

Using Point Mutations in rDNA for Differentiation of Bioraces of *Ditylenchus dipsaci* from the Czech Republic

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Abstract

Ditylenchus dipsaci is one of the most harmful parasitic nematodes in Central Europe. It is able to survive for long time in soil without its host plants and that is why it belongs to organisms with quarantine importance. Nothing is known about *D. dipsaci* distribution in the Czech Republic. The aim of the study was to collect samples of *D. dipsaci* from the Czech Republic and to identify them by molecular methods. Region of rDNA including 3' end of *18 S* gene, ITS1, *5,8 S* gene, ITS2 and 5' end of *26 S* gene was amplified using general primers designed according to the DNA sequence of *Caenorhabditis elegans*. The amplicon (900 bp) was analyzed by RFLP and SSCP. Restriction endonucleases Eco R1, Hinc II and Alu I can be used for differentiation of certain bioraces of *D. dipsaci*. At the same time methods for DNA extraction from plant material and contaminated soil were optimized.

Keywords: *Ditylenchus dipsaci*; DNA; SSCP; RFLP; PCR; biorace; differentiation

INTRODUCTION

Ditylenchus dipsaci is free living stem phytoparasitic nematode. There are many biologic races differing in host range. It belongs to pests of quarantine importance. It occurs on more than five hundred plant species, mainly on onion, garlic, lucerne, potato and other crops and weeds. The nematode causes swellings and deformations of above ground parts of plants and necroses of stems, bulbs and roots (SHURTLEFF & AVERRE 1997).

Ditylenchus dipsaci is resistant to low temperatures. Under the conditions of Central Europe it overwinters without any damages. It survives in dry material several years in anabiosis. It is one of the most harmful nematodes in temperate regions. It can completely destroy infected plant in absence of control. The key factor in *D. dipsaci* control is early specific diagnosis including monitoring of its occurrence, prognosis of its occurrence and indication to integrated control (BRZESKI 1998). For diagnosis morphology can be used for clear species identification but it is not utilizable for biorace differentiation. Biorace-specific

antibodies have also been raised. Different antigens from nematodes from *Tripolium repens*, *Medicago sativa* and *Narcissus* sp. Give different reactions with antibodies from *Tripolium pretense* biorace (GIBBINS & GRANDISON 1968). Differences in nucleic acid sequences can be also used for diagnostics. For *Ditylenchus* species and bioraces differentiation internal transcribed spacer 1 (ITS1) and *18 S* gene region are very important. They can be amplified in PCR 900 bp long. Amplicon can be used in subsequent methods revealing mutations both in palindromic sequences of restriction endonucleases and in other regions. Restriction cleavage was used for differentiation of *D. dipsaci*, *D. destructor* and *D. myceliophagus*. All three species differentiated using PCR amplification of rDNA and subsequent restriction cleavage (WENDT *et al.* 1993). We used similar method for *D. dipsaci* bioraces differentiation and single strand conformation polymorphism to finding point mutation together. This sensitive method is able to detect exchange of one base, which can cause change of secondary conformation of ssDNA. It needs some conditions to obtain satisfactory results (ORITA *et al.*

1989). Concentration of gel, AA:BA ration, strange of gel, amount of DNA loaded and temperature were optimized for a conditions of the laboratory (SAVOV *et al.* 1992). For obtaining of optimal large of DNA fragments before using SSCP its possible used restriction digestion (SHEFFIELD *et al.* 1993).

MATERIAL AND METHODS

Several methods of DNA extraction were optimized. First, DNA extraction from single nematodes had to be optimized. DNA extraction from small amount of material of sperm was used (LI *et al.* 1988; FOLKERTSMA 1997). Several steps of the method were changed for DNA extraction from single nematode. DNA extraction from soil was done using kit from Q-biogene. Samples of sterilized soil spiked with certain number of nematodes (200, 150, 100, 50 and 10 nematodes/500 mg of soil) were tested.

For amplification of rDNA cistron between 18 S and 26 S genes specific primers for *Caenorhobditis elegans* rDNA amplification primers published by FALLAS (1996) were used. Using these primers 18 S 5'-TTG ATT ACG TCC CTG CCC TTT-3' a 26 S 3'-GGA ATC ATT GCC GCT CAC TTT-5' 900 bp fragment of *D. dipsaci* can be amplified. PCR was done in MJ research PTC 200 thermocycler using following program: 1. 94°C 2', 2. 94°C 1', 3. 60°C 30'', 4. 72°C 1', 5. 30 × from the step 2, 6. 72°C 4', 7. 4°C, 8. end. 50 µl reaction mixture contained 1.5mM MgCl₂, 0.4mM each dNTP (Promega), 12.5 pmol of each primer, 1.5 U of Dynazyme (Finzymes), 1 × reaction buffer, 1 µl of extracted DNA. Product of PCR was visualized after electrophoresis in 1% agarose gel in TBE buffer on UV transiluminator 260 nm.

On the basis of sequence analysis some restriction endonucleases were chosen for digestion: Eco RI, Hinc II, Alu I, Taq I, Bam HI, Eco 321, Bsu RI, Hinf I, Eco 881, Hind III, Dra I, Msp I, Rsa I, Hin GI, Xho I, Bse GI.

Digestion was done in 200 µl eppendorf tubes. The mixture contained 10 µl of PCR product, 1 × buffer, 8 units of enzyme. The mixture was overlanged by 20 µl of mineral oil and incubated in appropriate temperature (37°C, 55°C or 65°C) for 9 h. Visualization was done as in the case of PCR product (see above).

For SSCP 5 µl of PCR product, 5 µl of deionized formamid and 2 µl of loading dye was used. This mixture was boiled for 5 minutes and then chilled on wet ice. After short spinning the mixture was loaded on a 10% nondenaturing polyacrylamide gel. Electrophoresis was done in 17°C and 66 V 17 h. Optimal length of fragment for SSCP is around 150 bp (SHEFFIELD *et al.* 1993). Thereby amplicon from PCR was also digested by several restriction endonucleases and finally Hinf I was chosen for this purpose. Then electrophoresis was done under the same conditions as above but only for 8 h. After electrophoresis gels were stained by Ag⁺ ions.

RESULTS AND DISCUSSION

Methods of DNA extraction from single nematode, plant material and soil where optimized. Extraction from soil enabled detection of 100–200 nematodes in 500 mg of soil.

D. dipsaci populations from garlic had specific and homogene patterns of restriction sites in RFLP and they could not be further differentiated neither by RFLP or by SSCP. *D. dipsaci* population from (A003, Stiglitz (5), Stiglitz (1), Lipůvka, 16708, Věstec, Havránek 2001, Sekanina-Žerůvky) pattern was very similar to that of garlic populations. On the other side populations from lucerne (Slovenia, Stiglitz, CR) had very different patterns in RFLP, SSCP and also in confinement RE-SSCP. Similar differences was also in population from onion.

In some cases bioraces of *D. dipsaci* can be differentiated by molecular methods. More populations of *D. dipsaci* should be analyzed to prove the utility of



Figure 1. Scheme of studied cistron rDNA *D. dipsaci*

these methods and to prove that obtained differences are consistent to each biotype.

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