Identification of PCN species (*Globodera rostochiensis*, *G. pallida*) by using of ITS-1 region's polymorphism

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ABSTRACT

Progressive methods of molecular analyses of DNA are routinely used in the fields of zoological and botanical taxonomy, pest management and plant breeding. Knowledge of species-composition in populations of potato cyst nematodes (*Globodera rostochiensis*, *G. pallida*) is very important for selection of appropriate measure of regulation PCN's occurrence. The molecular method for distinguishing of PCN species is described in this article. European populations of PCN – Šluknov (Ro1), Obersteinbach (Ro2), Harmerz (Ro5), Kalle (Pa2), Chavornay (Pa3), Delmsen (Pa3), and some cysts of unknown pathotype from Kašperské hory (K) locality were used. Species-specific sets of primers for ITS-1 (Internal Transcribed Spacer 1) amplification were designed on base of known sequences ITS-1 of both PCN species by using of freeware *Primers! for the World Wide Web*. By using of set Fro1-Rro1 was product 411 bp detected (only in cause *G. rostochiensis*), by using of set Fpa2-Rpa1 the product 239 bp was detected (only *G. pallida*). For these reasons the identity of the European populations was confirmed. Cysts of population K were identified as *G. pallida*.

Keywords: *Globodera rostochiensis*; *Globodera pallida*; ITS-1 region; PCR; DNA markers; species identification

Potato cyst nematodes (PCN) – *Globodera rostochiensis* (Wollenweber, 1923) Behrens, 1975 (yellow potato nematode) and *Globodera pallida* (Stone, 1973) Behrens, 1975 (white potato nematode) are very important pests of potato. They were included in EPPO file A2 among quarantine pests of worldwide importance. According to Bendezu et al. (1998) the losses of quantity and quality of tubers in Europe represent 300 millions EUR per year. The control of occurrence PCN is very difficult due to the existence of pathotypes Ro1-Ro5 and Pa1-Pa3 (Kort et al. 1977). Forasmuch as that a lot of genes of resistance to PCN in genus *Solanum* are known, there is a chance of elimination of losses in breeding and growing of resistant cultivars of potato. There are about 75% of resistant varieties against Ro1 in the present Czech assortment of potato cultivars (Vokál et al. 2000). In contrast is big deficiency of varieties resistant against *G. pallida* in the worldwide dimensions.

Detection of the species-occurrence of populations of PCN is one of the most important steps to selection of the optimal method of PCN's occurrence regulation. At the present time, the morphological methods of identification PCN species or differentiations of pathotypes on the special clones of *Solanum* genus are used.

These methods are pernickety for the reason of subjective influence of evaluator or they are very laborious. The alternative of these methods is using of molecular analyses of DNA by PCR (polymerase chain reaction). Roosien et al. (1993), Folkertsma et al. (1994), Blok et al. (1997) and Bendezu et al. (1998) used RAPD (random amplified polymorphic DNA) for differentiation of PCN species and pathotypes. Shields et al. (1996) studied PCN species by SPLAT-PCR (specific polymorphic locus amplification test). At the end of 90th of 20th century, the most of work was directed to study of variability of ITS-1 (internal transcribed spacer) of the rDNA cistron. This region is most genetically conserved source of interspecific variable. There is possible to encourage the variable by PCR or by restriction endonucleases digestion (Blok et al. 1998, Zouhar et al. 2000).

The main objective of this work is development of the precise and reliable molecular-genetic method of distinguishing of both PCN species and more precise detection of composition of PCN populations.

MATERIAL AND METHODS

The cysts of European populations of PCN were used. They are Ro1 (Šluknov), Ro2 (Obersteinbach), Ro5 (Harmerz), Pa2 (Kalle), Pa3 (Chavornai), Pa3 (Delmsen) and cysts of unknown pathotype of Kašperské hory (K) locality. The cysts were acquired in cooperation with The State Phytosanitary Administration of The Czech Republic at Prague-Ruzyně and The Department of Plant Protection of Faculty of Agronomy of The Czech University of Agriculture.

DNA was isolated from individual cysts by the following method: to freeze the cyst in liquid nitrogen for 5 minutes, to homogenize the cyst in 10 µl TE buffer (Sambrook et al. 1989) and incubate in 60°C water bath for 60 minutes. Centrifuge 10 minutes (10 000 rpm) and move supernatant into new sterile tube.

On the base of known sequences of PCN ITS-1, the species-specific primer sets by using freeware *Primers! for the World Wide Web* were designed. They were Fro1-Rro1 (5'-ACACATGCCCGCTGTGTATG-3' and 5'-AAA-

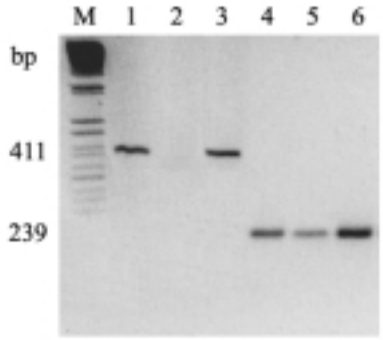

Figure 1. Identification of PCN species with described primer-sets

M – the leader – λDNA/*Eco*47I/*Ava*II
Lanes 1–3: *Globodera rostochiensis* (Ro1, Ro2, Ro5)
(Primer-set Fro1-Rro1)
Lanes 4–6: *Globodera pallida* (Pa2, Pa3, K)
(Primer-set Fpa2-Rpa1)

GATGGGAAAAAGCTGGCC-3') for *G. rostochiensis* identification and Fpa2-Rpa1 (5'-TCAACAATGTATG-GACAGCG-3' and 5'-GGCACGTACGACATGGAATA-3') for *G. pallida* identification. Composition of 25µl reaction mixture: 1 × PCR buffer, 10 ng of template DNA, 2.5mM of $MgCl_2$, 0.2µM of each primer, 100µM of dNTP and 0.7 units of *Taq* polymerase (FERMENTAS, Lithuania). Conditions of PCR: 5 × (94°C 90 s, 64°C 45 s, 72°C 90 s) 15 × (94°C 60 s, 61°C 45 s, 72°C 90 s) 15 × (94°C 60 s, 60°C 45 s, 72°C 90 s) 1 × 72°C 480 s (Thermocycler-Techne, GB). Amplified markers were analysed in 1.5% agarose electrophoretic gel and visualized by ethidium bromide (Sambrook et al. 1989).

RESULTS

DNA of *G. rostochiensis* (apart from Ro2) analysed by primer set Fro1-Rro1, offered 411 bp fragment by primer set Fpa2-Rpa1 did not offered any fragment. In reverse DNA of *G. pallida* analysed by set Fpa2-Rpa1, offered 239 bp fragment (Figure 1). Otherwise, the possibility of MULTI-PCR (using of both sets primers in one reaction) was verified (Figure 2). This variant is usable for fast determination of population structure. The presence of only one cyst different species in collection of cyst from particular locality is reliably detectable. Figure 2 present etalon of typical electrophoretic profiles of mixed populations of species PCN. In the case of lanes B7, B8 and B9 that correspond with mixed populations Ro2 and Pa, the ability to amplification of DNA of Ro2 pathotype was proved. The proof is the 274 bp fragment. Identity of markers (411 bp a 239 bp fragments) was verified by digestion with restriction endonucleases *Alu*I, *Hae*III, *Hha*I and *Rsa*I, (FERMENTAS, Lithuania) of which response places were in the sequences found. Another larger variable by digestion, which could nearly characterize different populations was not detected.

DISCUSSION

Described method of DNA extraction is very humble and undemanding relative to laboriousness or material demands. Although the DNA was not purified by phenol-chloroform (Folkertsma et al. 1994), there was no problem with amplification. Described primer sets and method make possible to reliably distinguish both PCN

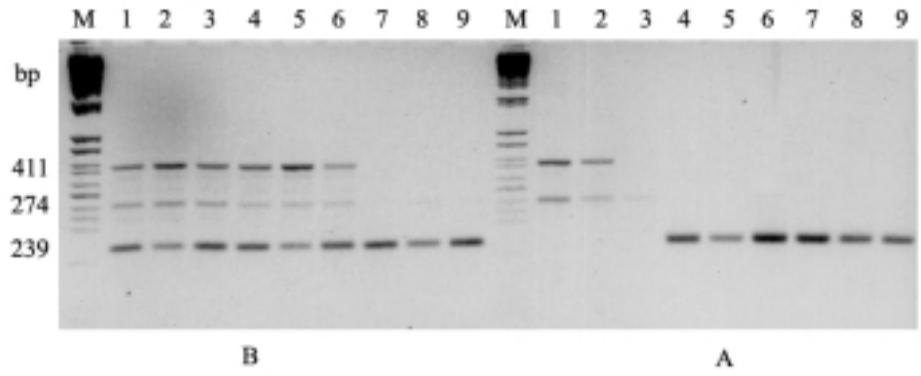

Figure 2. Etalons of populations (A) and mixed populations (B) of PCN analysed by MULTI-PCR

M – the leader – λDNA/*Eco*47I/*Ava*II
A) Lanes: 1) Ro1, 2) Ro5, 3) Ro2
Lanes 4–9: populations *Globodera pallida*
B) Lanes 1–6: mixed populations Ro1 + Pa, Ro5 + Pa
Lanes 7–9: mixed populations Ro2 + Pa

species by cross-test. Why did not amplify DNA of Ro2 pathotype? There is evidently some point mutation in the primer-complementary part of DNA. Zouhar et al. (2000) used three-primer system for distinguishing both species; this method was similar to ours. He used similar population as well to optimise the method. De Giorgi et al. (1994), Powers et al. (1997) and Blok et al. (1998) dealt with sequential analyses of ITS-1 of genus *Globodera* and others (*Xiphinema*, *Meoidogynae*). Shields et al. (1996) differentiated species of PCN by one primer-set with different length of amplified fragment of ITS-1. These results were not well reproducible. For this reason we had to design primer sets and made usable method of molecular analyse of ITS-1. Cited authors worked with relative longer fragment of cistron rDNA (about 1100 bp) that was achieved by using of universal primer-set for rDNA cistron amplification (Vrain et al. 1992) and obtained the distinguishing of species by digestion with restriction endonucleases. Both methods are mutually different but both reach common destination – proved distinguishment among species and detected their genetic linkage. Cited authors moreover used sequential analyses of the fragment to verifying of differences between species. Our attitude was suspiciously experimental, because we had to use only published sequences of ITS-1 of both species, but of different populations. The method of MULTI-PCR in combination with RFLP (Restriction fragment Length polymorphism) used Blok et al. (1998) to distinguish different populations of *Globodera pallida*.

CONCLUSION

Described method is usable for distinguishing of PCN species by cross-test or by MULTI-PCR. Using of cross-test distinguishes both species PCN, but only at the expense of higher laboriousness. Into reverse, the digestion with restriction endonucleases is not needed. It is possible to make only one analysis by cross-test or MULTI-PCR. MULTI-PCR is usable with benefit for amplification of multiple isolated DNA of the larger set of cyst by (Folkertsma et al. 1994) for detection of mixed populations of PCN. The both variants are usable in phytopathological diagnostic of these quarantine pests. At disparity with conventional methods of detection PCN species, this method is not affected by mistakes of subjective evaluation and facilitates processing of large number of biological material in the small space and at the relative short time.

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ABSTRAKT

Identifikace druhů háďátka bramborového (*Globodera rostochiensis*, *G. pallida*) s využitím polymorfismu ITS-1 regionu

Moderní metody molekulární analýzy DNA jsou běžně využívány v oblastech zoologické a botanické taxonomie, fytopatologie a tvorby odrůd. Znalost druhového složení populací háďátka bramborového (*Globodera rostochiensis*, *G. pallida*), významného škůdce brambor, má význam pro volbu vhodného opatření regulace jeho výskytu. V tomto příspěvku je popsána molekulárně genetická metoda odlišení druhů háďátka bramborového. Byly použity evropské populace – Šluknov (Ro1), Obersteinbach (Ro2), Harmerz (Ro5), Kalle (Pa2), Chavornay (Pa3), Delmsen (Pa3), a izoláty z oblasti Kašperských hor (K). Na základě známých sekvencí ITS-1 (Internal Transcribed Spacer 1) obou druhů byly pomocí volného programu *Primers! for the World Wide Web* navrženy druhově specifické sady primerů pro amplifikaci části ITS-1. Sada Fro1-Rro1 poskytovala produkt o velikosti 411 bp (pouze u *G. rostochiensis*), sada Fpa2-Rpa1 produkt o velikosti 239 bp (pouze u *G. pallida*). Na základě této skutečnosti byla potvrzena příslušnost evropských populací k deklarovaným druhům. Populace K byla identifikována jako *G. pallida*.

Klíčová slova: *Globodera rostochiensis*; *Globodera pallida*; ITS-1 region; PCR; DNA markery; identifikace druhu

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