Appendix

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Molecular and Biochemical Methods Used for the Identification of *Globodera* Species in Slovenia

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Abstract

Globodera species identification is based on specific morphological characteristics which are included in the majority of keys used for *Globodera* identification. To confirm and make more precise the morphometrical method, other methods have been developed. At Agricultural Institute of Slovenia, molecular and biochemical methods are applied. With the use of PCR-RFLP method we try to distinguish between *G. rostochiensis*, *G. pallida* and *G. tabacum*. Patterns of nematode DNA digested with restriction endonucleases and subjected to agarose gel electrophoresis are analysed. Differences in DNA sequence result in the number and size of fragments produced (RFLPs). For the differentiation of all three nematodes we have introduced a method for protein electrophoresis. Samples are compared after being separated by polyacrilamide slab gel electrophoresis. Proteins are visualised after silver staining.

Keywords: potato cyst nematode; *Globodera*; identification; PCR; isoelectric focusing (IEF) method

The potato cyst nematodes (PCN), *Globodera rostochiensis* and *Globodera pallida* are considered economically the most important plant parasitic nematodes of potato plants. Until now *G. rostochiensis* has been spread in Slovenia (Urek & Lapajne 2001), while *G. pallida* was intercepted only. A correct identification of *Globodera* species is therefore important to make a successful integrating management program. In this paper, molecular (PCR-RFLP) and biochemical (IEF) methods for *Globodera* species identification in Slovenia are described. PCR-rDNA-RFLP technique, used for routine identification of PCN, is based on differences in DNA sequences. Molecular markers, restriction fragments length polymorphism (RFLPs) of rDNA internal transcribed spacer (ITS) region, are species specific. Fragments are separated by size using agarose gel electrophoresis and stained with ethidium bromide, a dye which binds to DNA and fluoresces when exposed to UV light (Williamson 1991).

This contribution was presented in the panel discussion of the 6th Conference of European Foundation for Plant Pathology, Prague, Czech Republic, 8–14 September 2002.

Isoelectric focusing (IEF) method is applied for the separation of proteins in a pH gradient where the sample components migrate towards the anode or catode, until their net charges reach zero – their isoelectric point (pI). Proteins are driven by the electric field to their pI, which is estimated with calibration marker proteins. The method of miniaturised electrophoresis system (PhastSystem, Pharmacia) enables the study of small amounts of soluble proteins, even from a single female cyst (Karssen *et al*. 1995).

MATERIALS AND METHODS

Nematodes. Cysts of *Globodera rostochiensis* (originally isolated from samples taken in the field at Libeliče, Slovenia) and *Globodera pallida* (intercepted at Slovene-Italian border) were laboratory cultured on potato *Solanum tuberosum* plants. Extracted cysts were used to optimise both methods.

Method 1 – ITS-RFLP. For ITS-RFLP analysis, DNA of a single vital cyst is extracted using Promega Wizard purification kit. 1 µl of extracted DNA is added to each PCR reaction containing 10mM Tris-HCl pH 8.3, 25mM $MgCl_2$ (Promega), dNTP-mixture 10mM each, 1µM of Ferris forward and reverse (Ferris *et al*. 1995), 1U Taq Polymerase (Promega) and sterile distilled water up to 50 µl. The primers used enable the amplification of rDNA region starting near the end of the *18S* gene and terminating shortly into *28S* gene including the ITS regions and *5,8S* gene. The amplification is carried out with a thermocycler (A&B gene AMP PCR system 2700) and the reaction conditions are as follows: at 94°C for 2.5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 45 s, polymerisation at 72°C for 1 min, and a final extension cycle at 72°C for 2 min. 4 µl of PCR product is treated with 10 U restriction endonuclease. The reaction is carried out with five different restriction enzymes, Alu1, Rsa1, Msp1, Hinf1 and Mbo1. The mixture is incubated at 37°C overnight to digest the PCR product completely. The restriction fragments are separated on 2% agarose gel stained with ethidium bromide, visualised and photographed under UV light.

Method 2 – IEF. Before electrophoresis, dry cysts are pre-soaked in 1% glycerol for at least 12 h at room temperature. Single cyst is placed in 0.7 µl of 1% glycerol and crushed with small glass rod

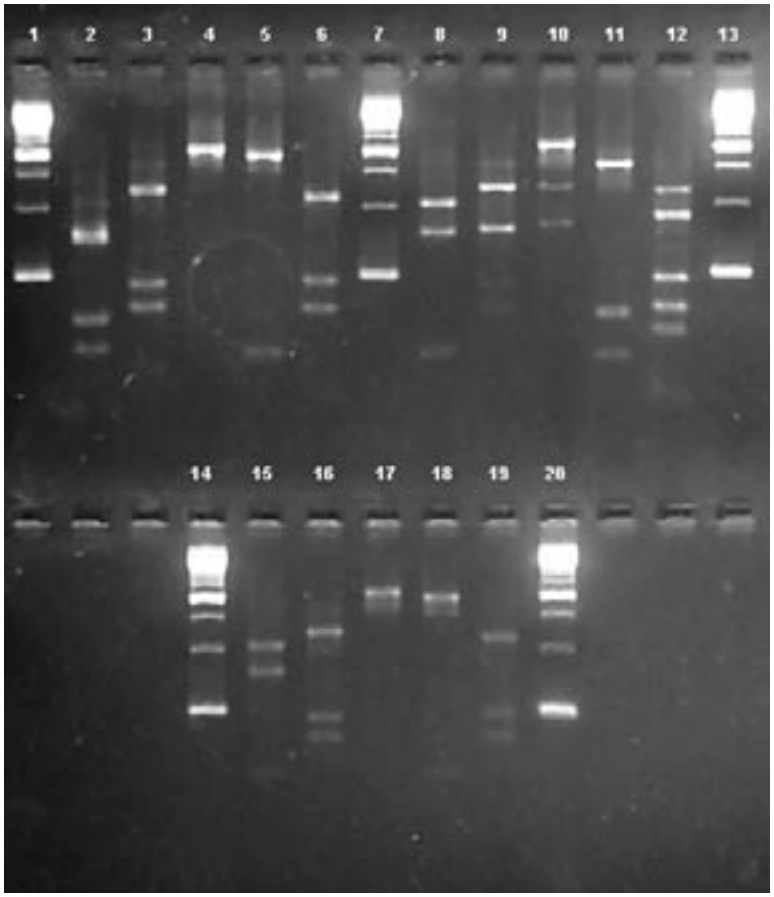

Figure 1. Restriction fragment length polymorphism of the ITS1, 5,8S and ITS2 rDNA region of *G. rostochiensis* (lines 2, 3, 4, 5, 6), *G. pallida* (lines 8, 9, 10, 11, 12) and *G. tabacum* (lines 15, 16, 17, 18, 19). Different restriction nucleases are used: Alu l (lines 2, 8, 15), Rsa l (lines 3, 9, 16), Msp l (lines 4, 10, 17), Hinf l (lines 5, 11, 18) and Mbo l (lines 6,12,19)

to release the egg contents. 0.3 µl of each sample is applied on gel. After electrophoresis the gel is silver stained with PhastGel silver kit.

RESULTS

Method 1 – ITS-RFLP analysis

RFLP patterns are used to distinguish between *Globodera* species. Digestion of PCR product with restriction endonucleases Alu l, Rsa l, Hinf l and Msp l, enable the definitive discrimination of *G. rostochiensis* from *G. pallida* while only Alu l discriminates between *G. rostochiensis* and *G. tabacum* (Figure 1).

Method 2 – IEF analysis

The pI of proteins is estimated with calibration marker proteins (Pharmacia broad pI calibration kit 3.5–9.3). *G. pallida* and *G. rostochiensis* show one major band at pl 5.7 and pl 5.9 respectively (Figure 2A and B) and one weaker band at pl 8.7 and pl 6.9 respectively (Figure 2C and D). To upgrade the differences between these bands samples it can be run on gels with more narrow pH range – PhastGel 5 – 8 (Karssen *et al*. 1995).

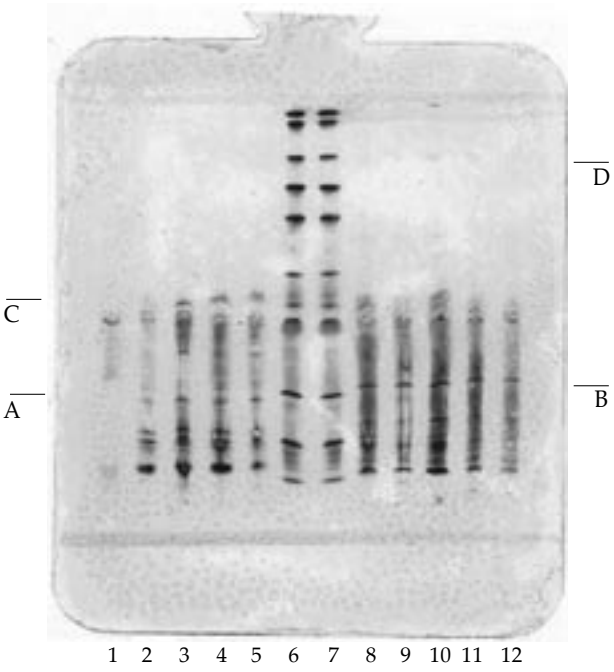

Figure 2. IEF pattern of *G. pallida* (lines 1, 2, 3, 4, 5), *G. rostochiensis* (lines 8, 9, 10, 11, 12) individual female cyst and broad pl calibration kit (lines 6, 7). IEF on PhastGel 3–9, followed by silver staining. A, B, C and D mark differences in pl

DISCUSSION

Molecular and biochemical methods have enabled the rapid and reliable identification of nematodes. However, for both methods it is essential to use fresh material (young, full cysts) which is sometimes not available. In such cases, only morphometrical methods can be used. We believe that combining morphometrical methods and at least one molecular or biochemical method is a guarantee for proper identification of *Globodera* species.

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