Analyses of *Globodera rostochiensis* and *G. pallida* populations from Serbia by morphometrics and real-time PCR

Short communication

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Infestation by potato cyst nematodes (PCN) is becoming an increasingly serious problem in Serbia. Potato is an important crop for Serbian agriculture with annual production of nearly one million tons (Marks & Rojancovski, 1998). Western Serbia is the major potato-producing area of the country. According to data supplied by Association of Potato Growers of Serbia, ware potatoes are grown on approximately 75.000 hectares and seed potatoes on 500 hectares with an average yield of 18 tons/ha. *Globodera rostochiensis* was detected in Serbia in 1999 for the first time (Krnjaic *et al.,* 2002) while *G. pallida* was detected five years later (Radivojević *et al.,* 2006). In this work the PCN from Serbia were analysed by morphometrical method and confirmed using a real-time PCR approach where species-specific ribosomal internal transcribed spacer (ITS) region was amplified and detected. In addition, PCN species identification was tested using empty cyst-shells.

Cysts of PCN nematodes were extracted from 7 locations in Serbia: Kladnica KP6- Kušići, Bašta KP1769 – Jagodnja, Brdo KP18806 - Jagodnja, Brdo KP 413 - Jagodnja, Milatovići KP 1646/2, Ponikve – Panića brdo and Vukovo Voće- Jagodnja. Nematodes were identified on the basis of cysts and larvae characters (distance from anus to vulval basin, vulval basin diameter, Granek's ratio, number of cuticular ridges, body length, stylet length, tail length and hyaline part of the tail). Juveniles were extracted from cysts, fixed in TAF (triethanolamin-formalin) mounting medium and prepared as semi-permanent microscopic slides. Morphometric characters were measured with an ocular micrometer.

Nematode DNA was extracted using the Wizard genomic DNA purification kit (Promega) from 10 to 15 vital cysts from each sample following the manufacturer's instructions with some modifications. DNA from 1, 5 and 10 empty cysts shells was isolated using Wizard genomic DNA purification kit (Promega) and QIAamp DNA Micro kit (Qiagen) for forensic case work samples following manufacturer's instructions, respectively. The presence of *G.*

rostochiensis and *G. pallida* was tested for each sample in separate reactions with species-specific primer pairs, and each test was performed in duplicate or triplicate. *G. rostochiensis* specific ribosomal internal transcribed spacer (ITS) region was amplified by primers ITS5 (White *et al.,* 1990) and PITSr3 (Bulman & Marshall, 1997), and in *G. pallida* by ITS5 (White *et al.,* 1990) and PITSp4 (Bulman & Marshall, 1997). DNA was amplified in 25 µl reaction mixtures composed of 12.5 µl Power sybr green PCR master mix (Applied Biosystems), 3 µl ITS5 (5 pM/µl), 3 µl PITSr3 (5 pM/µl) or PITSp4 (5 pM/µl), 2 µl of template DNA and additional water. Positive controls for each species, of *G. rostochiensis* and *G. pallida* DNA were also tested. PCR was performed in an Applied Biosystems 7500 Real Time PCR System. PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 5 sec and elongation at 72°C for 33 sec. Dissociation was measured by heating to 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. The C_t value, which is the number of PCR cycles needed to reach a minimum level of fluorescence associated with an exponential increase in PCR product, was calculated by the Sequence Detection Software v1.3 (Applied Biosystems). DNA concentration of template DNA isolated from viable cysts was measured with a DyNA Quant 200 fluorometer (Hoefer).

Morphometrical analyses of ten cysts and J2 larvae established the presence of *G. rostochiensis* in 6 soil samples (Bašta KP1769, Brdo KP18806, Brdo KP 413, Milatovići 1646/2, Panića Brdo and Vukovo Voće) and of *G. pallida* in a soil sample from Kladnica. Some cysts of the Kladnica population showed a greater distance between vulva-anus (70.2 µm) and Granek's ratio (4.8), which is not typical for the species. Finally, the number of cuticular ridges between vulva-anus was smaller (10-16) compared to the others (16-24). The

morphometrics were compared with *G. rostochiensis* (Stone, 1973a) and *G. pallida* (Stone, 1973b) and coincided with the characteristics indicated in the literature (Fleming & Powers, 1998). Real-time PCR analyses confirmed morphometrical identification. The peak of dissociation curve in the range of 86.7 to 87.1°C indicated the presence of *G. rostochiensis* whereas the peak at 83.3 – 83.7°C was consistent with the presence of *G. pallida*. Six of the seven samples tested positive for *G. rostochiensis* and one sample (Kladnica KP6 - Kušići) tested positive for *G. pallida*. Mixed PCN populations were not detected. In order to test whether the PCN species identification is possible from empty cyst-shells, DNA from empty cyst-shells was tested in a real-time PCR. No amplification was measured regardless of DNA extraction method or number of empty cysts in the initial sample.

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Table 1: Morphometrical characters of *Globodera* second stage juveniles and cysts from 7 locations from Serbia (n=10).

	Bašta 1769	Brdo 18806	Brdo 413	VukovoVoće	Milatovići 1676/2	Panića Brdo	Kladnica 6
Body length (µm)	426.10 ± 32.69 (369-452)	427.70 ± 33.43 (371-467)	426.30 ± 27.30 (386-462)	431.30 ± 27.23 (374-466)	457.30 ± 28.01 (432-498)	429.10 ± 27.90 (388-466)	467.50 ± 22.48 (417-488)
Stylet length (µm)	21.21 ± 0.67 (19.80-21.70)	21.64 ± 0.95 (19.80-22.77)	20.69 ± 0.72 (19.80-21.87)	21.38 ± 1.01 (19.80-22.27)	21.19 ± 1.06 (19.80-22.70)	21.04 ± 1.52 (19.80-22.27)	22.97 ± 1.39 (19.80-24.30)
Tail length (µm)	44.95 ± 4.79 (36.45-53.46)	45.92 ± 7.30 (36.45-60.75)	49.81 ± 7.35 (36.45-60.75)	50.20 ± 6.57 (41.31-58.32)	50.14 ± 4.22 (43.70-57.50)	52.24 ± 5.52 (43.74-60.75)	53.78 ± 5.59 (48.60-60.75)
Hyaline part of tail (µm)	22.35 ± 2.00 (19.44-24.30)	22.23 ± 2.28 (18.25-25.51)	25.12 ± 2.63 (20.65-29.16)	26.36 ± 2.22 (23.08-29.16)	24.70 ± 3.29 (20.60-29.16)	24.97 ± 3.74 (19.44-29.16)	27.24 ± 2.29 (24.30-29.16)
Vulval basin diameter (µm)	18.42 ± 2.14 (14.58-20.65)	17.37 ± 1.72 (14.58 -19.44)	16.54 ± 1.67 (14.58-19.44)	18.84 ± 3.43 (14.58-26.73)	18.58 ± 3.08 (14.58-21.87)	16.76 ± 1.97 (13.36-19.44)	21.86 ± 3.71 (14.58-26.73)
Distance from anus to vulval basin (µm)	55.88 ± 8.32 (44.95- 72.90)	60.99 ± 11.01 (46.17-77.76)	64.73 ± 12.48 (36.45-74.40)	66.58 ± 10.29 (48.60-77.77)	58.56 ± 15.34 (34.02-77.76)	56.13 ± 9.81 (38.32-70.47)	46.38 ± 12.08 (34.02-70.19)
Granek's ratio	3.03 ± 0.78 (2.31-4.61)	3.52 ± 0.67 (3.12-4.92)	3.93 ± 0.81 (2.50-4.66)	3.65 ± 0.90 (2.00-4.95)	3.29 ± 1.23 (1.55-5.33)	3.39 ± 0.82 (2.57-5.27)	2.25 ± 1.04 (1.33-4.81)
No. of cuticular ridges (min-max)	20-24	16-21	19-22	18-23	19-24	18-22	10-16

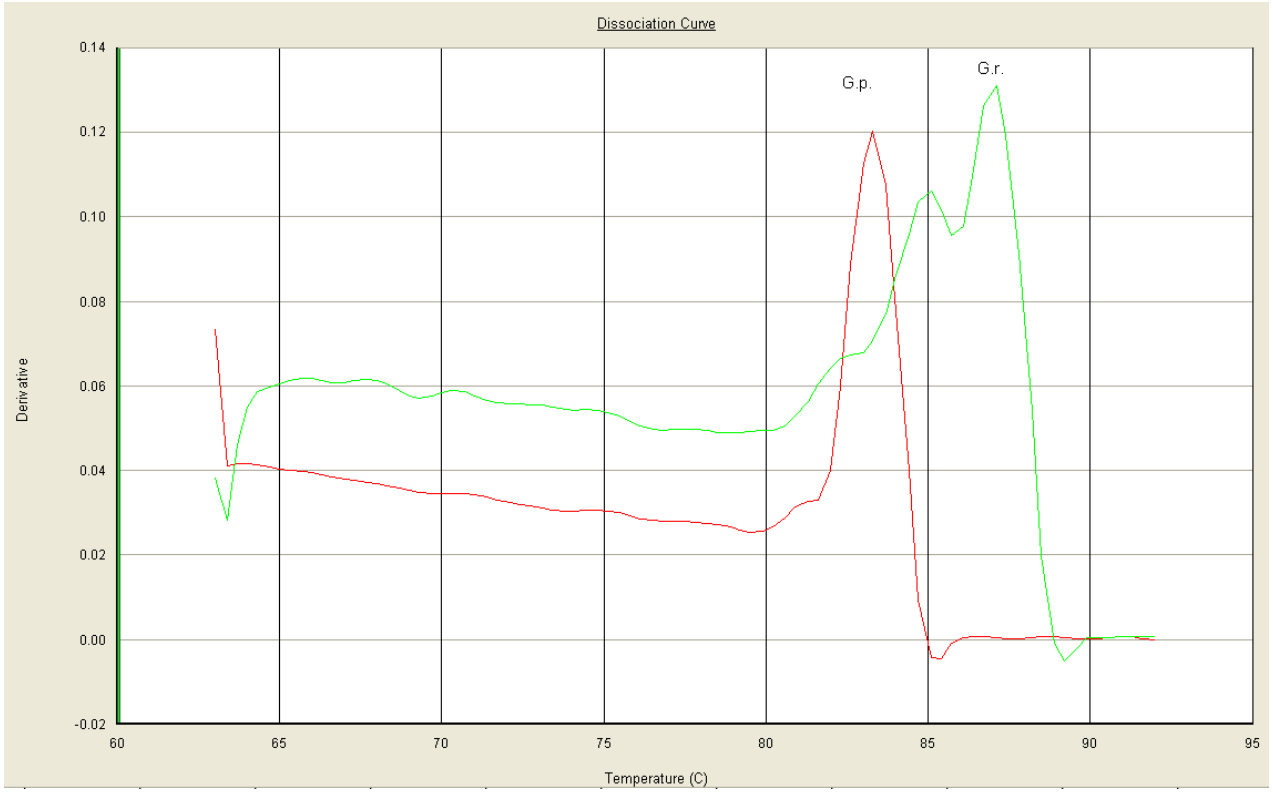

Figure 1: Dissociation curve with Tm value at 83.3ºC for *G. pallida* and Tm value at 87.1ºC for *G. rostochiensis*.