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Pratylenchus neglectus (Nematoda: Pratylenchidae) under the rhizosphere of *Brassica napus*

S. KUMARI

Crop Research Institute, Division of Plant Health, Drnovská 507, Ruzyně, 16106 Prague 6, Czech Republic,
E-mail*: kumari@vurv.cz*

Summary

Soil samples under the rhizosphere of *Brasicca napus* were collected from three localities (Bílé Podolí, Prague, Kylešovice). Two localities Prague and Kylešovice were positive for the presence of *Pratylenchus neglectus*. The species was identified using morphological features and the morphological identification was verified by using published species-specific primers and by sequencing 18S and 28S genes of ribosomal DNA.

Keywords: *Pratylenchus neglectus*; PCR; Ribosomal DNA; Czech Republic; Nematode

Introduction

Root lesion nematodes of the genus *Pratylenchus* are obligate endoparasites and significant pests in crop cultivation throughout many parts of the world (Nicol *et al*., 2004; Castillo & Vovlas, 2008). They are polyphagous in nature and feed on several crops and they can cause substantial yield losses. They feed, molt and reproduce primarily within the plant tissue. All motile stages are capable of feeding from the plant and they are able to move into the soil in search of new roots to invade. The nematode spends most of its time migrating through root tissues destructively feeding on plant cells. They simply suck out the plant cell cytoplasm using its stylet and moving ahead of the lesion. Plants with impaired root branching and cortical degradation caused by lesion nematodes become less capable of extracting nutrients, and water from soil and of yielding as well as healthy plants (Smiley & Machado, 2009). Root-lesion nematodes reproduce only on living roots of susceptible plant species and, as highly evolved parasites, they do not kill their hosts. These nematodes remain mobile and may move into and out of roots and may deposit eggs in soil as well as within root tissue. They are the second most important plant-parasitic nematodes after root knot nematodes (Jatala & Bridge, 1990). *P. neglectus* along with *P. thoreni* were associated with reduction of winter wheat and barley yield under field conditions (Taylor *et al*., 1999; Lasserre *et al.,* 1994; Smiley *et al*., 2005). Oilseed rape (*Brassica napus*) is often used as a break crop between cereal crops and in vitro tests of *Pratylenchus* on rape cultivar has shown that rape cultivar could be damaged by *Pratylenchus* from preceding cereal crop (Webb, 1990; Webb, 1996).

Morphological identification of *Pratylenchus* nematodes requires examination of several adult female specimens by an experienced taxonomist. Therefore, a simple, quick, reliable, and relatively inexpensive diagnostic DNA-based techniques have been developed to discriminate among different species of *Pratylenchus* (Al-Banna *et al*., 2004; Yan *et al*., 2008). The objective of this study was to describe *Pratylenchus neglectus* morphologically and verify their morphological identification by using published species-specific primers and by sequencing.

Materials and methods

The occurrence of *Pratylenchus neglectus* (Rensch 1924) Filipjev and Schuurmans Stekhoven 1941 associated with *Brassica napus* was examined from three localities (Bílé podolí, Prague, Kylešovice) in the Czech Republic. *Pratylenchus* was found in two localities Prague and Kylešovice Specimens for morphological and molecular analysis from the soil were extracted by Baermann funnel method. Small amount of soil (25 – 50 g) was placed on a tissue paper on a Baermann funnel for 24 – 48 hours. Nematodes for morphological study were heat killed, fixed in TAF, processed in slow glycerin process and mounted in anhydrous glycerin on slides. Photomicrographs were recorded with a digital camera linked to a computer and measurements were made with the aid of imaging software (Olympus DP-soft).

For specific amplification of *P. neglectus* two set of pri-

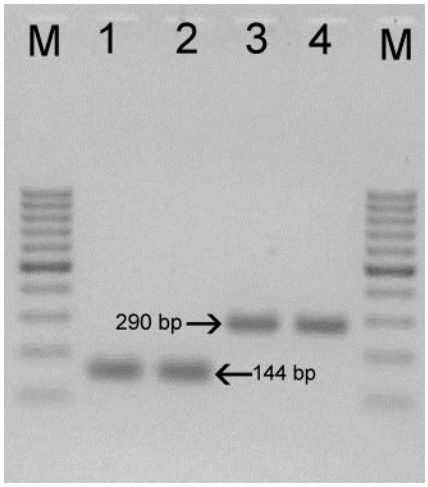

Fig. 1. Electrophoresis of the amplified products from single individuals of *Pratylenchus neglectus*: lane M – 100 bp DNA ladder (Fermentas); lane 1 and 2 with primers PNEG_F1+D3B5; lane 3 and 4 with primers PNEG+D3B

mers were used. Species-specific sense primers for first set was PNEG-F1 (5′-CGC AAT GAA AGT GAA CAA TGT C -3′) and antisense D3B5 (5′-AGT TCA CCA TCT TTC GGG TC-3′) (Yan *et al*., 2008). Species-specific sense primers for the second set was PNEG (5′-ATG AAA GTG AAC ATG TCC TC -3′) and antisense D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) (Al-Banna *et al.*, 2004). Total genomic DNA was extracted from single individuals with a rapid technique (Stanton *et al*., 1998). The PCR was performed in a 25 µl total volume containing 1 PCR bead (GE Healthcare, Buckinghamshire, UK), 20.5 µl double distilled sterile water, 2.0 µl each primer (10pmol/µl) (synthesized by Generi Biotech, Hradec Králové, Czech Republic), and to this 0.5 µl of DNA was added as a template for PCR. A negative control (sterilized water) was included in all PCR experiments. All PCR reactions were performed on a DNA Engine PTC–1148 thermal cycler (Bio-Rad). The DNA was subjected to a PCR with the following specifications: first denaturation for 3 min at 95°C, 41 cycles with 30 s at 95°C, 30 s at 62°C, 30 s at 72°C and final extension at 72°C for 10 min. For both primer sets annealing temperature used was 62°C. An aliquot (8µl) of each amplification reaction was mixed with 2.0 µl of 6x loading dye (Fermentas, MBI) and electrophoresed in high resolution 1.5 % agarose gel and run in TAE (Tris-Acetate-EDTA) buffer. The bands were visualized and photographed under UV (312 nm) after syber safe (1 µg/ml) binding to the DNA fragments. A 100 base pair marker (Fermentas) was included on gel.

Few alive specimens were preserved in IM NaCl for sequencing. Temporary mounts of four individual nematodes from two populations were made in a droop of IM NaCl containing glass beads and after taking photomicrographs the slides were dismantled, individual nematodes removed, and added to digest in 0.25M NaOH overnight. Total genomic DNA was extracted as described above. 18S gene of ribosomal DNA was amplified in two overlapping fragments and primer combination was 988F+1912R for the first fragment and 1813F+2646R for the second fragment (Holterman *et al*., 2006). D2/D3 expansion segments of 28S gene were amplified using D2A and D3B primers (De Ley *et al*., 1999). PCR reactions were performed as described above. The cycling conditions were: first denaturation for 3 min at 94°C, 40 cycles with 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and a final elongation step was run at 72°C for 10 min. DNA was purified using High Pure Product Purification kit (Roche Diagnostics GmbH, Manheim, Germany) and directly sequenced in both directions (Macrogen, Korea). Sequencher^{TM} 4.8 (Genes Codes. Corp., Ann Arbor, MI, USA) software was used to assemble and view sequences and check for base-calling errors. These four specimens for sequencing were also amplified with the both sets of species-specific primers.

Results and Discussion

Morphometrics of females are presented in Table 1. Females characterized by great variation in body length, width and tail shape. Body slightly curved ventrally when killed by gentle heat. Cutilce with fine annulations; lateral field with four incisures. Cephalic region low and flattened with massive sclerotization. Robust stylet with round, anteriorly flat or indented basal knobs. Median bulb oval to round very muscular. Female monodelphic, with anterior ovary functional. Postvulval uterine sac present. Vulav in posterior region. Tail variable in shape, conoid to subcylindroid. Males were not found.

Table 1. Morphometrics of females of *Pratylenchus neglectus* (Rensch 1924) Filipjev and Schuurmans Stekhoven 1941. Measurements in µm (in form): mean ± standard deviation (range).

Locality	Prague	Kylešovice
n	9	8
L	435 ± 78	410 ± 38
	(307 – 552)	(371 – 475)
a	26.1 ± 6.20	22.6 ± 2.29
	(15.4 – 36.8)	(19.4 – 26.2)
b	4.2 ± 0.39	4.1 ± 0.35
	(3.7 – 5.0)	(3.6 – 4.6)
c	21.5 ± 3.84	18.8 ± 3.02
	(12.3 – 25.0)	(14.4 – 24.0)
c´	2.06 ± 0.39	1.96 ± 0.34
	(1.43 – 2.67)	(1.45 – 2.45)
vulva	82 ± 1.96	81 ± 1.40
	(79 – 86)	(79 – 83)
stylet	21 ± 3.32	15 ± 1.07
	(13 – 17)	(14 – 17)
Tail length	21 ± 3.32	22 ± 3.88
	(16 – 25)	(16 – 28)
Body diameter at lip region	8 ± 0.83	8 ± 0.64
	(7 – 9)	(7 – 9)
at mid body	17 ± 3.26	18 ± 1.83
	(14 – 24)	(16 – 21)
at anus	10 ± 1.62	11 ± 1.19
	(9 – 14)	(10 – 13)

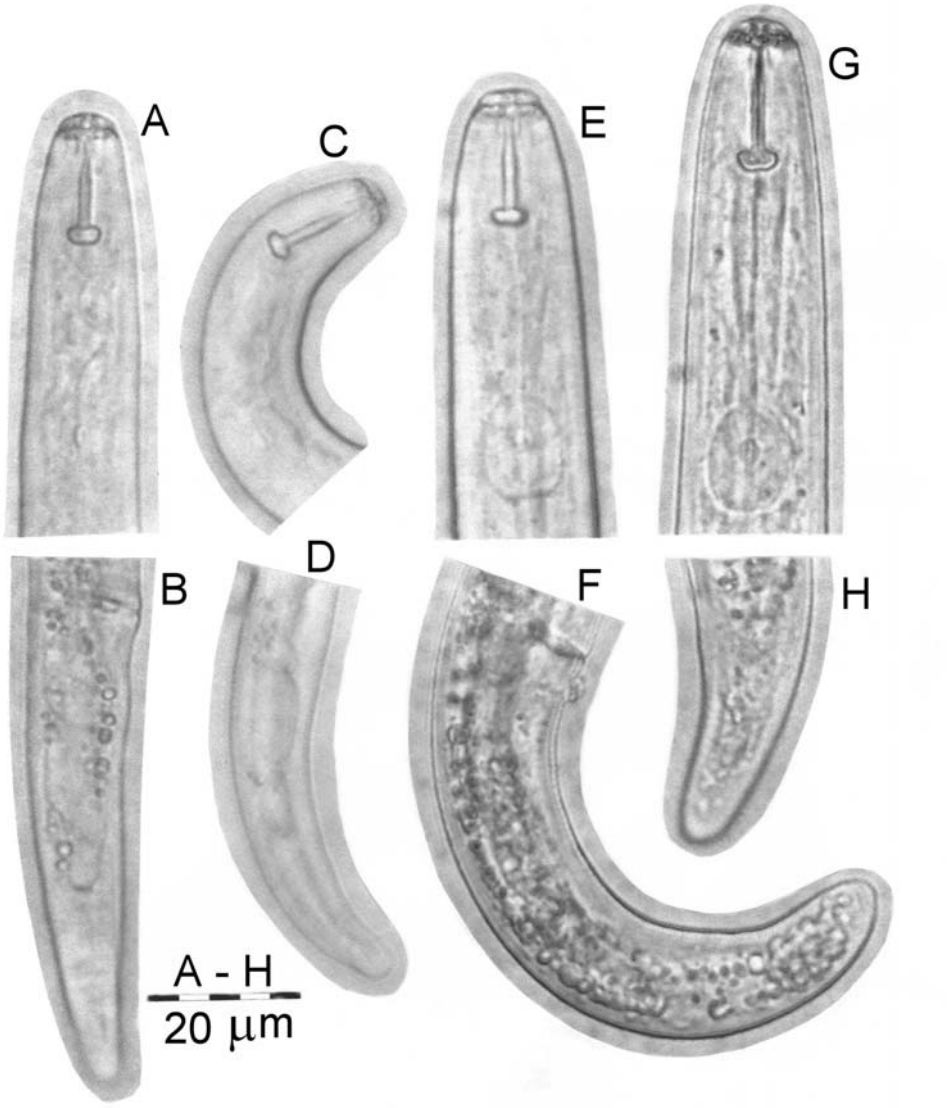

Fig. 2. Anterior and posterior regions of specimens from locality Prague (A – D) and Kylešovice (E – H) used for sequencing. A, B: Female; C, D: Juvenile; E, F: First female; G, H: Second female

Morphometrics of females of *P. neglectus* from two localities are similar to each other. Morphometrics of females are also within the minimum and maximum ranges given by Andrásy (2007) except radio 'b' which is shorter in both Czech populations. Morphological identification of both populations was reliably verified by PCR using two sets of published primers of ribosomal DNA (Al-Banna *et al*.; 2004, Yan *et al*., 2008). A single fragment of approximately 144 bp was amplified for all studied individuals with primers PNEG-F1+D3B5 and 299 bp with primers PNEG+D3B (Fig. 1). No PCR products were obtained in the negative control lacking DNA template or in the negative control containing DNA of *Aphelenchoides*, *Aphelenchus*, *Bitylenchus*, *Boleodorus*, *Helicotylenchus*, *Merilinus*, *Trophurus* and *Tylenchus.*

Photomicrographs of four individual (two per populations) used for sequencing 28S and 18S genes of ribosomal DNA are given in Fig. 2. These four specimens were also positive with the both sets of species-specific primers. Identical sequences were obtained from four individuals. The representative sequences were deposited in National Center for Biotechnology Information (NCBI) and their accession numbers are JQ303332 (18S gene) and JQ303333 (28S expansion segments). The obtained sequences were compared by Basic Local Alignment Search Tool (BLAST) and the first top hit of 18S sequence showed 1673/1676 nucleotides identity to *P. neglectus* accession number EU669923 (Holterman *et al.*, 2009) and 28S gene showed 648/656 identities also to *P. neglectus* accession number EU130854 (Subbotin *et al*., 2008).

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