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MOLECULAR CHARACTERIZATION OF PCN POPULATIONS FROM SERBIA

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The morphology of potato cyst nematodes (PCN) was until recently almost the only way to identify these quarantine organisms. In the last two decades, molecular analyses contributed to faster and more efficient identification of two *Globodera* species (*Globodera pallida* and *G. rostochiensis)* and allowed insight into the genetic structure of those parts that were practically inaccessible by morphological studies. Molecular characterization was performed in ITS1-5.8S-ITS2 region. The comparison was made with sequences of different foreign PCN populations via NCBI GenBank database. The results of molecular studies showed similarities and differences between local and foreign PCN populations in the part of genome that was studied.

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INTRODUCTION

Potato (*Solanum tuberosum* L.) is nowadays one of four major food crops in the world beside wheat, maize and rice. In the 16th century the Spanish conquistadores searching for the "treasure of the Andes" brought to Europe, besides gold, the potato along with its parasites – potato cyst nematodes (PCN): *Globodera pallida* (Stone) Behrens and *G. rostochiensis* (Wollenweber) Behrens.

PCN cause up to £300 M pound sterling worth of damage to the potato crop in the EU each year (RYAN *et al.,* 2000). Etymologically, the name of the genus is derived from Latin: globus = sphera and Greek: deras = skin (SIDDIQI, 2000), and it depicts the structure and shape of female body. The names of species are derived from Latin adjectives: pallidus = pale, indicating the white color of a young cyst, and rostochiensis = from the type locality Rostock in Germany.

The morphology of potato cyst nematodes was until recently almost the only way to identify these quarantine organisms. In the last two decades, molecular analyses contributed to faster and more efficient identification of two *Globodera* species and allowed insight into the genetic structure of those parts that were practically inaccessible by morphological studies. Number of papers describe sequences and phylogeny of PCN: SUBBOTIN *et al.,* 2000; GRENIER *et al.,* 2001; ŠIRCA and UREK, 2007; SKANTAR *et al.,* 2007; PICARD *et al.,* 2008; PLANTARD *et al.,* 2008; GRENIER *et al.*, 2010; MADANI *et al.,* 2010 etc.

ITS region has sufficient information to diagnose most nematode species (POWERS, 2004). This region is used as a "molecular marker" of different eukaryotic phyla (COLEMAN, 2007) and as a universal molecular method for identifying plant species (LINDER *et al.,* 2000).

In this study, the ITS1-5.8S-ITS2 region of PCN is used for confirmation of species identity of some local populations previously identified by morphological studies, (ORO *et al.,* 2010) and for subsequent phylogenetic analyses. The comparison was made with sequences of similar foreign PCN populations via NCBI GenBank database.

MATERIAL AND METHODS

The cysts of *Globodera* from Kladnica, Sanac and Milatovici were grown on susceptible potato varieties in a climate chamber at 15-25^{o}C for a 16 h photoperiod. The cyst extraction was done with the Spears apparatus (SPEARS, 1968) and collected on a 150 μm sieve. DNA was extracted from a single cyst with Dneasy Blood & Tissue Kit (Qiagen) in accordance with the manufacturer`s instructions. PCR was done with primers for sequencing: TW 81 and AB 28, as per SKANTAR *et al.,* 2007. Sequencing was performed in BMR Genomics, Padova, Italy.

The sequences of PCN from Kladnica, Sanac and Milatovici are deposited in NCBI GenBank DNA sequence database under numbers: HM 159428, HM 159429 and HM 159430, respectively. Sequences were aligned by using ClustalW module within MEGA 4 (TAMURA *et al.,* 2007). Phylogenetic analyses were

performed using MEGA 4, Phyml 4.2.4. (GUINDON and GASCUEL, 2003), and MrBayes 3.1.2. (HUELSENBECK and RONQUIST, 2005) computer softwares.

RESULTS AND DISCUSSION

Molecular characterization of PCN populations confirmed that those from Kladnica and Sanac were *G. pallida* and the population from Milatovici was *G. rostochiensis*.

The total number of sequenced nucleotides of *G. pallida* was 885. The ITS-1 region spanned from 1st to 516th nucleotide as a partial segment, 5.8 S was in the range from 517th to 675th as a complete segment, and the ITS-2 ranged from 676th to 885th nucleotide as a partial segment. Out of 883 sequenced nucleotides of *G. rostochiensis* (Milatovici population), the ITS-1 region was in the range from 1st to 514th as a partial segment, 5.8 S spanned from 515th to 673rd as a complete segment, and ITS-2 ranged from 674th to 883rd position as partial nucleotide segment.

Differences among local PCN populations in genetic sense were evident in 28 varying segments which were positioned in: 82nd place, 86th, 88th, 103rd, 114th, 169th, 178th, 179th, 192nd, 210th, 221st, 222nd, 294th, 303rd, 324th, 332nd, 347th, 348th, 428th, 451st, 473rd, 483rd, 484th, 501st, 756th, 763rd, 795th and 872nd place. Also, when comparing sequences of *G. rostochiensis* to *G. pallida,* two deletions were noticed: in 317th place there was a deletion of guanine, and deletion of adenine in the 493rd nucleotide position. The presence of 18 transition and 8 transversion pairs was detected: A / G (2), G / A (3), T / C (10), C / T (3) and T / A (3), A / T (4), C / G (1), respectively. Most variations occurred in ITS-1 region (24+2). ITS-2 region had four varying positions, and 5.8S none.

The content of nitrogen bases (shown in Table 1) varied within the same species and was similar among different species. The potato cyst nematodes had a higher percentage of guanine (27.7) and thymine (27.6), while the percentage of adenine (20.5) was the smallest. In contrast to insects, GC content was something higher than AT content. ITS-1 of insect DNA is AT rich, which can be explained by processes as biased occurrence or fixation of point mutation (DE LA RUA *et al.,* 2007). The relative transition/transversion ratio was 2.25. This is in accordance with Kimura-2-parameter model (KIMURA, 1980), Felsenstein`s model (FELSENSTEIN, 1985) and Hasegawa, Kishino and Yano model (HASEGAWA *et al.,* 1985) of sequence evolution.

Table 1. Content of nitrogen bases of local PCN

	T(U)%	C%	A%	G%	AT%	GC%	GC/AT
Gp Kladnica	27.7	24.2	20.5	27.7	48.2	51.9	1.08
Gp Sanac	27.7	24.2	20.5	27.7	48.2	51.9	1.08
Gr Milatovici	27.3	24.7	20.5	27.6	47.8	53.3	1.12
$\bar{x}$	27.6	24.4	20.5	27.7	48.1	52.4	1.09

The degree of similarity can be expressed as a percentage of direct matching of investigated populations (pairwise distances). Analyses of divergence among our PCN populations showed that Kladnica and Sanac populations share identical pattern. In comparison to Milatovici population there was a difference of 3.0%.

Divergence among local and foreign populations of *G. pallida* is shown in Table 2. The percentage of difference among investigated populations of *G. pallida* ranged from: 0.0% between local populations and populations of York, UK and Idaho, USA; 0.11% between our populations and those from Cusco (Peru) and Li-Pali (New Zealand); 0.23% between local populations and other populations from Peru (Puno and Amantani), 0.57% between local populations and the population from Risby (England); 1.37% to 1.71% between ours and populations from the Andes and Chile (Cassola, Apurímac, Porvenir, Terre de Feu); 1.82% between ours and the population from Huancabamba (Peru); to 2.50% between our populations and a population from Argentina. Genetic divergence among populations can be shown graphically by other phylogenetic methods such as Neighbour Joining, Maximum Likelihood, Bayesian inference, etc.

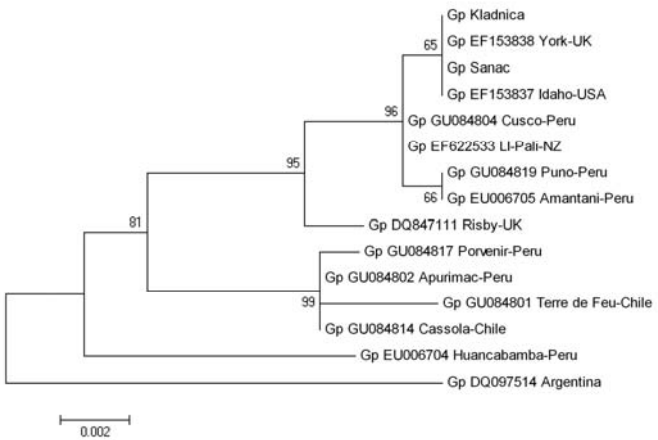
Table 2. Divergence among investigated populations of G. pallida calculated from pairwise distances

POPULATIONS	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Gp Kladnica														
2. Gp Sanac	0.00													
3. Gp DQ097514 Argentina	2.50	2.50												
4. Gp EU006704 Huancabamba-Peru	1.82	1.82	2.28											
5. Gp GU084804 Cusco- Peru	0.11	0.11	2.39	1.71										
6. Gp GU084817 Porvenir-Peru	1.48	1.48	2.39	1.48	1.37									
7. Gp GU084819 Puno-Peru	0.23	0.23	2.50	1.82	0.11	1.48								
8. Gp GU084802 Apurimac-Peru	1.37	1.37	2.28	1.37	1.25	0.11	1.37							
9. Gp EU006705 Amantani-Peru	0.23	0.23	2.50	1.82	0.11	1.48	0.00	1.37						
10. Gp GU084801 Terre de Feu-Chile	1.71	1.71	2.62	1.71	1.59	0.46	1.71	0.34	1.71					
11. Gp GU084814 Cassola-Chile	1.37	1.37	2.28	1.37	1.25	0.11	1.37	0.00	1.37	0.34				
12. Gp EF153838 York-UK	0.00	0.00	2.50	1.82	0.11	1.48	0.23	1.37	0.23	1.71	1.37			
13. Gp DQ847111 Risby-UK	0.57	0.57	2.39	1.71	0.46	1.14	0.57	1.02	0.57	1.37	1.02	0.57		
14. Gp EF622533 Li-Pali-NZ	0.11	0.11	2.39	1.71	0.00	1.37	0.11	1.25	0.11	1.59	1.25	0.11	0.46	
15. Gp EF153837 Idaho-USA	0.00	0.00	2.50	1.82	0.11	1.48	0.23	1.37	0.23	1.71	1.37	0.00	0.57	0.11

Phylogenetic relationships among the observed populations from the Graph 1 were obtained by the Neighbour Joining method (SAITOU and NEI, 1987).

Dendrogram was generated from 500 replicates; the percentage of replicate dendrograms in which taxa clustered together in the bootstrap test was shown next to clusters (FELSENSTEIN, 1985). Codon positions included all three positions + noncoding. All positions that contained gaps and missing data were eliminated from analyses (Complete deletion option). Phylogenetic analyses were performed using MEGA 4 (TAMURA *et al.,* 2007) computer software. The Neighbour Joining tree clustered local populations of *G. pallida* with populations from England (York) and America (Idaho) in a common subunit and also clustered them with populations from Cusco (Peru), Li-Pali (NZ), Amantani and Puno (Peru), in a common unit or clade. In the second clade were clustered Porvenir and Apurímac populations from Peru

and Terre de Feu and Cassola populations from Chile. Genetically, the most distant was a population from Argentina.



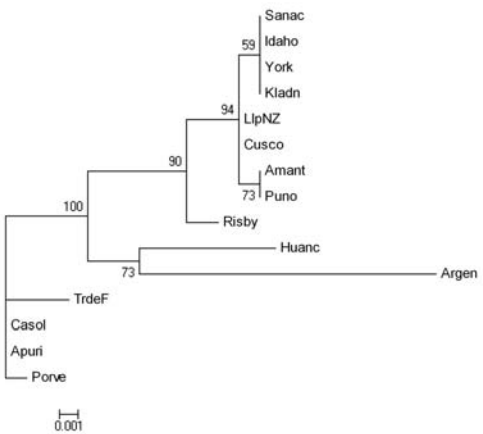
Graph 1. Neighbour Joining dendrogram of *G. pallida* populations

Phylogenetic relationships among the observed populations from the Graph 2 were obtained using the Maximum Likelihood method. The used model of nucleotide substitution was the model of Hasegawa, Kishino and Yano (HASEGAWA *et al*., 1985). Evaluation of dendrogram reliability was calculated by the bootstrap test of 100 replicates. Phylogenetic analyses were done using Phyml 4.2.4. and the graphic presentation of the dendrogram was created by exporting Newick format to MEGA 4. Dendrogram obtained by the Maximum Likelihood clustered Kladnica and Sanac populations with the populations from Idaho and York in the same cluster subunit, and also clustered them with populations from Cusco (Peru), Li-Pali (NZ), Amantani and Puno (Peru) as evolutionary nearest populations, in the same cluster unit. The most divergent population was the one from Argentina.

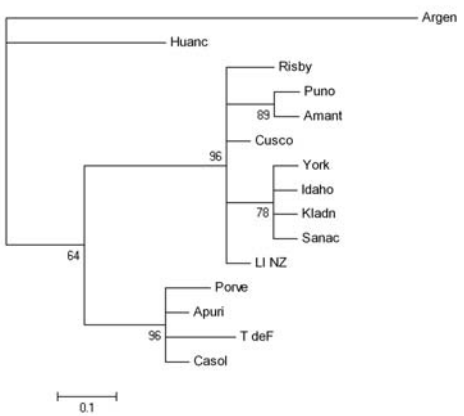
Phylogenetic relationships among the observed populations from the Graph 3 were obtained by Bayesian inference. Nucleotide evolution model was the General Time Reversible Model (TAVARE, 1986).

The dendrogram was created by 300 000 generations of MCMC (Markov Chain Monte Carlo), with the frequency of the sample of 100 and the average standard deviation of 0.007478 of achieved convergence. Phylogenetic analyses were performed using MrBayes 3.1.2. computer software. The graphic presentation of the dendrogram was created by exporting Newick format to MEGA 4. Dendrogram obtained by Bayesian inference, similarly to previous dendrograms, clustered Kladnica and Sanac populations with those from England (York) and America (Idaho) in the same subunit, while the populations from Cusco, Li-Pali,

Amantani, Puno and Risby as evolutionary nearest to them, clustered in the same unit. In another unit were clustered Porvenir and Apurímac populations from Peru and Terre de Feu and Cassola populations from Chile. Genetically most divergent was the population from Argentina.



Graph 2. Maximum Likelihood dendrogram of *G. pallida* populations



Graph 3. Bayesian dendrogram of *G. pallida* populations

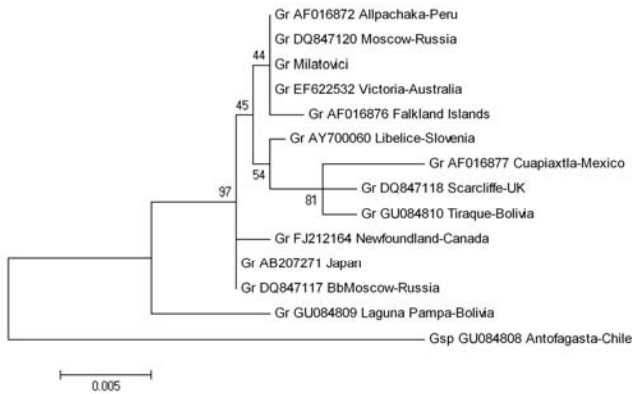
Divergence between local and foreign populations of *G. rostochiensis* calculated from pairwise distances is shown in Table 3. The difference among the investigated populations of *G. rostochiensis* ranged from: 0.0% between the local population from Milatovici and populations from Moscow (Russia), Victoria (Australia) and Alpachaka (Peru); 0.19% between our population from Milatovici and populations from Libelice (Slovenia), Falkland Islands, Japan and Bb Moscow (Russia); 0.75% between Milatovici population and those from Scarcliffe (UK) and Tiraque (Bolivia); to over 1% between Milatovici population and those from Cuapiaxtla (Mexico) and Laguna Pampa (Peru). The most divergent population was from Antofagasta (Chile).

Table 3. Divergence among investigated populations of G. rostochiensis calculated from pairwise distances

POPULATIONS	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Gr Milatovici													
2. Gr AY700060 Libelice-Slovenia	0.19												
3. Gr DQ847118 Scarcliffe-UK	0.75	0.56											
4. Gr AF016876 Falkland Islands	0.19	0.37	0.93										
5. Gr GU084809 Laguna Pampa-Bolivia	1.31	1.50	1.68	1.50									
6. Gr GU084810 Tiraque-Bolivia	0.75	0.56	0.37	0.93	1.68								
7. Gr AF016872 Allpachaka-Peru	0.00	0.19	0.75	0.19	1.31	0.75							
8. Gsp GU084808 Antofagasta-Chile	3.74	3.93	4.11	3.93	3.74	4.11	3.74						
9. Gr AF016877 Cuapiaxtla-Mexico	1.12	0.93	0.75	1.31	2.06	0.75	1.12	4.49					
10. Gr DQ847120 Moscow-Russia	0.00	0.19	0.75	0.19	1.31	0.75	0.00	3.74	1.12				
11. Gr DQ847117 BbMoscow-Russia	0.19	0.37	0.56	0.37	1.12	0.56	0.19	3.55	0.93	0.19			
12. Gr FJ212164 Newfoundland-Canada	0.37	0.56	0.75	0.56	1.31	0.75	0.37	3.74	1.12	0.37	0.19		
13. Gr AB207271 Japan	0.19	0.37	0.56	0.37	1.12	0.56	0.19	3.55	0.93	0.19	0.00	0.19	
14. Gr EF622532 Victoria-Australia	0.00	0.19	0.75	0.19	1.31	0.75	0.00	3.74	1.12	0.00	0.19	0.37	0.19

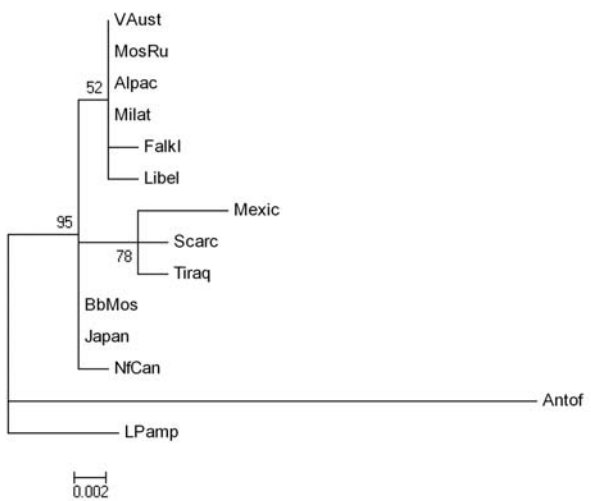
Genetic divergence among populations of *G. rostochiensis* is shown graphically using the Neighbour Joining method, Maximum Likelihood method and Bayesian inference.

Phylogenetic relationships among the observed populations from Graph 4 were obtained by Neighbour Joining method. Dendrogram was generated from 500 replicates; the percentage of replicate dendrograms in which taxa clustered together in the bootstrap test was shown next to clusters. Codon positions included all three positions + noncoding. All positions that contained gaps and missing data were eliminated from analyses (Complete deletion option). Phylogenetic analyses were conducted using MEGA 4 computer software. The obtained Neighbour Joining dendrogram clustered local population from Milatovici with populations from Alpachaka (Peru), Moscow (Russia), Victoria (Australia) and Falkland Islands in the same subunit, while the populations from Libelice (Slovenia), Scarcliffe (UK), Tiraque (Bolivia) and Cuapiaxtla (Mexico) were in another subunit. In the same clade, dendrogram clustered all of them with those from Japan, Bb Moscow (Russia) and Newfoundland (Canada). The most divergent population was the one from Antofagasta (Chile).



Graph 4. Neighbour Joining dendrogram of *G. rostochiensis* populations

Phylogenetic relationships among the observed populations from the Graph 5 were obtained using the Maximum Likelihood method. The used model of nucleotide substitution was the model of Hasegawa, Kishino and Yano. Evaluation of dendrogram reliability was calculated by the bootstrap test of 100 replicates. Phylogenetic analyses were performed using Phyml 4.2.4. computer software and the graphic presentation of the dendrogram was created by exporting Newick format to MEGA 4. Dendrogram obtained by the Maximum Likelihood method clustered population from Milatovici with populations from: Moscow (Russia), Victoria (Australia), Alpachaka (Peru), Libelice (Slovenia) and Falkland Islands.

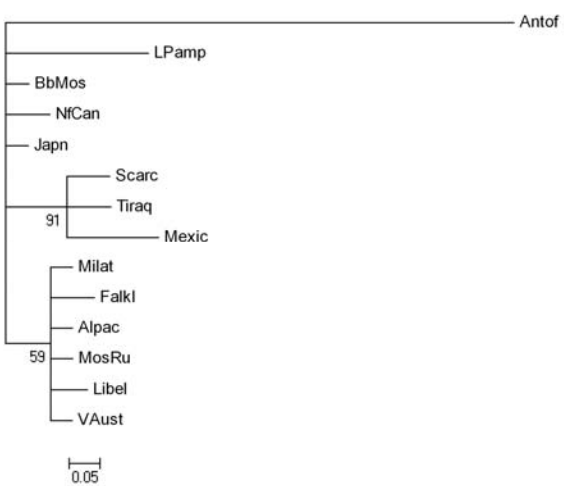


Graph 5. Maximum Likelihood dendrogram of *G. rostochiensis* populations

The separate subunit was formed by clustering of Scarcliffe (UK), Tiraque (Bolivia) and Cuapiaxtla (Mexico).

In the same cluster unit were populations from Newfoundland (Canada), Bb Moscow (Russia) and Japan. Laguna Pampa (Peru) and Antofagasta (Chile) populations were the most divergent populations.

Phylogenetic relationships among the observed populations from the Graph 6 were obtained by Bayesian inference. Nucleotide evolution model was the General Time Reversible Model. The dendrogram was created by 500 000 generations of MCMC (Markov Chain Monte Carlo), with the frequency of the sample of 100 and the average standard deviation of 0.007446 of achieved convergence. Phylogenetic analyses were performed using MrBayes 3.1.2. computer software. The graphic presentation of the dendrogram was created by exporting Newick format to MEGA 4. Dendrogram obtained by Bayesian inference generated two cluster subunits, grouping the population from Milatovici with populations from Moscow (Russia), Victoria (Australia), Alpachaka (Peru), Libelice (Slovenia) and the Falkland Islands into one subunit. As a separate subunit, dendrogram clustered the populations from Scarcliffe (England), Tiraque (Bolivia) and Cuapiaxtla (Mexico). Phylogenetically, most distant from all of them was the population from Antofagasta, Chile.



Graph 6. Bayesian dendrogram of *G. rostochiensis* populations

From the previous phylogenetic analyses, it could be assumed that possible ancestors of our PCN populations originate from Peru. The presence of *G. pallida* and *G. rostochiensis* in our country is not the result of direct import of infected potatoes from Latin America, but it is more likely that a country in Europe was a "transition host". Possible "transition host" for *G. pallida* could be England, where

the population from York has the same sequence. It is hard to imagine which country could be a "transition host" for *G. rostochiensis*, since there are no data on ITS sequences from Germany, the Netherlands, and other potato exporting countries.

The congruence of our populations with the population from Idaho (USA) in case of *G. pallida* and populations from Moscow (Russia) and Victoria (Australia) in case of *G. rostochiensis* could mean that these populations had a common "transition host", because it is not likely that the infected potato was imported from these countries.

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I z v o d

Morfologija cistolikih nematoda krompira (CNK) je do nedavno bila gotovo jedini način identifikacije ovih karantinskih organizama. Molekularne analize su u poslednje dve decenije doprinele bržoj i efikasnijoj identifikaciji dve *Globodera* vrste (*Globodera pallida* i *G. rostochiensis)* i omogućile uvid u genetičku strukturu onih delova koji su praktično bili nedostupni morfološkim studijama. Molekularna karakterizacija je urađena u ITS1-5.8S-ITS2 regionu. Poređenja su vršena sa sekvencama različitih stranih populacija CNK preko NCBI GenBank baze podataka. Rezultati molekularnih studija pokazuju sličnosti i razlike domaćih i stranih populacija CNK u proučavanom delu genoma.

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