% to 0.9% for *G. rostochiensis* Wollenweber, 1923 (unpubl. data, GenBank AF016865 to AF016881). Using PCR-RFLP of ITS2, Conole et al. (1999) found significant intraspecific variation in three *Metastrongylus* species. These levels of intraspecific variation do not approach those observed in this study; however, high levels of ITS variation have been observed in other invertebrates. For example, Marrelli et al. (1999) found up to 13.0% genetic variation among populations of the mosquito *Anopheles oswaldoi* Peryassu, 1921 and speculated that they may represent cryptic species. Although the sequence divergence of ITS1 from four populations of *L. biformis* was as high as 10.9%, they were indistinguishable morphologically and form the same cluster together with another morphologically distinct species, *L. paralongicaudatus*. The high rate of

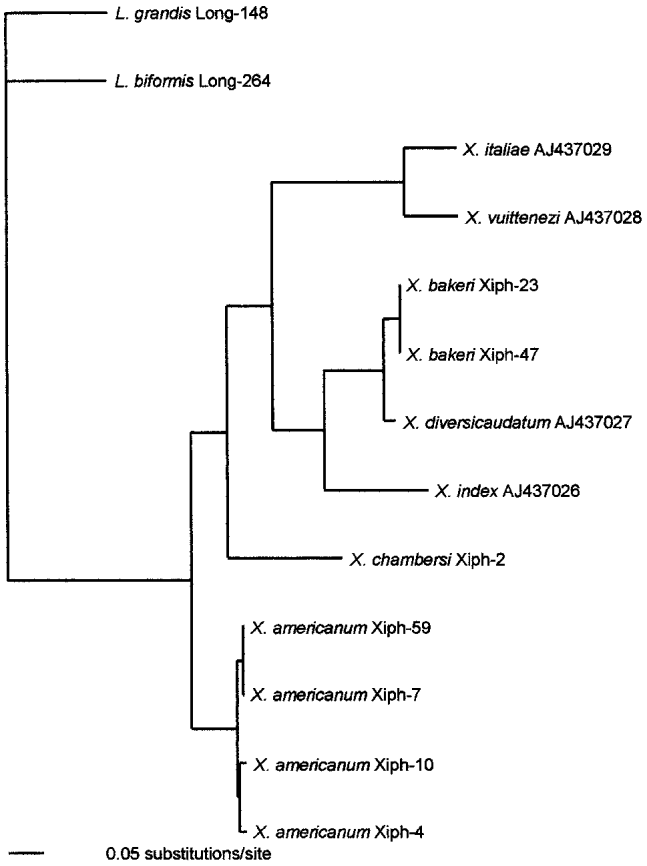

FIG. 3. Best maximum likelihood tree for ITS1 of *Xiphinema*. Branch lengths are proportional to the number of inferred changes.

sequence variation among *L. biformis* populations could be because of the amphimictic reproduction of this species. Based on our phylogenetic analysis and the extent of intraspecific genetic variation, the four *L. biformis* populations represent two distinct clades and quite possibly represent two sibling species. All of the other longidorids in this study are parthenogenetic.

The ITS1 regions of the rDNA repeat in *Longidorus* and *Xiphinema* were much longer than most of those previously published (Adams et al., 1998; Cherry et al., 1997; Ferris et al., 1999; Nguyen et al., 2001; Powers et al., 2001; Szalanski et al., 1997). Interestingly, the ITS1 sequences showed considerable variation in length among the *Longidorus* and *Xiphinema* species examined. The sequence from the type populations of *L. biformis* was 224 bp longer than that of *L. elongatus,* and the sequence of *X. chambersi* was about 470 bp longer than that of *X. americanum*. The sequence data revealed high levels of intraspecific sequence variation in *L. biformis* (0.6%–10.9%) and minor intraspecific variation in *L. diadecturus* (0.8%) and *X. americanum* (0.3%–1.9%). Identical sequences were obtained between two populations of *L. grandis* and between two populations of *X. bakeri*.

ITS1 rDNA sequences provided sufficient and useful information for reconstructing a phylogeny within the genus *Longidorus* and *Xiphinema,* but not for resolving relationships among species of the two genera because of the extent of nucleotide variation and insertions/deletions. However, it should be an ideal marker for population genetic studies of these taxa. In the future, other genetic markers, such as mtDNA and nuclear ribosomal 18S, should be evaluated for their utility in resolving plylogenetic relationships among longidorids.

LITERATURE CITED

Adams, B. J., A. M. Burnell, and T. O. Powers. 1998. A phylogenetic analysis of *Heterorhabditis* (Nemata: Rhabditidae) based on internal transcribed spacer 1 DNA sequence data. Journal of Nematology 30: 22–39.

Cherry, T., A. L. Szalanski, T. C. Todd, and T. O. Powers. 1997. The internal transcribed spacer region of *Belonolaimus* (Nemata: Belonolaimidae). Journal of Nematology 29:23–29.

Conole, J. C., N. B. Chilton, T. Jarvis, and R. B. Gasser. 1999. Intraspecific and interspecific variation in the second internal transcribed spacer (ITS-2) sequence for *Metastrongylus* (Nematoda: Metastrongyloidea) detected by high resolution PCR-RFLP. International Journal for Parasitology 29:1935–1940.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783–791.

Ferris, V. R., J. M. Ferris, J. Faghihi, and A. Ireholm. 1994. Comparisons of isolates of *Heterodera avenae* using 2-D PAGE protein patterns and ribosomal DNA. Journal of Nematology 26:144–151.

Ferris, V. R., S. A. Subbotin, A. Ireholm, Y. Spiegel, J. Faghihi, and J. Ferris. 1999. Ribosomal DNA sequence analysis of *Heterodera filipjevi* and *H. latipons* isolates from Russia and comparisons with other nematode isolates. Russian Journal of Nematology 7:121–125.

Hoste, H., N. B. Chilton, I. Beveridge, and R. B. Gasser. 1998. A comparison of the first internal transcribed spacer of ribosomal DNA in seven species of Trichostrongylus (Nematoda: Trichostrongylidae). International Journal for Parasitology 28:1251–1260.

Hoste, H., R. B. Gasser, N. B. Chilton, S. Mallet, and I. Beveridge. 1993. Lack of intraspecific variation in the second internal transcribed spacer (ITS-2) of *Trichostrongylus colubriformis* ribosomal DNA. International Journal for Parasitology 23:1069–1071.

Joyce, S. A., C. T. Griffin, and A. M. Burnell. 1994. The use of isoelectric focusing and polyacrylamide gel electrophoresis of soluble proteins in the taxonomy of the genus *Heterorhabditis* (Nematoda: Heterorhabditidae). Nematologica 40:601–612.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative study of nucleotide sequences. Journal of Molecular Evolution 16:111–120.

Lamberti, F., P. Kunz, J. Grunder, S. Molinari, F. De Luca, A. Agostinelli, and V. Radicci. 2001. Molecular characterization of six *Longidorus* species from Switzerland with the description of *Longidorus helveticus* sp. n. (Nematoda, Dorylaimida). Nematologia Mediterranea 29:181–205.

Marrelli, M. T., R. S. Malafronte, C. Flores-Mendoza, R. Lourenco-De-Oliveira, J. K. Kloetzel, and O. Marinotti. 1999. Sequence analysis of the second internal transcribed spacer of ribosomal DNA in *Anopheles oswaldoi* (Diptera: Culicidae). Journal of Medical Entomology 36:679–684.

Molinari, S., F. De Luca, F. Lamberti, and C. De Giorgi. 1997. Molecular methods for the identification of Longidorid nematodes. Nematologia Mediterranea 25:55–61.

Nasmith, C. G., D. Speranzini, R. Jeng, and M. Hubbes. 1996. RFLP analysis of PCR amplified ITS and 26S ribosomal rDNA genes of selected entomopathogenic nematodes (Steinernematidae, Heterorhabditidae). Journal of Nematology 28:15–25.

Nguyen, K. B., J. Maruniak, and B. J. Adams. 2001. Diagnostic and phylogenetic utility of the rDNA internal transcribed spacer sequences of *Steinernema*. Journal of Nematology 33:73–82.

Powers, T. O., A. L. Szalanski, P. G. Mullin, T. S. Harris, T. Bertozzi,

and J. A. Griesbach. 2001. Identification of seed gall nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. Journal of Nematology 33:191–194.

Swofford, D. L. 2001. PAUP*: Phylogenetic analysis using parsimony (*and other methods), ver. 4.0b10. Sunderland, MA: Sinauer.

Szalanski, A. L., D. D. Sui, T. S. Harris, and T. O. Powers. 1997. Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of ITS1. Journal of Nematology 29:255–267.

Szalanski, A. L., D. B. Taylor, and P. G. Mullin. 2000. Assessing nuclear and mitochondrial DNA sequence variation within *Steinernema* (Rhabditida: Steinernematidae). Journal of Nematology 32: 229–233.

Vrain, T. C., D. A. Wakarchuk, A. C. Levesque, and R. I. Hamilton. 1992. Intraspecific rDNA restriction fragment length (bp) polymorphism in the *Xiphinema americanum* group. Fundamental and Applied Nematology 15:563–573.

Wendt, K. R, T. C. Vrain, and J. M. Webster. 1993. Separation of three species of *Ditylenchus* and some host races of *D. dipsaci* by restriction fragment length (bp) polymorphism. Journal of Nematology 25:555–563.

Yang, Z. 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over sites: Approximate methods. Journal of Molecular Evolution 39:306–314.

Ye, W., and R. T. Robbins. 2003. *Longidorus grandis* n. sp. and *L. paralongicaudatus* n. sp. (Nematoda: Longidoridae), two parthenogenetic species from Arkansas. Journal of Nematology 35:375–387.

Ye, W., and R. T. Robbins. 2004. *Longidorus biformis* n. sp. and *L. glycines* n. sp. (Nematoda: Longidoridae), two amphimictic species from Arkansas. Journal of Nematology 36:1–13.