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Molecular and morphological exploration of a mixed population of two potato-parasiting nematode species, *Globodera rostochiensis* and *G. pallida*

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Summary

In this work, we report results of molecular and morphological analyses of a potato field population of *Globodera* (Nematoda: *Heteroderidae*) species, in Slovakia. Unexpectedly, our data show a mixed occurrence of two potato cyst nematode species, *Globodera rostochiensis* and *G. pallida*, in this locality. To our knowledge, this is the first report of mixed occurrence of these economically important plant-parasitic species in the same locality in the Central Europe. In addition, this finding reinforces the possibility of the cross-hybridization between these two nematode species that might result in a generation of new genotypes.

Keywords: Potato cyst nematode; *Globodera rostochiensis*; *Globodera pallida*; molecular diagnostics; morphology; population genetics; Slovakia

Introduction

The quarantine plant-parasitic nematodes are important biotic factors influencing commercial production of the agricultural commodities. Considering potatoes production, two quarantine cyst-forming nematode species are significant currently – *Globodera rostochiensis* and *Globodera pallida*. From these two species, *G. rostochiensis* is widespread in both Czech Republic and Slovakia, whereas *G. pallida* distribution is rather limited in both countries. It was firstly described from the Czech Republic in 2003 by Zouhar *et al*. and in 2009 in Slovakia by Hubinská *et al*. in 2009. The other population was recently described from the western region of Bohemia (Douda *et al*., 2012). It is crucial to precisely distinguish these species by phytosanitary diagnostic tools as the lack of resistant potato cultivars still exists for *G. pallida*, while occurrence of *G. rostochiensis* does not affect potato cultures significantly because nearly all contemporary potato cultivars are resistant or tolerant to this species.

Material and methods

Cysts of potato cyst nematode from a single locality in Central Slovakia were obtained from staff of the Central and Testing Institute in Agriculture of the Slovak Republic. Cysts were subjected to molecular and morphological analysis. DNA was isolated from embryon suspensions originating from cysts used for the previous morphological characterization. DNA was isolated using innuPREP Plant DNA Kit and isolation robot InnuPure® C16 (Analytik Jena) according to manual. DNA samples were used for amplification using multiplex PCR (Zouhar *et al*., 2000). This method allows distinguishing *G. rostochiensis* and *G. pallida* in one tube utilizing universal primer UNI 5'-GCAGTTGGCTAGGGATCTTC-3', *G. pallida* specific primer GPA1 5'-GGTGACTCGACGATTGCTGT-3' and *G. rostochiensis* specific primer GRO5A 5'-ATGTTGTAC GTGCCGTACCTT-3'. These primers amplify nuclear-encoded ribosomal DNA (rDNA), an DNA region that was shown to be highly valuable for classifying, molecular diagnostics and phylogeny reconstructions of diverse organisms at various taxonomic levels, including plant-parasitic nematodes (Marek *et al*., 2010; Douda *et al*., 2013). PCR protocol was as follows: initial denaturation 95 °C for 5 min followed by 34 cycles of PCR (60 s denaturation at 94 °C, 45 s annealing at 62 °C, and 60 s extension at 72 °C) and a 4 min final extension at 72 °C. As positive controls for PCR amplifications, genomic DNA extracted from *G. rostochiensis* (population Šluknov, pathotype Ro1, Czech Republic) and DNA from *G. pallida* (population Chavornay, pathotype Pa3, France) were used, while genomic DNA isolated from *Heterodera schachti*i served as negative control. PCR products were separated on a 1 % horizontal agarose electrophoresis and stained with ethidium bromide. The predicted length of the PCR products was 391 bp for *G. pallida* and 239 bp for *G. rostochiensis* (Fig. 1).

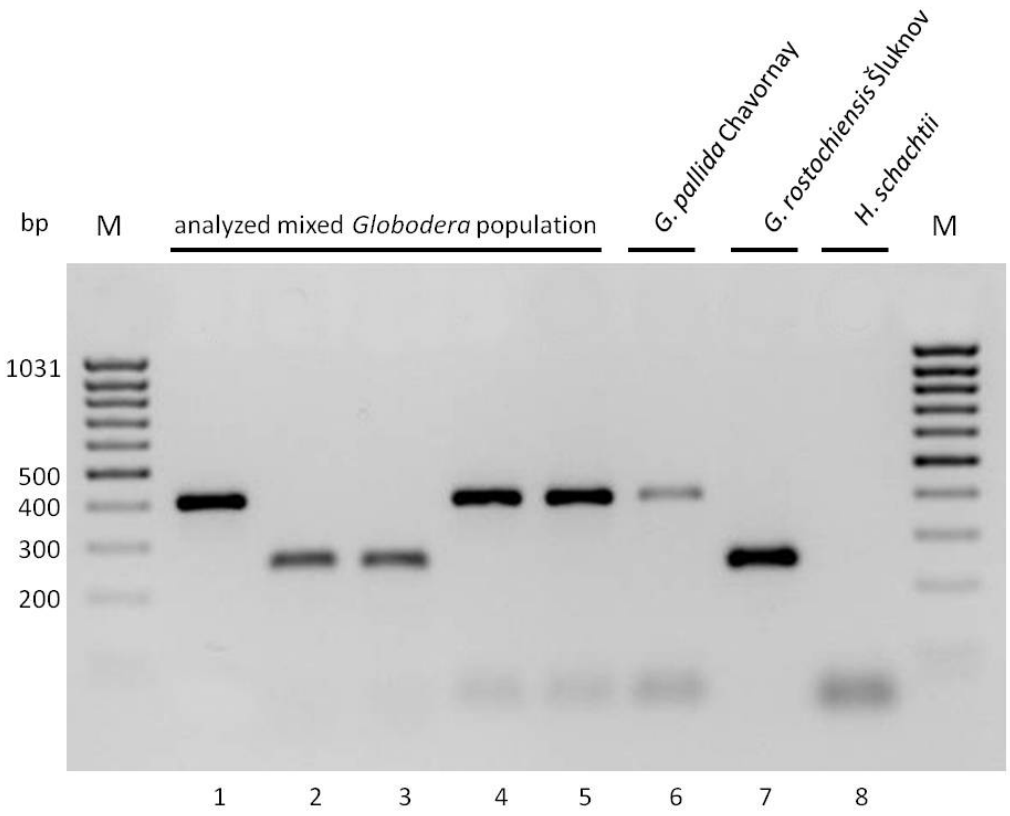

Fig. 1. Multiplex PCR-based amplification of ITS-rDNA region. Lines: 1 to 5 – genomic DNA isolated from individual cysts of the analyzed mixed *Globodera* population (Slovakia); 6 - genomic DNA from *G. pallida* (population Chavornay, pathotype Pa3, France) served as positive control, 7 – genomic DNA from *G. rostochiensis* (population Šluknov, pathotype Ro1, Czech Republic) served as positive control , 8. - genomic DNA from *Heterodera schachtii* served as negative control; M – DNA ladder (MassRuler Low range, Thermo Scientific)

Morphological diagnostics was performed utilizing cysts dissection and measuring using the light microscope. 2nd stage juveniles were obtained from cysts macerated overnight in hatching solution and measured as well. Following cyst morphological data were acquired: fenestra diameter, distance fenestra to anus, Granek's ratio (the vulva – anus distance divided by vulval basin diameter) and number of cuticular ridges between fenestra and anus. In the case of second stage juvenile following values were gained: whole body length (L), stylet length, tail length, and length of hyaline terminal part of tail. Nematological indexes a (body tength/largest body width), b (body length/pharynx length from head to pharyngeo-intestinal junction), b' (body length/pharynx length from anterior end to posterior end of glandular lobe), c (ration of body length to tail length) and c′ (ratio of tail length to body width at anus level) were calculated. All acquired data are summarized in Tables 1 and 2.

Results and discussion

Following morphological characteristics were described: *G. pallida* – juveniles slightly arcuate when relaxed. Lateral field with four lines, occasionally crossed by transverse

Table 1. Morphometrics of diagnostic characters of 2nd stage juveniles of *G. pallida* and *G. rostochensis* from Slovakia

Character	***G. pallida***		***G. rostochiensis***	
	n = 13		n = 32	
	Range	Mean	Range	Mean
Body length (µm)	420 – 540	489.0	400 – 630	486.0
Stylet length (µm)	21.3 – 23.2	22.9	18.8 – 23.5	21.6
Tail length (µm)	47.0 – 63.0	53.8	42.0 – 58.0	50.3
Hyaline terminal tail length (µm)	24.0 – 32.0	28.9	19.0 – 26.0	25.1
Pharynx length (µm)	94.0 – 105.0	102.6	79.0 – 99.0	94.5
Head end to pharyngeal lobe end (µm)	139.0 – 173.0	166.6	121.0 – 159.0	149.3
a	24.0 – 27.0	25.0	18.0 – 22.0	20.5
b	4.0 – 4.6	4.2	4.0 – 4.5	4.3
b′	2.6 – 3.0	2.8	2.2 – 3.1	2.7
c	7.9 – 9.3	8.7	7.1 – 9.1	8.8
c′	3.8 – 5.2	4.5	2.9 – 4.5	4.3

Table 2. Morphometrics of diagnostic characters of cysts of *G. pallida* and *G. rostochensis* from Slovakia

Character	***G. pallida***		***G. rostochiensis***	
	N = 8		N = 12	
	Range	Mean	Range	Mean
Cyst length (mm)	0.53 – 0.60	0.57	0.38 – 0.75	0.57
Cyst width (mm)	0.46 – 0.56	0.53	0.35 – 0.62	0.53
Fenestra diameter (µm)	21.8 – 25.6	24.3	11.5 – 22.9	17.6
Distance fenestra to annus (µm)	35 – 58	49.8	48 – 95	73.2
Distance fenestra to annus/fenestra diameter	1.8 – 2.5	–	2.5 – 5.9	–
Granek´s ratio	1.15 – 3.49	2.32	2.1 – 6.5	4.8
Number of cuticular ridges between vulva and annus	9 – 18	14	15 – 23	18

striae. Head offset, bearing 4 – 6 annuli, about 10 – 12 µm wide at base. Stylet knobs with distinct forward projections, about 4 – 5 µm across. Dorsal gland orifice 3.2 – 4.8 µm posterior to knobs. Metacorporeal valve 64 – 75 µm, excretory pore 92 – 112 µm. Hyaline terminal part of tail 48 – 53 % of total tail length. Terminus rounded. Eggs 112.8 – 115.5 µm long, 41.0 – 42.5 µm wide, length/width 2.3 – 2.7. *G. rostochiensis* - Juveniles slight to slightly arcuate when relaxed. Lateral field with four lines. Head slightly offset, bearing 4 – 5 annuli, about 9 – 11 µm wide at base and 4.2 – 4.8 µm high. Stylet knobs anteriorly flattened to rounded, without forward projec-

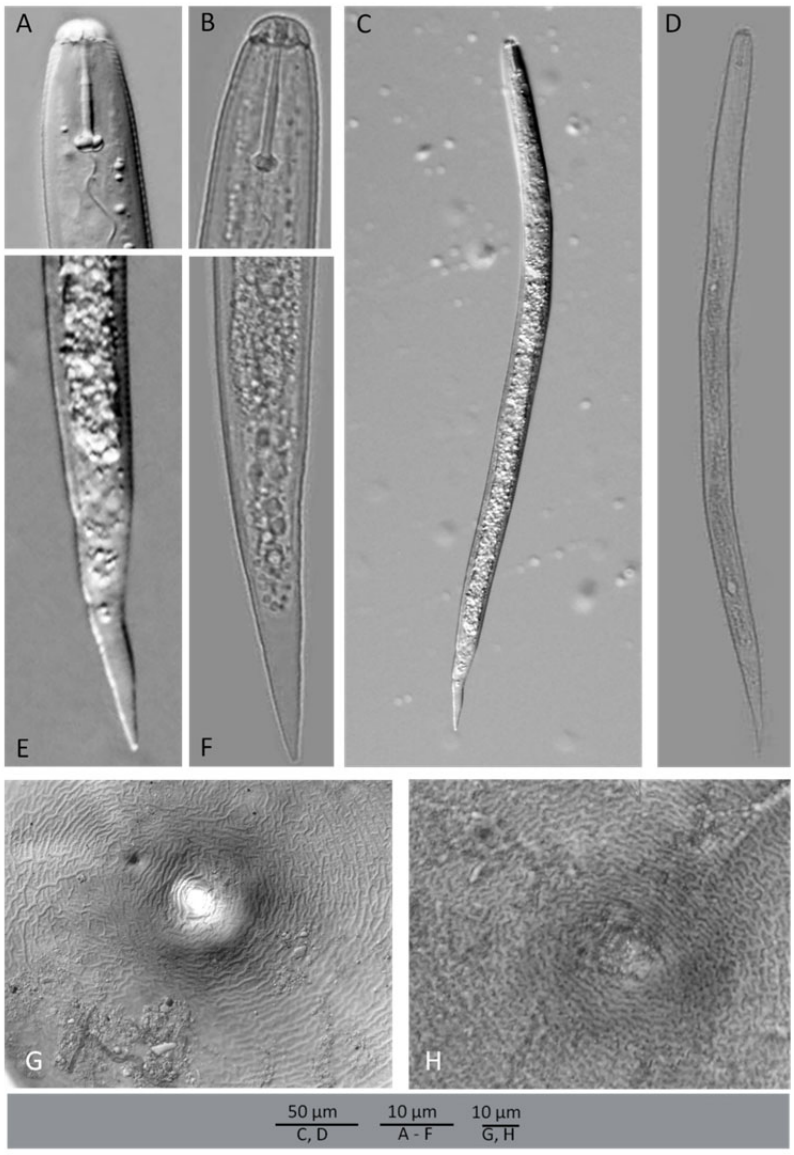

Fig. 1. Representative photographs of individual nematodes of the analyzed *Globodera* population: A) head, E) tail, C) 2nd juvenile, G) cyst fenestra of *G. pallida*; B) head, F) tail, D) 2nd juvenile, H) cyst fenestra of *G. rostochiensis*

tions, about 3.5 – 3.9 µm across. Dorsal gland orifice 4.3 – 6.5 µm posterior to knobs. Metacorporeal valve 63 – 73 µm, excretory pore 85 – 97 µm. Hyaline terminal part of tail 48 – 53 % of total tail length. Terminus rounded. Eggs 98.2 – 105.3 µm long, 45.0 – 49.6 µm wide, length/width 2.0 – 2.3.

From the molecular and morphological data acquired in this study, it is obvious that although cysts were extracted from soil samples originating from the same single locality, two potato cyst nematode species (*G. rostochiensis* and *G. pallida*) were present. It is the second reported occurrence of the mixed Potato cyst nematode in the Central Europe (Karnkowski *et al*., 2010). Although cross-hybridization between these two *Globodera* species is feasible (Brzeski, 1998), it seems to be rather unlikely event in field conditions. However, this possibility should be taken into account during diagnostics of these quarantine species, especially when considering numbers of cysts used for morphological description. In addition, molecular diagnostics-based tools were recently developed as single cyst DNA isolation or utilizing of multiplex PCR (Mullholland *et al*., 1996, Zouhar *et al*., 2000) should be used.

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