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DOI 10.1515/helmin-2015-0043

HELMINTHOLOGIA, 52, 3: 280 – 286, 2015

Characterization of *Pratylenchus crenatus* and *P. neglectus* (Nematoda: Pratylenchidae) associated with wheat crop

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Article info

Received November 10, 2014
Accepted July 7, 2015

Summary

The distribution of *Pratylenchus* species associated with wheat crops was investigated in Bohemian region of the Czech Republic. In total twelve localities were sampled. The populations were identified based on morphology and morphometrics, and further characterised based on sequences of the rDNA D2/D3 region and 18S gene. *Pratylenchus crenatus* was present in two localities and *P. neglectus* in five localities. At one locality both species were detected. Sequence analysis of 18S and D2/D3 region of three populations of *P. crenatus* reveal no variation while five populations of *P. neglectus* differ by 0 to 0.14 % (18S) and 0.17 to 0.50 % (D2/D3).
Keywords: *Pratylenchus crenatus*; *Pratylenchus neglectus*; PCR; ribosomal DNA; sequencing; nematode

Introduction

Root-lesion nematodes (RLN) of the genus *Pratylenchus* Filipjev, 1936 are migratory endoparasites of agricultural crops, considered among the most widespread and important nematode parasites in a variety of crops throughout the world (Castillo & Vovlas, 2007). The RLN rank third only to root-knot and cyst nematodes as having the greatest impact on crops worldwide (Castillo & Volvas, 2007; Jones *et al*., 2013). RLN are also a major limiting factor for cereal crop production throughout the world. Eight species of the genus *Pratylenchus* infest small grains (Rivoal & Cook, 1993; Nicol *et al*., 2004). Among them, *P. thornei* Sher and Allen, 1953, *P. neglectus* (Rensch, 1924) Filipjev and Schuurmans Stekhoven, 1941, *P. penetrans* (Cobb, 1971) Filipjev and Schuurmans Stekhoven, 1941 and *P. crenatus* Loof, 1960 have a worldwide distribution and can cause substantial yield losses (Taylor *et al*., 1999; Nicol *et al*., 2004).

RLN hatch from the egg as a second-stage juvenile and starts feeding on the plant. 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. After rupturing the epidermis they enter the cortex of roots, tubers and bulbs of plants, resulting in extensive root necrosis (Zunke, 1990; Taylor *et al*., 1999; Vanstone *et. al*., 1998) while providing avenues for secondary colonization by pathogenic microorganisms fungi and bacteria (Corbett, 1973). Their worldwide distribution, broad host ranges and interrelationships with other pests and diseases rank them among some of the most important soilborne pathogens affecting agricultural crops (Jones *et al*., 2013). *P. crenatus* has been reported in America, Asia, Europe, North America, Oceania and South and Central America; and *P. neglectus* has been reported in Africa, America, Asia, Europe, North America, Oceania and South and Central America (Castillo & Vovlas, 2007). *P. crenatus* and *P. neglectus* are found in a broad range of soil types from heavy clays to sandy soils and these two species occurs more frequently in acid than in neutral or alkaline soils. Vertical distribution of *P. crenatus* in different soil types was 70 % in the top 22 cm and in *P. neglectus* 94 % of specimens occur in the upper layers of soil up to 20 cm (Taylor and Evans, 1998) and both species are reported to be pathogenic to cereals crops and are associated with poor growth of cereals plants (Castillo & Vovlas, 2007).

Morphological methods can be time-consuming and impractical when rapid results are required. The use of molecular diagnostic tools like PCR with species specific primers (Al-Banna *et al*., 2004, Mekete *et. al*., 2011) and sequencing of diagnostic rDNA regions in combination with phylogenetic analyses contributes to overcome

Table 1. Primers used to amplify ribosomal DNA

Primer name	Direction	Primer sequence 5' - 3'	Reference
PNEG	forward	ATG AAA GTG AAC ATG TCC TC	Al-Banna *et al.*, 2004
PCR22_F	forward	AAA GCC TGA ATG CCC TGA G	Mekete *et al*., 2011
PCR22_R	reverse	AAA TTG AAA GAG GTC GGT CGT	Mekete *et al*., 2011
988F	forward	CTC AAA GAT TAA GCC ATG C	Holterman *et al*., 2006
1096F	forward	GGT AAT TCT GGA GCT AAT AC	Holterman *et al*., 2006
1912R	reverse	TTT ACG GTC AGA ACT AGG G	Holterman *et al*., 2006
1813F	forward	CTG CGT GAG AGG TGA AAT	Holterman *et al*., 2006
2646R	reverse	GCT ACC TTG TTA CGA CTT TT	Holterman *et al*., 2006
D2A	forward	ACA AGT ACC GTG AGG GAA AGT TG	De Ley *et al*., 1999
D3B	reverse	TCG GAA GGA ACC AGC TAC TA	De Ley *et al*., 1999

such a problem. The use of rDNA is potentially the most powerful method of nematode diagnosis. Ribosomal DNA (rDNA) sequence has been shown to be a reliable genetic marker for identification purposes, and a powerful tool to analyze genetic variation. The 28S D2/D3 rDNA fragment has been used frequently to characterise *Pratylenchus* populations (Handoo *et al*., 2001; Al-Banna *et al*., 2004; De Luca *et al*., 2004; Inserra *et al*., 2007; Subbotin *et al*., 2008).

Previously *P. neglectus* has been found under the rhizosphere of Brassica (Kumari, 2012) and *Pratylenchus* sp. has been found associated with hop plants (Cermak, 2011) in the Czech Republic, but despite the economical importance of RLN, there is a lack of knowledge about the diversity and distribution of RLN associated with cereal crops in the Czech Republic. Considering the economic significance of RLN in cereal crops a study was initiated to study the occurrence of these nematodes in the Czech Republic. Two species *P. crenatus* Loof, 1960 and *P. neglectus* (Rensch, 1924) Filipjev & Schuurmans Stekhoven, 1941 which were found in the latest survey of cereal crops, were described here. The specific objectives of the work were: 1) to characterise *P. crenatus* and *P. neglectus* based on morphology and morphometrics; 2) to amplify these species using species-specific published primers; 3) to sequence two (D2/D3 expansion segment of 28S and partial 18S gene) regions of ribosomal DNA.

Material and Methods

Soils samples were collected from the wheat growing areas in Bohemia, Czech Republic. Soil samples were taken at a depth of 0 – 30 cm and nematodes were extracted from soil by sieving on 1 mm, 150 µm and 75 µm and placing the residual on 99 µm and 56 µm sieve on a Baermann funnel from 24 – 48 hours (Brown and Boag, 1998). Nematodes for morphological study were heat killed, fixed in TAF, processed in slow glycerin process and mounted in anhydrous glycerin on slides (Courtney *et al*., 1955). 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. crenatus* primer set PCR22_F+PCR22_R and for *P. neglectus* primers set PNEG+D3B were used. Primer sequences and references to the primers are given in Table 1.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 (10 pmol/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

Table 2. NCBI accession numbers of representative individual specimen for ribosomal DNA

Species	Locality	isolate	18S	28S
P. crenatus	Chlumec	CRLN1	KM580535	KM580543
	Chvaletice	CRLN2	KM580536	KM580544
	Krymlov	CRLN3	KM580537	KM580545
P. neglectus	Brandýsek	CRLN4	KM580538	KM580546
	Kolinec	CRLN5	KM580539	KM580547
	Kozojedy	CRLN6	KM580540	KM580548
	Krymlov	CRLN7	-	-
	Suchdol	CRLN8	KM580541	KM580549
	Zásmuky	CRLN9	KM580542	KM580550

- not acquired

Table 3. Morphometrics of females of *P.crenatus* and *P. neglectus*. Measurements in µm (in form): mean ± standard deviation (range)

Species	***P. crenatus***	***P. neglectus***
Locality	**Chvaletice**	**Kolinec**
n	**13**	**31**
L	445 ± 21	494 ± 40
	(415 – 483)	(413 – 573)
a	26.8 ± 2.30	24.2 ± 2.08
	(23.0 – 30.1)	(19.2 – 27.9)
b	4.6 ± 0.31	4.25 ± 0.36
	(4.2 – 5.1)	(3.7 – 5.0)
c	19.7 ± 1.98	21.01 ± 2.81
	(17.2 – 23.1)	(16.0 – 26.7)
c′	2.26 ± 0.14	2.04 ± 0.32
	(2.00 – 2.50)	(1.60 – 2.70)
V	83 ± 1.12	81 ± 1.41
	(81 – 84)	(78 – 84)
o	17.5 ± 4.58	19.49 ± 4.07
	(13.3 – 23.1)	(12.5 – 26.7)
DGO	2 ± 0.51	3 ± 0.65
	(2 – 3)	(2 – 4)
Stylet length	14 ± 0.73	16 ± 1.50
	(13 – 15)	(14 – 19)
Metenchium length	6 ± 0.63	6 ± 1.15
	(5 – 7)	(5 – 9)
Telenchimum length	8 ± 0.28	10 ± 0.77
	(7 – 8)	(8 – 11)
m	40 ± 2.67	40 ± 4.43
	(38 – 47)	(31 – 50)
MB	49 ± 1.62	40 ± 6.36
	(47 – 53)	(31 – 59)
Excretory pore	69 ± 3.37	84 ± 4.56
	(64 – 74)	(75 – 94)
EP%L	15 ± 1.18	17 ± 0.91
	(13 – 17)	(15 – 19)
Pharynx length	97 ± 4.00	117 ± 9.07
	(91 – 105)	(96 – 136)
Pharynx to vulva	270 ± 20.35	284 ± 34
	(240 – 305)	(219 – 358)
head to vulva	368 ± 18.77	401 ± 36
	(338 – 400)	(329 – 480)
VL/VB	5 ± 0.51	5 ± 0.63
	(4 – 6)	(4 – 7)
Vulva – anus length	54 ± 4.48	69 ± 5.85
	(46 – 63)	(59 – 85)
Tail length	23 ± 2.82	24 ± 2.99
	(18 – 28)	(19 – 33)
Body diameter at lip region	7 ± 0.49	8 ± 0.58
	(6 – 8)	(7 – 9)
at mid body	16 ± 1.82	20 ± 1.59
	(13 – 20)	(17 – 22)
at vulva	15 ± 0.85	18 ± 2.07
	(13 – 16)	(14 – 23)
at anus	10 ± 1.04	12 ± 1.37
	(9 – 12)	(10 – 15)

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. An aliquot (6µl) of each amplification reaction was mixed with 1.5 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.

Additionally 18S gene and D2/D3 expansion segment of 28S gene of ribosomal DNA was amplified. 18S was amplified in two overlapping fragments and primer combination was 988F+1912R or 1096F+1912R for the first fragment and 1813F+2646R for the second fragment. D2/D3 expansion segments of 28S gene were amplified using D2A+D3B primers. 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) or by gel extraction kit (Qiagen) and directly sequenced in both directions (Macrogen, Netherlands). Sequencher™ 4.8 (Genes Codes. Corp., Ann Arbor, MI, USA) software was used to assemble and view sequences and check for base-calling errors. Sequences were deposited in Genbank and their accession numbers are given in Table 2.

Results and Discussion

A survey has been started in the Czech Republic to study plant parasitic nematodes associated with cereals crops. In the first survey 12 wheat growing localities were surveyed for the presence of plant parasitic nematodes and eight localities were found positive for the presence of *Pratylenchus* spp. Two localities were found positive for *P. crenatus* and five for *P. neglectus*. At one locality (Krymlov) both species were present. At Kolinec *P. neglectus* occurred with one more *Pratylenchus* sp. which will be described in a different work.

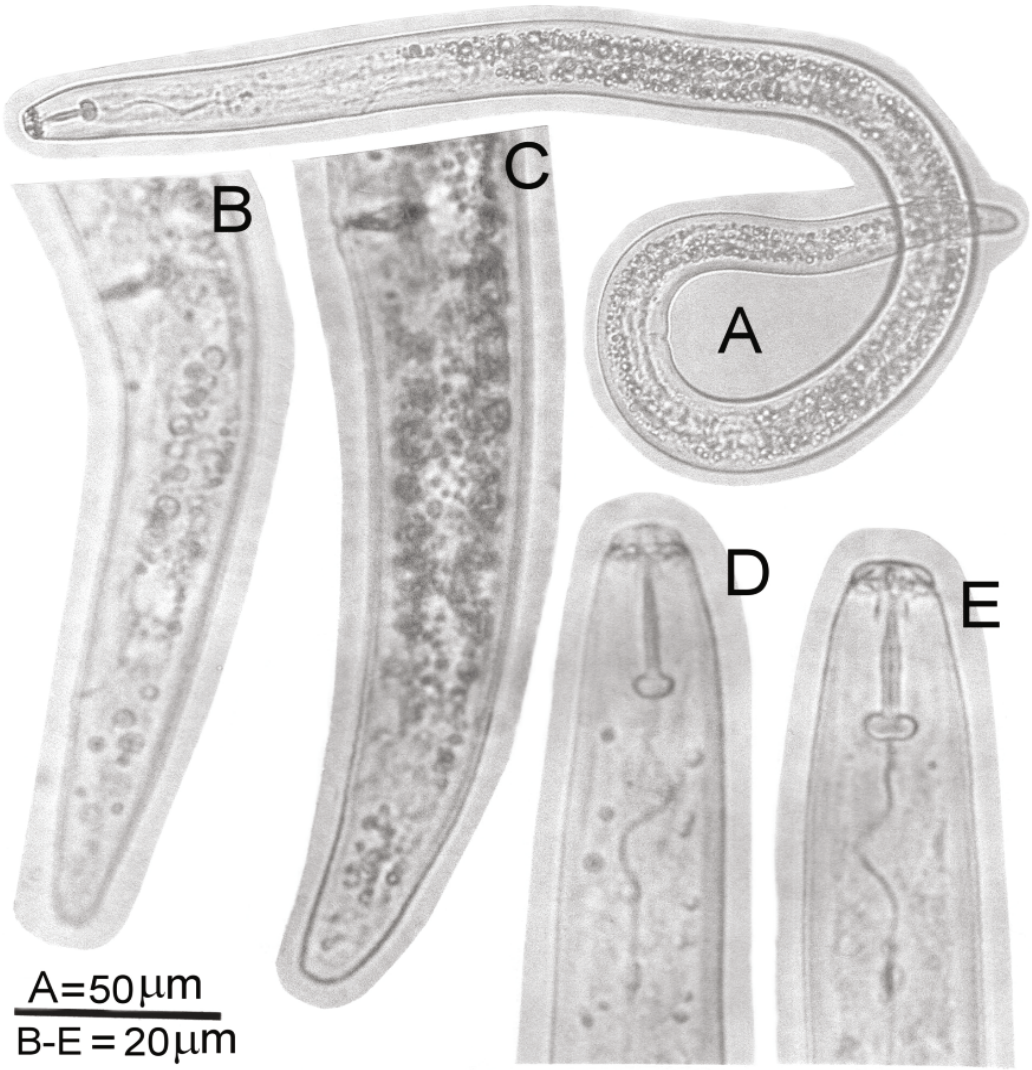

Fig. 1. Photomicrographs of females of *P. crenatus* (B: posterior region; D: anterior region) and *P. neglectus* (A: entire female; C: posterior region; E: anterior region)

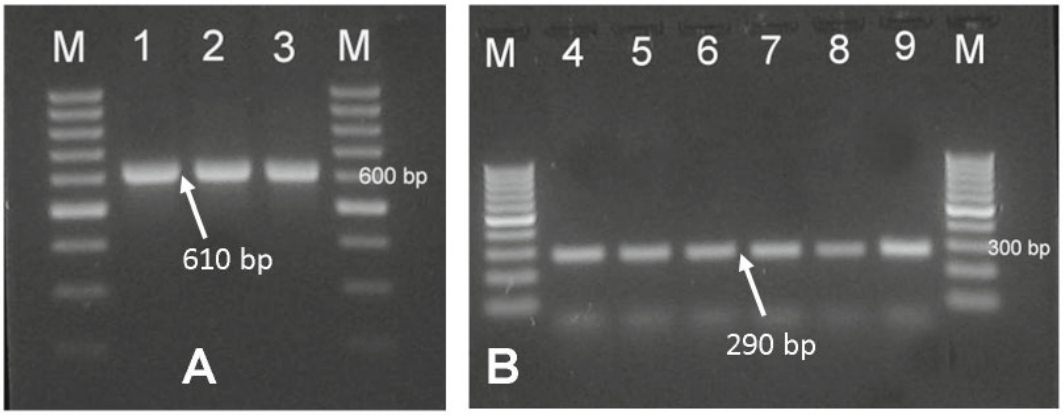

Fig. 2. Electrophoresis of the amplified products from single individual nematodes. A: *P. crenatus* 610bp, lane 1: Chlumec; lane 2: Chvaletice; lane 3: Krymlov. B: *P. neglectus* 290 bp, lane 4: Brandýsek; lane 5: Kolinec; lane 6: Kozojezdy; lane 7: Krymlov; lane 8: Suchdol; lane 9: Zásmuky and lane M:100 bp DNA ladder (Fermentas)

Morphometrics of females of *P. crenatus* and *P. neglectus* are given in Table 3 and photomicrographs are presented on Fig.1. Morphometrics of females of *P. crenatus* is within the minimum and maximum ranges given by Castillo and Vovlas (2007) except radio 'b' which is smaller 4.2 – 5.1 *vs* 4.9 – 7.9. All morphometrics of *P. neglectus* are within the minimum and maximum ranges given by Castillo and Vovlas (2007) and Fayazi *et al*. (2012) except ratio 'm' where minimum value is smaller 31 – 50 *vs* 40.8 – 48.5 (Fayazi *et al*., 2012). *P. crenatus* stylet knob width is 3±0.51 (3 – 4) µm where *P. neglectus* stylet width is 5±0.6 (4 – 6) µm; *P. crenatus* tail tip is crenate where as *P. neglectus* tail tip is without annulation.

In recent years it has become a standard to identify species on the basis of morphological and molecular data with more emphasis on molecular work. Therefore in this work species specific PCR was performed to confirm the morphological identification of the both species using published primers (Al-Banna *et al*.,2004; Mekete *et al.,* 2011). Moreover 18S and 28S partial genes of ribosomal DNA have been analyzed and compared. PCR with species specific primers set PCR22_F+PCR22_R for *P. crenatus* and primers set PNEG+D3B_R for *P. neglectus* yielded a fragment of approximately 610 bp and 290 bp respectively (Fig.2). No PCR products were obtained in the negative control lacking DNA template or in the negative control containing DNA of *Aphelenchus*, *Bitylenchus*, *Boleodorus* and *Helicotylenchus* (data not shown).

Sequence analysis of partial 18S gene of *P. crenatus* (accession numbers KM580535, KM5080536, KM580537) among the three Czech populations did not reveal any variation. 18S gene differs from other accession numbers AB905287, AB905288, AB905289, AB905290 (Kushida and Kondo, 2015) AY284610 (Holterman *et al*., 2006), EU130800 (Subbotin *et al*., 2008), EU669920, EU669921, EU669922 (Holterman *et al*., 2009) by 0.14 – 1.66 % and these twelve sequences (KM580535-KM580537, AB905287-AB905290, AY284610, EU130800, EU669920- EU669922) differ from each other 0 – 2.34 %. This sequence variability is too high for conservative 18S gene therefore one should be cautious when comparing 18S sequences of *P. crenatus*. In this study 18S gene and D2/D3 expansion segments and species-specific PCR were amplified from the same genomic DNA.

Sequence analysis of D2/D3 expansion segments of *P. crenatus* (accession numbers KM580543, KM580544, KM580545) among the three Czech populations also did not reveal any variation. The sequences of D2/D3 of *P. crenatus* differs from published sequences EU130852, EU130853 (Subbotin *et al*., 2008), HM921215 (Mekete *et al*., 2011) by 0.45 to 1.36 % and these six sequences (KM580543-KM580545, EU130852, EU130853, HM921215) varied by 0 – 1.82 %.

Sequences of 18S gene of *P. neglectus* among five populations (accession numbers KM580538-KM580542) from the Czech Republic differ by 0 – 0.14 %. These sequences differs from other sequences AB905298-AB905303 (Kushida and Kondo, 2015), EU130801, EU130802 (Subbotin *et al*., 2008), EU669923, EU669924 (Holterman *et al*., 2009), JQ303332 (Kumari, 2012), KC875378 (Rybarczyk-Mydłowska *et al*., 2015) from NCBI by 0 – 0.56 %. All these 17 (KM580538-KM580542, AB905298-AB905303, EU130801, EU130802, EU669923, EU669924, JQ303332, KC875378) sequences differ from each other 0 – 0.70 %.

Sequence analysis of D2/D3 expansion segments of *P. neglectus* (accession numbers KM580546-KM580550) differ by 0.17 – 0.50 %. The sequences of D2/D3 differs from other accession numbers EU130854, EU130855 (Subbotin *et al*., 2008), HM469438 (unpublished), JQ303333 (Kumari, 2012), JX046968, JX046969 (Wang *et al.,* 2012), JX261946, JX261947, JX261951 (Majd Taheri *et al.,* 2013) by 0.17 – 1.51 % and these 14 sequences (KM580546-KM580550, EU130854, EU130855, HM469438, JQ303333, JX046968, JX046969, JX261946, JX261947, JX261951) varied by 0.17 – 2.01 %.

The aim of this study was to identify two *Pratylenchus* species associated with wheat crop in the Czech Republic. There are over 60 named species of root lesion nematode (*Pratylenchus* spp.) which are distributed worldwide (Jones *et al.,* 2013). Because each species differs in host preference, it is essential that they be correctly identified. The ability to identify RLN responsible for crop damage is a fundament of plant nematology and for implementing adequate management strategies. It is important to distinguish between the different species of nematodes occurring in a specific region associated with a specific crop in order to estimate the extent of the damage to the crop and to make decisions on appropriate control measures, therefore morphological and molecular means were used to

identify the two *Pratylenchus* species (*P. crenatus* and *P. neglectus*) in the Czech Republic and the results confirmed their identification.

Acknowledgements

The work was supported by the National Agency of Agriculture Research of the Czech Republic, Project number QJ1230159.

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