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# Molecular diagnostics and variability of longidorid nematodes

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**Summary.** PCR-RFLP and sequencing approaches of ribosomal DNA are being used to study taxonomy, molecular identification and phylogeny of plant parasitic nematodes. In this paper, we discuss on the usefulness of ITS PCR-RFLP analysis to differentiate among longidorid species. In addition, we examined how well ITS PCR-RFLP differentiated between longidorid species, and how well sequencing of two different ribosomal regions, the ITS containing region and D1-D2 domains of the 26S rDNA, were able to infer phylogenetic relationships among those same species. These methods and their advantages in identifying longidorids and establishing their phylogenetic relationships are examined and discussed.

**Key words:** D1-D2 expansion segments, Internal Transcribed Spacers, ribosomal DNA.

## Introduction

Longidorid nematodes tend to be very conserved in gross morphology, making identification of individual species difficult even for specialists. Individual nematodes are identified and distinguished by their morphological features, the host they infect, their pathological effect on the host and/or their geographical origin. Several longidorid species are vectors of economically important plant viruses (Taylor and Brown, 1997) so that a correct identification of the species is of great importance. Identification criteria are often inadequate for the tiny egg and larval stages. The accurate identification of a species may have important implications for taxonomy and population biology as well as for disease management.

Molecular techniques are considered to be particularly useful where morphological characters lead to an ambiguous interpretation. The polymerase chain reaction (PCR) (Saiki *et al*., 1985) especially overcomes the limitations of morphological identification by its capacity to specifically amplify parasite DNA from minute amounts of material. Moreover, DNA is not influenced by environmental conditions, or by tissue, or by the developmental stage of an organism. The choice of the target region for amplification by PCR depends on the questions asked and the purpose the PCR is to serve. The nuclear ribosomal DNA (rDNA) cistron represents one of the most commonly used target regions to discriminate different nematode species (Zijlstra *et al*., 1995; Powers *et al*., 1997; Nadler *et*

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*al*., 2000; Waeyenberge *et al*., 2000; Lamberti *et al*., 2001; Lamberti *et al*., 2002; De Luca *et al*., 2004a, 2004b).

The rDNA of eukaryotes is a large multigene family consisting of tandemly arrayed sequence repeats usually found in clusters in specific chromosomes (Elder and Turner, 1995). Each rDNA repeating cistron is arranged as follows: an external non-coding region (ETS), a small ribosomal subunit (18S gene), an internal transcribed region (ITS1), the 5.8S gene, another internal transcribed spacer (ITS2), a large ribosomal subunit (26S or 28S gene) and finally an intergenic spacer region (IGS) (De Giorgi *et al*., 2002). The level of variability displayed by each of these regions is remarkably different (Schlotterer and Tautz; 1994; Elder and Turner, 1995). Within an individual cistron, the 26S, 18S and 5.8S regions are conserved in a broad range of different genera, while the ETS, ITS and IGS regions display sequence variations even between closely related species and consequently they can be used to establish phylogenetic relationships at different levels, ranging from those between distantly related organisms to those distinguishing between populations. However, even within a single gene there are regions with different degrees of variability. For instance, the 26S gene consists of both highly conserved and highly variable stretches (D expansions) (Clark *et al*., 1984). The D expansion segments diverge between closely related species and are suitable for examining relatively recent evolutionary events, as was also reported for nematode species (Al-Banna *et al*., 1997; Thomas *et al*., 1997; Blaxter *et al*., 1998; Duncan *et al*., 1999; Kaplan *et al*., 2000; Carta *et al*., 2001). Recent studies have demonstrated that internal transcribed spacers (ITSs) are accurate species markers because they vary between species, and between populations within a species. In particular ITS-1 and ITS-2 are commonly used for the molecular differentiation and identification of free-living, human, animal and plant parasitic nematodes (Powers *et al*., 1997; Blok *et al*., 1998; Hugall *et al*., 1999; Hung *et al*., 1999; Morales-Hojas *et al*., 2001; Elbadri *et al*., 2002; Otranto *et al*., 2003) because of the low level of intra-specific sequence variation combined with higher levels of inter-specific differences.

In this paper, we examined how well ITS PCR-RFLP differentiated between longidorid species, and how well sequencing of two different ribosomal regions, the ITS containing region and D1-D2 domains of the 26S rDNA, were able to infer phylogenetic relationships among those same species.

**PCR-RFLP and sequencing approaches for longidorid identification.**

The PCR amplification of a specific portion of DNA consists of three steps: denaturation, primer annealing and extension. The primers bind their complementary regions on both strands of the DNA and each primer performs the synthesis of a new strand complementary to the old one. This reaction is repeated many times, allowing the production of a large quantity of product starting from what is virtually a single DNA molecule. The PCR conditions are then modified to achieve optimum specificity, fidelity and amplification efficiency.

In our laboratory, we used the primer designed on the 3' end of the 18S and the reverse primer on the 5' end of the 26S to amplify the ITS-containing regions of various species of the plant-parasitic nematodes *Longidorus* and *Xiphinema*. The size of the ITS amplified product was the same for larval stages and for females.

The size of the amplified ITS-containing region of the *Longidorus* spp. studied (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 for *L. elongatus* and 1566 bp for *L. raskii* (Lamberti *et al*., 2001); that of the *Xiphinema* spp. was: 1500 bp for *X. citricolum*, *X. floridae* and *X. laevistriatum* belonging to the *Xiphinema americanum* group (Lamberti *et al*., 2002), 1804 bp for *X. vuittenezi,* 1913 bp for *X. diversicaudatum* and 2008 bp for *X. index*. The length variation of the ITS-containing regions was significant and corresponded to differences in the ITS1 and ITS2, since the 5.8S, and the 3' and 5' ends of the 18S and 26S respectively, were the same length in all species. The ITS1 varied from 632 to 1060 bp, and the ITS2 from 382 to 501 bp. The ITS1 of *L. macrosoma* and *L. helveticus*, at 1022 and 1060 bp respectively, were the longest ITS1 reported in nematodes to date. In eukaryotes, the ITS1 size is around 800 bp; only in *Schistosoma japonica*, *Exochomus quadripustulatus* and *Anopheles gambia* the ITS1 lengths are 1400, 2572 and 5500 bp respectively (Paskewitz *et al*., 1993; van Herwerden

*et al*., 1998; von der Schulenburg *et al*., 2001).

The data clearly demonstrated that congeneric species can be differentiated by the size of their ITS-containing region.

Further information is obtained when ITS-amplified products are examined directly by restriction digestion. The digested products separated in 2% agarose gel stained with ethidium bromide, were visualised under UV light and photographed. A characteristic RFLP profile was produced for each species, and no variation in profile was detected when multiple isolates were analysed. In our laboratory we used six different restriction enzymes *Alu* I, *Bam* HI, *Dde* I, *Hinf* I, *Rsa* I and *Xba* I, for *Xiphinema* species, and *Alu* I, *Ava* II, *Dde* I, *Eco* RI, *Hinf* I and *Rsa* I, for *Longidorus* species. Each restriction enzyme separated each *Longidorus* species from all the others, and from the *Xiphinema* species. Furthermore, genera and species exhibiting products of a similar size were all clearly differentiated from each other (Lamberti *et al*., 2001, Lamberti *et al*., 2002). This method thus proved a simple, highly sensitive and rapid means to discriminate longidorid nematodes, and the ITS regions represented accurate species markers.

Several methods have been explored to detect sequence variability in rDNA. Sequencing has made possible the identification of strain and species-specific markers, the identification of cryptic species (morphologically similar but genetically distinct) and the phylogenetic reconstruction of parasitic groups (Chilton *et al*., 1997; Blaxter *et al*., 1998). This method has detected sequence variation among the ITS regions of longidorid spp. Several nematode species such as *Longidorus helveticus*, *L. macrosoma*, *L. arthensis*, *L. profundorum*, *L. elongatus* and *L. raskii* showed high level of ITS inter-species variability making a proper multialignment difficult.

Direct sequencing of PCR products allows polymorphisms (one or more nucleotides present at one or more sequence positions) to be detected in the sequencing gel, but does not separate sequence variants. To overcome this problem, PCR products can be cloned into a plasmid vector and many clones sequenced in order to recover sequence variants present at low frequency. When a large number of clones are sequenced, they may contain PCR-induced errors. Therefore, a consensus sequence should be determined based on the sequencing of multiple clones derived from independently cloned PCR products.

In our study, the ITS-containing regions of *Longidorus* and *Xiphinema* spp. were cloned. The sequence analyses showed that there was little or no sequence variation between clones from individual nematodes. Several di- and trinucleotide microsatellites occurred both in ITS regions contributing to length variation in the number of repeats between species, and in individual nematodes. This phenomenon, called microheterogeneity, indicates the occurrence of more than one ITS in the genome of an individual nematode and has been described in nematodes (Powers *et al*., 1997; Blok *et al*., 1998; Hugall *et al*., 1999; Subbotin *et al*., 2000; Waeyenberge *et al*., 2000). This heterogeneity is due to the occurrence of microsatellites and the differences in the length and sequence of the ITSs (Zarlenga *et al*., 1996; van Herwerden *et al*., 1999; van Herwerden *et al*., 2000; von der Schulenburg *et al*., 2001). Simple or repetitive elements 7–12 nt long also occur in *Longidorus* spp. and are localised in the middle of both spacers, where there are no constraints.

A second region of the rDNA corresponding to the 5' end of the 26S of the rDNA was also amplified. This region contained the D1 and D2 expansion segments, which showed more variation than the core structure sequences (Clark *et al*., 1984). The sequencing of these domains in *Longidorus* spp. (De Luca *et al*., 2004a) revealed little or no sequence variation among clones from the same individual. A high level of similarity was recorded between species, allowing a correct alignment of the sequences, but with enough variation between sites for phylogenetic reconstruction.

The data clearly suggested that the D1-D2 domain was more suitable for inferring phylogenetic relationships among *Longidorus* species than ITS.

The ITS and D1-D2 sequences were used for phylogenetic reconstruction of the six species of *Longidorus*.

There are various procedures to establish the phylogenetic relationships among sequences: distance methods, parsimony and maximum likelihood. Genetic distances were obtained using the GTR and log-det methods. The GTR method calculated the distance between sequences without imposing any nucleotide substitution model, but estimating it from the data. The log-det method al-

lowed phylogenetic inference even in cases where the sequences showed a different base composition, as with *Longidorus* ITS. Based on these procedures, phylogenetic trees were constructed both by means of minimum evolution and by means of neighbour-joining approaches implemented with the PAUP*4.0b10 package (Swofford, 1998). In addition to these methods, the maximum parsimony and maximum likelihood methods were also performed using the PAUP package (Swofford, 1998).

Using *X. index* as the outgroup, we found that *Longidorus* spp. clustered in two groups, one of them containing *L. helveticus* and *L. macrosoma,* and the other *L. arthensis*, *L. profundorum, L. elongatus* and *L. raskii*, regardless of the region (ITS or D1-D2) or the means used for phylogenetic reconstruction (De Luca *et al*., 2004a). Within the latter *Longidorus* group *L. profundorum* always clustered with *L. arthensis*, while the position of *L. raskii* in the clusters changed depending on the region being examined: close to *L. profundorum* and *L. arthensis* when D1-D2 is examined, or as an outgroup of all other *Longidorus* species when the ITS region is investigated.

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## Literature cited

Al-Banna L., V. Williamson and S.L. Gardner, 1997. Phylogenetic analysis of nematodes of the genus *Pratylenchus* using nuclear 26S rDNA. *Molecular Phylogenetics and Evolution* 7, 94–102.

Altschul S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.

Blaxter M.L., P. De Ley, J.R. Garey, L.X. Liu, P. Scheldeman, A. Vierstraete, J.R. Vanfleteren, L.Y. Mackey, M. Dorris, L.M. Frisse, J.T. Vida and W.K. Thomas, 1998. A molecular evolutionary framework for the *phylum* Nematoda. *Nature* 392, 71–75.

Blok V.C., G. Malloch, B. Harrower, M.S. Phillips and T.C. Vrain, 1998. Intraspecific variation in ribosomal DNA in populations of the potato cyst nematode *Globodera pallida*. *Journal of Nematology* 30, 262–274.

Carta L.K., A.M. Skantar and Z.A. Handoo, 2001. Molecular, morphological, and thermal characters of 19 *Pratylenchus* spp. and relatives using D3 segment of the nuclear LSU rRNA gene. *Nematropica* 31, 193–207.

Chilton N.B., R.B. Gasser and I. Beveridge, 1997. Phylogenetic relationships of Australian strongyloid nematodes inferred from ribosomal DNA sequence data. *International Journal of Parasitology* 27, 1481–1494.

Clark C.G., B.W. Tague, V.C. Ware and S.A. Gerbi, 1984. *Xenopus laevis* 28S ribosomal RNA: a secondary structure model and its evolutionary and functional implications. *Nucleic Acids Research* 12, 6197–6220.

De Giorgi C., P. Veronico, F. De Luca, A. Natilla, C. Lanave and G. Pesole, 2002. Structural and evolutionary analysis of the ribosomal genes of the parasitic nematode *Meloidogyne artiellia* suggests its ancient origin. *Molecular and Biochemical Parasitology* 124, 91–94.

De Luca F., E. Fanelli, M. Di Vito, A. Reyes and C. De Giorgi, 2004b. Comparison of the sequences of the D3 expansion of the 26S ribosomal genes reveals different degrees of heterogeneity in different populations and species of *Pratylenchus* from the Mediterranean region. *European Journal of Plant Pathology*, in press.

De Luca F., A. Reyes, J. Grunder, P. Kunz, A. Agostinelli, C. De Giorgi and F. Lamberti, 2004a. Characterization and sequence variation in the rDNA region of six nematode species of the genus *Longidorus* (Nematoda). *Journal of Nematology*, in press.

Duncan L.W., Inserra R.N., W.K. Thomas, D. Dunn, I. Mustika, L.M. Frisse, M.L. Mendes, K. Morris and D.T. Kaplan, 1999. Molecular and morphological analysis of isolates of *Pratylenchus coffeae* and closely related species. *Nematropica* 29, 61–80.

Elbadri G.A.A., P. De Ley, L. Waeyenberge, A. Viersstraete, M. Moens and J. Vanfleteren, 2002. Intraspecific variation in *Radopholus similis* isolates assessed with restriction fragment length polymorphism and DNA sequencing of the internal transcribed spacer region of the ribosomal RNA cistron. *International Journal for Parasitology* 32, 199–205.

Elder J.F. and B.J. Turner, 1995. Concerted evolution of repetitive DNA sequences in eukaryotes. *Quarterly Review Biology* 70, 297–320.

Hugall A., J. Stanton and C. Moritz, 1999. Reticulate evolution and the origins of ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. *Molecular Biology and Evolution* 16, 157–64.

Hung G.C., N.B. Chilton, I. Beveridge, X.Q. Zhu, J.R. Lichtenfels and R.B. Gasser, 1999. Molecular evidence for cryptic species within *Cylicostephanus minutus* (Nematoda: Strongylidae). *International Journal for Parasitology* 29, 285–291.

Kaplan D.T., W.K. Thomas, L.M. Frisse, J.L. Sarah, J.M. Stanton, P.R. Speijer, D.H. Marin and C.H. Opperman. 2000. Phylogenetic analysis of geographically diverse *Radopholus similis* via rDNA sequence reveals a monomorphic motif. *Journal of Nematolology* 32(2), 134–142.

Lamberti F., F. De Luca, S. Molinari, L.W. Duncan, A. Agostinelli, M.I. Coiro, D. Dunn and V. Radicci. 2002. Stud-

ies on some species of the *Xiphinema americanum* group (Nematoda, Dorylaimida) occurring in Florida. *Nematologia Mediterranea* 30, 31–44.

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.

Morales-Hojas R., R.J. Post, A.J. Shelley, M. Maia-Herzog, S. Coscaron and R.A. Cheke, 2001. Characterisation of nuclear ribosomal DNA sequences from *Onchocerca volvulus* and *Mansonella ozzardi* (Nematodi: Filarioidea) and development of a PCR-based method for their detection in skin biopsies. *International Journal for Parasitology* 31, 169–177.

Nadler S.A., E.P. Hoberg, D.S.S. Hudspeth and L.G. Rickard, 2000. Relationships of *Nematodirus* species and *Nematodirus battus* isolates (Nematoda: Trichostrongyloidea) based on nuclear ribosomal DNA sequences. *Journal of Parasitology* 86(3), 588–601.

Otranto D., E. Tarsitano, D. Traversa, F. De Luca and A. Giangaspero, 2003. Molecular epidemiological survey on the vectors of *Thelazia gulosa, Thelazia rhodesi* and *Thelazia skrjabini* (Spirurida: Thelaziidae). *Parasitology* 127, 365–373.

Paskewitz S.M., D.M. Wesson and F.H. Collins, 1993. The internal transcribed spacers of ribosomal DNA in five members of the *Anopheles gambiae* species complex. *Insect Molecular Biology* 2, 247–257.

Power T.O., T.C. Todd, A.M. Burnell, P.C.B. Murray, C.C. Fleming, A.L. Szalanski, B.A. Adams and T.S. Harris, 1997. The internal transcribed spacer region as a taxonomic marker for nematodes. *Journal of Nematology* 29, 441–450.

Saiki R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich and N. Arnheim, 1985. Enzymatic amplification of a β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 230, 1350–1354.

Schlotterer C. and D. Tautz, 1994. Chromosomal homogeneity of *Drosophila* ribosomal arrays suggests intrachromosomal exchanges drive concerted evolution. *Current Biology* 4, 777–783.

Subbotin S.A., P.D. Halford, A. Warry and R.N. Perry, 2000. Variations in ribosomal DNA sequences and phylogeny of *Globodera* parasitising solanaceous plants. *Nematology* 2, 591–604

Swofford D.L., 1998. *PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4.0 beta 4a*. Sinauer Associates, Sunderland, MA, USA.

Taylor C.E. and D.J.F. Brown, 1997. *Nematode Vectors of Plant Viruses*. CAB International, Wallingford, Oxon, UK, 286 pp.

Thomas W.K., J.T. Vida, L.M. Frisse, M. Mundo and J.G. Baldwin, 1997. DNA sequences from formalin-fixed nematodes: integrating molecular and morphological approaches to taxonomy. *Journal of Nematology* 29, 250–254.

van Herwerden L., D. Blair and T. Agatsuma, 1998. Intra- and inter-specific variation in nuclear ribosomal internal transcribed spacer 1 of the *Schistosoma japonicum* species complex. *Parasitology* 116, 311– 317.

van Herwerden L., D. Blair and T. Agatsuma, 1999. Intra- and interindividual variation in ITS1 of *Paragonimus westermani* (Trematoda: Digenea) and related species: implications for phylogenetic studies. *Molecular Phylogenetics and Evolution* 12, 67–73.

van Herwerden L., R.B. Gasser and D. Blair, 2000. ITS-1 ribosomal DNA sequence variants are maintained in different species and strains of *Echinococcus*. *International Journal for Parasitology* 27, 601–5.

von der Schulenburg J.H.G., U. Englisch and J.W. Wagele, 1999. Evolution of ITS1 rDNA in the Digenea (Platyhelminthes: Trematoda): 3' end sequence conservation and its phylogenetic utility. *Journal of Molecular Evolution* 48, 2–12.

von der Schulenburg J.H.G., J.M. Hancock, A. Pagnamenta, J.J. Sloggett, M.E.N. Majerus and G.D.D. Hurst, 2001. Extreme length and length variation in the first ribosomal Internal Transcribed spacer of ladybird beetles (Coleoptera: Coccinellidae). *Molecular Biology and Evolution* 18(4), 648–660.

Waeyenberge L., A. Ryss, M. Moens, J. Pinochet and T.C. Vrain, 2000. Molecular characterization of 18 *Pratylenchus* species using rDNA restriction fragment length polymorphism. *Nematology* 2, 135–142.

Zarlenga D.S., R.A. Aschenbrenner and J.R. Lichtenfels, 1996. Variations in microsatellite sequences provide evidence for population differences and multiple ribosomal gene repeats within *Trichinella pseudospiralis*. *Journal of Parasitology* 82, 534–538.

Zijlstra C., A.E.M Lever, B.J. Uenk and C.H. Van Silfhout, 1995. Differences between ITS regions of root-knot nematodes *Meloidogyne hapla* and *M. chitwoodi*. *Phytopathology* 85, 1231–1237.

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