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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF POTATO CYST NEMATODE POPULATIONS IN SERBIA

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Abstract - Quarantine species such as potato cyst nematodes *Globodera rostochiensis* and *G. pallida* are present in Serbia since 1999 and 2005, respectively. These nematodes are sibling species and their morphological identification is complex due to their morphometric overlap. The cysts from the localities of Kladnica, Šanac, Gojna Gora and Milatovići were grown on susceptible potato varieties and their morphological differences have been discussed. To avoid ambiguities in species morphological designation a duplex PCR method was chosen for a rapid and accurate species identification. The whole procedure, from DNA extraction to DNA isolation, can be performed in a single day.

Keywords: Potato cyst nematodes, populations, morphology, duplex PCR

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INTRODUCTION

The year 2008 was designated the International Year of the Potato (FAO UN) signifying the importance of this plant in food production. The most important potato parasites are *Globodera rostochiensis* and *G. pallida* and both have quarantine status and are present in Serbia since 1999 and 2005, respectively. The species originate from the area around Lake Titicaca in Peru where the potato was grown 1200 years before Christ.

Potato cyst nematodes cause up to £300M sterling worth of damage to the potato crop in the EU each year (Ryan et al., 2000).

For these reasons an accurate identification is essential for the phytosanitary system of every country. The morphological identification of potato cyst nematodes is based on the combination of morphological and morphometric characteristics of cysts and invasive larvae (J2). *Globodera rostochiensis* and *G. pallida* are sibling species and their morphometric features can overlap among different populations, which can lead to their incorrect identification.

The aim of this study was to confirm the identity of potato cyst nematodes (PCN) by molecular methods for morphologically identified populations and to find a simple and reliable molecular method for species identification, since molecular analyses for plant parasitic nematodes have not been performed in Serbia before.

MATERIALS AND METHODS

Nematodes

The cysts of *Globodera* from Kladnica, Šanac, Gojna Gora and Milatovići, Serbia, were grown on susceptible potato varieties in a climatized chamber at 15-25°C for a 16 h photoperiod from 2006 to 2009.

The cyst extraction was done by elutriation with the Spears apparatus (Spears, 1968) and collected on a 150 μm sieve while invasive larvae were obtained by cutting vital cysts under dissecting microscope.

Morphological characteristics

For morphological study the cysts and larvae were fixed in formalin-glycerol fixative (Hooper, 1970), mounted on glycerol and observed with a light microscope. Morphometric characteristics were measured with an eyepiece micrometer. The cysts were air dried and gold plated for scanning electron microscopy studies. The morphological identification comprises larval stylet length and stylet knob shape, cyst vulval basin diameter, distance between vulva and anus, Granek´s ratio (the vulva – anus distance divided by vulval basin diameter) and number of cuticular ridges in perineal area (EPPO Standards, 2004).

DNA extraction

DNA was extracted with DNAzol™ (MRC, Inc.) using 10 cysts in 10 µl doubly distilled water and 100 µl DNAzol was added. The procedure was done in accordance to the manufacturer´s instructions except that all reagents were used in lower quantities, i.e. 50 µl of 100% ethanol, 100 µl of 75% ethanol and 25 µl of 8mM NaOH.

DNA amplification and separation of PCR products

Amplification was done with both sets of primers used in one reaction according to Vejl et al., (2002). The PCR master mix contained reagents as in Subbotin et al., (2001).

The positive controls were PCN populations previously identified by molecular methods: for *G. pallida* population Javor (Radivojevic et al., 2006) and for *G. rostochiensis* population Ponikve (Radivojevic et al., 2001), and a negative control was without nematode DNA. The marker was a DNA Ladder mix #SMO331. Electrophoretic 1.5% agarose gel was stained with ethidium bromide and visualized under UV light.

RESULTS

Differences between the PCN populations were analyzed by measuring the larval morphological characteristics: stylet length (fig. 1), body length, tail and hyaline tail length (fig. 2) and cyst morphological characters: cyst size (fig. 3), vulval basin diameter, the distance between the vulva and anus (fig. 4), Granek´s ratio and the number of ridges in perineal area (fig. 5).

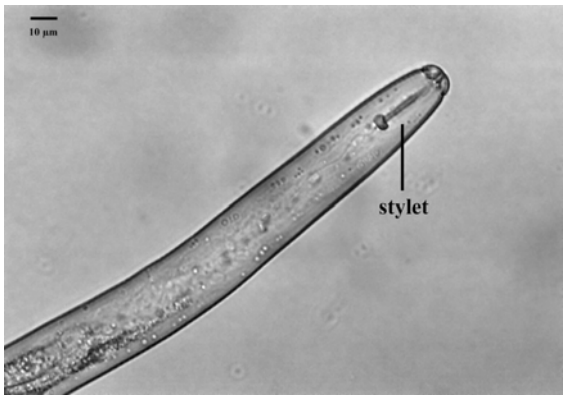

Fig. 1. Larval head and stylet

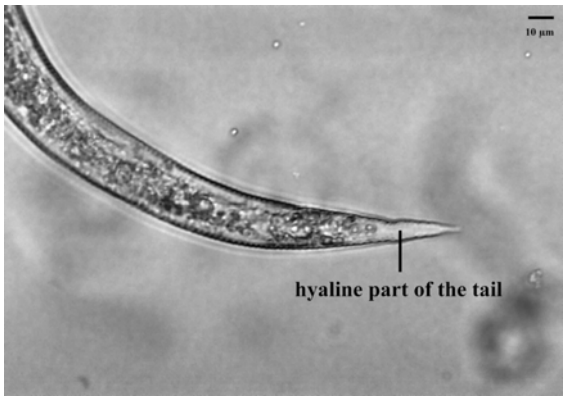

Fig. 2. Larval tail and hyaline part of the tail

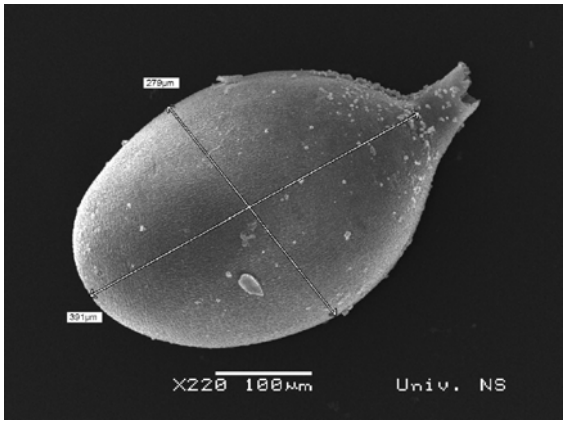

Fig. 3. SEM of cyst

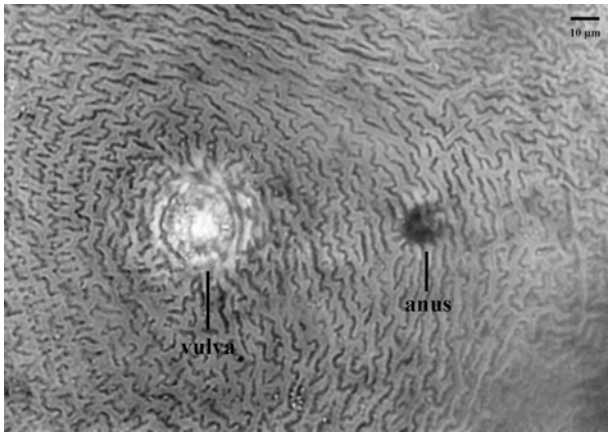

Fig. 4. Vulva and anus of cyst

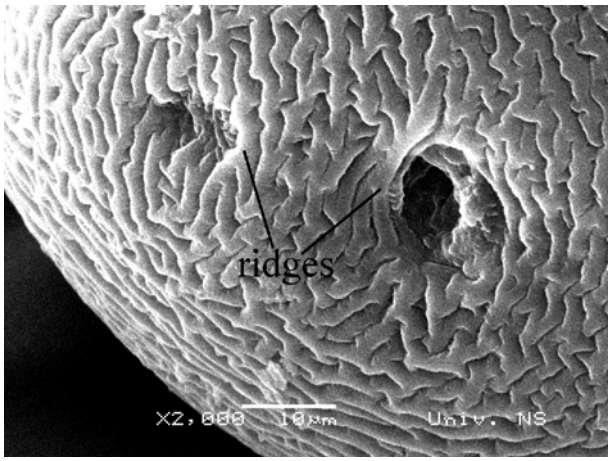

Fig. 5. SEM of ridges in perineal area

The morphometrics of the cysts and larvae from the different populations, based on 30 specimens, are given in Tables 1-4.

All the shown morphological values from the different populations and different species are very close. The characteristics that vary most are cyst size and larval length. On average, the shortest larvae are from the Kladnica population (439.74 µm) and the longest are from Milatovići (471.87 µm). The larvae from Kladnica have the highest mean stylet value (25.25 µm), and the specimens from Milatovići have the lowest mean stylet value (21.28 µm). On average, the larvae from Šanac have the longest tail (50.72 µm) and the Gojna Gora larvae have the shortest tail (47.70 µm).

Comparison of the mean morphological values of the Serbian populations and type populations Lincolnshire of *G. pallida* (Stone, 1973b) and Rostock of *G. rostochiensis* (Stone, 1973a) are shown in the Figs. 6 and 7. With respect to the type population, the closest to *G. rostochiensis* is the population from Milatovići. The mean stylet and tail length from Šanac is the closest to the *G. pallida*-type population. The Kladnica population has a longer stylet than the type population of *G. pallida,* but other morphological character values are within the range of the type population. The mean stylet length value of the Gojna Gora population is closer to *G. rostochiensis* than to *G. pallida*.

As for the morphometrics of cysts, the specimens from Milatovići have the highest values of Granek´s ratio, number of ridges and vulva-anus distance, which fits well with the *G. rostochiensis* description. The specimens from Kladnica have the lowest values of Granek´s ratio, number of ridges and vulva-anus distance and they are within the range observed for the *G. pallida* type population.

Regarding Granek´s ratio and the number of ridges in the perineal area, the closest to the *G. pallida* type population are the Gojna Gora and Šanac populations.

Duplex PCR revealed that the Kladnica, Šanac and Gojna Gora localities were *G. pallida* (lanes 3, 4 and 5) and Milatovići was *G. rostochiensis* (lane 6) (Fig. 8). The two sets of primers amplified products of the 239 bp fragment for *G. pallida* and the 274 bp fragment for *G. rostochiensis* in the ITS-1 region.

DISCUSSION

The taxonomic features of the two sibling species *G. pallida* and *G. rostochiensis* overlap among different populations. The characteristics that vary most are larval length and cyst size. The mean Granek´s ratio is the most reliable morphological characteristic for species morphological designation. Comparing the values of the different populations from Serbia to type population values resulted in the unambiguous

Table 1. Morphometrics of larvae and cysts from Kladnica population

	Larvae (J2)			Cysts		
Characters	range	mean	SD	range	mean	SD
J2 body length (µm)	396.90-529.20	439.74	33.27	-	-	-
J2 stylet length (µm)	22.40-27.20	25.25	0.98	-	-	-
J2 tail length (µm)	44.80-56.00	50.56	3.08	-	-	-
J2 hyaline tail length (µm)	19.20-41.60	27.28	4.85	-	-	-
cyst length (µm)	-	-	-	329.00-744.00	566.90	90.89
cyst width (µm)	-	-	-	233.00-698.00	517.30	102.83
vulval basin diameter (µm)	-	-	-	16.00-32.00	22.83	4.62
distance vulva-anus (µm)	-	-	-	27.20-73.60	43.15	9.86
Granek´s ratio	-	-	-	1.00-2.92	1.94	0.49
number of ridges	-	-	-	8-14	9.97	1.79

species designation of the Kladnica, Šanac and Milatovići populations. Some extreme values of Granek´s ratio, number of ridges, vulva-anus distance and stylet length from Gojna Gora were closer to *G. rostochiensis* than to *G. pallida*. This could be why the Gojna Gora population is described as *G. rostochiensis* by Krnjaic et al. (2006). The PCR method avoided such ambiguities in nematode diagnostics. There are some other advantages of PCR and used reagents.

Using two sets of primers in the same reaction enables species identification in only one step without the need for subsequent analysis.

Although DNAzol is not usually used for plant parasitic nematodes, it enables reliable results, since the extracted DNA is visible as a precipitate.

Table 2. Morphometrics of larvae and cysts from Šanac population

	Larvae (J2)			Cysts		
Characters	range	mean	SD	range	mean	SD
J2 body length (µm)	378.00-516.60	455.91	33.27	-	-	-
J2 stylet length (µm)	20.80-25.60	23.63	1.24	-	-	-
J2 tail length (µm)	43.20-56.00	50.72	3.62	-	-	-
J2 hyaline tail length (µm)	22.40-35.20	28.64	3.55	-	-	-
cyst length (µm)	-	-	-	310.00-651.00	508.00	79.81
cyst width (µm)	-	-	-	279.00-682.00	487.22	87.37
vulval basin diameter (µm)	-	-	-	19.20-43.20	27.20	5.82
distance vulva-anus (µm)	-	-	-	40.00-105.60	59.36	15.50
Granek´s ratio	-	-	-	1.18-3.67	2.27	0.57
number of ridges	-	-	-	9-22	13.17	3.41

Table 3. Morphometrics of larvae and cysts from Gojna Gora population

	Larvae (J2)			Cysts		
Characters	range	mean	SD	range	mean	SD
J2 body length (µm)	359.10-485.10	444.99	32.98	-	-	-
J2 stylet length (µm)	20.80-24.00	22.77	1.09	-	-	-
J2 tail length (µm)	35.20-56.00	47.07	4.84	-	-	-
J2 hyaline tail length (µm)	17.60-30.40	23.36	3.16	-	-	-
cyst length (µm)	-	-	-	372.00-744.00	521.32	77.92
cyst width (µm)	-	-	-	310.00-713.00	479.47	89.45
vulval basin diameter (µm)	-	-	-	20.80-56.00	30.45	7.26
distance vulva-anus (µm)	-	-	-	32.00-124.80	61.65	20.00
Granek´s ratio	-	-	-	1.05-3.90	2.09	0.69
number of ridges	-	-	-	8-25	12.90	3.54

Table 4. Morphometrics of larvae and cysts from Milatovići population

	Larvae (J2)			Cysts		
Characters	range	mean	SD	range	mean	SD
J2 body length (µm)	409.50-516.60	471.87	21.59	-	-	-
J2 stylet length (µm)	19.20-27.20	21.28	2.19	-	-	-
J2 tail length (µm)	44.80-54.44	50.19	2.51	-	-	-
J2 hyaline tail length (µm)	20.80-35.20	25.92	3.48	-	-	-
cyst length (µm)	-	-	-	403.00-744.00	615.00	66.82
cyst width (µm)	-	-	-	372.00-744.00	587.53	82.06
vulval basin diameter (µm)	-	-	-	12.80-25.60	19.15	2.89
distance vulva-anus (µm)	-	-	-	44.80-84.80	61.23	10.26
Granek´s ratio	-	-	-	2.23-6.63	3.25	0.76
number of ridges	-	-	-	13-21	16.23	2.40

The DNA need not to be frozen, which speeds up the diagnostic process. The whole procedure (from DNA extraction to DNA visualization) can be performed in a single day.

In the article of Vejl et al., (2002) it was shown that *G. pallida* amplified 239 bp fragments for the Kalle, Chavornai and Delmsen populations and *G. rostochiensis* obtained 411 bp amplimers for Šluknov and Harmerz, and 274 bp amplimers for the Obersteinbach population. As we obtained two 274 bp products for both the Ponikve and Milatovići populations, this could indicate a possible genetic similarity of the two Serbian

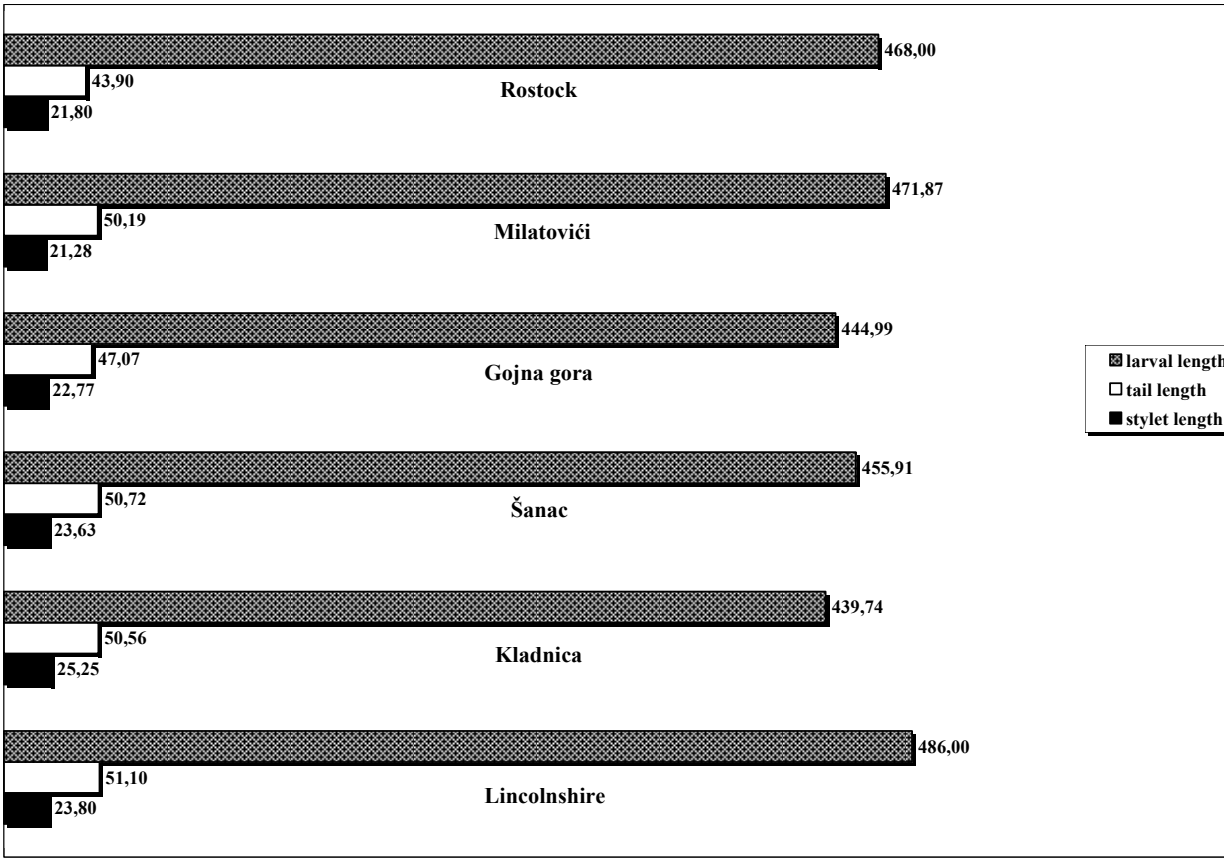

Fig. 6. Comparison of mean larval characteristics (in micrometers) from different populations

populations to the German one. This hypothesis can be resolved by nucleotide sequencing, which will be the subject of further investigations.

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REFERENCES

EPPO Standards PM 7/40. (2004). Diagnostic protocols for regulated pests: *Globodera rostochiensis* and *Globodera pallida*. EPPO bulletin, 34, 309-314.

Hooper, D. J. (1986): Handling, fixing, staining and mounting nematodes. In: Laboratory methods for work with plant and soil nematodes, (Ed. J.F. Southey), 59-81. Ministry of Agriculture, Fisheries and Food.

Krnjaic, D., Oro, V., Gladovic, S., and N. Trkulja. (2006). Distribution of potato cyst nematodes in Serbia. XXVIII International Symposium, Blagoevgrad-Bulgaria. Programme and Abstracts, 134.

Radivojevic, M., Krnjaic, D., Krnjaic, S., Bacic, J., Subbotin, S.A., Madani, M., and *M. Moens.* (2001). Molecular methods confirming the presence of *Globodera rostochiensis* (Wollenweber, 1923) in Yugoslavia. Russian Journal of Nematology, 9, 139-141.

Radivojevic, M., Krnjaic, D., Grujic, N., Oro, V., Gladovic, S., and *M. Madani*. (2006). The first record of potato cyst nematode *Globodera pallida* (Stone, 1973) from Serbia, 58th International Symposium on Crop Protection. Programme and abstracts, 203.

Ryan, N.A., Duffy, E.M., Cassell, A.C., and *P.W. Jones* (2000).: The effect of mycorrrhizal fungi on the hatch of potato cyst nematodes. *Applied Soil Ecology*, **15**, 233-240.

Spears, J.F. (1968). The Golden Nematode Handbook - Survey, Laboratory, Control and Quarantine Procedures. Agriculture Handbook 353. USDA, Agricultural Research Service. Washington, D.C., 1-82.

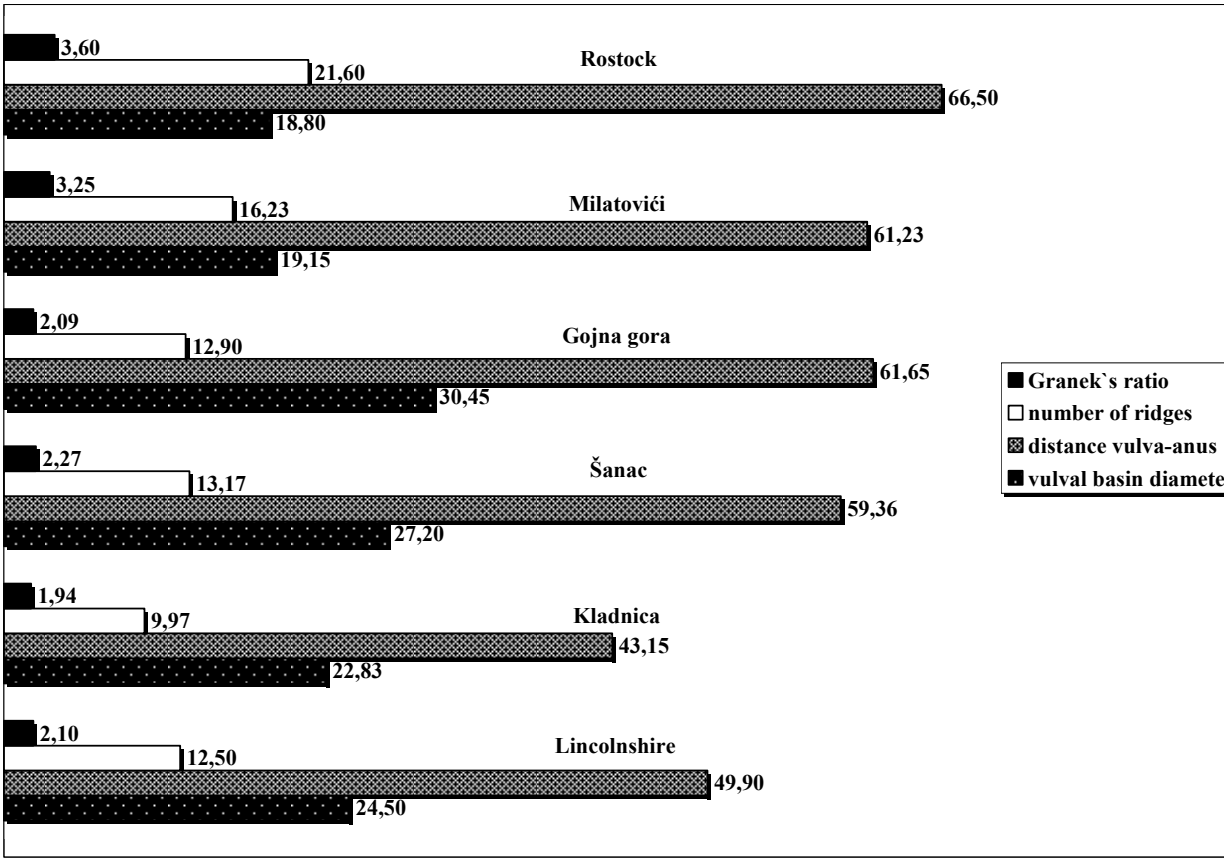

Fig. 7. Comparison of mean cyst characteristics (in micrometers) from different populations

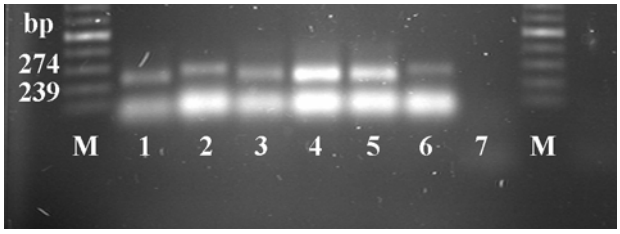

Fig. 8. PCR products of *G. pallida* and *G. rostochiensis* populations: 1) Javor, 2) Ponikve, 3) Kladnica, 4) Šanac, 5) Gojna Gora, 6) Milatovići, 7) Negative Control, M) DNA Ladder mix #SMO331.

Stone, A.R. (1973a). *Heterodera rostochiensis.* C.I.H. Descriptions of plant-parasitic nematodes, Set 2, No. 16. CAB International, Wallingford.

Stone, A.R. (1973b). *Heterodera pallida.* C.I.H. Descriptions of plant-parasitic nematodes, Set 2, No. 17. CAB International, Wallingford.

Subbotin, S.A., Peng, D. and *M. Moens*. (2001). A rapid method for the identification of the soybean cyst nematode *Heterodera* glycines using duplex PCR. *Nematology*, **3**, 365-371.

Vejl, P., Skupinova, S., Sedlak, P. and *J. Domkarova*. (2002). Identification of PCN species (*Globodera rostochiensis, G. pallida*) by using of ITS-1 region polymorphism. *Rostlinna vyroba*, **48**, 486-489.

МОРФОЛОШКА И МОЛЕКУЛАРНА ИДЕНТИФИКАЦИЈА ПОПУЛАЦИЈА ЦИСТОЛИКИХ НЕМАТОДА КРОМПИРА У СРБИЈИ

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Карантинске врсте као што су цистолике нематоде кромпира *Globodera rostochiensis* и *G. pallida* су присутне у Србији од 1999. год. одн. 2005. год. Ове нематоде су сестринске врсте и њихова морфолошка идентификација је сложена због морфометријског преклапања. Цисте са локалитета Кладница, Шанац, Гојна Гора и Милатовићи су гајене на осетљивим сортама кромпира и њихове морфолошке разлике су анализиране. Да би се избегле нејасноће у морфолошком одређивању врста, изабран је duplex PCR метод за брзу и прецизну специјску идентификацију. Целокупна процедура, од екстракције ДНК до њене визуализације, може бити урађена за један дан.