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Identification of populations of potato cyst nematodes from Russia using protein electrophoresis, rDNA-RFLPs and RAPDs

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Summary. Twenty nine potato cyst nematode populations from Russia, five populations from England and one population each from the Ukraine, New Zealand, Germany and The Netherlands were compared using protein electrophoresis, restriction fragment length polymorphism of ribosomal DNA (rDNA-RFLP) and random amplified polymorphic DNA (RAPD) techniques. All populations from Russia were identified as *Globodera rostochiensis* and RAPD analysis revealed substantial genomic diversity.
Key words: molecular taxonomy, RAPD, rDNA-RFLP, *Globodera rostochiensis,* Russia, protein electrophoresis, identification.

The potato cyst nematodes (PCN), *Globodera rostochiensis* and *G. pallida*, are considered the most economically important nematode pests of potatoes and they are the subject of strict quarantine regulations in many countries. After introduction from South America into Europe during the last century, PCN successfully colonized the temperate zones of the world (Brodie *et al.,* 1993). In the former USSR, PCN were first found near Vilnus in Lithuania in 1948, possibly having been introduced from Germany during World War II (Efremenko, 1961). The area of infestation widened considerably to include regions of eastern Russia, where PCN were found in 914 ha of farmland by 1960. Subsequently, PCN were found in Latvia, Estonia, Byelorussia and the Leningrad and Kaliningrad regions (Efremenko, 1961; Borovkova, 1963, 1967). Currently, only *G. rostochiensis* is widely distributed in the republics of the former Soviet Union, including the Far East Region, where it is registered as an important quarantine pest. PCN have been reported from 51 regions of Russia and the area affected is estimated to be 41148 ha, including more than 140000 private fields and 288 farms (Yudina *et al.,* 1998); only pathotype Ro1 has been identified (Borovikova, 1981; Guskova *et al.,* 1981, Solovieva *et al.,* 1989).

Various biochemical tests have been used to identify and separate species and populations of PCN: isoelectric focusing (IEF) (Fleming & Marks, 1983; Fox & Atkinson, 1984) and two-dimensional gel electrophoresis (Bakker *et al.,* 1992) of proteins, random amplified polymorphic DNA (RAPD) (Roosien *et al.,* 1993; Folkertsma *et al.,* 1994; Burrows *et al.,* 1996; Blok *et al.,* 1997; Thiéry *et al.,* 1997; Bendezu *et al.,* 1998; Conceição *et al.* 1998), restriction fragment length polymorphism (RFLP) of ribosomal DNA (Fleming *et al.,* 1993; Thiéry & Mugniéry, 1996) and other diagnostic methods (Jones *et al.,* 1997). Some of these techniques are suitable for discrimination of these species in routine assays. However, in Russia, identification of different PCN populations was done mainly by traditional approaches based on microscopic observations of differences in morphological characters of vulval cones and juveniles. The aim of the present work was to identify PCN populations from Russia by biochemical and molecular techniques and also to use RAPDs to examine the genomic variation between these populations and defined populations from Europe and New Zealand.

MATERIALS AND METHODS

Nematodes. Cysts of the 29 populations of PCN from different regions of Russia and one population from the Ukraine were collected from small fields (0.2-0.4 ha) in which potatoes had been grown and were kept dry at room temperature in plastic specimen tubes. Populations of *G. rostochiensis* from England and New Zealand and *G. pallida* from

England, Germany, The Netherlands and New Zealand (Table 1) were used for comparisons.

Isoelectric focusing (IEF). Ten cysts containing eggs and juveniles were soaked overnight in distilled water, transferred into a plastic homogenizer (Biomedix, UK) containing 10 µl of water and homogenised. The homogenised samples were centrifuged and 1 µl of supernatant fluid was taken for electrophoretic study. The remainder was frozen and stored at -20 °C for PCR-RAPD analysis. Isoelectric focusing (IEF) gels (pH range 5-8) were run on a Phast System (LKB Pharmacia) and then stained (Phast System Silver Stain Technique for IEF media) using standard methods (LKB Pharmacia Phast System Owners Manual). One standard *G. pallida* (population P4) and *G. rostochiensis* (population R32) (Table 1) and six other samples were run concurrently on each gel. The tracks on the gels were examined for the presence or absence of species-specific diagnostic bands (Fleming & Marks, 1983).

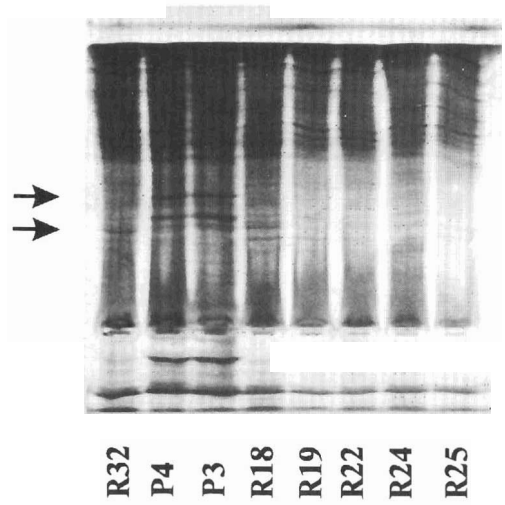

Fig. 1. Polyacrylamide gel showing comparison of two populations of *G. pallida* and six populations of *G. rostochiensis* (for population codes see Table 1). Diagnostic bands for *G. rostochiensis* (pI 5.7) and *G. pallida* (pI 5.9) are arrowed.

rDNA-RFLPs. Four to six cysts were placed in a sterile Eppendorf tube containing 18 µl of worm lysis buffer (500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 10 mM DTT, 4.5% Tween 20, 0.1% gelatin), homogenised and 2 µl of proteinase K (600 µg/ml) were added. The tubes were incubated at 65 °C for 1 h and then at 95 °C for 10 min. After centrifugation (1 min; 16 000 rpm), 6 µl of supernatant was added to a PCR reaction mixture containing 10 µl PCR incubation buffer with 25 mM $MgCl_2$ (Appligene, B &L Systems), dNTP-mixture 5 µM each (Eurogentec), 1.5 µM of each primer (synthesised by Eurogentec), 0.8U *Taq* Polymerase (Appligene, B&L Systems) and double distilled water added to a final volume of 100 µl. Primers AB 28 (5'-ATATGCTTAAGTTCAGCGGGT-3') and TW 81 (5'-GTTTCCGTAGGTGAACCTGC-3') for the internal transcribed spacer (ITS) region (Joyce *et al.*, 1994) were used in this study. Amplification was carried out in a GeneE New Brunswick Scientific DNA thermal cycler. A control without nematode DNA was always included. PCR amplification conditions were: denaturation at 94 °C for 1 min, annealing at 60 °C for 1.5 min, and extension at 72 °C for 2 min, repeated for 35 cycles. This was followed by an incubation period of 5 min at 72 °C. After DNA amplification, 5 µl of the products were analysed on a 1% agarose gel. Seven µl of each PCR-product were digested with one of the following four restriction enzymes: *Alu*I, *Bsp*143I, *Hin*fI and *Rsa*I in the corresponding buffer according to the manufacturer's instructions. The resulting fragments were separated on a 1.5% agarose gel, stained with ethidium bromide, visualised on a UV transilluminator and photographed using Polaroid type 667 film. The procedures for obtaining and digestion of PCR products were repeated several times to determine consistency of results.

RAPD. Eleven µl of DNA extraction buffer (100mM Tris-HCl, pH 8.5, 100mM NaCl, 25mM EDTA), 9 µl homogenised samples and 80 µl of 6% Chelex were mixed and incubated at 56 °C for 40 min with final heating at 96 °C for 8 min. After centrifugation, 1 µl of supernatant was taken for PCR. Each PCR amplification reaction was prepared on ice and consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM dNTP, 0.5 µM primer, 1U *Taq* polymerase (Promega Corporation) to a final volume of 25 µl. All RAPD reactions were performed at least twice. A control without nematode DNA was always used. Five 10-mer primers (Operon Instruments, USA), selected from thirteen primers originally tested, were used for this study: OPA-3 (5'-AGTCAGCCAC-3'), OPE-6 (5'-AAGACCCCTC-3'), OPE-19 (5'-ACGGCGTATG-3'), OPK-4 (5'-CCGCCCAAAC-3'), OPK-16 (5'-GAGCGTCGAA-3'). The thermal cycler (Omnigene, Hybaid) conditions used were: 1 min 94 °C, 1 min 35 °C and 1 min 70 °C for 40 cycles.

Amplification products were separated by electrophoresis in 1X TAE buffered 1.3% agarose gels (0.04 M Tris acetate, 0.001M EDTA (TAE), pH 7.8 containing ethidium bromide at a final concentration of 0.02 µM/ml as the DNA stain; DNA was visualised and photographed as above.

Table 1. Populations, origins and methods of identification of potato cyst nematodes used in this study.

Code	Country	Origin	Methods of identification	Species
R1	Ukraine	Kiev region, Borodjanskii district, s. Neloshaev	IEF, RFLP, RAPD	*G. rostochiensis*
R2	Russia	Tula region, Jasnogorskii district, s. Chertovoe	IEF,RFLP	*G. rostochiensis*
R3	Russia	Primorskii region, Chernigovskii district, s. Dmitrievka	IEF, RAPD	*G. rostochiensis*
R4	Russia	Karelija, uknown	RAPD	*G. rostochiensis*
R5	Russia	Primorskii region, Nadezhdinskii district, s. Devjatyi Val	RFLP, RAPD	*G. rostochiensis*
R6	Russia	Voronezh region, Ramonskii district, s. Stupino	IEF	*G. rostochiensis*
R7	Russia	Kaliningrad region, Gur'evskii district, s. Lugovoe	IEF, RFLP	*G. rostochiensis*
R8	Russia	Primorskii region, Khasan district, s. Khasan	IEF, RAPD	*G. rostochiensis*
R9	Russia	Kaliningrad region, Pravdinskii district, s. Zheleznodorozhmyi, 1	IEF, RFLP, RAPD	*G. rostochiensis*
R10	Russia	Kursk region, Ryl'skii district, s. Terekhovka	IEF, RFLP, RAPD	*G. rostochiensis*
R11	Russia	Vologda region, Cherenobetskii district, s. Suda-Menenoe	IEF, RFLP	*G. rostochiensis*
R12	Russia	Novgorod	IEF	*G. rostochiensis*
R13	Russia	Belgorod region, Volokonovskii region, unknown	IEF	*G. rostochiensis*
R14	Russia	Primorskii region, s. Volno-Nadezhdinskoe	RAPD	*G. rostochiensis*
R15	Russia	Vladimir region, Gus'-Krustal'nyi district, s. Uljakhino	IEF, RFLP	*G. rostochiensis*
R16	Russia	Jaroslavl region, Nekrasovskii district, s. Levashovo	IEF, RAPD	*G. rostochiensis*
R17	Russia	Jaroslavl region, Rostovskii district, s. Priimkovo	IEF, RFLP	*G. rostochiensis*
R18	Russia	Jaroslavl region, Jaroslavskii district, s. Schedrino	IEF	*G. rostochiensis*
R19	Russia	Jaroslavl region, Gavrilo-Jamskoi district, s. Kuzminskoe	IEF	*G. rostochiensis*
R20	Russia	Jaroslavl region, Nekrasovskii district, s. Likhoobrazovo	IEF, RFLP	*G. rostochiensis*
R21	Russia	Tula region, Schekinskii district, s. Reitinovka	IEF, RFLP	*G. rostochiensis*
R22	Russia	Tver region, Bol'sheborskii district, s. Stepan'kovo	IEF, RFLP	*G. rostochiensis*
R23	Russia	Belgorod region, Ivnjanskii district, s. Sukholotino	IEF	*G. rostochiensis*
R24	Russia	Belgorod region, Korochanskii district, s. Annovka	IEF	*G. rostochiensis*
R25	Russia	Khabarovsk region, Komsomol'sk na Amure	IEF, RAPD	*G. rostochiensis*
R26	Russia	Kaliningrad region, Polleskii district, s. Alleevka	IEF, RFLP, RAPD	*G. rostochiensis*
R27	Russia	Kaliningrad region, Pravdinskii district, s. Zheleznodorozhmyi, 2	IEF, RFLP, RAPD	*G. rostochiensis*
R28	Russia	Pskov, Pristan-2	IEF, RFLP	*G. rostochiensis*
R29	Russia	Kaliningrad region, Gyr'evskii district, s. Zaozernoe	IEF, RFLP	*G. rostochiensis*
R30	Russia	Vologda region, Vologodskii district, s. Raboche-Krestjanskii	IEF	*G. rostochiensis*
R31	New Zealand	Lincoln	IEF, RAPD	*G. rostochiensis*
R32	England	Lansane (Ro1)	IEF, RFLP, RAPD	*G. rostochiensis*
R33	England	Scarcliffe (Ro1)	RFLP, RAPD	*G. rostochiensis*
R34	England	Terrington, West Norfolk (Ro1)	RAPD	*G. rostochiensis*
P1	Germany	Delmsen	IEF, RAPD	*G. pallida*
P2	New Zealand	Lincoln	IEF, RFLP	*G. pallida*
P3	The Netherlands	unknown	IEF, RAPD	*G. pallida*
P4	England	Nocton (Pa2)	IEF, RFLP	*G. pallida*
P5	England	Leverton (Pa2)	RFLP, RAPD	*G. pallida*

Amplified DNA bands were recorded as a binary matrix of 0 and 1 corresponding to the absence or presence of individual bands. The data were used to produce a similarity matrix using the formula of Nei and Li (1979). The matrix was used then to produce a dendrogram using the unweighted pair group method with arithmetic averaging (UPGMA). The Neighbor and Consense programmes in Phylogeny Inference Package (PHYLIP, version 3.5) (Felsenstein, 1993) were used to generate the 100 trees and produce a consensus tree to give a measure of the degree of support for population groupings.

RESULTS

Isoelectric focusing analysis. Twenty six samples of PCN from Russia, one from the Ukraine and six from other countries including two samples with typical *G. rostochiensis* and *G pallida* banding profiles produced consistent banding patterns on mini-IEF gels over the pH range 5.0-8.0 (Fig. 1). Species identification was not judged by the intensity of the bands obtained but by the presence or absence of diagnostic bands for *G. pallida* (pI 5.7) or *G. rostochiensis* (pI 5.9). From the specific protein profiles,

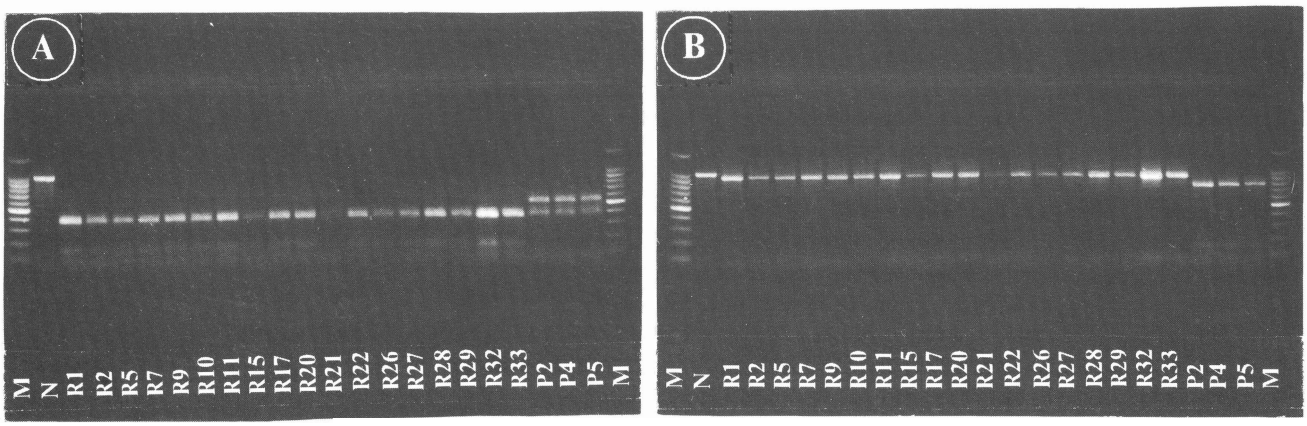

Fig. 2. Restriction fragments of the amplified ITS region of 18 populations of *G. rostochiensis* and 3 of *G. pallida*. A: *Alu*I; B: *Hin*fI. (M - 100bp DNA ladder; N - undigested PCR product; for population codes see Table 1).

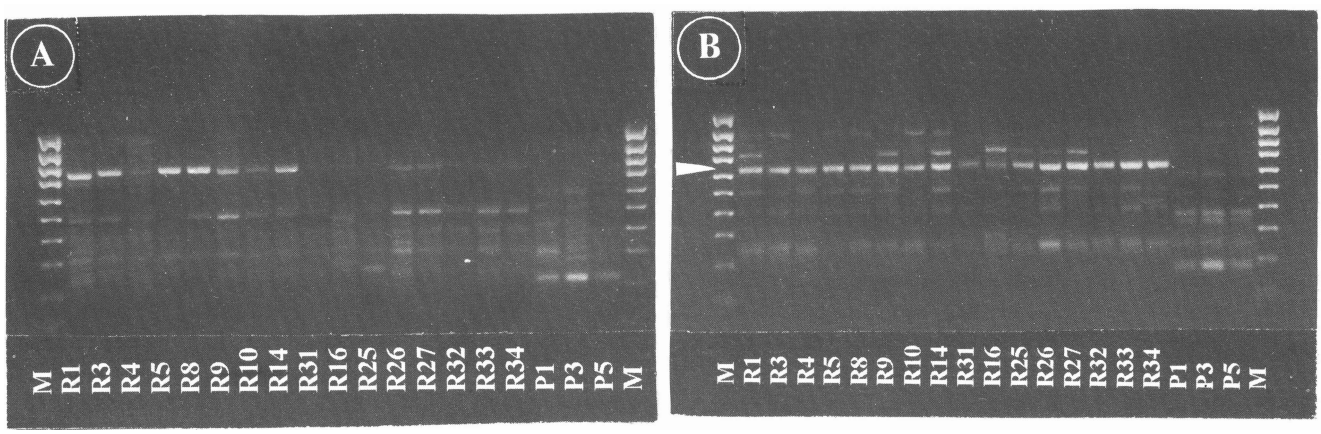

Fig. 3. RAPD patterns of 16 populations of *G. rostochiensis* and 3 populations of *G. pallida* using the following primers: A: OPA-3; B: OPK-4. (M - 100bp DNA ladder; for population codes see Table 1); the *G. rostochiensis*-specific band at 630 bp is arrowed.

all the samples from Russia and the Ukraine were *G. rostochiensis*.

rDNA-RFLPs. The amplification of the ITS region of each PCN population gave one fragment of approximately 1.0 kb (Fig. 2). No PCR products were obtained in the negative control lacking DNA template. All four enzymes used in this study: *Alu*I (Fig. 2A), *Bsp143*I, *Hin*fI (Fig. 2B) and *Rsa*I generated polymorphism and clearly distinguished *G. pallida* from *G. rostochiensis*. This method did not reveal any intraspecific polymorphism within either species of PCN. Samples from Russia and the Ukraine gave banding patterns identical to *G. rostochiensis*.

RAPD. Five 10-mer primers were used to study eleven PCN populations from Russia, four from England and one each from Germany, Ukraine, New Zealand and The Netherlands. Two examples of RAPD patterns obtained in this analysis are shown in Fig. 3A, B. All primers generated specific banding patterns for each species. For example, using primer OPK-4, a diagnostic fragment of approximately 630 bp (Fig. 3B) was present in all populations of *G. rostochiensis*, but absent from *G. pallida*, distinguishing these two species. The five primers generated numerous bands; fourty six of them were selected for scoring as present or absent. Figure 4 shows the dendrogram indicating considerable dissimilarity between *G. rostochiensis* and *G. pallida*. The majority of the *G. rostochiensis* populations appear to fall into a single large group; however, two Russian populations (R25 and R16) were separate. Differences between these two populations and other *G. rostochiensis* were observed, eg. using primer OPA-3 (Fig. 3A) the DNA fragment of approximately 760bp was not amplified in populations R16 or R25.

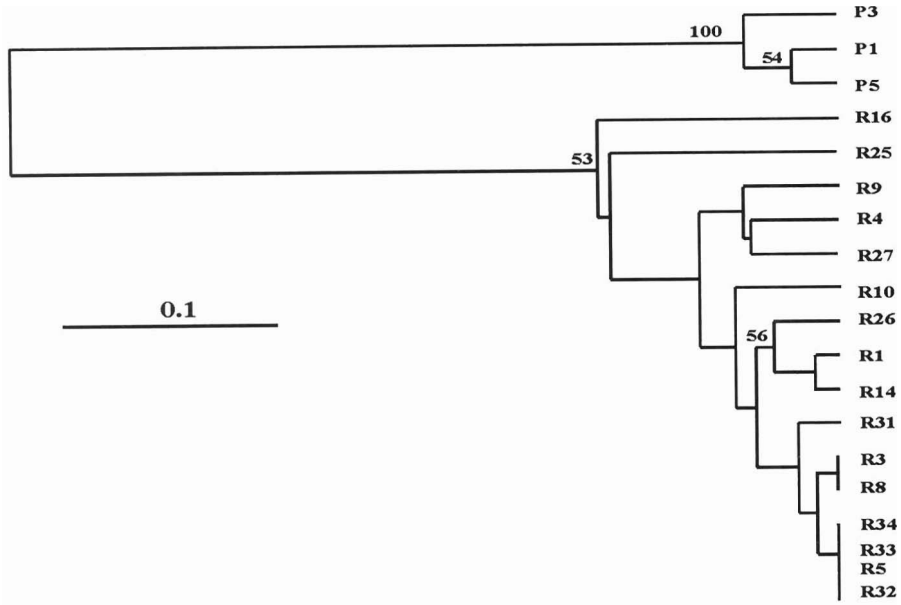

Fig. 4. Similarity dendrogram of PCN populations constructed from the genetic distance based on 46 random amplified polymorphic DNA fragments, using the UPGMA method. Bootstrap values (more than 50%) are given on appropriate clusters (for population codes see Table 1).

DISCUSSION

The results of biochemical studies of thirty PCN populations from the former Soviet Union confirmed previous morphological identifications, by scientists and plant quarantine inspectors, that only *G. rostochiensis* is present. Detailed morphometric and protein electrophoresis studies by Guskova & Makovskaja (1982) of populations from the North-west region of Russia also did not reveal any evidence of *G. pallida* in Russia. Although it is possible that *G. pallida* has not been introduced into the former Soviet Union, there are alternative, more likely, explanations for the absence of this species in the samples analysed. In the UK and other European countries, the increased distribution of *G. pallida* over *G. rostochiensis* in recent years is due not only to the availability of potato cultivars with full resistance to *G. rostochiensis* only, but also to the use of nematicides, which control *G. pallida* poorly (Whitehead *et al.*, 1984) partly because of its slower hatch and greater persistence (Halford *et al.*, 1995; Perry, 1998). In Russia, high densities of PCN are found mainly in private fields, which occupy more than 60% of all infected areas, but no effective control measures are implemented; samples used in the present work were collected from such fields. The introduction of resistant cultivars and the application of nematicides primarily occurs only in potato breeding agrofirms and the regions with isolated fields infected with PCN (Efremenko & Dudik, 1987; Solovieva *et al.*, 1989). Thus, if *G. pallida* has been introduced into Russia, the absence of control strategies in private fields may tend to favour increase in populations of *G. rostochiensis* rather than *G. pallida*, whereas the reverse would be likely to occur in commercial enterprises.

The genetic variation between populations of *G. rostochiensis* observed in the RAPD study seems to be greater than in *G. pallida*; this contrasts with the data obtained by Folkertsma *et al.* (1994), Burrows *et al.* (1996) and Blok *et al.* (1997). However, the *G. rostochiensis* populations in the present work were collected from more widespread geographical regions than in other studies and only a limited number of *G. pallida* populations were included. Perhaps a more detailed RAPD study of Russian populations is needed and a greater number of RAPD bands should be used for the evaluation of the real degree of genetic variation within *G. rostochiensis* populations.

Although several populations showed evidence of their geographical separation [for example, populations from Jaroslavl region (R16) and Komsomolsk na Amure (R25) were clearly distinguished from others and English populations also grouped together], there was no good correlation between RAPD data groupings and the geographical origin of populations. There was no significant genetic variation between populations from the Primorskii region (Russian Far East) and some west Russian populations and it is possible that the Primorskii populations were brought recently from the west regions. In general, however, the results demonstrate a large variation between Russian populations of *G. rostochiensis*. There are several possible explanations for this variation. The introduction of new PCN

genotypes could have accompanied the increase during recent years of potato trade with West European countries, Poland and South America. Some variation also could have been induced by selection pressures from local environmental conditions, specific plant protection measures and potato cultivars. It has been shown that even different susceptible host genotypes can have a genetic effect in relation to breeding for PCN resistance and, therefore, cannot be assumed to be selectively neutral (Phillips & Dale, 1982).

Thus, our preliminary results have shown relatively large genomic variations between Russian populations of *G. rostochiensis* compared with previous work. Detailed biological and molecular studies are needed to create a full picture of the distribution of different PCN populations in Russia.

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Субботин С.А., Халфорд П.Д., Перри Р.Н. Идентификация российских популяций картофельных цистообразующих нематод методами электрофореза белков, рДНК-RFLPs и RAPDs.

Резюме. Проведено сравнение 29 популяций картофельных цистообразующих нематод из России, 5 популяций из Великобритании и по одной популяции из Украины, Новой Зеландии, Германии и Нидерландов с использованием метода электрофореза белков, анализа различий в длине рестрикционных фрагментов рибосомальной ДНК и анализа полиморфизма при случайной амплификации фрагментов ДНК. Все популяции из России идентифицированы как *Globodera rostochiensis.* Предварительные данные указывают на относительно высокое геномное разнообразие среди популяций этой нематоды из России.