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Analysis of ITS sequences of nuclear rDNA and development of a PCR-based assay for the rapid identification of the stem nematode *Ditylenchus dipsaci* (Nematoda: Anguinidae) in plant tissues

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Summary

Ditylenchus dipsaci, the stem nematode, is a migratory endoparasite of over 500 species of angiosperms. The main method of control of *D. dipsaci* is crop rotation, but the presence of morphologically indistinguishable host races with different host preferences makes rotation generally ineffective. Therefore, a sensitive, rapid and reliable as well as cost effective technique is needed to identification of *D. dipsaci*. The objective of this study was to determine whether the ribosomal gene cluster, namely the whole rDNA cistron, could provide useful nucleotide sequences for DNA diagnostics of *D. dipsaci*. The region of rDNA including 3´end of 18S gene, ITS1, 5.8S, ITS2 and 5´end of 28S gene was amplified using general primers designed according to the DNA sequence of *Caenorhabditis elegans*. No length-polymorphism was detected within the ITS rDNA citron of tested host races of *D. dipsaci*. The PCR products (967 bp) of three representative host races of *D. dipsaci* were cloned, sequenced using an automatic sequencing system, and the sequences aligned and compared. No significant homology between *D. dipsaci* and other nematode and plant organisms was found. The sequences of ITS regions were used to design two pairs of primers, PF1-PR1 and PF-PR2, each of which was subsequently shown to be specific for the amplification of predicted-size fragments from genomic DNA of *D. dipsaci*. The primer pairs were further tested using as template DNA extracted from healthy plant hosts and from other nematodes but no amplification was observed. The PCR protocol was shown to be quite sensitive (10 pg of genomic DNA) and able to specifically detect *D. dipsaci* in artificially infested plant tissue.

Key words: *Ditylenchus dipsaci*; DNA-diagnostics; sequence alignment; plant-parasitic nematodes; polymerase chain reaction (PCR); rRNA gene cluster

Introduction

Ditylenchus dipsaci (Kühn) Filipjev, the stem nematode, is a migratory endoparasite of over 500 species of angiosperms (Fortuner, 1982). *D. dipsaci* is prevalent in wide range of climatic conditions, where moisture regimes enable nematode infection, multiplication and dispersal (Smith *et al.*, 1992). The main method of control of *D. dipsaci* is crop rotation, but the presence of morphologically indistinguishable host races with different host preferences makes rotation difficult (Wendt *et al.*, 1993). The races exhibit varying degrees of reproductive isolation, such as partial or complete reproductive incompatibility (Eriksson, 1974). Most populations from cultivated plants (*Fragaria* sp., *Medicago sativa*, *Trifolium pratense*) have 2n = 24 chromosomes, while the giant race from *Vicia faba* has 2n = 48 – 60 chromosomes and population from *Cirsium setosum* has 2n = 52 chromosomes (Subbotin *et al.*, 2004). Therefore, a sensitive, rapid and reliable as well as cost effective technique is needed to detect and precisely determine *D. dipsaci* in soil samples and plant tissues. The classical morphological diagnosis is based mainly on tail shape and size, relative length of stylet and post-vulval sac, and number of cuticular lateral lines (Fortuner, 1982). This method is time-consuming, not always reliable, a larger number of nematode individuals is required and only an experienced person can do it. During the last several years DNA-diagnostic methods like RFLP (Curran *et al.*, 1985), DNA hybridization (Burrows & Perry, 1988), RAPD (Esquibet *et al.*, 1998; Folkertsma *et al.*, 1994; Williamson *et al.*, 1997 and Zhang *et al.*, 1998), allele specific-PCR (Zouhar *et al.*, 2000) and SCAR (Zijlstra, 2000) have been developed for plant-parasitic nematodes. Analysis of DNA is a more direct measure of variability than is protein analysis (Sambrook *et al.*, 2001), and the application of Southern blot hybridization provided precise identification of three

species of *Ditylenchus* but did not differentiate host races of *D. dipsaci* (Palmer *et al.*, 1991). Moreover, the use of DNA technologies overcomes the metabolic state variation (Fullaondo *et al.*, 1999). A practical PCR-based assay with specific primers for rapid and reliable determination *D. dipsaci* in plant tissues is not available.

The ribosomal gene cluster, namely the whole rDNA cistron, has became a useful DNA region for classifying eukaryotes at various taxonomic level. The rDNA is multicopy, tandemly repeated array according in the nucleolar organizer region at one or several chromosomal sites (Szalanski *et al.*, 1997). The rDNA coding genes vary in evolutionary conservation from most-conserved 18S, 5.8S, to least-conserved 28S. The spacer regions including ETS and ITS are more variable than the gene regions and are generally used for phylogenetic analyses (Cherry *et al.*, 1997; Fallas *et al.*, 1996; Subbotin *et al.*, 2004). In addition, portions of the ITS 1 transcript may play a role in the maturation of nuclear RNAs (van Nues *et al.*, 1994), suggesting that nucleotide changes in one portion of the molecule could affect other nucleotide sites (Adams *et al.*, 1998). The PCR-RFLP analysis of PCR amplified ITS and 26S ribosomal RNA genes of selected entomopathogenic nematodes was done by Nasmith *et al.* (1996). The ITS region was also found to be useful in differentiating members of the plant-parasitic nematode genera *Globodera* and *Heterodera* (Blok *et al.*, 1998; Ferris *et al.*, 1995; Ferris *et al.*, 1999; Subbotin *et al.*, 1999).

The objectives of this study were sequence analysis of ITS ribosomal DNA in selected races of *D. dipsaci* and development of a PCR-based assay for the rapid and sensitive identification of *D. dipsaci* in plant tissues.

Materials and Methods

Nematode isolates: The nematode isolates used are listed in Table 1. Some populations were derived directly from the field and others had been in culture for a number years. *Bursaphelenchus xylophilus* was maintained on *Botrytis cinerea* culture in the laboratory.

DNA extraction: DNA was extracted from nematode-infested plant tissue or nematode suspension after elution in a Baermann funnel. Approximately 10 individuals or 0.5 – 1.0 g of plant tissue artificially inoculated with 10 individuals were crushed in liquid nitrogen using mortar and pestle and homogenized in 300 µl lysis buffer (100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 200 mM NaCl, 0.2 % SDS and 0.4 mg/ml proteinase K). The mixture was incubated for 1 h at 37°C with shaking and finally denaturated 5 min at 85°C. The homogenate was mixed 1:1 with phenol (pH 8.0)-chloroform-isoamylalcohol (25:24:1), vortexed for 15 min and centrifuged at 7 000 x *g*. Each lysate (water phase) was transferred to a new tube, an equal volume of chloroform was added, and the extraction was repeated. DNA was precipitated with an equal volume of isopropanol in -20°C overnight or in liquid nitrogen for 20 min and centrifuged at 10 000 × *g* for 10 min. The supernatant was removed and the remaining pellets were vacuum-dried. The pellets for each sample were resuspended in 50 µl TE (10 mM Tris, 1.0 mM EDTA (pH 8.0) or ddH_2O). DNA was stored at -20°C. The working stock of DNA for PCR amplification was diluted to approximately 50 ng/µl after quantification using the Helios Gamma spectrophotometer (ThermoSpectronic, USA).

Universal primer design and PCR amplification: The region on of rDNA including 3´end of 18S gene, ITS1, 5.8S, ITS2 and 5´end of 28S gene was amplified using general primers designed according to the DNA sequence of *Caenorhabditis elegans*. The DNA sequence of rDNA region of *C. elegans* was obtained from GenBank (*Accession No.* X03680). All PCR reactions were performed in 25 µl volumes including: 50 ng of DNA, 200 µM dNTPs, 10 pmol each primer, 1.5 mM $MgCl_2$ and 1.5 U Taq DNA polymerase (Fermentas, Lithuania). Amplification conditions were as follows: an initial denaturation at 94°C for 3 min, after which 30 cycles of denaturation (2 min at 94°C), primer annealing (30 sec at 60°C) and primer extension (2 min at 72°C). Amplification reactions were conducted at least twice, in two separate experiments, for each nematode isolate. Negative controls were included in all PCR amplifications to test for contaminats in the reagents. Amplification was carried out in an automated thermal cycler (PTC 200 MJ Research Inc, USA). Aliquots (5.0 µl) of PCR products were analysed by electrophoresis in 1.2 % (w:v) agarose gels, with 1xTBE buffer, stained with ethidium bromide (0.5 µg/ml) and photographed under ultraviolet (UV) light. The length of the DNA fragments was estimated by comparison with MassRuler 100 bp DNA ladder (Fermentas, Lithuania).

Cloning and sequencing: The PCR products (967 bp) amplified by universal primer pair (S18 and S26) were directly cloned to pTZ57 vector using 3´-A overhangs generated by Taq polymerase (InsT/Aclone™PCR Product Cloning Kit, Fermentas, Lithuania) following the protocol provided by the supplier and transformed into *E. coli* DH5. Clones were checked for the rDNA insert by PCR, and plasmid was prepared with the Perfectprep Plasmid Mini Kit (Eppendorf, Germany). Fragments were sequenced using an automatic sequencing system (ABI Prism 377, Perkin Elmer, USA). Multiple sequence alignments and comparisons were performed using the computer package ClustalW 1.64 with default options and then optimized manually. The partial ITS sequences for several isolates of *D. dipsaci* were obtained from the GenBank (*Accession No.* AF396319-AF396323). The sequence data were also compared with other nucleotide sequences available throught the National Center for Biotechnology Information (NCBI, USA) databases.

PCR with specific primers: Two primer pairs, PF1-PR1 and PF2-PR2, targeting the ITS regions of *D. dipsaci*, were designed using the software program Primer3 (Whitehead Institute, Cambridge, USA) and synthetized (Generi-Biotech, Czech Republic). The two primer pairs were tested

Table 1. Sources, geographic origin and host or substrate of nematode isolates used in this study

Species	Host or substrate	Source	Origin
Ditylenchus dipsaci	*Allium sativum*	P. Havranek, UP Olomouc	Czech Rep.
Ditylenchus dipsaci	*Medicago sativa*	O. Douda, VURV Prague	Czech Rep.
Ditylenchus dipsaci	*Cichorium inthybus*	G. Urek, AI Ljubljana	Slovenia
Globodera pallida	*Solanum tuberosum*	V. Gaar, SRS Prague	Czech Rep.
Bursaphelenchus xylophilus	*Botrytis cinerea*	M. Mota, ICAM Evora	Portugal
Rhabditis spp.	*Clay soil*	V. Gaar, SRS Prague	Czech Rep.

Table 2. Details of primers used in this study

Primer	Sense	Sequence	Author
S18	Forward	5´-TTGATTAGGTCCCTGCCCTTT-3´	This study
S26	Reverse	5´-TTTCACTCGCCGTTACTAAGG-3´	This study
PF1	Forward	5´-AACGGCTCTGTTGGCTTCTAT-3´	This study
PR1	Reverse	5´-ATTTACGACCCTGAGCCAGAT-3´	This study
PF2	Forward	5´-TCGCGAGAATCAATGAGTACC-3´	This study
PR2	Reverse	5´-AATAGCCAGTCGATTCCGTCT-3´	This study

for the amplification of a fragment of known size, according to the sequences of the ITS-rDNA region of *D. dipsaci*. Optimal conditions for specific PCR amplification were determinated in a total reaction volume of 25 µl, with 50 ng of DNA as template, and containing 200 µM dNTPs, 10 pmol each primer, 1.5 mM $MgCl_2$ and 1.5 U Taq DNA polymerase (Fermentas, Lithuania). A negative control without template was used in each PCR experiment. Amplification was carried out as described above except that the annealing temperature was increased to 62°C for primer pair PF1-PR1 or to 63°C for primer pair PF2-PR2. Aliquots (5.0 µl) of the PCR products were resolved by electrophoresis in 1.2 % agarose gels and visualised as aleady stated above.

Primer specifity and sensitivity: The specifity of the primer pairs PF1-PR1 and PF2-PR2 was tested by attempting amlification using as templete the genomic DNA of some plant-parasitic (*Globodera pallida, Bursaphelenchus xylohilus*), saprophytic and free-living nematodes (*Rhabditis* ssp.). The DNA of these nematodes was extracted using the same protocol as that described above for *D. dipsaci*. The specifity of these primers was also examined using garic (*Allium sativum* cv. Alan), onion (*Allium cepa* cv. Augusta), chicory (*Cichorium inthybus* cv. Jupiter), alfalfa (*Medicago sativa* cv. Jarka) and carrot (*Daucus carota* cv. Rubina) DNA. The DNA was extracted from the bulbs, peioles and stems, according to the protocol of Bai *et al.* (1997).
Sensitivity was tested by preparing serial dilutions of puified *D. dipsaci* DNA in sterile distilled water, from 100 ng to 1 pg, and checking for amplification with the established PCR protocol.

Results

PCR amplification: The S18 and S26 primers non-specifically amplified the entire length of the ITS rDNA, including 3´end of 18S gene, ITS1, 5.8S, ITS2 and 5´end of 28S rRNA genes. Gel electrophoresis of PCR products from *D. dipsaci, G. pallida, B. xylophilus* and *Rhabditis* ssp. always yielded a single bands approximately 970, 1230, 1070 and 900 bp long, respectively (Fig. 2). The fragment size has the same length in all examined isolates of *D. dipsaci*, when estimated on agarose gels. No PCR product was obtained in the negative controls.

Sequencing and multiple sequence alignments: The ITS regions of three isolates of *D. dipsaci* were sequenced, exact size of DNA fragments were determined 967 bp, and no variation in length was observed. The sequences of three host races of *D. dipsaci* were compared to each other and to all other sequences from the GenBank database. In all examined sequences originating from *D. dipsaci* an extreme ITS rDNA similarity was found, with exception of

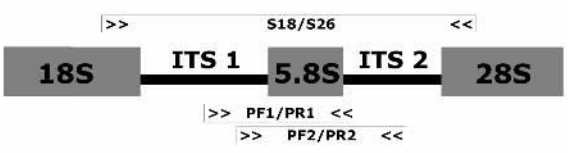

Fig. 1. Diagram with arrows (>>, <<) indicating amplified regions of nuclear ribosomal DNA, with universal primer pair S18-S26 and primer pairs PF1-PR1 and PF2-PR2 for specific PCR amplication of the stem nematode *D. dipsaci*. ITS – internal transcribed spacer. 18S, 5.8S and 28S are rRNA genes

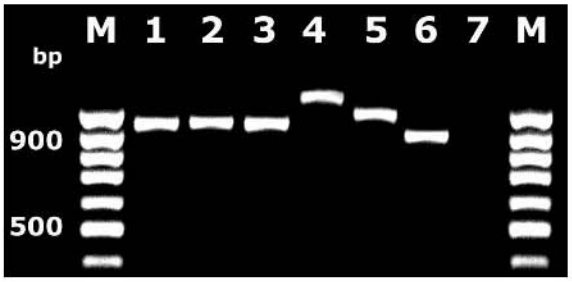

Fig. 2. Agarose gel with PCR amplification products of ITS1-5.8S-ITS2 region of rDNA of selected nematode species using universal S18 and S26 primers. Lanes: 1, 2 and 3 – garlic, alfalfa and chicory isolates of *D. dipsaci*, 4 – *G. pallida*, 5 – *B. xylophilus*, 6 – *Rhabditis* spp., 7 – negative control of sterile distilled water, M – MassRuler 100 bp DNA ladder (Fermentas, Lithuania). A fragment of 967 bp is observed at all isolates of *D. dipsaci*

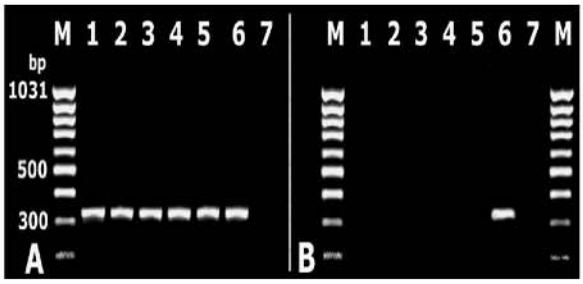

Fig. 4. Gel electrophoresis of the PCR products obtained with primer pairs PF1-PR1, using as template DNA extracted from plant tissue artificially infested with 15 individuals of *D. dipsaci* (A), or healthy plant tissue (B). Lanes: 1 – garlic bulb, 2 – onion bulb, 3 – chicory petiole, 4 – alfalfa stem, 5 – carrot bulb, 6 – positive control with genomic DNA of *D. dipsaci*, 7 – negative control of sterile distilled water, lanes M – MassRuler 100 bp DNA ladder (Fermentas, Lithuania)

Fig. 3. Agarose gel of the PCR products using specific primer pairs PF1-PR1 (A) and PF2-PR2 (B). Lanes: 1,2 and 3 – *D. dipsaci* (isolates from garlic, chicory and alfalfa), 4 – *G. pallida*, 5 – *B. xylophilus*, 6 – *Rhabditis* spp., 7 – negative control of sterile distilled water, M – MassRuler 100 bp DNA ladder (Fermentas, Lithuania). A fragment of 327 bp for PF1-PR1 primer pair and 396 bp for PF2-PR2 primer pair bp are observed with all isolates of *D. dipsaci*, while no fragment was amplified with other nematodes

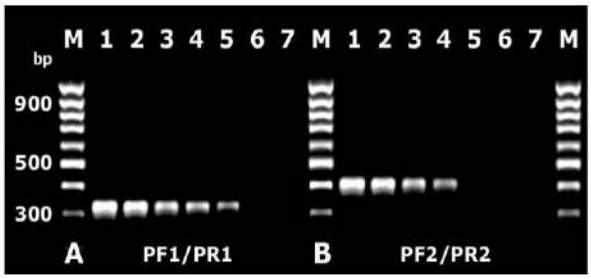

Fig. 5. Gel electrophoresis of the PCR products obtained with primer pairs PF1-PR1 (A) and PF2-PR2 (B), using tenfold dilution series of DNA template. Lanes: 1 to 6, 100 ng to 1 pg of *D. dipsaci* genomic DNA, 7 – negative control of sterile distilled water lanes, M – MassRuler 100 bp DNA ladder (Fermentas, Lithuania). The detection threshold is observed to be 10 pg for PF1-PR1 primer pair and 100 pg for PF2-PR2 primer pair

the giant race from *Vicia faba* (*Ac. No.* AF396323) and the population from *Cirsium setosum* (*Ac. No.* AF396322). The amount of shared sequence identity among the different isolates from *Medicago sativa*, *Cichorium intybus*, *Allium sativum*, *Trifolium pratense* and *Fragaria* sp. showed a low degree of variability. Two isolates from *Medicaco sativa* (Alf1 and Alf2) from diverse geographic locations differed at one nucleotide position (G-A transition). However, the differences between the remaining two isolates varied from 30 substitution and 1 deletion events between the giant race from *Vicia faba* and an alfalafa isolate (Alf2) to 34 substitutions and 2 deletions between the population from *Cirsium setosum* and the second alfalfa isolate Alf2). The aligned ITS sequences of recent known isolates of *D. dipsaci* are illustrated in Table 3.

No significant homology of evolutionary divergent ITS regions between *D. dipsaci* and other plant-parasitic and saprophytic nematodes was found. Furthermore, they did not show any particular homology with any plant organisms in the database (data not shown).

Specific primer design: On the basis of the sequence data of the ITS regions, the primer pairs PF1-PR1 and PF2-PR2 were designed to amplify specific DNA fragments using genomic DNA from all isolates of *D. dipsaci*. The sequences of these primers are shown in Table 2 and their location in the rRNA gene cluster in Fig. 1. Primer lengths were 21 bp and G+C content was about 48 % for each primer. To accomodate the molecular characteristics of the primers selected, appropriate PCR conditions were optimalized (see Materials and methods).

PCR experiments with the specific primer pairs and using as template genomic DNA produced bands in accordance with those predicted from the sequence analysis for all *D. dipsaci* isolates. The primer pair PF1-PR1 specifically amplified a fragment of 327 bp in all *D. dipsaci* isolates (Fig. 3). No fragment was amplified using genomic DNA of other tested nematodes (Fig. 3). The PF2-PR2 primer pair specifically amplified a fragment of 396 bp in all *D. dipsaci* isolates, and no fragment in other tested nematodes (Fig 3). Moreover, the primer pairs PF1-PR1 and PF2-PR2 did

Table 3. Multiple alignment ITS1-5.8S-ITS2 sequences of eight isolates of *D. dipsaci*. The start (>) and the end (<) of the ITS1, 5.8S and ITS2 are indicated. In the aligned sequences, asterisk = match, dash = gap. The localization of the primer pairs PF1-PR1 and PF2-PR2 designed for the identification of *D. dipsaci*, are indicated by black frames. The sense of each primer is accordance to the arrow (►, ◄) reported above the sequences. Abbreviations: Alf, *Medicago sativa*; Chic, *Cichorium inthybus*; Gar, *Allium sativum*; Tri, *Trifolium pratense*; Fra, *Fragaria* sp.; Vic, *Vicia faba*; Cir, *Cirsium setosum* are indicator of food sources of nematode isolates. The sequences Alf1, Tri, Fra, Vic and Cir were obtained from the GenBank, *Accession No.* AF396319-AF396323

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          18S  < ▼ >  ITS 1
Alf1      TCGATCAACCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTAAAATGAGGAAT 60
Chic      TCGATCAACCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTAGAATGAGGAAT 60
Alf2      TCGATCAACCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTAGAATGAGGAAT 60
Gar       TCGATCAACCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTAGAATGAGGAAT 60
Tri       TCGATCAACCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTAGAATGAGGAAT 60
Fra       TCGATCAACCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTAGAATGAGGAAT 60
Vic       TCGATCAACCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTTGAATGAGGAAT 60
Cir       TCGATCAACCAAAACACTAGGAATTGGACCTGGCTGGACCTTTTCCGTAGAATGAGGAAT 60
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Alf1      TCATTCTTACAGCCAATAGTCCAAGAGGGTGCCGTGATATTGGCACGATGCTCACTGGTG 120
Chic      TCATTCTTACAGCCAATAGTCCAAGAGGGTGCCGTGATATTGGCACGATGCTCACTGGTG 120
Alf2      TCATTCTTACAGCCAATAGTCCAAGAGGGTGCCGTGATATTGGCACGATGCTCACTGGTG 120
Gar       TCATTCTTACAGCCAATAGTCCAAGAGGGTGCCGTGATATTGGCACGATGCTCACTGGTG 120
Tri       TCATTCTTACAGCCAATAGTCCAAGAGGGTGCCGTGATATTGGCACGATGCTCACTGGTG 120
Fra       TCATTCTTACAGCCAATAGTCCAAGAGGGTGCCGTGATATTGGCACGATGCTCACTGGTG 120
Vic       TCATTCTTACAGCCAATAGTCCAAGAGAGTGCCGTGATATTGGCATGGTGCTTACTGGTG 120
Cir       TCATTCATACAGCCAATAGTCCAAGAGGGTGCCGTGATATTGGCATGGTGCTTACTGGTG 120
          ****** ******************** ***************** * **** *******
                                                        PF1 ►
Alf1      ATGTCCCCACCGGTTTGCATGCTTATTCTTGGGCGAAAAAACGGCTCTGTTGGCTTCTAT 180
Chic      ATGTCCCCACCGGTTTGCATGCTTATTCTTGGGCGAAAAAACGGCTCTGTTGGCTTCTAT 180
Alf2      ATGTCCCCACCGGTTTGCATGCTTATTCTTGGGCGAAAAAACGGCTCTGTTGGCTTCTAT 180
Gar       ATGTCCCCACCGGTTCGCATGCTTATTCTTGGGCGAAAAAACGGCTCTGTTGGCTTCTAT 180
Tri       ATGTCCCCACCGGTTTGCATGCTTATTCTTGGGCGAAAAAACGGCTCTGTTGGCTTCTAT 180
Fra       ATGTCCCTACCGGTTGGCATGCTTATTCTTGGGCGAAAAAACGGCTCTGTTGGCTTCTAT 180
Vic       ATGTCCCCACTGGTTTGCATGCTTATTCTTGGGCGAAAAAACGGCTCTGTTGGCTTCTAT 180
Cir       ATGTCCCCACCGGTTTGCATGCTTATTCTTGGGCGAAAAAACGGCTCTGTTGGCTTCTAT 180
          ******* ** **** ********************************************

Alf1      GGTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTTGAAGAGAACTGGCACGTGG 240
Chic      GGTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTTGAAGAGAACTGGAACGTGG 240
Alf2      GGTTCTCTGAGCAGTTGTATGCCTACGTCCGTGCCTGCGTTGAAGAGAACTGGCACGTGG 240
Gar       GGTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTTGAAGAGAACTGGCACGTGG 240
Tri       GGTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTTGAAGAGAACTGGCACGTGG 240
Fra       GGTTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTTGAAGAGAACTGGCACGTGG 240
Vic       GATTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGCGTTGAAGAGAATCGACAGGTGG 240
Cir       GATTCTCTGAGCAGTTGTATGCCTACGTCCGTGGCTGTGTTGAAGAGAATCGGCAGGTGG 240
          * ********************************** ***********  *  * ****
                    18 S  < ▼ >  PF2 ►      ITS 1
Alf1      TCTTCGTGATCGCGAGAATCAATGAGTACCGGTTAGGTGCCGCCAACAAAAACCCCATTT 300
Chic      TCTTCGTGATCGCGAGAATCAATGAGTACCGGTTAGGTGCCGCCAACAAAAACCCCATTT 300
Alf2      TCTTCGTGATCGCGAGAATCAATGAGTACCGGTTAGGTGCCGCCAACAAAAACCCCATTT 300
Gar       TCTTCGTGATCGCGAGAATCAATGAGTACCGGTTAGGTGCCGCCAACAAAAACCCCATTT 300
Tri       TCTTTGTGATCGCGAGAATCAATGAGTACCGGTTAGGTGCCGCCAACAAAAACCCCATTT 300
Fra       TCTTCGTGATCGCGAGAATCAATGAGTACCGGTTAGGTGCCGCCAACAAAAACCCCATTT 300
Vic       TCTTCGTGATCGCTAGAATCAATGAGTACCAGATAGGTGCCGCCAACAAAAACCCCATTT 300
Cir       TCTTCGTGATCGCTAGAATCAATGAGTACCGGTTAGGTGCCGCCAACAAAAACCCCATTT 300
          **** ******** ***************** * ****************************
                       ITS 1  < ▼ >  5.8S
Alf1      TTGAACTTTTTTACAAGAAAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGAT 360
Chic      TTGAACTTTTTTACAAGAAAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGAT 360
Alf2      TTGAACTTTTTTACAAGAAAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGAT 360
Gar       TTGAACTTTTTTACAAGAAAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGAT 360
Tri       TTGAACTTTTTTACAAGAAAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGAT 360
Fra       TTGAACTTTTTTACAAGAAAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGAT 360
Vic       TTGAACTTTTTTACAAGAAAACATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGAT 360
Cir       TTGAACTTTTTTACAAGAAA-CATTTCTAGTCTTATCGGTGGATCACTCGGTTCATAGAT 359
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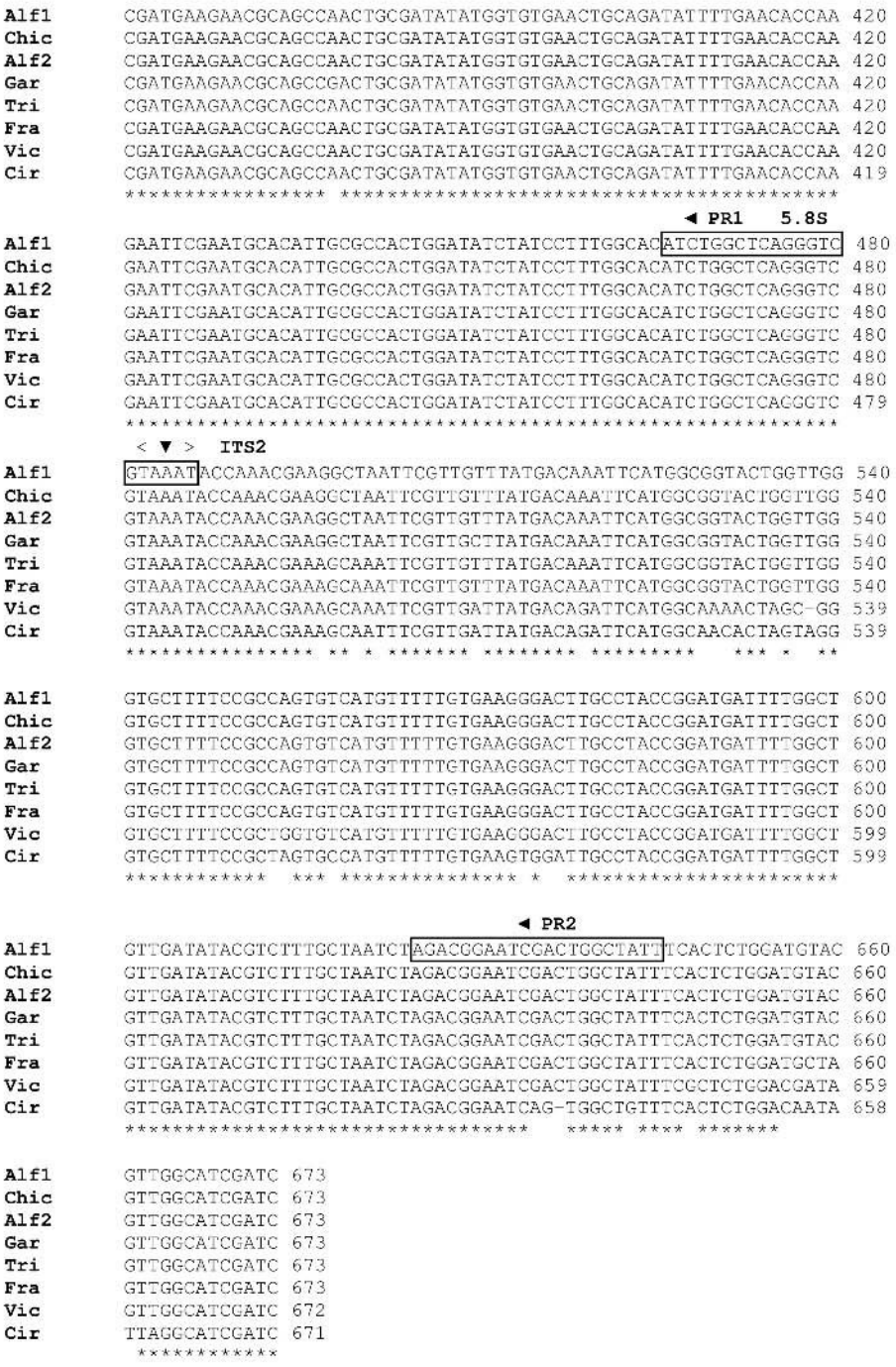

not amplify any fragment when the genomic DNA of any plant hosts was used as template (Fig 4).

Sensitivity of PCR detection: The primer pair PF1-PR1 was able to amplify the appropriate fragment from 100 ng down to 10 pg of DNA template, whereas primer pair PF2-PR2 only from 100 ng down 20 pg of DNA template (Fig. 5).

Discussion

The ITS regions of rRNA genes can revel diagnostic differences at the species level among most nematode taxa studied thus far (Powers *et al.*, 1997). Although the host races of *D. dipsaci* examined in this study generally originated from different geographic locations and different food sources, bioinformatic analysis of sequenced ITS

regions and ITS sequences from GenBank did not reveal significant differences with the exception of the giant race from *Vicia faba* and a population from *Cirsium setosum*. The results of our study also prompted some speculations on the structure of the biological species *Ditylenchus dipsaci*. Analysis of ITS sequences confirmed existence of several nucleotide autapomorphies for the giant race from *Vicia faba*, and a population from *Cirsium setosum* as proposed by Subbotin *et al.* (2004). Moreover, the high level of their sequence dissimilarities from *D. dipsaci sensu stricto* also support the suggestion by Sturhan and Brzeski (1991) and Subbotin *et al.* (2004) that probably for these isolates should be considered a distinct species status. Similar results were obtained by using restriction analysis of ITS regions (Wendt *et al.*, 1993), RAPD analysis (Esquibet *et al.*, 1998) and AFLP analysis (Esquibet *et al.*, 2003).

The two pairs of primers, designed and used with PCR conditions established here showed the potential of this method as a diagnostic test for the stem nematode *D. dipsaci* in plant tissue. The results showed that each of the PCR primer pairs PF1-PR1 and PF2-PR2 amplified a specific fragment from the ITS region of *D. dipsaci*. No amplification was observed with these primers when DNA from other nematodes and plants was used. Thirty cycles of PCR amplification using specific primers produced a sufficient amount of the predicted-size fragments (327 bp for PF1-PR1 and 396 bp for PF2-PR2) to visualise them on the ethidium bromide-stained gels, when one-fifth of the PCR reaction volume (5.0 µl) was loaded on the gel. In these conditions the detection threshold was found to be 10 pg for PF1-PR1 primer pair and 100 pg for PF2-PR2 primer pair of nematode genomic DNA, which are an acceptable limit of detection. Since rRNA gene cluster is tandemly multiple-repeated array in eukaryotes genome, our PCR-based method is more sensitive and reliable than oligonucleotide primers derived from AFLP markers developed by Esquibet *et al.* (2003). However, SCAR primers developed by Esquibet *et al.* (2003) can be used as a tool for differentiate normal and giant type of the stem nematode *D. dipsaci*. Nevertheless, further tests with DNA from other nematodes of *Anguinidea* family species are needed to verify specificity of our-developed method. Some authors have developed specific DNA probes for detection of *D. dipsaci* by Southern-blot technique (Palmer *et al.*, 1991; Wendt *et al.*, 1993). Probes were labelled radioactively in each case. Although radioisotope labelling could be avoided using non-radioactive detection methods (Allefs *et al.*, 1990), relatively large amounts of DNA are required for hybridization.

It thus seems that this PCR-based method can be employed when the phytosanitary laboratories are asked to verify the health of the batch of plant materials. Moreover, in the case of quarantined fields or green-houses this PCR assay would serve to ensure that the quarantine procedures had been effective. As our data showed, the PCR-based detection of *D. dipsaci* in plant tissue would also markedly speed up this process and indicate in a short time whether specific precautions are required to prevent the spread of this stem nematode.

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