Detection and Differentiation of the Potato Cyst Nematodes *Globodera rostochiensis* and *Globodera pallida* by PCR*

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Abstract

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The potato cyst nematodes (PCN) *Globodera rostochiensis* and *G. pallida* were detected and differentiated by PCR using several specific primers situated to a small region between the internal transcribed spacer 1 (ITS 1) and the 5.8 S ribosomal RNA gene region. The method is relatively fast (7 h or less) and very specific. We were able to detect and identify PCN from single cysts with viable eggs and also from single mature eggs.

Key words: potato cyst nematode; *Globodera rostochiensis; Globodera pallida;* detection; PCR

The potato cyst nematodes *Globodera rostochiensis* (Wollenweber, 1923) (GRO) and *G. pallida* (Stone, 1973) (GPA) are closely related plant pests capable to cause severe yield losses in potato crops. Both are listed as EPPO quarantine pests. Whereas some potato varieties are resistant to GRO pathotypes, no varieties are completely resistant to GPA (ANONYM 1996). It means that a field once contaminated by GPA is excluded from potato cultivation for a relatively long period. It is, therefore, crucial for the Plant Protection Service to be able to distinguish the two species of PCN. Several methods exist to do this: the classical diagnostics using analysis of certain metric characters like length of stilets or number of lines between anus and vulva of cysts (FRANCO 1978; GOLDEN 1986) is time-consuming, not always convincing, a larger number of nematodes is required and only an experienced person can do it. Electrophoresis of species-specific proteins (BAKKER *et al.* 1988), 2D-protein gels (BAKKER & GAMMERS 1982; ROOSIEN *et al.* 1993) and IEF (FLEMING & MARKS 1982) is possible. During the last several years molecular methods like RFLP (CURRAN *et al.* 1985), DNA hybridisation (BURROWS & PERRY 1988), RAPD (FOLKERTSMA *et al.* 1994) and AP PCR (ROOSIEN *et al.* 1993) have been developed for PCN diagnosis. The problem is that all these more modern methods are either too complex and time-consuming, or interpreting the results is difficult. Several suppliers offer monoclonal antibodies for ELISA, but an assay still takes about 15 h. PCR offers a relatively facile possibility to detect nematodes in a short time (VRAIN & MCNAMARA 1994). Recently, very effective PCR methods of PCN differentiation were published (MULHOLLAND *et al.* 1996; SHIELDS *et al.* 1996). We adapted these methods for the routine PCN diagnosis in our reference diagnostic laboratory.

MATERIAL AND METHODS

Potato Cyst Nematodes: Cysts of PCN pathotypes GRO 1 (Šluknov), GRO 1 (Hannover), GRO 2/3 (Obersteinbach), GRO 4, GRO 5 (Harmerz), GPA 2 (Kalle), GPA 3 (Delmsen) and GPA 3 (Chavornay) were obtained from Ing. Gaar from the State Phytosanitary Administration and from Mr. Brož from the Potato Research Institute Havlíčkův Brod.

DNA Extraction: Cysts were crushed in liquid nitrogen with mortar and pestle and homogenized in extraction buffer (1 cyst per 20 µl of 50mM Tris HCl pH 8.0, 0.7M NaCl, 10mM EDTA, 1% CTAB and 20 mM β-mercaptoethanol). The homogenate was heated to 60°C for 2 h, mixed 1:1 with chloroform – isoamylalcohol (24:1), vortexed for 10 min and centrifuged 10 min at 8000 rpm. An equal volume of isoamylalcohol was added to the supernatant and DNA was precipitated at –25°C for 9–12 hrs or in liquid nitrogen for 10 min. Then it was centrifuged (10 min at 7000 rpm) and the pellet was washed with 40 µl of 80% ethanol with 10mM LiCl and 1mM Tris. After 15 min the microtubes were centrifuged 10 min at 7000 rpm. The

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pellets were dried under vacuum and resuspended in 10 µl water.

DNA from single cysts was extracted after crushing the cysts in microtubes with a minishaker in the presence of 20 µl of extraction buffer. Crushed cysts were 5 times frozen in liquid nitrogen and thawed in a water bath at 60°C. DNA from single mature eggs was extracted after crushing of cysts with a glass rod under a microscope. Single mature eggs were transferred by a micromanipulator into microtubes with 20 µl of extraction buffer. The microtubes were then frozen and thawed as described above. In both cases DNA was extracted with the method outlined above.

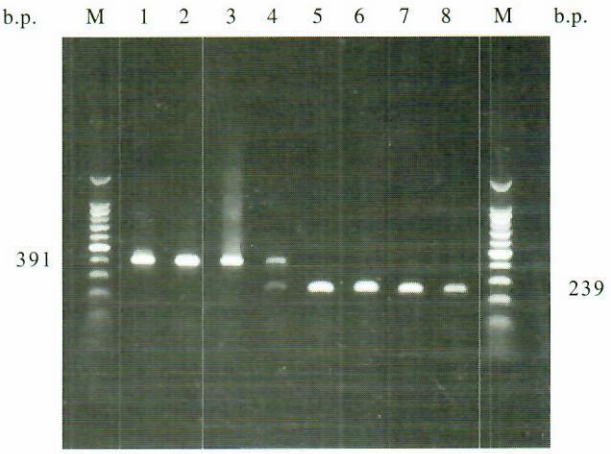
A simplified protocol of DNA extraction was also used. Cysts were crushed in liquid nitrogen and homogenized in water, TE, CTAB extraction buffer or directly in the appropriate buffer for DNA polymerase. After heating at 60°C for 2 h the extracts were centrifuged (10 min at 7000 rpm) and used in PCR. The shortest method of DNA extraction was to crush cysts in water, TE, CTAB extraction buffer or appropriate buffer for DNA polymerase, boiling for 2 or 5 min and centrifuging (10 min at 7000 rpm).

Primers: As knowledge of sequences of DNA of both species of PCN is very limited we were confined to a small region of the genome between the interial transcribed spacer 1 (ITS 1) region and the 5.8 S ribosomal RNA gene sequence published by MULHOLLAND *et al.* (1996). We, therefore, used the same three primer system, e.g., one universal downstream primer which binds to both species (UNI, 5´-GCAGTTGGCTAGCGATCTTC-3´), one primer which binds to GPA (GPA 1, 5´-GGTGACTCGACGATTGCTGT-3´) giving product 391 b.p., and another which binds to GRO only (GRO 1, 5´-TGTTGTACGTGCCGTACCTT-3´ or GRO 1* with one A added to the 5´end) giving a product 238 or 239 b.p. long, respectively. Later we designed primers situated in the same region: GRO 2 (5´-GCCAACGGAGGAAGCAC-3´) and GPA 2 (5´-ACCAACGGAGGTGGCAC-3´), both giving a product 356 b.p. long and GRO 3 (5´-CGCCTTGCAGATATGCTAAC-3´) giving a product 271 b.p. long. All these primers are used in combination with primer UNI, GRO 2 and GPA 2 in separated reactions and GRO 3 with GPA 1.

PCR: 25 µl reactions contained 10 pmol of each primer, 2.5 units of Taq DNA polymerase (Promega), Taq Gold DNA polymerase (Perkin-Elmer), Stoffel fragment of Taq polymerase (Perkin-Elmer), Dynazyme (Finnzymes) or Tfl DNA polymerase (Promega), 0.2mM of each dNTP, 1.5mM $MgCl_2$ for Taq DNA polymerase, 4mM $MgCl_2$ for Stoffel fragment, 1.5mM $MgSO_4$ for Tfl polymerase, 1× appropriate buffer and usually 1 µl of DNA extract. The only exception was in the case of single eggs, where total extracted DNA was taken. PCR amplification was performed in a MJ Research PTC 150 cycler usually for 35 cycles of 94°C for 1 min, 62°C for 30 s and 72°C for 1 min. These cycles were followed by a 72°C incubation for 5 min and for Taq Gold polymerase preceded by a 95°C incubation for 5 min. 5 µl aliquots were removed from the reaction and subjected to electrophoresis on a 1% agarose gel. Products were visualised by staining with ethidium-bromide. A 100 b.p. ladder (Promega) was used as molecular size standard.

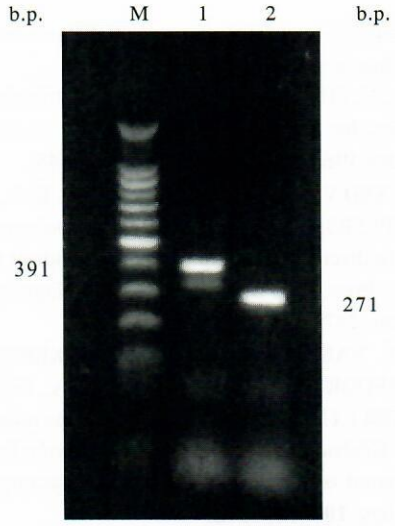
RESULTS

The three primers system with primers UNI, GRO 1* and GPA 1 was able to detect and differentiate all isolates of *G. rostochiensis* and *G. pallida* in DNA extracts both from several cysts (Fig. 1) and from one cyst. We were also able to detect PCN species from only one egg, but in some cases the PCR failed. PCNs were also succesfully identified in simple extracts in TE, CTAB extraction buffer and appropriate buffer for DNA polymerase (both after heating at 60°C for 2 h and boiling for 2 min), but not in aqueous extracts (data not shown). Boiling of extracts for 5 min substantially reduced product formation. Initially we used primer GRO 1 and annealing temperature 55°C, but under these conditions in GRO DNA extracts a 391 b.p. fragment thought to be specific for GPA was also present, even when using Stoffel fragment of DNA polymerase or hot start with Taq Gold polymerase. This band disappeared when primer GRO 1* was used after a higher annealing temperature of 62°C. All DNA polymerases tested (Taq, Taq Gold, Tfl, Stoffel fragment of Taq, Dynazyme) were under these conditions able to generate the expected products for both species. Another three primers system with UNI, GRO 3 and GPA 1 primers was also successful in distinguishing between both species of PCN (Fig. 2). Likewise, the primers GRO 2 and GPA 2 in combination with UNI in separated reactions gave the expected products (Fig. 3).



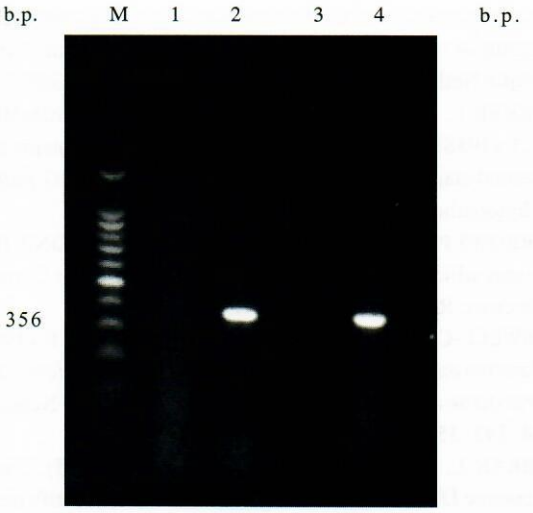
Lane M – molecular marker; Lane 1 – DNA GPA 3 (Delmsen); Lane 2 – DNA GPA 3 (Chavornay); Lane 3 – DNA GPA 2 (Kalle); Lane 4 – Mix DNA GRO 1:GPA 2 1:2; Lane 5 – DNA GRO 5; Lane 6 – DNA GR0 4; Lane 7 DNA GRO 2,3; Lane 8 – DNA GRO1

Fig. 1. PCR products from reaction using primers GRO 1*, GPA1, UNI



Lane M – molecular marker; Lane 1 – DNA GPA 2; GRO 3, GPA 1, UNI; Lane 2 – DNA GRO 1; GRO 3, GPA 1, UNI

Fig. 2. PCR products from reactions using primers GRO 3, GPA 1, UNI



Lane M – molecular marker; Lane 1 – DNA GRO 1; GPA 2+UNI; Lane 2 – DNA GPA 2; GPA 2+UNI; Lane 3 – DNA GPA 2; GRO 2+UNI; Lane 4 – DNA GRO 1; GRO 2+UNI

Fig. 3. PCR products from reactions using primers GRO 2, GPA 2, UNI

DISCUSSION

A PCR method for species-specific discrimination of PCN was adapted for routine use in our reference diagnostic laboratory. Compared with other methods it is more specific and unambiguous results are obtained in a shorter time of about 7 hrs when classical DNA extraction is used or even less if the cyst extract is boiled for 2 min. An annealing temperature of 55°C (MULHOLLAND *et al.* 1996) was used at the beginning of our study, but we encountered nonspecific fragments in GRO extracts. MULHOLLAND *et al.* (1996) faced this problem by using the Stoffel fragment of Taq DNA polymerase which lacks the 5′–3′ exonuclease activity so that lesions in its path are not repaired. In our hands the Stoffel fragment did not improve the specifity of PCR. We also tried hot start PCR using Taq Gold polymerase, again without success. Therefore, we designed primer GRO 1* with one A added to the 5′end and gradually raised the annealing temperature to 62°C. This worked well with all DNA polymerases used in these tests. We thus suppose that the problem lay in nonspecific annealing of GPA 1 primer to GRO DNA. As the sequence of PCN DNA at sites where specific primers bind differs only in one or two bases, respectively, nonspecific binding can also occur if the annealing temperature is not high enough. According to our experience, in most cases an annealing temperature $T_a = 2(A + T) + 4(C + G)$ is the best.

One cyst with viable eggs was quite sufficient to reliably distinguish between both species of PCN. We were also able to detect the PCN species from only one mature egg, but for unknown reasons it sometimes failed even though PCN DNA was present in extracts as revealed by spectrophotometer and also by RAPD with several random primers known to match with PCN DNA. Similar difficulties were also encountered by WILLIAMSON *et al.* (1997) with juveniles of *Meloidogyne* sp. Perhaps the specific sequence of DNA amplified in this system is rather rare and can be lost if such a small amount of DNA has to be extracted.

The primers GPA 2 and GRO 2 also gave specific products in separated reactions, but a three primers system to differentiate PCNs has a substantial advantage in that an internal control is built into the reaction. It means that either a band specific for GRO or a band specific for GPA must be present. Moreover it is less expensive and less laborious.

For DNA extraction all methods were about equally suitable, with the only exception of extraction with water. Similar results with water extraction were obtained by MULHOLLAND *et al.* (1996) who reported low yield or failure of certain PCR amplifications. We have also tried a recommended method of treating aqueous extracts with a resin like Chelex-100 (CASWELL-CHEN *et al.* 1992), but use of TE or DNA polymerase buffer for extraction works equally well and is quite simple. Extraction with DNA polymerase buffer may be advantageous if the whole amount of extracted DNA is to be taken for the PCR reaction as was the case with single mature eggs.

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Souhrn

ZOUHAR M., RYŠÁNEK P., KOČOVÁ M. (2000): **Detekce a diferenciace druhů háďátek** *Globodera rostochiensis* **a** *Globodera pallida* **metodou PCR.** Plant Protect. Sci., **36**: 81–84.

Oba druhy háďátek, *Globodera rostochiensis* a *Globodera pallida*, byly detekovány a rozlišeny metodou PCR (polymerázová řetězová reakce) při použití specifických primerů situovaných do oblasti mezi interním přepisovaným spacerem 1 (ITS 1) a 5.8 S genem pro ribozomální RNA. Použitá metoda je relativně rychlá (7 hodin i méně v závislosti na způsobu extrakce DNA) a vysoce specifická. K detekci postačuje jediná živá cysta a případně jediné zralé vajíčko.

Klíčová slova: háďátko bramborové; *Globodera pallida*; *Globodera rostochiensis*; detekce; PCR

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