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FULL RESEARCH PAPER

Identification of *Ditylenchus* species associated with Fabaceae seeds based on a specific polymerase chain reaction of ribosomal DNA-ITS regions

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Abstract A technique based on the use of specific primers for polymerase chain reaction (PCR) was developed for the identification of the stem and bulb nematode belonging to the *Ditylenchus dipsaci* species complex. The internal transcribed spacer region ITS1 and ITS2, the gene 5.8 S and part of genes 18 S and 26 S of twenty populations of the *D. dipsaci* species complex belonging to both *D. dipsaci sensu stricto* and *Ditylenchus* sp. B (corresponding to populations of giant individuals associated to *Vicia faba*) and three congeneric species were amplified with two universal ribosomal primers. PCR-amplified DNA samples were digested with five restriction enzymes in order to reveal some polymorphism allowing the identification of *D. dipsaci* populations associated with Fabaceae seeds. The polymorphism among species was confirmed by the sequencing of the PCR products. A primer (DdpS2) was designed in a region conserved in all populations of both *D. dipsaci sensu stricto* and *D.* sp. B studied in the present work. The other Anguinidae species (except a few species from Central Asia associated to Asteraceae and *D.* sp. G associated to *Plantago maritima*) differ in two to four nucleotides at the 3′ extremity of this region. This sequence portion coincides with a *Tsp*EI restriction site. In combination with a primer located in the ribosomal region, this first primer is a good candidate for identification by PCR of populations of the *D. dipsaci* species complex found in Fabaceae seeds. A second primer (DdpS1) was designed in a similar way and was specific to *D. dipsaci sensu stricto.* The utility of these two sets of primers is discussed against the background of quarantine regulation.

Keywords Diagnostics · *Ditylenchus* · ITS · Pathogen · PCR · Seed

Abbreviations
rDNA Ribosomal DNA
SCAR Sequence characterized amplified regions

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Introduction

The species of the *Ditylenchus* genus are difficult to differentiate due to the high degree of morphological similarity between them (Wendt et al. 1993). The most important species for agriculture is the stem and bulb nematode *Ditylenchus dipsaci*. It is an

obligatory endoparasite species, able to grow in over 500 species of plants belonging to different families; most hosts are found among Fabaceae, Liliaceae, and Chenopodiaceae (Caubel and Pedron 1976). In the past, up to 30 host-races have been distinguished within *D. dipsaci* (Ait Ighil 1983; Hooper 1984; Sturhan and Brzeski 1991). This variability has led to several authors considering this taxa as a 'species complex'. A recent study by Subbotin et al. 2005) recognized two groups within the '*D. dipsaci* complex'. The first group contains diploid ($2n$ = 24) populations of normal size associated with various crops and named '*D. dipsaci sensu stricto*'. The second (paraphyletic) group contains several (at least 6) polyploid species: *Ditylenchus* sp. B associated with *Vicia faba* (Fabaceae) and previously called the 'giant race' due to the larger size of the nematodes, *Ditylenchus* sp. C, D, E and F associated with various Asteraceae, and *Ditylenchus* sp. G associated with *Plantago maritima* (Plantaginaceae).

The populations of *D. dipsaci sensu stricto* associated with alfalfa are the only widely recognized nematodes parasitizing the shoots of alfalfa and may cause serious damage and mortality in seedlings and older plants (Caubel and Pedron 1976; Gray et al. 1994). However, because the chrysanthemum nematode *Aphelenchoides ritzemabosi* also attacks alfalfa shoots, the presence of a mixture of *A. ritzemabosi* and *D. dipsaci* in alfalfa seeds may confound diagnosis (Gray et al. 1994; Milano de Tomasel and McIntyre, 2001).

Ditylenchus dipsaci belongs to the list of A2 pests regulated as quarantine pests in the EPPO region (European & Mediterranean Plant Protection Organization–www.eppo.org). The absence of an effective means of control necessitates the use of healthy certified seeds. Currently, specific identification of nematodes after isolation is only based on morphological and morphometric analysis. This method is time-consuming and requires experienced analysts in nematode identification. *Ditylenchus dipsaci*-specific molecular probes have been developed from the cloning of restriction fragments of genomic DNA by Palmer et al. (1991). However, the sequences of these probes were not described. The use of PCR amplification has brought significant advances in the identification of pests species in general and several approaches have been used for the *D. dipsaci* species complex. Some approaches are based on non-defined target DNA (Esquibet et al. 1998, 2003), whilst others are focused on the internal transcribed spacers (ITS 1 and ITS 2) of ribosomal RNA situated between the 18 S gene and 28 S gene (Wendt et al. 1993; Leal-Bertioli et al. 2000; Powers et al. 2001; Subbotin et al. 2005; Marek et al.2005). Ribosomal DNA sequences have been commonly used in phylogenetic studies and therefore as targets to differentiate species by polymerase chain reaction (PCR)-based methods (Ferris et al. 1993). The growing number of ITS sequences in open-access databases such as GenBank allow a general increase in the knowledge of phylogenetic relationships between races, species, genera or families that can be widely used and compared. Analysis of internal transcribed spacer (ITS) polymorphism has solved taxonomical problems at the intra-generic level for many phytopathogenic nematodes such as *Ditylenchus* spp. (Wendt et al. 1993; Subbotin et al. 2005), *Anguina* spp. (Powers et al. 2001; Subbotin et al. 2004) or *Xiphinema* spp. and enables nematode detection based on species-specific PCR methods (Zijlstra 2000; Amiri et al. 2002; Wang et al. 2003). PCR using specific primers from ribosomal genes have been recently developed for detection and quantification of *D. dipsaci sensu stricto* (Marek et al. 2005; Subbotin et al. 2005). A multiplex PCR using SCAR primers based on AFLP polymorphic bands has been also used to differentiate the giant race from normal populations (Esquibet et al. 2003) but no primers were available for identifying populations of the *D. dipsaci* species complex associated with Fabaceae seeds, belonging either to *D. dipsaci sensu stricto*, or to *Ditylenchus* sp. B.

The aim of this study was to develop a PCR-specific assay for those last two species. Because the two groups appear to be divergent (Sturhan and Brzeski 1991;Esquibet et al. 2003; Subbotin et al. 2004, 2005), an in-depth investigation of the polymorphisms must be conducted in order to determine sequence regions that are conserved between those two groups but divergent in other related *Ditylenchus* species. A combination of PCR and RFLP analyses was carried out to characterize ITS regions of 20 isolates of the *D. dipsaci* species complex (both from *D. dipaci sensu stricto* and *D.* sp. B). After a first investigation of the polymorphism based on the use of restriction enzymes, nucleotide sequences of strains representative of each species were obtained

and aligned in order to design specific primers. Experimental PCR conditions were also defined to allow identification of nematodes belonging to the *D. dipsaci* species complex extracted from seeds.

Materials and methods

Nematode strains

Twenty populations of the *D. dipsaci* species complex from different geographical areas and one population each of *D. myceliophagus, D. africanus, D. destructor, Anguina tritici* and *Aphelenchoides ritzemabosi* were used in this study. They are listed in Table 1 with their characteristics and origin. The nematodes were extracted from either fresh or dried plant tissues. *Ditylenchus myceliophagus* was maintained monoxically on the fungus *Agaricus bisporus*, growing on malt-agar medium 4.5 g l^{-1} (Cayrol 1970).

DNA isolation

Stock genomic DNA of different strains belonging to the *D. dipsaci* species complex was prepared as a control for the first step of this study. DNA was extracted according to the phenol–chloroform procedure (Sambrook et al. 1989). Following ethanol precipitation, DNA was resuspended in TE buffer [(0.01 M Tris (pH 8.0), 0.001 M EDTA (Ethylene Diamine Tetra-acetic Acid)] and stored at −20°C.

Preparation of DNA template for PCR from single individuals

A fast method was developed to obtain DNA from single individuals of the *D. dipsaci* species complex suitable for use in PCR. Single nematodes were hand-picked, transferred onto a glass slide under a dissection microscope and crushed when dry by gentle pressure with the tip of a micropipette. The crushed nematode was recovered carefully with 13.7 µl of sterile distilled water and transferred into a 0.2 ml Eppendorf tube. The tube was immediately placed in ice. The crushed suspension was stored at −20°C and used for further study. The PCR reaction was performed directly from fresh or frozen crushed suspension.

ITS rDNA amplification

Partial 18 S-ITS1-5.8-ITS2-26 S rDNA was amplified by PCR using the primer sequences 5′-TTGAT-TACGTCCCTGCCCTTT-3′ (rDNA1 primer) from the 18 S rDNA and 5′-TTTCACTCGCCGTTAC-TAAGG-3′ (rDNA2 primer) from the 26 S rDNA (Vrain et al. 1992). PCR was performed in a 0.2 ml tube with a total volume of 20 µl of reaction mixture containing 0.5 µM forward and reverse primer, 200 µM each dNTP, one unit of Taq DNA polymerase (Qbiogene SA, France), and 5 ng of genomic DNA in a 1 × amplification buffer (75 mM Tris–HCl buffer at pH 9, supplemented with 20 mM $(NH_4)_2SO_4$, 0.01% (wt/vol) Tween 20, and 5 mM $MgCl_2$). PCR was carried out in a thermocycler (GeneAmp PCR System 9600; applied Perkin-Elmer inc Foster City, CA) programmed for 40 cycles consisting of a denaturing step at 94°C for 30 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 45 s. These cycles were preceded by an initial denaturation step at 94°C for 1 min and ended with a final extension at 72°C for 2 min. The PCR products were analysed by electrophoresis through 1.5% agarose gel in a 0.5 × Tris–borate–EDTA buffer, stained with ethidium bromide, and visualized under UV light (300 nm).

Digestion with restriction enzymes of amplified products

To assess sequence variability within and between species, amplified rDNA-ITS products of the *D. dipsaci* species complex, *D. myceliophagus*, and *Anguina tritici* isolates were digested with restriction endonucleases *Hae*III, *Hinf*I, *Msp*I, *Sau*3AI, *Taq*I and *Tsp*EI (Qbiogene, S.A. France). Reactions were carried out following the manufacturer's recommendations. Digestions were performed in a final volume of 20 µl with 15 µl of PCR products and 5 units of enzymes. Restriction fragments were separated on 3% Nusieve GTG agarose gels (FMC BioProducts, Rockland, ME) and visualized as previously described. A 1 kb molecular weight ladder (Eurogentec) was used as the size standard.

Table 1 Populations of nematodes tested in this study with PCR results

Species	GenBank Accession numbers	Code	Host plant	Origin	Source	PCR results with specific primers	
						Set 1	Set 2
Ditylenchus dipsaci sensu stricto	AY703059	Dd.F.1.1[a]	*Vicia faba*	France	G. Caubel	+	+
	AY703060	Dd.A.1.1[a]	*Vicia faba*	Australia	M. Scurrah	+	+
	NS	Dd.A.1.2	*Avena sativa*	Australia	M. Scurrah	+	+
	AY703061	Dd.F.1.2[a]	*Beta vulgaris*	France	V. Delannoy	+	+
	AY703062	Dd.F.1.3[a]	*Medicago sativa*	France	G. Caubel	+	+
	AY703063	Dd.F.1.4[a]	*Medicago sativa*	France	G. Caubel	+	+
	AY703064	Dd.DZ[a]	*Vicia faba*	Algeria	G. Caubel	+	+
	AY703065	Dd.TK[a]	*Allium cepa*	Turkey	S. Neman	+	+
	NS	Dd.F.1.5	*Secale cereale*	France	G. Caubel	+	+
	AY714535	Dd.SI.1[a]	*Daucus carota*	Sicily	G. Caubel	+	+
Ditylenchus sp. B	AY714536	Dd.T.1[a]	*Vicia faba*	Tunisia	G. Caubel	+	–
	AY714538	Dd.M.1.1[a]	*Vicia faba*	Morocco	F. Abbad Andaloussi	+	–
	AY714537	Dd.M.1.2[a]	*Vicia faba*	Morocco	F. Abbad Andaloussi	+	–
	NS	Dd.M.1.3	*Vicia faba*	Morocco	F. Abbad Andaloussi	+	–
	AY714539	Dd.M.1.4[a]	*Vicia faba*	Morocco	F. Abbad Andaloussi	+	–
	AY714540	Dd.M.1.5[a]	*Vicia faba*	Morocco	F. Abbad Andaloussi	+	–
	NS	Dd.F.2.1	*Vicia faba*	France	M. Esquibet	+	–
	AY714541	Dd.F.2.2[a]	*Vicia faba*	France	G. Caubel	+	–
	NS	Dd.F.2.3	*Vicia faba*	France	G. Caubel	+	–
	NS	Dd.Sy	*Vicia faba*	Syria	S. Bekal	+	–
Ditylenchus africanus	NS	Da.SA	*Arachis hypogaea*	South Africa	A. Mc Donald	–	–
Ditylenchus destructor	DQ151459	Ddes.F.3[a]	*Solanum tuberosum*	France	G. Caubel	–	–
Ditylenchus myceliophagus	DQ151458	Dm.F.4[a]	*Agaricus bisporus*	France	E. Panchaud Mattei	–	–
Anguina tritici	DQ151460	Atri F.5[a]	*Triticum aestivum*	Syria	R. Rivoal	–	–
Aphelenchoides ritzemabotsi	NS	Aphr.F.6	*Medicago sativa*	France	G. Anthoine	–	–

Set 1: DdpS1/rDNA2, Set 2: DdpS2/rDNA2

[a] strain whose rDNA was sequenced in this study

NS: no ITS DNA sequence available

DNA sequencing

rDNA regions were sequenced for 14 populations of the *D. dipsaci* species complex (eight of normal size and belonging to *D. dipsaci sensu stricto* and six of giant size associated with *Vicia faba* and belonging to *Ditylenchus* sp. *B*) and two strains of each other species (*D. myceliophagus, D. destructor* and *Anguina tritici)* (Table 1). PCR products were purified by a Qiagen Kit (Qiagen Hilden, Germany) and sequenced automatically with an ABI 377 DNA sequencer with Taq Dye Deoxy terminator sequencing kit (Applied Biosystems). The rDNA1 and rDNA2 primers were used for sequencing reactions.

Sequence analysis

ITS sequences were aligned using ClustalW (Thompson et al. 1994) with several sequences of the ITS region of the *D. dipsaci* species complex and related species. Virtual enzymatic restriction was performed with Seqweb Version 1.1 (Wisconsin Package Version 10) from the GCG software.

Primer design and PCR conditions for specific identification of *D. dipsaci*

In addition to DNA sequences obtained in this study, data on partial 18 S-ITS1-5.8 S-ITS2–partial 26 S sequences from other strains belonging to the genera *Ditylenchus, Anguina* (mostly from a recent study of the different genera of the Anguinidae family, Subbotin et al. 2004), and *Aphelenchoides* were obtained from GenBank (Table 2). After alignment, differences in nucleotides between these sequences and data obtained by PCR-RFLP were used to design PCR primers putatively specific to *D. dipsaci sensu stricto* (primer DdpS1) or specific to both *D. dipsaci sensu stricto* and *Ditylenchus* sp. B (primer DdpS2). Several putative specific primers were manually selected and tested.

Validation of the specific primers designed

To the defrosted suspension was added a PCR mixture containing 2 µl 10 × Qbiogene buffer with 25 mM $MgCl_2$ 0.2 µl 20 mM dNTPs Mix, 1 U Taq polymerase (5 U μl^{-1}) and one or two set primers. For a 'simplex' PCR the set of primers (DdpS2/rDNA2) was used at 0.5 µM for both the putative species-specific and the universal primer. For a duplex PCR using set1 and set2 primers (DdpS2/rDNA2 and DdpS1/rDNA2 respectively), DdpS1 was used at 0.5 µM, DdpS2 and rDNA2 at 1 µM. PCR conditions were the same as those described previously for ITS amplification. Negative control was performed with PCR mix- reaction without DNA. To assess the reproducibility of PCR test, specific amplifications were repeated several times.

Results

Length of PCR products of the ITS DNA region

A single amplification product of approximately 900 bp corresponding to the entire length of the ITS region (ITS1, 5.8 S ribosomal gene ITS2, Fig. 2)

Table 2 References of aligned sequences

Species and populations	Genbank Accession number	Host plant	Origin	Source
D. dipsaci	NI	*Allium sativum*	Brasilia	Leal-Bertioli et al. (2000)
D. dipsaci « A »	AF 396321	*Fragaria* sp.	Russia	Subbotin et al. (2004)
D. dipsaci « A »	AF 396320	*Medicago sativa*	Estonia	Subbotin et al. (2004)
D. dipsaci « A »	AF 396319	*Trifolium pratense*	Estonia	Subbotin et al (2004)
D. dipsaci « B »	AF 396323	*Vicia faba*	Morocco	Subbotin et al. (2004)
D. dipsaci « C »	AF 396322	*Cirsium setosum*	Russia	Subbotin et al. (2004)
D. destructor	AF 363110	*Solanum tuberosum*	NI	Powers et al. (2001)
A. tritici	AF 363107	*Triticum aestivum*	Mexico	Powers et al. (2001)
A. tritici	AF 363108	*Triticum aestivum*	Mexico	Powers et al. (2001)
A. tritici	AF 363109	*Triticum aestivum*	USA	Powers et al. (2001)

plus a small partial portion of rDNA (18 S and 26 S) was obtained for tested DNA samples originating from all the strains listed in Table 1.

After sequencing of those PCR products and alignment, a length difference of 31 nucleotides was observed between the 14 sequences of the *D. dipsaci* species complex and the *D. myceliophagus* sequence. Among the *D. dipsaci* species complex sequences, a single nucleotide difference in length between *D. dipsaci sensu stricto* and *Ditylenchus* sp. B was observed. Analysis of additional sequences published by Subbotin et al. (2004, 2005) confirmed this difference of at least one base between these two species (data not shown).

PCR-RFLP on the ITS region

Two enzymes out of the six tested did not produce any polymorphism between the samples studied: the four-base restriction enzyme *Hae*III which does not cut the DNA amplicons of any population, and the four-base restriction Sau3AI which cut at six sites for all the populations (data not shown). The enzymes *Hinf*I (Fig. 1A), *Taq*I (Fig. 1B), *Msp*I (Fig. 1C) and *Tsp*EI (data not shown) clearly separated the four species studied: *D. dispsaci sensu stricto*, *Ditylenchus* sp. B, *D. myceliophagus* and *Anguina tritici*. *Hinf*I, *Taq*I, *Msp*I, and *Tsp*EI provided the same restriction pattern within a given species of the *Ditylenchus dipsaci* species complex, but a different restriction pattern between the four species. (Fig. 2)

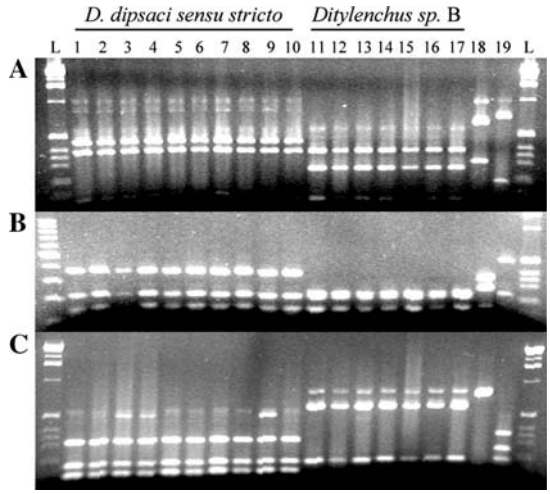

Fig. 1 Enzyme restrictions digests of ribosomal DNA amplified with universal primers (rDNA1 and rDNA2) from 1–17: *D. dipsaci* species complex (1–10: *D. dipsaci sensu stricto*, 11–17: *Ditylenchus* sp. B*)*, 18: *D. myceliophagus*, 19: *Anguina tritici*. **A**: *Hinf* I, **B**: *Taq*I, and **C**: *Msp*I. L: molecular weight markers (**A** and **C**: 1 kb Eurogentec, **B**: 1 kb Eurogentec (left) and 100 pb Promega (right))

DNA sequencing and species-specific primers

Sequencing of ITS regions for eight populations of *D. dipsaci sensu stricto,* six populations of *Ditylenchus* sp. B and three populations of other species (Table 1) was carried out to confirm the absence or the presence of restriction sites revealed by PCR-RFLP and to design species-specific primers. Sequence alignment of populations of the *D. dipsaci* species complex from diverse geographical origins displayed some mutations within species. Alignment of all sequences (including GenBank sequences of *D. dipsaci* and other species; Table 2) confirmed the presence of two main polymorphic regions. The first one (see Box 1 in Fig. 3A) differed between *D. dipsaci sensu stricto* populations and other *Ditylenchus* species. This region overlapped a *Tsp*EI restriction site and corresponded to two and four nucleotides mismatches for *D. myceliophagus* and *Anguina tritici* respectively. The second polymorphic region (see Box 2 in Fig. 3B) allowed the differentiation between *D. dipsaci sensu stricto* and *Ditylenchus* sp. B.

A first primer named DdpS2, was designed in order to have its 3′ extremity falling exactly on the two to four nucleotides mismatches observed when comparing *D. dipsaci sensu stricto*, *Ditylenchus* sp. B with other species (Fig. 3A; Table 3). The second primer named DdpS1 was designed on the *Taq*I restriction site region which shows two to three nucleotide mismatches between *D. dipsaci sensu stricto* and *Ditylenchus* sp. B (Fig. 3B; Table 3). These primers were used with reverse primer rDNA2 (‘universal’ oligonucleotide designed by Vrain et al. 1992), to amplify the DNA of the *D. dipsaci* species complex. The expected size of the PCR product was 707 bp for the first primer set (DdpS2/rDNA2) and 517 bp for the second set (DdpS1/rDNA2).

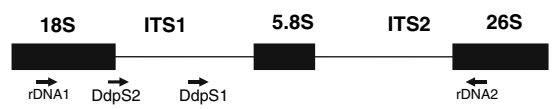

Fig. 2 Positions of primers in rDNA sequence

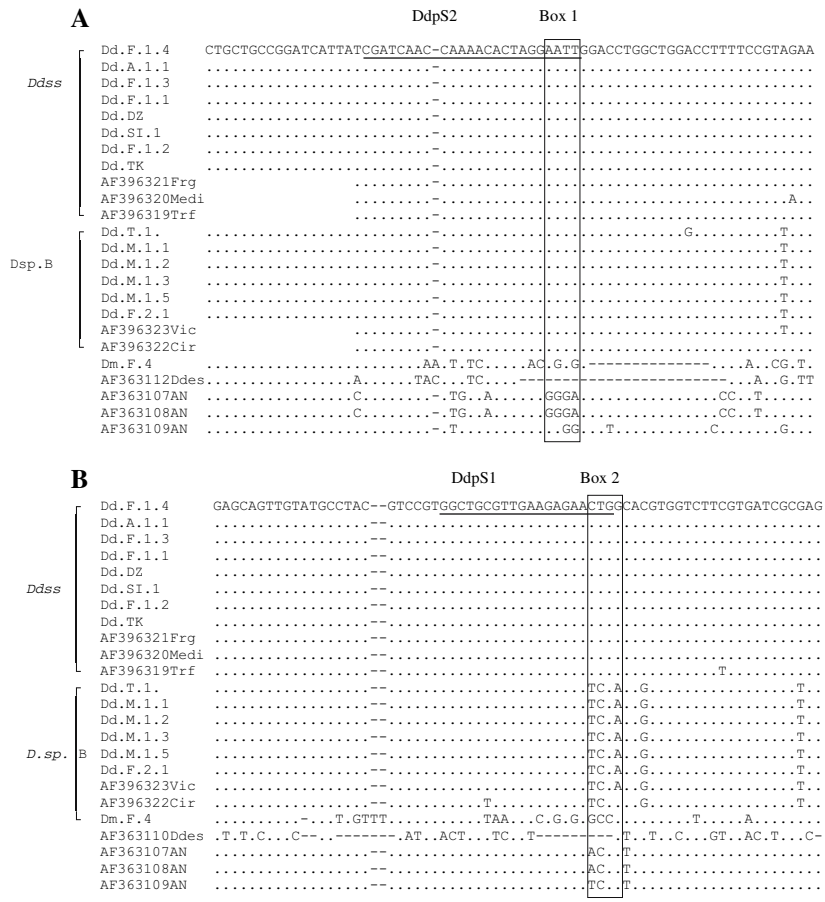

Fig. 3 Sequence alignment of rDNA fragments from *Ditylenchus dipsaci* and closely related species (bars '-' for gap and points '.' for identical nucleotide). Underlined characters indicate the sequence of the specific primers : DdpS2 (**A**) and DdpS1 (**B**) Critical restriction enzyme positions are in box. Box1 indicates the *TspE1* restriction site revealing some polymorphism between *D. dispsaci* and all the other Anguinidae species. Box 2 indicates the *Taq*I restriction site revealing some polymorphism between *D. dispsaci sensu stricto versus D*.sp. B

Table 3 Primer sequences

Primer	5′-3′ Sequence	Reference
rDNA1	TTGATTACGTCCCTGCCCTTT	Vrain et al. (1992)
rDNA2	TTTCACTCGCCGTTACTAAGG	Vrain et al. (1992)
DdpS2	CGATCAACCAAAACACTAGGAATT	This study
DdpS1	TGGCTGCGTTGAAGAGAACT	This study

Conditions for specific amplification

Validity of primer specificity was tested with genomic DNA extracted from all populations listed in Table 1 and with individually crushed nematodes. Standard conditions described for PCR of ITS regions were not modified for this specific amplification, except that the annealing temperature was increased to 60°C. In PCR using the two primers DdpS2/rDNA2, all populations of the *D. dipsaci* species complex provided a single 707 bp amplicon (Fig. 4A). DNA extracted from *D. myceliophagus*, *D. destructor*, *Anguina tritici* and *Aphelenchoides ritzemabotsi* does not amplify using these primers (Fig. 4A). In the PCR duplex case using the primers DdpS2/DdpS1/rDNA2, a single 707 pb amplicon was observed for all populations of the *D. dipsaci* species complex except the *D. dipsaci sensu stricto* which provided two amplicons (707 bp and 517 bp) (Fig. 4B).

Discussion

The rDNA internal transcribed spacer has been useful for diagnosis and phylogenetic relationships between

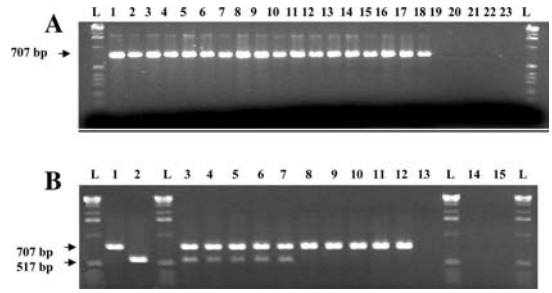

Fig. 4 PCR amplification of *D. dipsaci* DNA with species-specific primers.(**A**) Simplex PCR using the primers set 1 (DdpS2/rDNA2) on the *D. dipsaci* species complex and other species. 1–18: *D. dipsaci* species complex. 1–10, *D. dipsaci sensu stricto*, 11–18: *Ditylenchus* sp. B. 19: *D. myceliophagus.* 20: *D. destructor;* 21: *Anguina tritici*; 22: *Aphelenchoides ritzemabotzi.* 23 negative control without DNA. (**B**) Duplex PCR using the primers set1 and 2 on the *D. dipsaci* species complex and other species. 1, Dd.F.1.1 DNA amplified with DdpS2/rDNA2 primer combination and 2, Dd.F1.2. DNA amplified with DdpS1/rDNA2 primer combination. 3–15: PCR duplex, 3–7: *D. dipsaci sensu stricto*, 8–12: *Ditylenchus* sp. B. 13: negative control without DNA. 14*: D. myceliophagus*. 15: *Anguina tritici.* L: DNA ladder (1 kb Eurogentec)

various taxa of plant parasitic nematodes (Ferris et al. 1993). PCR–RFLPs are particularly suitable for population identification. Unfortunately, this strategy does not allow the identification of samples containing more than one species. The aim of this study was to characterize the ITS region of the *D. dipsaci* species complex in order to provide tools for identification of populations recognized as being associated with Fabaceae seeds.

Our first approach, a restriction study carried out on the rDNA region, clearly identified four enzymes (*Hinf*I, *Msp*I, *Taq*I, and *Tsp*EI) that enabled differentiation of 14 populations of the *D. dipsaci* species complex from *D. myceliophagus* and *Anguina tritici*. When considering *D. dipsaci sensu stricto* and *Ditylenchus* sp. B as separate species, very low intraspecific polymorphism was detected with these endonucleases.

Sequence data on these 14 populations confirmed the mismatches between the different species of *Ditylenchus*. Alignment of additional sequences published by Powers et al. (2001) and Subbotin et al. (2004) available in databanks or described by Leal-Bertioli et al. (2000) indicated that some substitutions differentiating *D. dipsaci sensu stricto* from *Ditylenchus* sp. *B* and located in a *Taq*I restriction site are conserved (Box 2, Fig. 3). This conserved polymorphism has been used to design the specific primer DdpS1. Moreover, 13 new sequences of *D. dipsaci sensu stricto*, to which should be added three sequences from Marek et al. (2005) and three new sequences of *Ditylenchus* sp. B have been recently described in Subbotin et al. (2005). In silico analyses confirmed that our DdpS1 primer should amplify all populations of *D. dipsaci sensu stricto* but no other species, such as *Ditylenchus* sp. B or any more distantly related Anguinidae species, due to the 2 nucleotides mismatch at the 3′ end of our oligonucleotide. These results confirm a wide divergence between the two taxa *D. dipsaci sensu stricto* and *Ditylenchus* sp. B as highlighted by several authors (reviewed in Subbotin et al. 2005).

Until recently, EPPO considered *D. dipsaci, Ditylenchus* sp. B and *D. dipsaci sensu stricto* as quarantine nematodes. A new primer set has been developed to amplify both taxa which are of great economic importance. A similar approach was used for the primer construction, and the *TspE*1 restriction site was selected to design the primer called DdpS2. The restriction site is conserved in all populations of *D. dipsaci sensu stricto* and *Ditylenchus* sp. B investigated, while there are at least two mutations located in this site in all other species. The PCR primer set (DdpS2/rDNA2) was successfully used to amplify various populations of *D. dipsaci sensu stricto* and *Ditylenchus* sp. B regardless of their geographic origin (e.g. Algeria, France, Morocco, Syria). The numerous additional sequences of the *D. dipsaci* species complex provided by the study of Subbotin et al. (2005) or Marek et al. (2005) allow confirmation of the utility of our DdpS2 primer on a larger set of populations or species. All the 16 additional sequences of *D. dipsaci sensu stricto* as well as the three additional *Ditylenchus* sp. B sequences match perfectly our 24 base-oligonucleotide DdpS2 primer, while *Ditylenchus* sp. D and E or more distantly related species of Anguinidae (e.g. *Subanguina radicicola, Anguina* sp) show a 2 (or more) base mismatch at the 3′ end of the oligonuclotide.

This in silico analysis also revealed that the DdpS2 primer should also amplify *Ditylenchus* sp. C (from Russia and associated with *Cirsium setosum*), sp. *F* (from Central Asia and associated with *Pilosella* sp.) and sp. G (from Germany and associated with *Plantago maritima*) as well as five species of the

Mesoanguina genus (*M. kopetdaghica, M. mobilis, M. picridis, M. pharangii* and *M. varsobica*, all associated with Asteraceae from Central Asia except *M. mobilis* from Australia) and one species of the *Heteroanguina* genus (*H. ferulae* associated with Apiaceae from Central Asia). Because of the geographic distribution of these species (Central Asia or Australia), or the specific range of *Ditylenchus* sp. G (confined to *Plantago maritima*, a species restricted to salt mud on the seashore), we are confident that our DdSp2 primer should not provide false positive results which might suggest the presence of the quarantine species '*D. dipsaci*' (*D. dipsaci sensu stricto* and *Ditylenchus* sp. B) in the analysis of nematodes extracted from Fabaceae seeds produced in Europe.

All these *Mesoanguina* species form a well supported group in the Anguinidae phylogeny described by Subbotin et al. (2005), and with *H. ferulae*, these two taxa are the sister groups of *Ditylenchus* sp. B and C. As already discussed by Subbotin et al. (2005), our results highlight the need for a revision of the Anguinidae family, with a description of all unnamed species of *Ditylenchus*. This would be especially useful for *Ditylenchus* sp. B (the giant, polyploid populations associated with *Vicia faba*), which, as a distinct species, could be considered as a new quarantine pest.

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