Conversion of sequence-characterized amplified region (SCAR) bands into high-throughput DNA markers based on RAPD technique for detection of the stem nematode *Ditylenchus dipsaci* in crucial plant hosts

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ABSTRACT

Ditylenchus dipsaci, the stem nematode, is a migratory endoparasite of over 500 species of angiosperms. The main method of *D. dipsaci* control is crop rotation, but the presence of morphologically indistinguishable host races with different host preferences makes rotation generally ineffective. Therefore, a sensitive, rapid, reliable, as well as cost effective technique is needed for identification of *D. dipsaci* in biological samples. This study describes the development of species-specific pairs of PCR oligonucleotides for detection and identification of the *D. dipsaci* stem nematode in various plant hosts. Designed DIT-2 primer pair specifically amplified a fragment of 325 bp, while DIT-5 primer pair always produced a fragment of 245 bp in all *D. dipsaci* isolates. Two developed SCAR primer pairs were further tested using template DNA extracted from a collection of twelve healthy plant hosts; no amplification was however observed. The developed PCR protocol has proved to be quite sensitive and able to specifically detect *D. dipsaci* in artificially infested plant tissues.

Keywords: *Ditylenchus dipsaci*; stem nematodes; quarantine organism; SCAR; diagnostics; detection; specific PCR; RAPD

The plant-parasitic nematodes are the most important pests around the world. Many species of the plant-parasitic nematodes cause high losses of crop yield and many of them have a quarantine status. The stem and bulb nematode *Ditylenchus dipsaci* (Kühn 1857) Filipjev 1836 is a migratory endoparasite nematode of over five hundred vascular plant species. The stem nematode *D. dipsaci* is prevalent in a wide range of climatic conditions, where moisture regimes enable nematode infection, multiplication and dispersal. The main method of *D. dipsaci* control is crop rotation, but the presence of morphologically indistinguishable host races with different host preferences makes rotation difficult (Wendt et al. 1993). The biological races exhibit different degrees of reproductive isolation, such as partial or complete reproductive incompatibility (Erikson 1974). Each biological race is able to complete its life cycle only on a specific plant host. Among the most important plant hosts of *D. dipsaci* in Central Europe are bulb vegetables (onion, garlic and leek), carrot, alfalfa, clover, sugar beet, chicory, potatoes, strawberry, ornamental bulb plants (*Narcissus* spp.), and also many common weeds. Symptoms on host plants are not always specific as for appearance. Early infested plants and low infested seeds show no symptoms. Nematodes cause swellings and distortion of aerial plant parts and necrosis or rotting of stem bases, bulbs, tubers and rhizomes. The

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stem nematode is resistant to low temperatures. In Central Europe this nematode overwinters without any damages. The stem nematode *D. dipsaci* is one of the most destructive plant-parasitic nematodes in the temperate climate zone. The host plants could be completely destroyed without a crop control (Brzecki 1998). The pivot element in the control of the stem nematodes should be fast, accurate and reliable diagnostics, including monitoring of its geographic occurrence. Diagnostic methods of plant-parasitic nematodes are usually based on anatomy and morphology characteristics comparable with the original descriptions. Diagnostics using light microscopy technique is time-consuming and it depends too strongly on personal experiences (Ebrahimi et al. 2004).

During the last several years DNA-diagnostics or population study methods including RFLP (Curran et al. 1985), DNA hybridization (Burrows and Perry 1988), RAPD (Folkertsma et al. 1994, Williamson et al. 1997, Esquibet et al. 1998 and Zhang et al. 1998), allele specific-PCR (Zouhar et al. 2000), SCAR (Zijlstra 2000) and RAPD (Samal et al. 2003, Sedlak et al. 2004), have been developed for characterization and identification of plant-parasitic nematodes. Specific PCR assays based on ribosomal RNA gene cluster for detection of *D. dipsaci* were developed (Marek et al. 2005, Subbotin et al. 2005). Nevertheless, Subbotin et al. (2004) characterized nucleotide sequences of evolutionary divergent ITS1-5.8S-ITS2 cistron for a number of gall-forming and stem nematodes. These results showed a high ITS rDNA sequence homology among the stem nematode *D. dipsaci* and several members of *Heteroanquina*, *Mesoanquina* and *Subanquina* genera. Therefore, an alternative molecular detection technique is needed to be developed for a reliable molecular detection and determination of the stem nematode *D. dipsaci* in biological materials, such as host plant tissues, soil samples etc. Alternative DNA markers for a differentiation of giant and normal types of *D. dipsaci* were previously characterized by SCAR and AFLP experimental approaches (Esquibet et al. 1998, Esquibet et al. 2003). However, these authors did not verify the developed SCAR and AFLP markers for routine molecular diagnostics purposes.

Hence, the main aim of the present study was the production of the appropriate collection of species-specific RAPD markers, their conversion to specific PCR-based assay for the rapid and sensitive identification of *D. dipsaci* normal type of biological races in plant organs and tissues of majority plant hosts in Central Europe.

MATERIAL AND METHODS

Nematodes isolates and plant hosts. Populations of nematodes from free field cultures were determined by morphological characters and cultivated on corresponding host plants. In this report geographically different origin of *D. dipsaci* populations were used (Table 1). Plants were placed in a cultivation chamber with controlled temperature and day length. Nematode inoculation was done by suspension of nematodes mix with dicarboxymethylcellulose (saturated solution) of final concentration 2%. Temperature was decreased on

Table 1. Origin of biological races of *Ditylenchus dipsaci* used in this study

Species	Host plant	Study code	Geographic origin	Origin
Ditylenchus dipsaci	*Allium sativum*	As-B	Blatnice	Czech Republic
	Allium sativum	As-M	Marefy	Czech Republic
	Allium sativum	As-Bo	Boškůvky	Czech Republic
	Allium sativum	As-L	Litomyšl	Czech Republic
	Allium sativum	As-O	Olomouc	Czech Republic
	Medicago sativa	Ms-P	Piešťany	Slovakia
	Cichorium inthybus	Ci-L	Ljubljana	Slovenia
	Allium cepa	Ac-B	Bari	Italy
	Daucus carota	Dc-B	Bari	Italy
	Vicia faba	Vf-B	Bari	Italy

14–16°C for a better penetration and multiplication of nematodes for two weeks. Nematodes were extracted from sliced plants with the Baerman funnel and several times rinsed in sterile water; subsequently they were used for DNA isolation.

DNA isolation and purification. DNA was isolated from nematodes, nematode-infested plant tissue and nematode free plant tissue (Table 4). In this part of work biological races of *D. dipsaci* normal type originated from garlic, carrot, alfalfa and chicory were used. For the isolation approximately 10 individuals or 0.5–1.0 g plant tissue of artificially inoculated plant with 10 individuals were used. These kinds of biological material were crushed in microtube containing glass beads (250 µm diameter) using mini grinder (Pellet Pestle ®Motor, Sigma) and homogenized in 300 µl lysis buffer (100mM Tris-HCl (pH 8.0), 5mM EDTA, 200mM NaCl, 0.2% SDS and 0.4 mg/ml proteinase K). The mixture was incubated for 1 h at 55°C with shaking and finally denatured for 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 × g. Each lysate (water phase) was transferred to a new tube to which equal volume chloroform was added and extraction was repeated. DNA was precipitated with 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 discarded and the remaining pellets were vacuum-dried. The pellets for each sample were resuspended in 50 µl TE (10mM Tris, 1mM EDTA (pH 8.0) or ddH_2O. DNA was stored at –20°C for a long-term storage. The working stock of DNA for RAPD amplification was diluted to approximately 50 ng/µl after quantification using the Helios Gamma spectrophotometer (ThermoSpectronic, USA).

RAPD fingerprinting protocol. Isolated nematodes' DNA was used for the amplification by three random primer sets (Operon Technology sets A, B and C). All RAPD reactions were performed in 25 µl volumes including: 100 ng of DNA, 200µM dNTPs, 20 pmol primer, 1.5mM $MgCl_2$ and 1.5 U Taq DNA polymerase (Fermentas, Lithuania). Amplification conditions were as follows: an initial denaturation at 94°C for 2 min, after which 2 cycles of denaturation (1 min at 94°C), primer annealing (1 min at 35°C) and primer extension (2 min at 72°C) after 34 cycles of denaturation (30 sec at 94°C), primer annealing (30 sec at 35°C) and primer extension (1.5 min at 72°C), final extension (25 min at 72°C). Amplification was performed in the thermal cycler (PTC 200 MJ Research Inc. USA). Aliquots (5.0 µl) of RAPD products were separated by the horizontal electrophoresis in 1.5% (w:v) agarose gel, with 1 × TBE buffer, stained with ethidium bromide (0.5 µg/ml) and analysed under ultraviolet (UV) light. The length of the DNA fragments was estimated by comparison with MassRuler 100 bp DNA ladder (Fermentas, Lithuania).

RAPD fragments selection and cloning. From obtained RAPD fingerprints different fragments were selected. These bands were cut and eluated from agarose gel by using the QIAEX II Gel Extraction Kit 150 (Qiagen). DNA fragments were directly cloned to pTZ57R/T vector using 3'-A overhangs generated by the Taq polymerase (InsT/Aclone™PCR Product Cloning Kit, Fermentas, Lithuania), following the protocol provided by the supplier and transformed into *E. coli* DH5. Blue-white selection was used for detection of positive bacterial colonies. Plasmid isolation from overnight-cultivated bacterial cultures was made with the Perfectprep Plasmid Mini Kit (Eppendorf, Germany). Plasmids were cleaved by restriction endonucleases (KpnI and PstI or BamHI and EcoRI) for verification of predicted cloned DNA fragment. Fragments were sequenced using the automatic sequencing system (ABI Prism 377, Perkin Elmer, USA). The sequence data were compared with other nucleotide sequences available throughout the National Center for Biotechnology Information (NCBI, USA) databases.

Primer design and PCR amplification. The acquired sequences were used for primer designing by means of PRIMER3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) in consideration of availability in routine diagnostics. For the verification of primers ability to amplify predicted fragment length, primers were tested with nematode DNA. Temperature gradient – thermal cycler (PTC 200 MJ Research Inc. USA) was used for optimization of PCR reaction conditions. Primers specificity was checked with DNA isolated from twelve host plants. Optimal conditions for specific PCR amplification were determined in a total reaction volume of 25 µl, with 50 ng of DNA as template, and containing 200µM dNTPs, 10 pmol each primer, 1.5mM $MgCl_2$ 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 (1 min at 94°C), primer annealing (30 sec at the optimal temperature for concrete pair of primers; temperatures are shown in Table 2) and primer extension (1 min at 72°C). For a positive

DNA amplification of plant hosts genomic DNA, Rbcl primer pair was used, which specifically amplified partial sequence of plant chloroplast gene for ribulose-1,5-bisphosphate carboxylase. Aliquots (5.0 µl) of the PCR products were resolved by electrophoresis in 1.2% agarose gels and visualized as already stated above.

RESULTS AND DISCUSSION

DNA profiling by RAPD approach

To find species-specific RAPD-fragments, RAPD-PCRs were performed with genomic DNA of some plant-parasitic nematodes, e.g. *Meloidogyne fallax* and *M. hapla*, *M. chitwoodi* (Zijlstra 2000). In our experiments, three sets of random primers (Operon technology sets A, B and C) were analyzed in RAPD reactions using *D. dipsaci* genomic DNA to find identical DNA bands among all tested biological races. Generated RAPD patterns were largely characteristic for each of biological races. Moreover, we also uncovered inter- and intra-population genetic diversity by experimental RAPD approach (data not shown). Nevertheless, we identified six requisite uniform DNA markers for all tested biological races of *D. dipsaci* generated by OPA-18, OPA-19, OPA-20 and OPB-16 random decameric primers. Using the primers OPA-18, OPA-19, OPA-20, OPB-16, following fragments – 955 bp, 522 bp and 1103 bp, 703 bp and 1007 bp, and 951 bp – were amplified strongly from all proved biological races of *D. dipsaci*, respectively (Figures 1–4). These 955, 522, 1103, 703, 1007 and 951 bp fragments were chosen for their uniformity, strong intensity and size, which would facilitate their cloning and sequencing. None of the RAPD fragments selected as specifically or more highly PCR amplified in DNA samples was polymorphic between the number of different *D. dipsaci* isolates.

Characterization of selected RAPD fragments

The selected species-specific RAPD fragments were isolated, cloned and sequenced. Each obtained sequence was compared by the BLASTN algorithm with sequences within the GenBank, DDJB and EMBL databases. The closest database similarities for each RAPD sequence are shown in Table 3. Three RAPD markers were similar to bacterial organisms, one marker matched to *Bubalus bubalis* microsatellite BBMS27and last two show no significant similarity to any sequences from the GenBank database. From the obtained sequences, six specific primer pairs were designed by using Primer3 software. Nucleotide sequences of specific primers conversed from RAPD markers and its PCR product size and established PCR conditions are shown in Table 2.

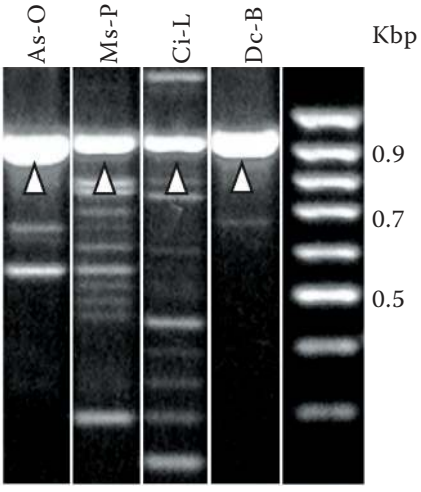

Figure 1. DNA fingerprint of four *Ditylenchus dipsaci* biological races after PCR with primer OPA-18. Bands that were sequenced are marked. The MassRuler 100 bp DNA ladder (Fermentas, Lithuania) was used as molecular marker

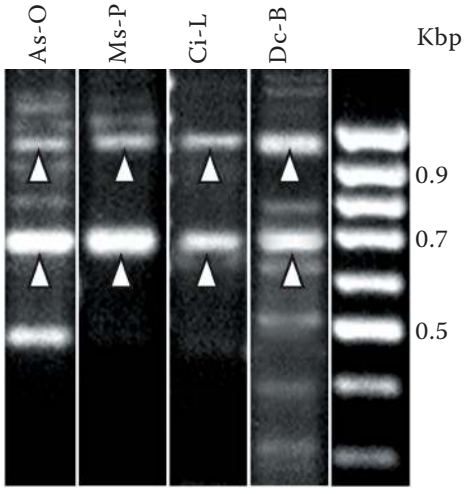

Figure 2. DNA fingerprint of four *Ditylenchus dipsaci* biological races after PCR with primer OPA-20. Bands that were sequenced are marked. The MassRuler 100 bp DNA ladder (Fermentas, Lithuania) was used as molecular marker

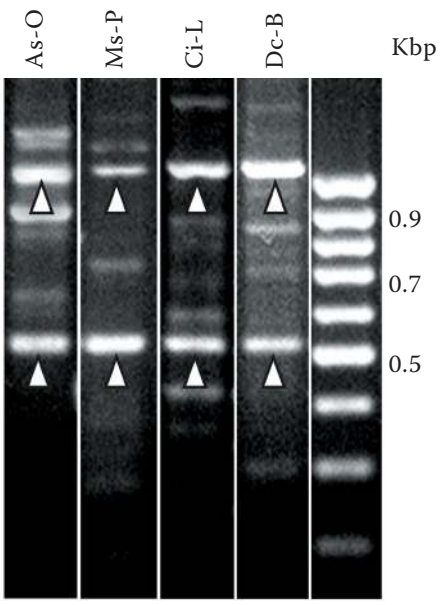

Figure 3. DNA fingerprint of four *Ditylenchus dipsaci* biological races after PCR with primer OPA-19. Bands that were sequenced are marked. The MassRuler 100 bp DNA ladder (Fermentas, Lithuania) was used as molecular marker

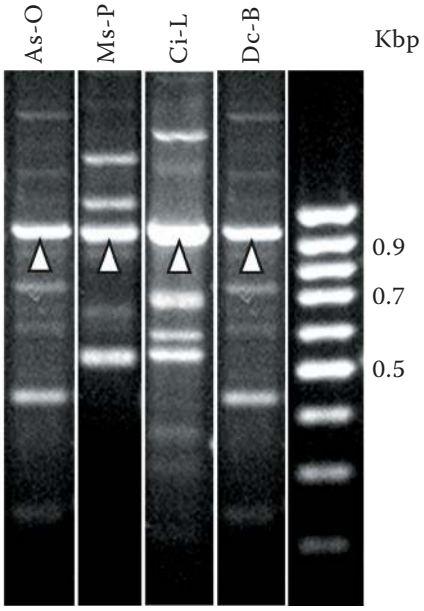

Figure 4. DNA fingerprint of four *Ditylenchus dipsaci* biological races after PCR with primer OPB-16. Bands that were sequenced are marked. The MassRuler 100 bp DNA ladder (Fermentas, Lithuania) was used as molecular marker

Development of specific PCR-based assay

The specificity of the designed primer pairs was tested by attempting amplification using genomic DNA of all isolates of proved *D. dipsaci* biological races as template. Only primer pairs DIT-2 and DIT-5 generated species-specific PCR products for all isolates of our *D. dipsaci* collection (Table 1). The DIT-2 primer pair specifically amplified a fragment of 325 bp, while DIT-5 primer pair always produced a fragment of 245 bp in all *D. dipsaci* isolates.

Unlike, other four primer pairs (DIT-1, DIT-3, DIT-4 and DIT-6) produced unspecific and non-

Table 2. Designed primers and PCR conditions used in this study

RAPD marker	RAPD product size (bp)	Specific PCR oligonucleotides		Specific PCR product size (bp)	PCR condition	
		sense	sequence 5′→3′		cycles No.	annealing temperature (°C)
DIT-1$_{OPA-18}$	955	forward reverse	ACGTGGTGGATACGGCTATTT CATTTCGAGACGCACATTCTC	460	30	58
DIT-2 $_{OPA-19}$	522	forward reverse	GCAATGCACAGGTGGATAAAG CTGTCTGTGATTTCACGGTAGAC	325	30	60
DIT-3 $_{OPB-16}$	951	forward reverse	GAATAATCAGCAGAGCGGTGA ATTCGATCACCTGTCCCACTT	449	30	57
DIT-4 $_{OPA-20}$	703	forward reverse	TTATCATGTTGGGGCTCTGTC CGGTCCAAAGGTGAACAAA	568	30	62
DIT-5 $_{OPA-19}$	1103	forward reverse	GAAAACCAAAGAGGCCGTAAC ACCTGATTCTGTACGGTGCAA	245	30	60
DIT-6 $_{OPA-20}$	1007	forward reverse	GAACAACCAGAATGGCGGTAT TGTACCTGGGTATTGCCTTTG	892	30	59

Table 3. Results of bioinformatic analyses of sequences acquired from RAPD

RAPD marker	BLASTN Bioinformatic predicted sequence homology	E-value
DIT-1_{OPA-18}	AY059069.1, *Serratia marcescens* aconitase gene	1e-24
DIT-2_{OPA-19}	CP000152.1, *Burkholderia* sp. 383 chromosome 2	0.001
DIT-3_{OPB-16}	No significant homology	
DIT-4_{OPA-20}	No significant homology	
DIT-5_{OPA-19}	AE008693.1, *Salmonella typhimurium* LT2	7e-11
DIT-6_{OPA-20}	AY912156.1, *Bubalus bubalis* microsatellite BBMS27	2e-04

reproducible PCR products with the use of genomic DNA of *D. dipsaci*. Therefore, these primer pairs were not suitable for a reliable molecular detection of *D. dipsaci* and were rejected. Moreover, the specificity of primer pairs DIT-2 and DIT-5 was also examined using template genomic DNA of twelve plant hosts important for central Europe climate conditions (Table 4). The primer pairs DIT-2

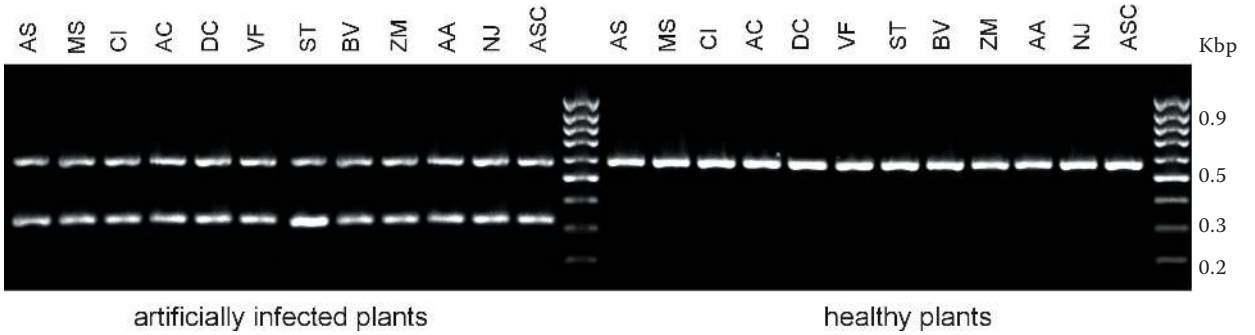

Figure 5. PCR products from twelve different healthy and artificially infected plant species described in Table 4 amplified by primer pairs DIT-2 and Rbcl. Species-specific primer pair DIT-2 generated the fragment of 325 bp length and Rbcl which specifically amplified partial sequence of plant chloroplast gene for ribulose-1,5-bisphosphate carboxylase approximately 590 bp. Biological race (study code As-B) was used in this experiment. Generally, all the isolates described in Table 1 and used for the same assay provided identical results (data not shown). The MassRuler 100 bp DNA ladder (Fermentas, Lithuania) was used as molecular marker

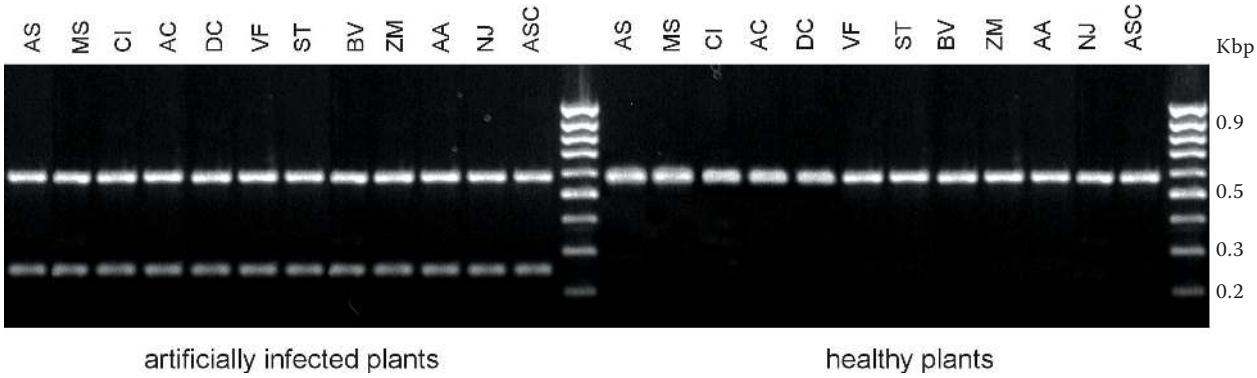

Figure 6. PCR products from twelve different healthy and artificially infected plant species described in Table 4 amplified by primer pairs DIT-5 and Rbcl. Species-specific primer pair DIT-5 generated the fragment of 245 bp length and Rbcl which specifically amplified partial sequence of plant chloroplast gene for ribulose-1,5-bisphosphate carboxylase approximately 590 bp. Biological race (study code As-B) was used in this experiment. Generally, all isolates described in Table 1 and used for the same assay provided identical results (data not shown). The MassRuler 100 bp DNA ladder (Fermentas, Lithuania) was used as molecular marker

Table 4. Plant species used in this study for testing specificity of DIT-2 and DIT-5 primer pairs

Plant species	Study code
Allium sativum	AS
Medicago sativa	MS
Cichorium inthybus	CI
Allium cepa	AC
Daucus carota	DC
Vicia faba	VF
Solanum tuberosum	ST
Beta vulgaris	BV
Zea mays	ZM
Allium ampeloprasum porrum	AA
Narcissius juncifolius	NJ
Allium schoenoprasum	ASC

and DIT-5 did not amplify any fragment where the genomic DNA of any plant hosts was used as template (Figures 5 and 6). Thirty cycles of PCR amplification using specific primers produced a sufficient amount of the predicted-size fragments (325 bp for DIT-2 and 245 bp for DIT-5) to visualize them on the ethidium bromide-stained gels, with one-fifth of the PCR reaction volume (5.0 µl) loaded on the gel. Previously, we described a PCR-based technique for sensitive identification of the stem nematode *D. dipsaci* in plant material (Marek et al. 2005). This technique was based on evolutionary divergent ITS1 and ITS2 sequences of the ribosomal RNA gene cluster (rDNA). The rDNA is multicopy, tandemly repeated array according in the nucleolar organizer region at one or several chromosomal sites (Szalanski et al. 1997), and is therefore very suitable for DNA amplification methods. However, Subbotin et al. (2004) pointed on the closest ITS rDNA sequence similarities between the stem nematode *D. dipsaci* and some species of *Heteroanquina*, *Mesoanquina* and *Subanquina* genera, which complicates development of molecular detection based on the rDNA cistron. Hence, this report shows the usefulness of converting alternative RAPD markers into reliable SCAR markers. This study is the only second report of SCAR analysis on *D. dipsaci*. Esquibet et al. (1998) did previous SCAR analyses on the stem nematode *D. dipsaci*, but the authors did not solve DNA-diagnostics problems. In this study, we developed the first species-specific PCR oligonucleotides useful for a sensitive and reliable detection and identification of the stem nematode *D. dipsaci* in plant host tissues. Some authors developed specific DNA probes for detection of *D. dipsaci* by the Southern-blot technique (Palmer et al. 1991, Wendt et al. 1993). In each case, these probes were labeled radioactively; although radioisotope labeling 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 greenhouses this PCR assay would serve to ensure whether 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. In this study SCAR primers were designed to identify *D. dipsaci*, but the method offers great possibilities to create markers at species level, serving a much wider range of identification levels and facilitating genetic, molecular biology and phytopathological research.

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