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Characterization of *Longidorus caespiticola* (Nematoda: Longidoridae) from the Czech Republic

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Summary

Longidorus caespiticola was found for the first time from the rhizosphere of apple with two other *Longidorus* sp. at Žlunice, Czech Republic. Females and males were analyzed morphologically and morphometrically. Four regions (18S, ITS1, ITS2, D2-D3 expansion segments of 28S) of ribosomal DNA and one region (*cox*1) of mitochondrial DNA were sequenced and analyzed and compared with a population from the Slovak Republic. The partial mitochondrial cytochrome c oxidase subunit 1 (*cox*1) gene showed relatively high genetic variation compared with ribosomal DNA between the Czech and Slovakian populations.

Keywords: *Longidorus caespiticola,* Mitochondrial DNA; Nematode; Ribosomal DNA; Sequencing

Introduction

Longidorus are root-ectoparasite nematodes of wild and agricultural plants. They are important to agriculture as direct parasites and also as vectors of plant viruses (Taylor & Brown, 1997). Though these nematodes are economically important pests, therefore an extensive study of these nematodes is being carried out in the Czech Republic. Previous surveys yielded six known species (*Longidorus elongatus*, *L. euonymus*, *L. helveticus*, *L. intermedius*, *L. leptocephalus, L. poessneckensis*) of genus *Longidorus* in the Czech Republic (Erbenová, 1977, Kumari & Decreamer 2007; Kumari *et al*., 2009; Kumari & Subbotin, 2012). During recent nematological surveys *L. caespiticola* Hooper, 1961 was found only at one site for the first time in the Czech Republic. *L. caespiticola* was originally described from Rothamsted, UK (Hooper, 1961). Later, this species was reported from other European countries Belgium, Bulgaria, France, German, Italy, Russia, Slovakia, Slovenia, Spain, Switzerland, The Netherlands and UK (Sturhan, 1963; Hoof, 1966; Taylor & Brown, 1976; De Waele & Coomans, 1990; Andres *et al.,* 1991; Lišková & Brown, 1998; Širca & Urek, 2009; Peneva *et al.,* 2012).
In recent years it has become a standard to identify species on the basis of morphological and molecular data because combination of morphological and molecular methods is considered as a perspective approach in diagnostics of this nematode group. Therefore in this study *L. caespiticola* was identified by morphological and morphometrical means. Objectives of this study were to characterize a population of *L. caespiticola* morphologically from the Czech Republic and to sequence and analyze four markers (18S, ITS1, ITS2 and 28S) of ribosomal DNA and one marker (cytochrome *c* oxidase subunit1 - *cox*1) of mitochondrial DNA. This study is one of a series of works on characterization of Longidoridae in the Czech Republic.

Materials and methods

Soil samples were taken at a depth of 0 – 40 cm and nematodes were extracted from soil by sieving on 1 mm and 150 µm and placing the residual on 99 µm sieve on a Baermann funnel from 24 – 48 hours. Nematodes were heat killed, fixed in TAF, processed by a slow glycerin method 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).
Individual nematodes of *L. caespiticola* from the Czech Republic and Slovak Republic were stored in 1M NaCl and used to extract DNA. Total genomic DNA was extracted according to a rapid method by Stanton *et al*. (1998). Four regions (18S, ITS1, ITS2 and D2/D3 expansion segments of 28S) of ribosomal and one region (*cox*1) of mitochondrial DNA were amplified and sequenced. All regions and genes were amplified from the total genomic DNA of the same single specimen. Primer sequences and references to the primers are given in Table 1.

Table 1. Primers used to amplify ribosomal and mitochondrial DNA

Region	Primer name	Direction	Primer sequence 5′ - 3′	Reference
18S	988F	forward	CTC AAA GAT TAA GCC ATG C	Holterman *et al.*, 2006
18S	1912R	reverse	TTT ACG GTC AGA ACT AGG G	Holterman *et al*., 2006
18S	1813F	forward	CTG CGT GAG AGG TGA AAT	Holterman *et al.,* 2006
18S	2646R	reverse	GCT ACC TTG TTA CGA CTT TT	Holterman *et al.*, 2006
ITS1	pXb101	forward	TTG ATT ACG TCC CTG CCC TTT	Vrain *et al*., 1992
ITS1	rDNA1.58S	reverse	ACG AGC CGA GTG ATC CAC CG	Cherry *et. al*., 1997
ITS2	WDF	forward	AGA CAC AAA GAG CAT CGA CT	Kumari *et al*., 2009
ITS2	pXb481	reverse	TTT CAC TCG CCG TTA CTA AGG	Vrain *et al*., 1992
D2/D3	D2A	forward	ACA AGT ACC GTG AGG GAA AGT TG	De Ley *et al*., 1999
D2/D3	D3B	reverse	TCG GAA GGA ACC AGC TAC TA	De Ley *et al*., 1999
*cox*1	COIF	forward	GAT TTT TTG GKC ATC CWG ARG	He *et al*.,2005a
*cox*1	XIPHR1	reverse	ACA ATT CCA GTT AAT CCT CCT ACC	Lazarova *et al*., 2006

The 18S gene was amplified in two fragments. Primer combination was as follows: first fragment 988F+1912R and second fragment 1813F+2646R. The PCR was performed in a 25 µl total volume containing 1 PCR bead (GE Healthcare, Buckinghamshire, UK), 20.0 µl or 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 or 1.0 µl of DNA was added as a template for PCR. A negative control (sterilized water) was included in all PCR experiments. The cycling profile for all markers of ribosomal DNA was as follows: first denaturation for 3 min at 94 ºC, 40 cycles with 30 s at 94 ºC, 30 s at 55 ºC, 30 s at 72 ºC and final extension at 72 ºC for 10 min. The cycle profile for mtDNA was as described by He *et al*. (2005a): 95 ºC for 10 min, 5 cycles at 94 ºC for 30 s, 45 ºC for 40 s, and 72 ºC for 1 min, and further 35 cycles at 94 ºC for 30 s, 37 ºC for 30 s, and 72 ºC for 1 min, followed by an extension at 72 ºC for 10 min. All PCR reactions were performed in a DNA Engine PTC-1148 thermal cycler (Bio-Rad).

Aliquots of PCR were analysed by gel electrophoresis and the remaining products were purified using High Pure Product Purification kit (Roche Diagnostics GmbH, Mannheim, Germany) and sequenced in both directions using each primer pair one forward and one reverse (Macrogen, Netherlands). SequencherTM 4.8 (Genes codes. Corp., Ann Arbor, MI, USA) was used to assemble and view each sequence and check for base-calling errors. Sequences were deposited in Genbank and their accession numbers are given in Table 2. Nucleotide variation and A+T content was calculated by MEGA 5 (Tamura *et al.,* 2011).

Table 2. Accession number of *Longidorus caespiticola*

Country	**Czech Republic**	**Slovak Republic**
Locality	**Žlunice**	**Gajary**
Specimen	**30L**	**64L**
18S	KJ567466	KJ567467
ITS1	KJ567468	KJ567469
ITS2	KJ567470	KJ567471
D2/D3	KJ567472	KJ567473
*cox*1	KJ567474	KJ567475

Results

L. caespiticola was found for the first time from the rhizosphere of apple with two other *Longidorus* sp. at Žlunice, Czech Republic. Morphometrics of males and females are given in Table 3 and photomicrographs are presented in Fig.1. Morphometrics of juveniles are not given because it occurred with two other *Longidorus* species, which makes it difficult to separate juveniles with certainty. Body 5.8 to 7.3 mm (female) and 5.7 to 7.7 mm (male) long and robust; when killed by heat it forms an open C-shape with slightly greater curvature in the posterior half. Cephalic region smoothly rounded and continuous with the neck contour. The shape of amphidial pouch elongate, funnel shaped (not lobed). Odontostyle 90 – 104 µm (female) and 93 – 103 µm (male) long and not heavily sclerotized. It forms a simple junction with the odontophore. Guiding ring 33 – 38 µm from the oral opening. Vulva about median in position (48 to 54 %) with genital tracts amphididelphic. Tail dorsally convex hemispherical to bluntly conoid, with a bluntly rounded terminus. Mail tail is similar in shape to that of the female but more curved. Tails of both sexes are shorter than anal body width. Male possesses 12 – 18 pairs of copulatory supplements. Spicule 78 – 92 µm long. Male are as common as female. The identification codes according to the polytomous key for *Longidorus* (Chen *et al.,* 1997) for the Czech population are A-34, B-23, C-3, D-1, E-4, F-34, G-1, H-1, I-2.

Fragment of 1639 and 1678 (18S), 818 and 814 (ITS1), 627 and 606 (ITS2), 823 and 807 (D2/D3) and 364 and 353 (*cox*1) nucleotides were sequenced for Czech and Slovakian populations respectively. Identical sequences were obtained for the Czech and Slovakian population for 18S, ITS2 and D2/D3 region. Two nucleotides (G↔T and G↔A) difference was found for ITS1 region and relatively high variability (10 nucleotides) was found for partial *cox*1 gene among the Czech and Slovakian populations (Fig. 2). The sequences of *cox*1 were aligned unambiguously without gap. Czech populations differ from Slovakian population by 3.14 % (total sites 319). Within the 319 bp alignment (except primer sequences) there were 10 variable sites (9 transitions and 1 transversion), eight of which

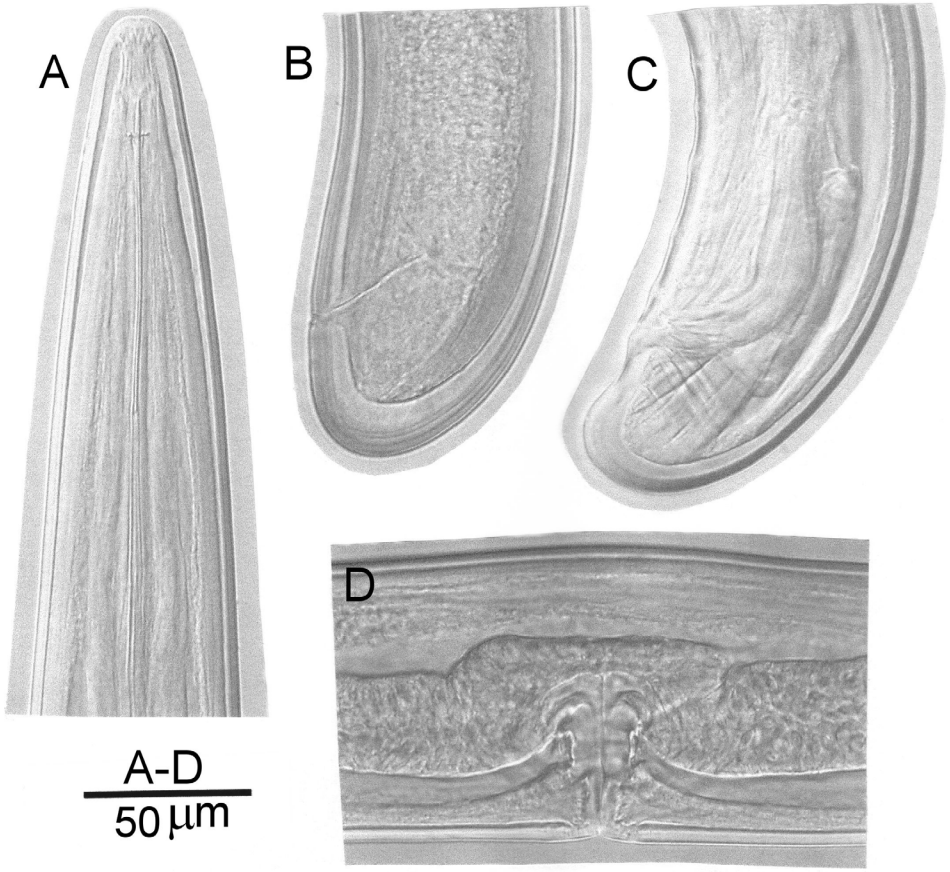

Fig.1. *Longidorus caespiticola* Hooper, 1961. A: Female anterior; B: female posterior; C: male posterior; D: vulva

occurred at third codon position sites and two at first codon position. The 10 variable nucleotide sites among *cox*1 sequence variants represented transitions C↔T (*n*=7) and A↔G (*n*=2); and transversion G↔C (*n*=1) (Fig. 2). Whilst most (*n*=9) nucleotide changes were silent, the transition at position 138 (G↔A) resulted in a change in the *cox*1 amino acid sequence for a valine to an isoleucine. The A+T content was 51.8 % at the first codon, 59.2 % at second codon and 53.9 % at the third codon.

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                10        20        30        40        50        60
      .. ..| ... .|. ... |.. ..| ... .|. ... |.. ..| ... .|. ... |.. ..| ... .|. ... |
CR    GA TTT TTT GGG CAT CCW GAG GTG TAT ATT CTC ATT CTG CCC GGG TTT GGC TTG GTC AGA C
SR    .. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .
            COIF >

CR    AT GCC GTT GTT TTA GTT AGA GGT AAA GAG GTC CCG TTC GGT GCC CCG GGC ATA TTC CTG G
SR    .. ... ... ... ... ... ... ..C ... ... ... ..C ... ... ... ... ... ... ... ... .

CR    CA ATT GCC AGG ATT GGC GTT TTA GGG TGC GCA GTG TGG GCC CAT CAC ATG TTT AGG GTA G
SR    .. ... ... ... ... ... A.. ... ... ... ... ... ... ... ..C ... ... ... ... ... .

CR    GC ATA GAC ATG GAC ACT CGG CTA TAC TTC ACA GCA GCC TCT ATA ATC ATT GCT GTC CCA A
SR    .. ... ... ... ... ..C ... T.. ... ... ... ... ... ... ..T ... ... ... ... ... .

CR    CC GGG ATT AAA GTC TTT AGT TGA TTA GCT TCC TTT AGA GGA AGC TTA GTA TTA ATA AAA C
SR    .T ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .

CR    CA GTT CAA ATG TGG ATT CTA GGG TTT TTA TTT CTT TTT ACG GTA GGA GGA TTA ACT GGA A
SR    .G ... ... ... ... ... ... ... ... ... ..C ... ... ... ... ... ... --- --- -
                                                          < XIPHR1
CR    TT GT
SR    -- --
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Fig. 2. Alignment of parital *cox*1 gene from the Czech Republic (CR) and Slovak Republic (SR). Italic and underlined are the sequences of the oligonucleotide primers used in the PCR. Square indicates nucleotides which resulted in a change in amino acid sequence

Table 3. Morphometrics of females and males of *Longidorus caespiticola*. Measurements in µm (in form): mean ± standard deviation (range)

Specimens	Females	Males
n	9 ♀	14 ♂
L	6417 ± 504 (5783 – 7253)	6340 ± 563 (5723 – 7704)
a	70.1 ± 5.75 (63.1 – 78.3)	87 ± 6.48 (76.4 – 100.0)
b	11.9 ± 1.77 (9.2 – 13.6)	12.5 ± 1.13 (10.9 – 14.8)
c	161 ± 8.11 (151.2 – 176.9)	156.2 ± 21.48 (128.4 – 208.2)
c′	0.64 ± 0.05 (0.55 – 0.72)	0.74 ± 0.05 (0.67 – 0.83)
V/spicule	51 ± 1.68 (48.3 – 53.5)	86 ± 4.10 (78 – 92)
Odontostyle	96 ± 5.17 (90 – 104)	98 ± 2.44 (93 – 103)
Odontophore	54 ± 2.85 (51 – 58)	59 ± 4.28 (54 – 67)
Total stylet length	150 ± 6.94 (141 – 161)	157 ± 4.52 (151 – 167)
Oral aperture to guide ring	35 ± 1.79 (33 – 38)	35 ± 1.34 (33 – 38)
Tail length	40 ± 2.76 (35 – 43)	41 ± 3.77 (37 – 48)
Length of hyaline tip	15 ± 1.80 (13 – 19)	12 ± 1.45 (9 – 14)
Number of supplements	–	15 ± 1.65 (12 – 18)
Body diam. at lip region	14 ± 1.12 (13 – 16)	14 ± 0.95 (12 – 15)
at guiding ring	32 ± 1.69 (30 – 35)	32 ± 1.33 (29 – 34)
at vulva/mid body	92 ± 8.73 (83 – 111)	73 ± 6.57 (65 – 88)
at anus	62 ± 4.88 (55 – 69)	55 ± 2.82 (52 – 61)
at beginning of hyaline tip	44 ± 2.59 (41 – 49)	32 ± 2.59 (28 – 36)

Discussion

Morphometrics of the Czech populations (female) compared with the type (Hooper, 1961), Belgian (Sturhan, 1963) and Slovenian (Širca & Urek, 2009) populations. Compare to type population body length range is within in the range of type population but the mean length is shorter 6417 *vs* 6700 µm. Distance from oral aperture to guiding ring is similar but odontostyle length is shorter 96 *vs* 110 µm. Ratio 'a' and 'b' are similar but ratio 'c' is much higher 161 *vs* 103. Compare to Belgian population body length is shorter 6417 *vs* 7580 µm. Ratio 'a' (70.1 *vs* 90) and 'b' (11.9 *vs* 14.6) are shorter. Ratio 'c′' is also shorter 161 *vs* 169 but within the minimum and maximum range of Belgian population. Odontostyle length is longer 96 *vs* 92 µm and tail length is shorter 40 vs 46 µm. Compare to Slovenian population body length (6417 *vs* 7840 µm), odontostyle (96 *vs* 102.5 µm) tail length (40 *vs* 44.6 µm) are shorter. All ratios 'a' (70.1 *vs* 96), 'b' (11.9 *vs* 15.5), 'c' (161 *vs* 176) and 'c′' (0.64 *vs* 0.74) are lower.

Morphometrical comparison of the Czech population with the type, Belgian and Slovenian shows a greater range of over-lapping character for *L. caespiticola*.

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 (Coomans *et al.*, 2012). Therefore in this work five markers of ribosomal and mitochondrial DNA have been analyzed and compared. Analysis of partial 18S gene, ITS2 and D2/D3 of Czech and Slovakian populations did not reveal any variation, whereas ITS1 showed nucleotide changes at two sites. It has been known that mitochondrial DNA evolves faster than ribosomal DNA and has more discriminate power in the separation of closely related species (Morgan & Blair 1998). Partial *cox*1 gene of mitochondrial DNA assessed 3.14 % (10 bp) nucleotide variability among two populations of *L. caespiticola*.

Sequences obtained in this study were compared with the sequences of *L. caespiticola* already deposited in Genbank. There are 6 sequences (accession numbers KF242344, HM447030, AY601567, AF480081, AF480080, AF480079) of D2/D3 and one sequence (accession number KF242280) of 18S gene are available in Genbank (Rubtsova *et al.,* 2001; He *et al.,* 2005b; Širca & Urek, 2009; Subbotin *et al.,* 2014). Sequences of D2/D3 region is 100 % (identities 774/774 nucleotides) identical to accession number AF480081 (Country: Belgium, Vliermaal) and differs by 5 – 20 nucleotides (0.68 to 2.04 %) from the other five published sequences. The sequences of 18S are 100 % identical to KF242280 (identities 850/850 nucleotides).

Subbotin *et al.* (2014) on the basis of restriction enzyme profile of D2/D3 expansion segment of 28S gene and *in silico* analysis predicted three profiles for *L. caespiticola*: type A (Slovenian population), type B (Russian population) and type C (UK, German and Brussegem, Belgian populations). They hypothesized that *L. caespiticola* might represent a species complex but further studies of the morphological characters in these three groups will be needed to confirm this hypothesis. Hundred percent identity of D2/D3 sequence with a sequence (accession number AF480081) from Vliermaal, Belgium indicated that Czech and Slovakian populations of *L. caespiticola* belong to type A.

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