Journal of Nematology 36(2):147–152. 2004.

Characterization and Sequence Variation in the rDNA Region of Six Nematode Species of the Genus *Longidorus* (Nematoda)

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Abstract: Total DNA was isolated from individual nematodes of the species *Longidorus helveticus, L. macrosoma, L. arthensis, L. profundorum, L. elongatus,* and *L. raskii* collected in Switzerland. The ITS region and D1-D2 expansion segments of the 26S rDNA were amplified and cloned. The sequences obtained were aligned in order to investigate sequence diversity and to infer the phylogenetic relationships among the six *Longidorus* species. D1-D2 sequences were more conserved than the ITS sequences that varied widely in primary structure and length, and no consensus was observed. Phylogenetic analyses using the neighbor-joining, maximum parsimony and maximum likelihood methods were performed with three different sequence data sets: ITS1-ITS2, 5.8S-D1-D2, and combining ITS1-ITS2+5.8S-D1-D2 sequences. All multiple alignments yielded similar basic trees supporting the existence of the six species established using morphological characters. These sequence data also provided evidence that the different regions of the rDNA are characterized by different evolution rates and by different factors associated with the generation of extreme size variation.

Key words: Concerted evolution, D1-D2 expansion segments, heterogeneity, internal transcribed spacers, phylogenetic analysis, ribosomal DNA.

Nematodes belonging to the genus *Longidorus* Micoletzky (Nematoda, Dorylaimida) are ectoparasites that spend their entire life cycle outside the host plant roots. Juvenile stages and adults are found in the soil in all seasons, and adults can survive for 1 to 2 years. *Longidorus* spp. are of special scientific and economic interest because they directly damage the roots of the host plant and because some species are vectors of economically important plant viruses (Taylor and Brown, 1997). These nematodes are morphometrically similar, making identification difficult. The ability to accurately identify and distinguish between species belonging to the same genus has important implications for studying taxonomy and population biology, as well as disease management. Moreover, correct identification is necessary for quarantine and phytopathological purposes.

Ribosomal DNA coding sequences are among the most conserved gene families and have received considerable attention with respect to nematode identification and evolutionary and phylogenetic studies (Blaxter et al., 1998). These sequences consist of several hundred tandemly repeated copies of the transcription unit, which encodes for 18S, 5.8S, and 26S genes with two internal transcribed spacers, ITS1 and ITS2. Depending on the level of investigation, researchers have chosen different regions: large subunit ribosomal DNA (LSUrDNA), small subunit (SSUrDNA), or ITS region. The 18S and 26S genes and the spacers differ greatly in their rate of evolution; they can reveal phylogenetic relationships ranging from distantly related organisms to the level of populations (Hillis and Dixon, 1991). The organization of the 26S rDNA is peculiar due to the presence of conserved and highly variable stretches (D expansions). The D expansion segments also have been demonstrated to diverge between closely related species (González-Lamothe et al., 2002; Kaplan et al., 2000; McLain et al., 2001; Olson and Littlewood, 2002; Thomas et al., 1997). The ITS sequences vary both among species and among populations within a species (Nadler et al., 2000; Nishikawa et al., 1999; Odorico and Miller, 1997; Rouland-Lefevre et al., 2002; Schlotterer et al., 1994; von der Schulenburg et al., 2001).

It is commonly accepted that the level of intraspecific sequence variation among ribosomal sequences is low due to their concerted evolution, i.e. single repeats in the multigene family evolve in concert, resulting in the homogenization of all the repeats in an array (Elder and Turner, 1995). Although this seems to be usual, sequence variation in ITS1 and ITS2 regions within species and individuals of free-living, human, animal-, and plant-parasitic nematodes has been reported (Blok et al., 1998; Elbadri et al., 2002; Hugall et al., 1999; Hung et al., 1999; Morales-Hojas et al., 2001).

In this study, we report the molecular characterization and the evolutionary relationship among the six species of *Longidorus* collected in Switzerland during a survey carried out in 1996 to 2000 (Lamberti et al., 2001), using the ITS regions as well as the 5.8S rDNA genes and the D1-D2 expansion segments of the 26S gene.

Materials and Methods

Soil samples were collected from the rhizosphere of orchard trees (mainly apple and cherry) in different localities of Switzerland (Lamberti et al., 2001). Nematodes were extracted by sieving and centrifugal flotation methods (Coolen and D'Herde, 1977).

Received for publication 30 April 2003.

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This work was partially supported by a grant from the Italian Ministry of Scientific and Technologic Research (MURST), Plan for the Development of Research Networks, Law 488/92, Cluster C03, Project 2.

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This paper was edited by Andrea Skantar.

DNA extraction, amplification, and sequencing: In order to avoid cross-contamination between the different species, the analyses were carried out at different times, using a single species each time. Fifteen individual nematodes of each *Longidorus* species were handpicked and each one placed on a glass slide in 3 µl of the lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% Tween 20) with 90 µg/ml proteinase K and then cut into 3 to 5 pieces by using a sterilized syringe needle under a dissecting microscope. The suspension was recovered and transferred into a cold 0.5-ml microcentrifuge tube. Samples were incubated at 60°C for 1 hour and then at 95°C for 10 minutes. The crude DNA extracted from each individual nematode was directly amplified. The ribosomal DNA region spanning from the 3′ end of 18S rDNA to the 5′ end of 26S rDNA, and including the ITS1 and ITS2 regions and the 5.8S rDNA, was amplified using forward primer 18S (5′ TGATTACGTCCCTGCCTTT 3′) and reverse primer 26S (5′ TTTCACTCGCCGTTACTAAGG 3′). In addition, the D1-D2 expansion segment of the 26S rDNA was amplified using forward primer FOR (5′GCATATCAATAAGCGGAGGAAA) and reverse primer REV (5′ GGTCCGTGTTTCAAGACG) in five individual nematodes of each species.

PCR reactions were performed in 100 µl containing 0.2 mM of each dNTP, 20 pmols of each primer (10 µM), and 2.5 units of Taq DNA polymerase (ROCHE, Germany). PCR cycling conditions used for amplification of both segments were identical: hot start (95 °C/5 minutes) and then 35 cycles of 95 °C/50 seconds, 55 °C/50 seconds, and 72 °C/1 minute 30 seconds followed by a post-amplification extension at 72 °C for 7 minutes. The size of amplification products was determined by comparison with the molecular weight marker Ladder 100 (Fermentas, St. Leon-Rot, Germany) following electrophoresis of 10 µl on a 1% agarose gel. PCR products from four individual nematodes for each ribosomal region (D1-D2 and ITS containing region) were purified from agarose gel using the High Pure PCR elution kit (ROCHE, Germany) and ligated and cloned into pGEM-T Easy vector (Promega, France) according to the manufacturer's instructions.

From the resulting eight plates, two clones for each individual nematode were sequenced using an ABI Prism 377 sequencer (PE Applied Biosystem, Foster City, CA) and dye terminator sequencing reagents at the University of Padova (CRIBI, Italy). Both strands of each clone were sequenced with either general primers or sequence-specific primers. A BLAST (Basic Local Alignment Search Tool) search at NCBI (National Center for Biotechnology Information) was performed using *Longidorus* sequences obtained as queries to confirm their nematode origins (Altschul et al., 1997). The availability of many sequences for each species allowed us to identify the most representative sequence for each species. The sequences obtained were deposited at the EMBL database with their accession numbers listed in Table 1.

Phylogenetic analysis: Three data sets of sequences (ITS1+ITS2, 5.8S+D1-D2 expansion segment of the 26S, and the supergene obtained after concatenating all the above sequences, ITS1-ITS2+5.8S-D1-D2) were multialigned using the PILEUP program from GCG (1994). The corresponding sequences of the plant-parasitic nematode *Xiphinema index* (De Luca, unpubl. data) were used as an outgroup and added to the *Longidorus* alignment profile. The alignment of the sequences was obtained using the default parameters of PILEUP and corrected by visual inspection with the aid of pairwise alignments performed using the GAP program from GCG (Genetic Computer Group, Madison, WI). In the case of ITS sequences, nine computer alignments (data not shown) were created with different combinations of gap creation penalty values (1, 3 and 8) and gap extension penalty values (3, 5, and 8) as previously described for Steinernema and Heteroderidae (Nguyen et al., 2001; Subbotin et al., 2001). All of these alignments were used for phylogenetic analyses.

Phylogenetic relationships among sequences were established using different procedures: neighbor-joining GTR (NJ), maximum-parsimony (MP), maximum likelihood (ML), and minimum evolution GTR (ME). Genetic distances were obtained using the Markov stationary method, also called the GTR method, implemented in the PAUP 4.0b10 package (Swofford, 1998). The GTR method calculates the distance between sequences without imposing any kind of nucleotide substitution model, but estimating it from the data. The minimum evolution and neighbor-joining procedures were also applied to the distance matrix obtained using the log-det method, implemented in the PAUP 4.0b10 package (Swofford, 1998). The log-det method allows phylogenetic inference even in cases in which sequences show different base composition, as it is the case of ITS regions. Trees were obtained for all three different data sets, and bootstrap values were based on 1,000 replicates in order to assess the degree of support for each branch on the trees.

TABLE 1. EMBL accession numbers for ITS regions and D1-D2 rDNA sequences.

Species	EMBL Accession no.	
	ITS1-5.8S-ITS2	D1-D2
L. arthensis	AJ549981	AJ549999
L. elongatus	AJ549986	AJ549997
L. helveticus	AJ549985	AJ549996
L. macrosoma	AJ549978	AJ549994
L. profundorum	AJ549988	AJ549991
L. raskii	AJ549983	AJ550000
X. index	AJ549984	AJ550001

Results and Discussion

Characterization of the ribosomal DNA region: The six species of *Longidorus* under consideration have been previously identified by morphometric parameters as *Longidorus macrosoma, L. profundorum, L. elongatus, L. arthensis, L. raskii,* and the new species *L. helveticus*. The PCR-RFLP results revealed length differences and species-specific restriction patterns for the ITS regions of the species studied (Lamberti et al., 2001).

The length of the ITS regions (including 193 bp of the 3′ end of the 18S and 98 bp of the 5′ end of the 26S) was: 1985 bp for *L. helveticus,* 1967 bp for *L. macrosoma,* 1835 bp for *L. arthensis,* 1827 bp for *L. profundorum,* 1624 bp for *L. elongatus,* 1566 bp for *L. raskii*. The sequence analysis revealed that the length variation of this rDNA segment was due to differences in the ITS regions (ITS1 and ITS2, Table 2) since the 5.8S rDNA (Table 2) and the 3′ and 5′ ends of 18S and 26S, respectively, were constant in length in all six species.

The other PCR segment of the rDNA we obtained corresponds to the 0.8 kb located at the 5′ end of the 26S rDNA (Table 2). This fragment contains the D1 and D2 expansion domains that are less constrained than the core-structure sequences (Clark et al., 1984; Hancock and Dover, 1988). The alignment of the sequences confirmed that variability is restricted to the D1 and D2 expansions, the latter being more variable than the D1 domain (multialignment not shown). Moreover, the D2 expansion is about 500 bp in length, longer than that of *C. elegans* (286 bp) (Ellis et al., 1986) and *Scottnema lindsayae* counterparts (392 bp) (Courtright et al., 2000).

Intra-individual and intra-species variation: Recent studies have revealed rDNA sequence microheterogeneity, that is, the presence in individual nematodes of more than one ITS pattern in their genome (Blok et al., 1998; Gasser et al., 1998; Heise et al., 1999; Hugall et al., 1999; Powers et al. 1997; Subbotin et al., 2000; Waeyenberge et al., 2000; Zjilstra et al., 1995). The main causes of heterogeneity are the presence of repeated sequences and differences in length and sequence in both ITSs and 26S rDNA (Conole et al., 2001; Depaquit et al., 2002; Harris and Crandall, 2000; Leignel et al., 1997; van Herwerden et al., 1999, 2000; von der Schulenburg et al., 2001; Zarlenga et al., 1996).

There was little or no sequence variation between clones from the same individual in either the ITS or D1-D2 regions that could be explained in terms of natural variation within populations (Subbotin et al., 2000). Most of the nucleotide variations observed are transitions, with few transversions and indels (insertion/deletion). It is possible that these differences are due to nucleotide misincorporations during PCR. However, empirical evidence indicates that PCR misincorporations occur at very low rates.

Several microsatellites are present in both ITS regions of *Longidorus* species as reported for trematodes, dipterans, mites, and humans (Depaquit et al., 2002; Harris and Crandall, 2000; Kumar et al., 1999; van Herwerden et al., 1998, 1999; von der Schulenburg et al., 2001). In this study, small di- and trinucleotide repeats, or degenerate versions of repeats such as $(TA)_n$, $(GA)_n$, $(CT)_n$, and $(TTC)_n$, were detected (not shown). Intra-individual heterogeneity was always observed at the dinucleotide $(TA)_n$, the repeat number of which was ≤ 7, and resulted in the main polymorphic microsatellite. Stretches of $(A)_n$, $(G)_n$, and $(T)_n$ were also considered to represent microsatellites (Conole et al., 2001). The presence of microsatellites contributes to length variation in the number of repeats between species and also within single nematodes, (Table 2) but does not hinder the identification of these species and does not alter the relationships between species.

The intra-specific variations in length of both ITS and D1-D2 regions are reported in Table 1. *Longidorus elongatus* and *L. arthensis* showed two ITS1s differing by 2 bp corresponding to the addition or loss of one (AA) and (TA), respectively, whereas *L. raskii* had two different ITS2 differing by 4 bp in length. *Longidorus profundorum,* indeed, showed three different D1-D2 sequences, differing from each other by about 16 and 50 bp; the remaining species differed by just 1 bp. Intra-individual difference in 5.8S was detected only for *L. macrosoma* at alignment position 5, where an insertion of C is present.

Inter-species variation: The 5.8S rDNA gene was 154 bp in length for all six species of *Longidorus* (Table 1). Only five variable nucleotide sites were detected within the six species (48, 53, 107, 125, 146 positions), and all are transitions (G-A, T-C).

Table 2. Lengths (L, in bp) and G+C contents (in %) of sequenced rDNA regions of the six *Longidorus* species.

	ITS1		ITS2		5.8 S		D1-D2	
Species	L (bp)	G+C (%)	L (bp)	G+C (%)	L (bp)	G+C (%)	L (bp)	G+C (%)
L. macrosoma	1022	44	501	42	154	54	824	52
L. helveticus	1060	46	481	46	154	54	824	50
L. profundorum	896	48	485	40	154	54	808; 824	50
L. elongatus	798; 800	42	382	50	154	55	807; 808	54
L. arthensis	996; 998	44	395	40	154	54	812	50
L. raskii	632	42	491; 495	40	154	53	808; 809	50

The D1-D2 expansion domains are characterized by the presence of many indels (1–10 bp), despite the overall high degree of similarity. *Longidorus helveticus* and *L. macrosoma* always showed a high percent of similarity (97%). Indeed, *L. arthensis* and *L. profundorum* showed the same D1-D2 sequence, suggesting that these species could have arisen relatively recently and are quite closely related phylogenetically. The GC content ranged from 50% to 54% (Table 1).

In contrast, there were significant length differences in the ITS1 that varied between the species from 632 to 1060 bp, whereas the ITS2 varied from 382 to 501 bp (Table 2). The GC content of the ITS1 ranged from 42% to 48% and from 40% to 50% for ITS2 (Table 2). The ITS1 of *L. macrosoma* and *L. helveticus,* 1022 and 1060 bp in length, respectively, are the longest ITS1s in nematodes reported to date. The majority of eukaryotes usually show ITS1 lengths of no more than 800 bp; the only exceptions are found in *Schistosoma japonica* (Platyhelminthes), *Exochomus quadripustulatus* (Coleoptera), and *Anopheles gambiae* (Insecta) with ITS1 sizes of 1400, 2572 and 5500 bp, respectively (Paskewitz et al., 1993; van Herwerden et al., 1998; von der Schulenburg et al., 2001). In these species, long repetitive elements have been identified and can occupy up to 40% of the spacer length. In coccinellids these elements were confined to the middle of the spacer where the lack of constraints may have favored the rise of repetitions (von der Schulenburg et al., 2001). In both spacers of *L. macrosoma, L. helveticus,* and *L. profundorum* several repetitive elements of about 7 to 12 nt have also been identified. These elements are reiterated twice, immediately adjacent or separated from each other by a few nucleotides. They are also localized in the middle of both spacers and do not show any interspecific similarities as observed in coccinellids. In *L. arthensis, L. raskii,* and *L. elongatus,* indeed, simple sequence repeats were present and the tetranucleotide element CGGG was repeated many times and dispersed along both strands in both spacers and in the 5.8S gene in both orientations.

These findings clearly show that the internal transcribed spacers of *Longidorus* species are free to diverge, undergoing mainly slippage events during replication, which result in short di- and tri-nucleotide repeats, and transposition and crossing-over events, which are represented by many indels of varying sizes. Both of these molecular processes can lead to concerted evolution among rDNA repeats of a species at a given locus. Concerted evolution describes the ability of individual repeats in a multigene family to evolve in concert rather than independently, showing a great similarity to each other within a species, but accumulating differences between species (Elder and Turner, 1995). In contrast, in arthropods the rDNA is present at several different loci and is not subject to interlocus recombination, resulting in a less efficient homogenization process that generates locus-specific variants of the rDNA.

Sequence information of the ITS regions revealed high sequence divergence among the six species of *Longidorus*. Species within the same genus, sharing similarities in morphological characters, were expected to be genetically more similar to each other than to species of other genera. However, the only blocks of similarities among the six ITS sequences were located at the 5′ and 3′ ends of both spacers (Table 3). These conserved blocks (<10 bp) suggest the presence of functional constraints that are essential for accurate spacer maturation and biogenesis of the functional ribosome as described in yeast, drosophilid dipterans (Insecta), and digeneans (Platyhelmintes) (Lalev and Nazar, 1999; Schlotterer et al., 1994; von der Schulenburg et al., 1999; Weaver et al., 1997).

Phylogenetic analysis: Computer alignments of the ITS sequences of *Longidorus* were obtained using different values of gap creation and gap extension penalties as detailed in Materials and Methods. The results of this comparison showed that the high sequence divergence increased noise levels, thus reducing the bootstrap values and the phylogenetic resolution among the six *Longidorus* species. In contrast, the manual alignment revealed an overall degree of similarity and bootstrap values that were significantly higher than those provided by the computer program.

The alignment of the coding sequences of the ribosomal DNA, including 5.8S plus the D1-D2 expansion domains, is 1019 bp long with 111 parsimony informative sites (Table 4). The alignment of the ITS regions was 2189 bp long, including 1005 bp variable but uninformative sites, and 672 bp variable and informative sites (Table 4). Because the most accurate inference of phylogenetic relationships is likely to be attained when multiple traits are considered together, the 5.8S, D1-D2

TABLE 3. Conserved sequences at the 5′ and 3′ ends of ITS1 and ITS2 among the six *Longidorus* species.

	ITS1		ITS2	
Species	5′ end	3′ end	5′ end	3′ end
L. macrosoma	ACGAGCTAA	GAGATTC	TAAGAGAAC	GTTCGACG
L. helveticus	ACGAGCGAAA	GAGATTC	TAAGAAAAC	TATCGACG
L. profundorum	ACGAGCTA	GAGATTC	TAAGAATAT	GTTTCGATA
L. elongatus	ACGAGCTAAA	GAGATTC	TAAGAATAT	TTTATACA
L. arthensis	ACGAGCAAA	GAGATTC	TAAGAAAAT	GTTCAGTA
L. raskii	ACGAGCTAA	GAAGATTC	TAAGAGAAC	TATCGAAA

TABLE 4. Number of variable and constant sites in *Longidorus* species: the total number of sites used for coding (5.8S+D1-D2), noncoding (ITS1+ITS2), and combined sequences (5.8S+D1-D2+ITS1-ITS2).

	5.8S+D1-D2	ITS1+ITS2	5.8S+D1-D2+ITS1+ITS2
Length	1019	2189	3134
Constant sites	747	512	1175
Variable but uninformative	161	1005	1163
Variable and informative	111	672	796

expansion domains, ITS1, and ITS2 sequences were combined in a single multi-alignment. The alignment of the combined sequences was 3134 bp long, including 1175 bp variable and uninformative, and 796 bp variable and informative (Table 4). The phylogenetic analyses with NJ, MP, ML, and ME approaches carried out on the three different data sets yielded trees with similar topologies (Fig. 1). The six *Longidorus* species can be separated into two groups: the first included *L. helveticus* and *L. macrosoma,* and the second included the remaining four species. Using the *X. index* as an outgroup, the clustering of *L. helveticus* and *L. macrosoma* in all trees was supported by a strong bootstrap value (96–100), suggesting that *L. helveticus* is more closely related to *L. macrosoma* than to the other species. In the ITS1-ITS2 tree (Fig. 1B), *L. raskii* slightly changed position relative to the other two trees (Fig. 1A, C). The ITS data caused *L. raskii* to cluster separately from the group including *L. profundorum, L. arthensis,* and *L. elongatus,* suggesting that *L. raskii* is distant from these species. This difference may be explained by a higher mutational saturation in the shorter ITS region relative to the other regions examined. Regarding the relative position of *L. elongatus, L. arthensis,* and *L. profundorum,* only in the case of the ITS tree we have obtained a resolved phylogeny for the three species that was supported by high bootstrap values with all approaches. In this case, *L. profundorum* clusters first with *L. arthensis,* while *L. elongatus* is the most distinct of the three. The other two trees also give support to a close relationship of these three species.

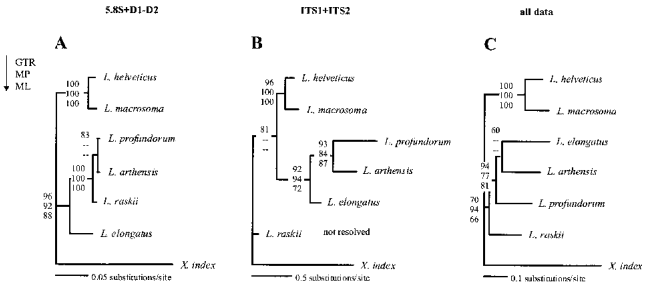

FIG. 1. Phylogenetic trees describing the evolutionary relationships among six species of *Longidorus.* Branch lengths are proportional to the distances as derived from the distance matrix obtained using the GTR method from PAUP. Bootstrap values, based on 1,000 replications, for Neighbor-Joining GTR (GTR), Maximum Parsimony (MP), and Maximum Likelihood (ML) are shown from top to bottom at each node. Three different data sets have been considered for the analysis: 5.8S+D1-D2 region of the 26S (A), ITS1+ITS2 (B), and all data available, i.e., 5.8S+D1-D2+ITS1-ITS2 (C).

Due to ease of alignment, we conclude that the 26S rDNA D1-D2 expansion region was more useful for establishing the phylogenetic relationships among *Longidorus* species than was ITS. The high length and sequence variability found in the ITS regions of *Longidorus* species in the present study suggest that a variety of poorly understood factors are involved in the evolution of these regions in nematodes. Thus, the ITS regions appear better suited for differentiation of species than for phylogenetic analysis of Longidorids.

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