European Journal of Plant Pathology (2005) 111: 39–46

Identification of *Globodera rostochiensis* and *G. pallida* in the Ukraine by PCR

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Accepted 12 June 2004

Key words: diagnostics, internal transcribed spacer, phylogenetics, potato cyst nematodes

Abstract

Multiplex polymerase chain reaction was used to identify the potato cyst nematodes in soil samples from the Ukraine. The results show the occurrence of *Globodera pallida* in the Uzhhorod region (Zakarpats'ka oblast'), where only *G. rostochiensis* had been previously reported. In the mixed potato cyst nematode (PCN) populations, *G. pallida* was less prevalent (2–5%) than *G. rostochiensis* (95–98%). A phylogenetic analysis based on ribosomal DNA internal transcribed spacer sequences showed that the Ukrainian population of *G. pallida* had >99% sequence identity with other *G. pallida* pa2/3 isolates from Europe. This study has demonstrated that polymerase chain reaction-mediated amplification of specific regions of the potato cyst nematode genome is not only highly effective as a species diagnostic tool but is also a sensitive method which can be used for taxonomic purposes with cyst collections which vary in age.

Abbreviations: ITS – internal transcribed spacer; PCN – potato cyst nematode; rDNA – ribosomal DNA

Introduction

Potatoes are one of the most important crops in the Ukraine cultivated in a broad range of agro-ecological environments. Since the first introduction of potatoes in the 18th century the total area planted has increased to over 1.5 million hectares. However, the yields per hectare are still lower than those reported in adjacent potato-producing countries such as the Russian Federation and Poland. Problems in the potato industry can be seen from the structure of potato production over the last decade: annually about 16,980 Mt of potatoes are produced, of which 34.5% is sold as table potato, 25.5% for forage potato, 24.2% for seed, and 15.8% processed and lost during storage (Tesluk, 2002). The application of high plant-health standards is a key objective to improve production in the potato industry. There is also a need to improve regulatory control either to prevent the introduction of pests or to control those that are already established and which adversely affect potato production. One such established pest is the economically important obligate parasite, potato cyst nematode (PCN), which is a major problem worldwide.

In the Ukraine, PCN was first identified in 1963 in the western part of the country in the Cernivtsi region, in fields at the Research Station where potato breeding material as well as seed potato from different institutions of the former USSR had been screened for resistance to *Synchitrium*

endobioticum. It is thought that PCN had been introduced together with potatoes sent from Byelorussia or the Baltic Republics (Estonia, Latvia, and Lithuania) (Nikitin, 1969), where PCN had been introduced in 1948–1953 (Marks and Rojancovski, 1998).

To prevent the distribution of PCN to other regions of the Ukraine, regulatory controls were imposed on the Cernivtsi region (Nikitin, 1972). Although infested fields were held under quarantine and treated with chloropicrin, surveys revealed the spread of PCN to other regions (Sigareva and Miroshnik, 1994; Miroshnik, 1996; Pylypenko, 1998). Ongoing research indicates that in the Ukraine PCN may cause significant yield losses, depending on population density and susceptibility of potato cultivars. Populations of 55 eggs and juveniles per gram soil were associated with a loss of 3.3% of total yield of resistant and 37.7% of susceptible cultivars; and populations of 121 eggs and juveniles per gram soil were associated with losses of 16.9% and 63.3%, respectively (Pylypenko, 1999).

Currently PCN is present in 13 regions of the Ukraine comprising a total infested area of approximately 5529 ha (Ukrainian General State Inspection on Quarantine of Plant, 2003; Table 1). Such significant distribution is associated with lack of nematicides in the Ukrainian market, the increased monoculture of potatoes in most small private holdings representing 98% of potato-growing areas and poor seed production of nematode-resistant potato cultivars. The use of nematode-resistant potato cultivars constitutes the most important component of the Ukrainian strategy for PCN management. In 2000, a work conference was organized by the Ukrainian Academy of Agrarian Sciences, Institute of Potato Researches and including by invitation the General State Inspection on Quarantine of Plant and the Institute of Plant Protection, to formulate new approaches to control PCN by using nematode-resistant cultivars. The conference resulted in a programme consisting of breeding strategies that targeted a wide resistance base to PCN and surveys of all potato-growing areas for the correct identification of PCN species. The latter involves routine soil sampling usually done manually. The common sampling procedure for PCN is for one sample of 250 cm^3 ha^{-1}, consisting of 50 cores of 5 cm^3 of soil. Cysts are extracted from the air-dried soil, utilizing the principles of the Fenwick-can procedure (Fenwick, 1940). After cyst extraction local quarantine inspectors identify cysts to genus, but trained taxonomists are relied on to make the final identification at the central quarantine laboratory. However, in both cases identification is based on examination of morphological features so that it is time-consuming and frequently unreliable because of morphological similarity of the two PCN species, *Globodera rostochiensis* and *G. pallida*. The only species identified to date in the Ukraine has been *Globodera rostochiensis* (Sigareva and Miroshnik, 1994; Pylypenko, 1998, 1999).

The key to future success of national management strategies involving the use of resistant cultivars is the unambiguous identification of PCN species (Marks and Fleming, 1985). This paper reports on studies conducted to identify Ukrainian populations of PCN by polymerase chain reaction (PCR). This technique offers a highly effective means of detecting inter- and intra-specific variation and for identification of nematodes (Jones et al., 1997; Powers and Fleming, 1998).

Material and methods

Nematode isolates

A soil survey was conducted in 13 out of 25 regions of the Ukraine by the General State Inspection on Quarantine of Plant and Institute of Plant Protection. Population 60 was collected in 1975; populations 56, 57, 58 in 1978; populations 52, 53, 54, 55 and 59 in 1983 and the remaining populations from 1996 to 2003. Soil samples were collected to a depth of 15–20 cm and up to 50 subsamples (each 5 cm^3) per hectare were combined, mixed and a representative sample of 250 cm^3 was selected. The soil samples were placed immediately in strong plastic bags, labeled and transported to the Department of Nematology for storage in the dark at 7–12 °C. For subsequent analyses, cysts from each population were extracted using a Fenwick can (Fenwick, 1940), dried and placed into 2 ml tubes and stored at room temperature in the dark. A total of 60 populations were collected (Table 1). Reference samples used were populations *G. rostochiensis* Ro1 Scotland and *G. pallida* Pa2/3 Lindley England (Blok et al., 1997).

Table 1. The distribution of potato cyst nematodes in Ukraine (Ukrainian General State Inspection on Quarantine of Plant, 2003) together with information on the codes for the Ukrainian populations used in this study

Region of Ukraine	The distribution of PCN in the different property farms (Ha)			Populations of PCN used in this study	
	Farms (Ha)	Small holdings (Ha)	Total area infested within region	Number of populations collected within region	Population codes
Cherkasy	–	181.5	181.5	1	59
Chernihiv	30.0	1293.2	1323.7	7	9–14, 51
Ivano-Frankivs'k	0.3	5.4	5.7	1	48
Khmel'nyts'kyi	2.0	32.6	34.6	2	49, 50
Kyiv	–	61.5	61.5	22	1–3, 25–43
L'viv	115.0	781.0	896.0	5	20–24
Rivne	–	638.3	638.3	4	52–55
Sumy	–	780.2	780.22	5	4–8
Ternopil'	1.0	21.5	22.5	3	56–58
Vinnytsia	–	17.7	17.7	1	60
Luts'k (Voluns'ka oblast')	–	1173.3	1173.3	3	44–46
Uzhhorod (Zakarpats'ka oblast')	7.0	14.3	21.3	5	15–19
Zhytomyr	253.0	160.6	372.6	1	47
Total	408.3	5161.0	5528.8	60	

Morphological identification of juveniles

Stylet lengths of heat fixed juveniles were determined using an Olympus BX50 microscope. Measurements were taken for 10 *G. rostochiensis*, 10 *G. pallida* and 9 juveniles from samples from two populations from the Uzhhorod region.

DNA extraction from bulked cysts

Bulk samples of cysts of approximately 1mg were prepared by mixing populations collected within one region (Table 1). DNA was extracted from the bulks using the DNeasy Tissue Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. DNA was quantified using a nanodrop spectrophotometer (Labtech International, UK) and DNA dilutions made at 2 ng μl^{-1} and stored at −20 °C.

DNA extraction from single juveniles

Cysts were crushed and presoaked in sterile water and after 2 days juveniles were collected. Crude lysates of DNA were prepared following a modified method of Uehara et al. (1998). A single juvenile nematode was cut in 13 µl of worm lysis buffer (50 mM KCl, 10 mM Tris–HCl: pH 8.0, 2.5 mM $MgCl_2$, 60 µg ml^{-1} proteinase K, 0.45% NP40, 0.45% Tween20 and 0.01% gelatin) on a glass slide under a dissecting microscope. The solution was transferred by pipette to a 1.5 ml vial, and 7 µl of additional worm lysis buffer added. After incubation at −80 °C for 20 min, the solution was incubated at 65 °C for 70 min and then 95 °C for 15 min.

PCR

Primers PITSr3 5′-AGCGCAGACATGCCGCA-A3′ and PITSp4 5′-ACAACAGCAATCGTCGA-G-3′ (Bulman and Marshall, 1997) in combination with the forward primer 5′-CGTAACAAGGT-AGCTGTAG-3′ (Ferris et al., 1993) were used to distinguish between *G. rostochiensis* and *G. pallida*. PCR amplification reactions were carried out using PureTaq Ready-To-Go-PCR Beads (Amersham Bioscience, UK) in 25 µl reactions comprised of sterile water, each primer (10 pmol), nematode DNA extracted from cysts (4 µl of 2 ng μl^{-1}) or from single juveniles (2 µl) in a 0.2 ml tube containing a PCR bead. Amplification conditions were

an initial denaturation step at 94 for 3 min followed by 35 cycles (40 cycles for single nematodes) of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. A final extension cycle (72 °C, 5 min) was used to terminate the reaction. The PCR program was performed using a Perkin-Elmer 9700 thermal cycler (Applied Biosystems, Warrington, UK). The PCR products were separated by electrophoresis in Tris-Borate-EDTA (TBE) buffered 1% agarose gels with ethidium bromide and visualized with UV illumination.

Sequencing

In order to obtain sequence covering the ITS1 and ITS2 regions amplification products were produced from population 16 using forward primer 5′-CGTAACAAGGTAGCTGTAG-3′ and reverse primer 5′-TCCTCCGCTAAATGATATG-3′ (Ferris et al., 1993) which are located in the 18S and 28S genes, respectively. The same reaction components and amplification conditions as described above were used. PCR products were purified using QIAquick PCR Purification kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The PCR products were sequenced by cycle sequencing using a DNA sequencing kit (Perkin-Elmer ABI PRISM Big Dye; Applied Biosystems) according to the manufacturer's instructions, and the sequencing reactions were run on an automatic sequencer (ABI 377; Applied Biosystems). Sequence was determined using the above forward and reverse primers, and 5.8SF 5′-CGGTGGATCACTCGGCTCGT-3′ and 5.8SR 5′-ACGAGCCGAGTGATCCACCG-3′ primers. Sequence information was assembled using DNASIS software (Hitachi Software Engineering) and subjected to a database search using BLAST® (Altschul et al., 1990) or FASTA. The sequence data were aligned using the MultAlin program (Devereaux, 1984, Thompson et al., 1994). Similarities between sequences were obtained using the DNADIST program from the PHYLIP (version 3.6) package (Felsenstein, 1989) and using the default settings. A dendrogram was produced from these similarities using the NEIGHBOR programme with the neighbour joining method. Trees were drawn with the TREEVIEW program (Page, 1996). The sequence accession number for the Ukrainian population is AJ606687.

Results

Detection of G. pallida *from the Ukraine using PCR*

All bulk DNA extracts produced an amplification product of the same size as *G. rostochiensis*. The DNA extract from the bulk prepared from populations 15, 16 and 19 collected from the Uzhhorod region produced an additional product of the same size as *G. pallida* (Figure 1, Lane 6). Individual nematodes from each population collected within that region were subjected to PCR. The individuals from populations 16 and 18 produced amplification products of the same size as *G. pallida* (Figure 2, Lanes 3–6 and 8); the individuals from populations 15 and 17 produced amplification products the same size as *G. rostochiensis* (Figure 2, Lanes 2 and 7). *Globodera pallida* was detected in samples taken from the villages Uzhok and Zhornava situated within the Uzhhorod region (Zakarpats'ka oblast') of the Ukraine.

Sequence analysis of the rDNA ITS region

Sequence of partial 18S, ITS1, 5.8S, ITS2 and partial 28S sequences from *G. rostochiensis* and *G. pallida* were obtained from Blok et al. (1998) and Subbotin et al. (2000) and compared to the sequence obtained from the PCR product from the Ukrainian population 16 which was the same size as the *G. pallida* control. A sequence of 938 bp was obtained and aligned with the published sequences. The sequence from the Ukrainian population had between 99.03% and 99.57% similarity with other *G. pallida* sequences other than *G. pallida* P5A where the similarity was 97.8%. The similarity to *G. rostochiensis* was 96.82% (Table 2; Figure 3).

PCR analyses from 25-year old cysts

DNA was amplified from juveniles obtained from 25-year old cysts (populations 56, 57, 58) using primers PITSr3, PITSp4 and the forward primer. The product size was the same as produced from control *G. rostochiensis*. A BLAST search showed that the sequence of the 324 bp PCR product showed 99% identity to six *G. rostochiensis* sequences and 96% identity to seven *G. pallida* sequences.

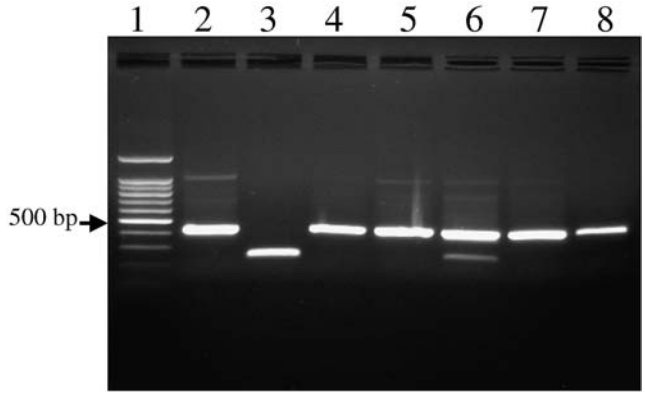

Figure 1. Molecular differentiation of Ukrainian PCN. Multiplex polymerase chain reaction using primers PITSr3, PITSp4 and the forward primer: Lane 1, Molecular weight markers (M) = 100 bp DNA ladder (Promega, Southampton, UK); Lane 2, *G. rostochiensis* Ro1; Lane 3, *G. pallida* Pa2/3; Lane 4, PCN from Chernihiv region (populations 9, 10, 11); Lane 5, PCN from Chernihiv region (populations 12, 13); Lane 6, PCN from Uzhhorod region (Zakarpats'ka oblast') (populations 15, 16, 19); Lane 7, PCN from Uzhhorod region (Zakarpats'ka oblast') (populations 17); Lane 8, PCN from L'viv region (population 22).

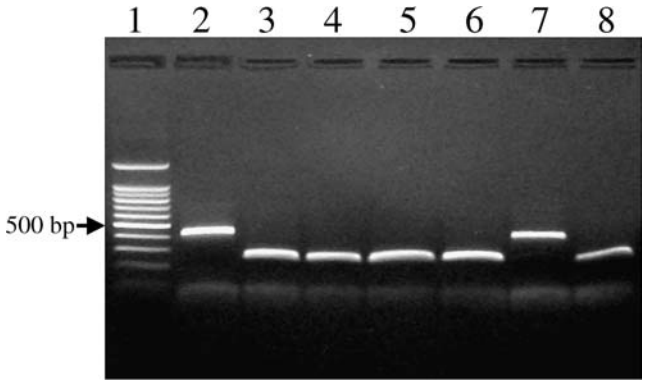

Figure 2. Molecular differentiation of single juveniles of PCN from Ukraine. Examples of the multiplex polymerase chain reaction using primers PITSr3, PITSp4 and the forward primer: Lane 1, Molecular weight markers (M) = 100 bp DNA ladder (Promega, Southampton, UK); Lane 2, Juvenile 15–11 (from population 15 number 11); Lane 3, Juvenile 16–8; Lane 4, Juvenile 16–9; Lane 5, Juvenile 16–11; Lane 6, Juvenile 16–18; Lane 7, Juvenile 17–10; Lane 8, Juvenile 18–1.

Morphological examination of putative G. pallida *specimens*

Stylet measurements of heat fixed juveniles from the three villages where *G. pallida* was identified were compared to *G. pallida* and *G. rostochiensis* juveniles for populations from the UK. The mean (±SD) stylet lengths for the control *G. pallida* and *G. rostochiensis* juveniles were 22.65 (0.34) and 21.15 (0.47) μm, respectively and for the Ukrainian *G. pallida* 22.44 (0.63). The mean values for the *G. pallida* and Ukrainian nematodes where not significantly different ($P = 0.36$) but both differed from the mean *G. rostochiensis* measurement ($P = 0.001$).

Discussion

It is thought that potatoes were first planted in the north-west regions of Ukraine situated close to Byelorussia, Poland and the Austria-Hungarian Empire about 250 years ago. Because of suitable environmental and agronomical conditions in almost all other regions of the country, the area planted rapidly increased (Tesluk, 2002). This may

Table 2. Similarity matrix comparing sequence derived from Ukrainian population 16 with sequences obtained from *G. rostochiensis* and *G. pallida* populations

G. rostochiensis Ro1	100							
G. pallida Pa1	97.38	100						
G. pallida Pa2/3	97.27	99.46	100					
G. pallida P5A2	97.49	98.38	98.27	100				
G. pallida Halton	97.27	99.47	99.68	98.27	100			
G. pallida Luffness	97.27	99.47	99.68	98.27	100	100		
G. pallida P4A	97.27	99.68	99.68	98.27	99.79	99.79	100	
Ukraine	96.82	99.03	99.46	97.83	99.57	99.57	99.36	100
	Ro1	Pa1	Pa2/3	P5A	Halton	Luffness	P4A	Ukraine

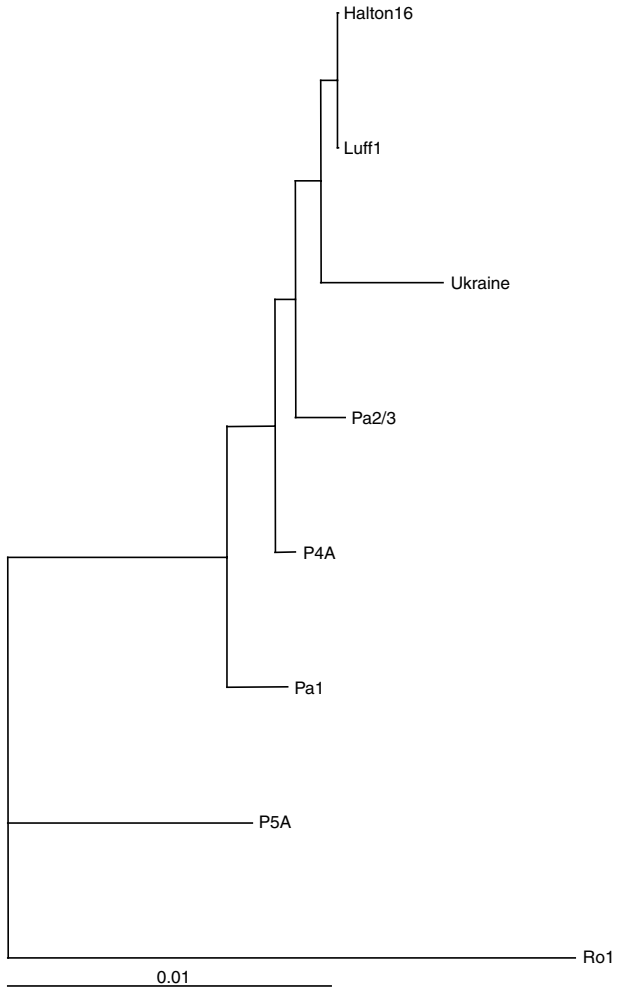

Figure 3. Phylogenetic tree showing the relationships between *G. rostochiensis*, *G. pallida* and Ukrainian population of PCN based on sequence obtained between the 18S and 28S genes of the ribosomal cistron.

explain why the most serious pests and pathogens of this crop, such as *Leptinotarsa decemlineata, Synchytrium endobioticum* and *Globodera rostochiensis*, are widespread in these north-west regions of the Ukraine and their further distribution has increased coincident with the cultivation of potatoes.

The results presented here give evidence of the occurrence of *G. pallida* in the Uzhhorod region (Zakarpats'ka oblast') of Ukraine, where only *G. rostochiensis* had been previously reported. The species composition within populations from this area shows that *G. pallida* is the less prevalent species compared to *G. rostochiensis* (Figure 1, Lane 6). The origin of *G. pallida* is not known. Introductions from neighboring countries where it has already been identified such as Poland, Hungary (Marks, Rojancovski, 1998) either with *G. rostochiensis* or independently may have occurred. Potato cultivars resistant to *G. pallida* have not been grown in the Uzhhorod region (Zakarpats'ka oblast') and *G. pallida* may be a recent introduction in this region.

Amplification products were produced from 20 to 25 year-old cysts with sequence matching *G. rostochiensis* indicating the persistence of target DNA in this material. This study has demonstrated that PCR-mediated amplification of specific regions of the PCN genome is not only highly effective as a species diagnostic tool but also a sensitive method which can be used for taxonomic purposes with cyst collections which vary in age. In practice, cyst samples which are collected, processed and stored can be subjected to PCR diagnostic analyses after considerable periods of time without the requirement of expensive maintenance on host plant.

Acknowledgements

The authors gratefully acknowledge General State Inspection on Quarantine of Plant of Ukraine for assistance in conducting a survey and collecting soil samples. The authors would like to thank Dr. D. Trudgill for help with morphological identification of PCN. Dr. Pylypenko was supported by a study visit from the Royal Society and Dr. Taketo was supported by an OECD fellowship.

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