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

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Abstract *Longidorus helveticus* was found at two out of 285 sampling sites for the first time in the Czech Republic. Females, males and juvenile stages were analyzed morphologically and morphometrically. The morphological identification of samples was verified by polymerase chain reaction using a species specific primer. Four markers of ribosomal DNA (18S, ITS1, ITS2, D2-D3 expansion segments of 28S rRNA) and two markers of mitochondrial DNA (*cox1* and *nad4*) were sequenced and analyzed and compared with published gene sequences of other populations of *L. helveticus*. The partial mitochondrial cytochrome c-oxidase subunit 1 gene and partial nicotinamide dehydrogenase subunits 4 gene showed relatively high genetic variation within the species compared with ribosomal DNA markers.

Keywords Mitochondrial DNA · Nematode · PCR · Ribosomal DNA

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Introduction

Plant parasitic nematodes of the genus *Longidorus* are polyphagous root-ectoparasites of many plants including various agricultural crops and trees. From 143 species presently described in the genus *Longidorus,* 73 species have been reported in Europe (Decraemer and Robbins 2007). To date, only four species of *Longidorus* were found and characterized from the Czech Republic (Kumari and Decraemer 2007; Kumari et al. 2009). Species identification of longidorid nematodes is based primarily on study of morphology; however, high morphological variability leads to considerable overlapping of many characters among species, and thus creates difficulties in identification. Combination of morphological and molecular methods is considered as a prospective approach in diagnostics of this nematode group.

Plant-parasitic nematodes belonging to family Longidoridae are economically important pests, therefore, an extensive study of these nematodes is being carried out in the Czech Republic. During several nematological surveys, *Longidorus helveticus* Lamberti, Kunz, Grunder, Molinari, De Luca, Agostinelli, Radicci 2001 was found in two localities for the first time in the Czech Republic. *Longidorus helveticus* was originally described from the rhizosphere of sweet cherry in Switzerland (Lamberti et al. 2001). Later, this species was found in Germany, Serbia, Slovenia and Slovakia (Hübschen et al. 2004; Barsi and De Luca 2005; Širca and Urek 2009; Lišková and Šalamún 2010).

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Molecular characterization of different populations of this species using RFLP and sequencing of rRNA gene was carried out by some researchers (Lamberti et al. 2001; De Luca et al. 2004; He et al. 2005; Kumari et al. 2009; Širca and Urek 2009). This study is one from a series of works on characterization of Longidoridae in the Czech Republic.

Objectives of this study were: 1) to characterize the two populations of *L. helveticus* morphologically from the Czech Republic; 2) to verify the morphological identification by polymerase chain reaction with a specific primer; and 3) to sequence and analyze four markers (18S, ITS1, ITS2 and 28S) of ribosomal DNA and two markers (cytochrome *c* oxidase subunit–*cox1* and nicotinamide dehydrogenase subunits 4–*nad4*) of mitochondrial DNA.

Materials and methods

Morphological study

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 a 99 μm sieve on a Baermann funnel from 24 to 48 h (Brown and Boag 1988). 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).

PCR and sequencing

Individual nematodes of *L. helveticus* from the Czech Republic were stored in 1 M NaCl and used to extract DNA. Total genomic DNA was extracted according to a rapid method by Stanton et al. (1998). DNA of the Serbian population was the same as used by Kumari et al. (2009). Species specific reverse primer Lhel1 and universal forward primer GenF were used to amplify three populations of *L. helveticus* (Table 1). The PCR reaction 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. The cycling profile was as follows: 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 final extension at 72°C for 10 min.

Six regions (18S, ITS1, ITS2, D2/D3 expansion segments of 28S, *cox1* and *nad4*) of ribosomal and mitochondrial DNA were also amplified and sequenced. Primer sequences and references to the primers are given in Table 1. Partial *cox1* gene of both populations (Chodovlice and Silničná) was amplified by primers COIF and COIR. For the population from Silničná only one specimen was amplified with this primer pair, therefore from this single sequence new primers sil_F+sil_R were designed by using the online software PRIMER3. Primers F400+R400 for the *nad4* gene were designed from the sequence of *X. americanum* accession number AY382608. This primer pair (F400+R400) amplified *nad4* gene only for the population from Chodovlice and no amplification was observed for individuals from the localities Silnična and Serbia. A new primer pair RDF+RDR was designed from the sequences of individuals from the locality Chodovlice. Primer RDF was designed using the online software PRIMER3 and primer RDR from the NCBI primer designing tool. Even though the new primer pair RDF+RDR amplified all three populations (two Czech and one Serbian), sequences which were already made by primers F400+R400 were used for the individuals from Chodovlice. Sequences from the localities Silničná and Serbia were sequenced by primers RDF+RDR. The 18S gene was amplified in two fragments. Primer combination was as follows: first fragment 988F 1912R and second fragment 1813F 2646R. PCR mix was for rDNA and mtDNA was same as described above. The cycling profile for all markers of ribosomal DNA was also same as above. The cycle profile for mtDNA was as described by He et al. (2005): 95°C for 10 min, 5 cycles at 94°C for 30 s, 45°C for 40 s, and 72°C for 1 min, and further 35 cycles at 94°C for 30 s, 37°C for 30 s, and 72°C for 1 min, followed by an extension at 72°C 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, Korea). Sequencher™ 4.8

Table 1 Primers used to amplify ribosomal and mitochondrial DNA

Gene	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	GenF	Forward	TTG ATT ACG TCC CTG CCC TTT GT	Hübschen et al. (2004)
ITS1	Lhel 1	Reverse	CCG CAT CTC TTT ATT TCC GAC CAT CAA CC	Hübschen et al. (2004)
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)
cox1	COIF	Forward	GAT TTT TTG GKC ATC CWG ARG	He et al. (2005)
cox1	XIPHR2	Reverse	GAT CAT AAT GAA AAT GTG CCA C	Lazarova et al. (2006)
cox1	sil_F	Forward	TTC TTA TCC TCC CTG GTT TT	This study
cox1	sil_R	Reverse	ATA ATG AAA ATG TGC CAC CA	This study
nad4	F400	Forward	TGA TAT AAG AAT TAG TCC AA	This study
nad4	R400	Reverse	ACT AGT GGA TCT ATG ATT TT	This study
nad4	RDF	Forward	CTA CTT TTC TGA ATT TTC TT	This study
nad4	RDR	Reverse	GAT TTT AGC TAG GTT GCT C	This study

(Genes codes Corp., Ann Arbor, MI, USA) was used to assemble and view each sequence and check for base-calling errors. Number of nematodes sequenced for each region and their accession numbers are given in Table 2.

Sequence and phylogenetic analysis

The newly obtained sequences for each gene were aligned using ClustalX 1.83 (Thompson et al. 1997) with default parameters with corresponding published gene sequences of *L. helveticus* and closely related species (Palomares-Rius et al. 2008). Pairwise distance was calculated by MEGA5 (Tamura et al. 2011). Sequence alignments were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) under the GTR+I+G model as described by Palomares-Rius et al. (2008).

Table 2 Number of individual nematodes sequenced and in brackets accession number of representative individual specimen

Region	Chodovlice	Silničná	Serbia
18S	2 (JN627408)	2 (JN627409)	*
ITS1	1 (JN627410)	1 (JN627411)	1 (JN627412)
ITS2	n/a	4 (JN627413)	*
D2/D3	4 (JN627414)	4 (JN627415)	*
cox1	10 (JN627416)	10 (JN627417)	*
nad4	6 (JN627418)	3 (JN627419)	3 (JN627420)

n/a not acquired; * Kumari et al. 2009

Results

Morphological analysis

L. helveticus was found for the first time from the rhizosphere of *Carpinus betulus L.* and *Acer platanoides L.* in a forest soil with a mixture of population of *L. intermedius* Kozlowska and Seinhorst, 1979, *Xiphinema dentatum* Sturhan, 1978 and *Xiphinema* sp. at Silničná and from the rhizosphere of *Prunus avium* L. at Chodovlice in a mixture of *Longidorus* sp. The two populations of *L. helveticus* consisted of females, males and all four juvenile stages. Morphometrics of the population from the localities Silničná

Table 3 Morphometrics of *Longidorus helveticus*. Measurements in μm (in form): mean±standard deviation (range)

Locality	Silničná				Silničná	Chodovlice	Silničná	Chodovlice
Specimens	J1=16	J2=8	J3=14	J4=9	Females=17	Females=3	Males=6	Males=10
L	1681±192 (1452–2051)	2877±320 (2428–3308)	3684±410 (2920–4179)	5867±485 (5222–6893)	7442±726 (6537–8952)	7569±687 (6914–8285)	6786±775 (6012–8114)	6979±673 (6044–8396)
a	49.6±3.18 (42.2–55.3)	48.8±4.30 (41.1–56.3)	57.0±8.90 (45.4–76)	63.6±3.35 (59.2–68.9)	68.6±5.41 (59.8–77.4)	65.4±3.37 (61.7–69.2)	69.3±5.23 (60.1–75.1)	71.7±7.47 (63.0–90.3)
b	5.2±0.72 (4.6–6.9)	8.6±2.87 (6.1–14.8)	8.5±1.02 (6.5–10.2)	11.5±1.20 (9.5–13.9)	12.3±1.51 (9.5–15.5)	13.5±0.85 (12.7–14.4)	12.1±1.00 (10.5–13.5)	12.0±1.46 (10.0–15.0)
c	34.3±3.26 (26.0–40.6)	80.9±10.32 (70.4–97.8)	98.6±9.65 (81.6–113.0)	147.4±18.01 (119.4–181.4)	179.7±21.28 (145.3–213.2)	196.9±14.61 (181.9–211.1)	172.8±32.01 (138.2–231.8)	161.0±17.94 (137.4–195.2)
c	1.90±0.12 (1.65–2.15)	0.80±0.11 (0.63–0.95)	0.71±0.07 (0.59–0.83)	0.63±0.05 (0.55–0.72)	0.63±0.05 (0.52–0.70)	0.58±0.01 (0.57–0.59)	0.66±0.07 (0.56–0.76)	0.69±0.03 (0.63–0.75)
V/spicule	—	—	—	—	52.0±1.70 (49.4–56.0)	49.8±1.17 (48.5–50.8)	99±3.50 (96–106)	97±4.75 (87–102)
Replacement odontostyle	87±4.83 (75–98)	107±2.77 (101–110)	120±4.10 (112–127)	137±4.09 (132–144)	—	—	—	—
Odontostyle	78±3.64 (72–87)	88±2.72 (85–93)	103±3.25 (98–110)	123±3.76 (117–128)	135±4.50 (129–142)	131±4.16 (126–134)	132±5.17 (126–137)	132±4.74 (125–138)
Odontophore	38±3.66 (32–45)	46±2.30 (42–50)	50±3.67 (43–56)	58±4.48 (49–65)	67±3.27 (62–73)	60±3.51 (56–63)	68±5.66 (64–79)	63±5.91 (50–69)
Total stylet length	116±5.77 (104–125)	135±4.50 (127–141)	153±5.29 (143–161)	181±4.18 (175–187)	202±6.08 (191–214)	190±4.51 (186–195)	199±8.50 (190–213)	194±8.53 (177–207)
Oral aperture to guide ring	24±1.89 (21–30)	28±1.04 (27–30)	31±1.38 (29–34)	37±1.59 (34–38)	43±1.89 (40–47)	44±1.73 (42–45)	42±1.86 (39–44)	43±2.06 (39–45)
Pharyngeal bulb length	77±7.59 (68–95)	98±8.64 (89–110)	108±5.30 (99–116)	125±9.47 (115–142)	144±5.46 (132–151)	147±4.58 (142–151)	134±7.80 (121–144)	133±6.62 (124–146)
Pharyngeal bulb diam.	19±1.83 (17–23)	28±4.00 (24–35)	31±3.20 (25–36)	36±3.78 (29–42)	39±5.47 (30–53)	36±1.53 (34–37)	39±3.82 (35–44)	37±3.44 (32–41)
Tail length	49 ±3.54 (45–57)	36±3.45 (31–40)	37±1.82 (33–40)	40±4.14 (36–50)	42±2.62 (37–46)	38±0.58 (38–39)	40±2.88 (35–44)	44±3.14 (39–49)
Length of hyaline tip	25±3.51 (20–33)	11±1.31 (9–13)	12±1.53 (10–15)	13±0.87 (12–15)	17±1.84 (13–20)	18±0.58 (17–18)	13±1.33 (11–15)	14±1.70 (12–16)
Number of supplements/ mucro length	16±2.42 (12–21)	—	—	—	—	—	14±0.82 (13–15)	14±1.23 (13–16)

Table 3 (continued)

Locality	Silničná				Silničná	Chodovlice	Silničná	Chodovlice
Specimens	J1=16	J2=8	J3=14	J4=9	Females=17	Females=3	Males=6	Males=10
Body diam. at lip region	9±0.83	12±0.92	14±0.76	17±1.20	19±1.28	20±1.15	19±1.55	20±0.52
	(7–10)	(11–14)	(14–16)	(15–19)	(17–22)	(20–21)	(17–21)	(20–21)
at guiding ring	18±1.18	25±1.58	29±1.48	36±1.22	41±2.59	42±1.53	40±1.17	41±1.51
	(16–21)	(24–29)	(27–33)	(34–37)	(38–46)	(40–43)	(38–41)	(38–43)
at base of pharynx	33±2.66	53±4.52	61±5.20	76±3.57	89±7.38	82±3.21	84±7.47	82±5.63
	(30–38)	(48–59)	(53–70)	(71–81)	(77–105)	(78–84)	(77–94)	(74–91)
at mid body/at vulva	33±3.16	59±5.76	69±7.19	92±7.68	109±7.39	115±3.51	98±6,46	98±5.42
	(30–41)	(50–69)	(56–80)	(80–102)	(95–120)	(112–119)	(90–108)	(91–109)
at anus	26±2.50	45±4.49	53±3.99	64±3.52	67±4.28	66±2.00	60±2.42	63±3.43
	(23–31)	(39–52)	(47–60)	(58–69)	(58–74)	(64–68)	(57–63)	(58–69)
at beginning of hyaline tip	17±1.69	28±3.04	35±2.93	43±1.87	46±4.17	48±1.15	36±2.16	40±2.32
	(14–20)	(25–35)	(31–40)	(40–46)	(37–54)	(47–49)	(33–39)	(37–45)

and Chodovlice are given in Table 3 and photomicrographs are presented in Fig. 1. Morphometrics of juveniles from Chodovlice are not given because it occurred with another *Longidorus* species, which makes it difficult to separate juveniles with certainty.

Molecular analysis

PCR with a species specific primer (Lhel1) and universal primer (GenF) yielded a single fragment of 368 bp for two samples of *L. helveticus* from the Czech Republic and one sample from Serbia (Fig. 2). This primer pair (Lhel1+GenF) amplified partial 18S gene and partial ITS1 region. No PCR products were obtained in the negative control lacking DNA template or from samples containing DNA of *L. caespiticola* Hooper, 1961, *L. distincuts* Lamberti, Choleva & Agostinelli, 1983, *L. intermedius* Kozlowska & Seinhorst 1979, *L. iuglandis* Roca, Lamberti & Agostinelli, 1984, *L. juglandicola* Lišková, Robbins & Brown, 1997, *L. juvenilis* Dalmasso, 1969, *L. leptocephalus* Lamberti, Choleva & Agostinelli, 1983, *L. pisi* Edward, Misra & Singh, 1964, two different *Longidorus* sp., *Paralongidorus* sp., *X. diversicaudatum* (Micoletzky, 1927) Thorne, 1939 or *X. vuittenezi* Luc, Lima, Weischer & Flegg, 1964 (data not shown).

Phylogenetic relationships of *L. helveticus* with closely-related *Longidorus* based on analyses of the D2-D3 of 28S rRNA, ITS1 rRNA and *cox1* are given in Figs. 3 and 4. In all phylogenetic trees *L. helveticus* from the Czech Republic formed a highly supported clade with other populations of this species. *Longidorus macrosoma* was a sister taxon for *L. helveticus.*

Sequences (18S, ITS1, ITS2, *cox1* and *nad4* gene) of two populations of *L. helveticus* from the Czech Republic were compared with the one population from Serbia studied by Kumari et al. (2009) and other populations of this species from GenBank. Identical sequences were obtained for all individuals studied from the same population for 18S, ITS2, *cox1* and *nad4* gene. Sequences of 18S rRNA gene of both Czech populations were identical to that of the Serbian population (EF538759).

ITS1 sequence, which was amplified and sequenced with one species-specific Lhel1 and universal primers GenF, from the locality Silničná was identical to Serbian population. Sequences from Chodovlice showed polymorphisms at three sites. ITS2 sequence was obtained only for the population from Silničná

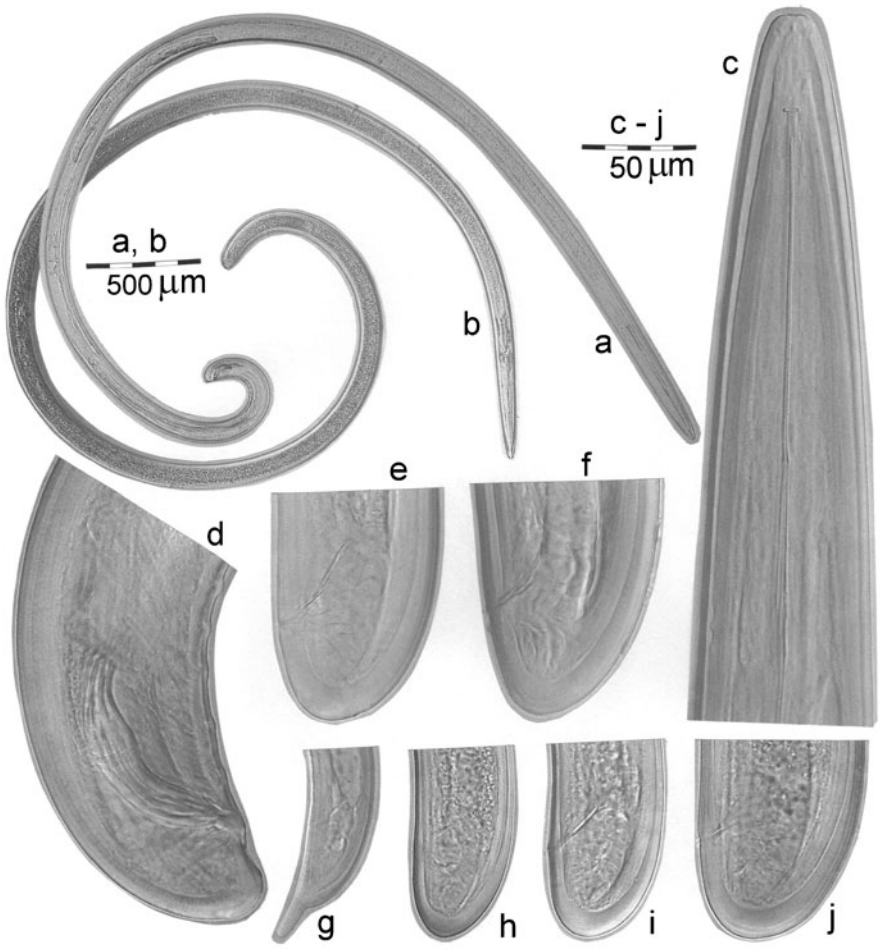

Fig. 1 *Longidorus helveticus.* **a**: Entire male; **b**: Entire female; **c**: Female anterior; **d**: Male posterior; **e**, **f**: Female posterior; **g**–**j**: tail region of J1, J2, J3 and J4 respectively

and the sequences differ by two nucleotides from the Serbain population (EU444015). Sequences of D2-D3 region for the two Czech populations differed by two nucleotides. Sequence of population from Silničná was identical to the Serbian population (EF538753). Sequence divergence for all *L. helveticus* ranged from 0 to 4 nucleotides (0–0.5%) for D2-D3 region.

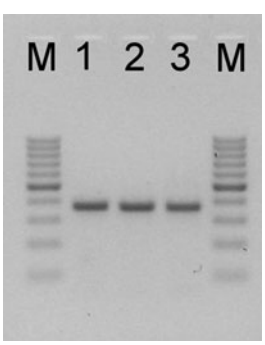

Fig. 2 Electrophoresis of the amplified products from single specimens of *L. helveticus* from two localities from the Czech Republic and one from Serbia: lane M: 100 bp DNA ladder (Fermentas); lane 1: Chodovlice; lane 2: Silničná; lane 3: Serbia

The lengths of partial *cox1* gene sequenced for *L. helveticus* were 384 bp for the population from Silničná and 437 bp for the population from Chodovlice. Difference in length of the *cox1* gene is due to the different primers used (see "Materials and methods"). The population from Chodovlice was amplified using primers COIF+COIR and Silničná by using primers sil_F+sil_R. Ten specimens per population were sequenced and identical sequences were obtained for all individuals studied from the same population for the *cox1* gene. Inter-population genetic variation was found among these two populations. Alignment of these two populations and one population from Serbia (EF538747) from the Genbank is given in Fig. 5. The sequences were aligned unambiguously without gaps.

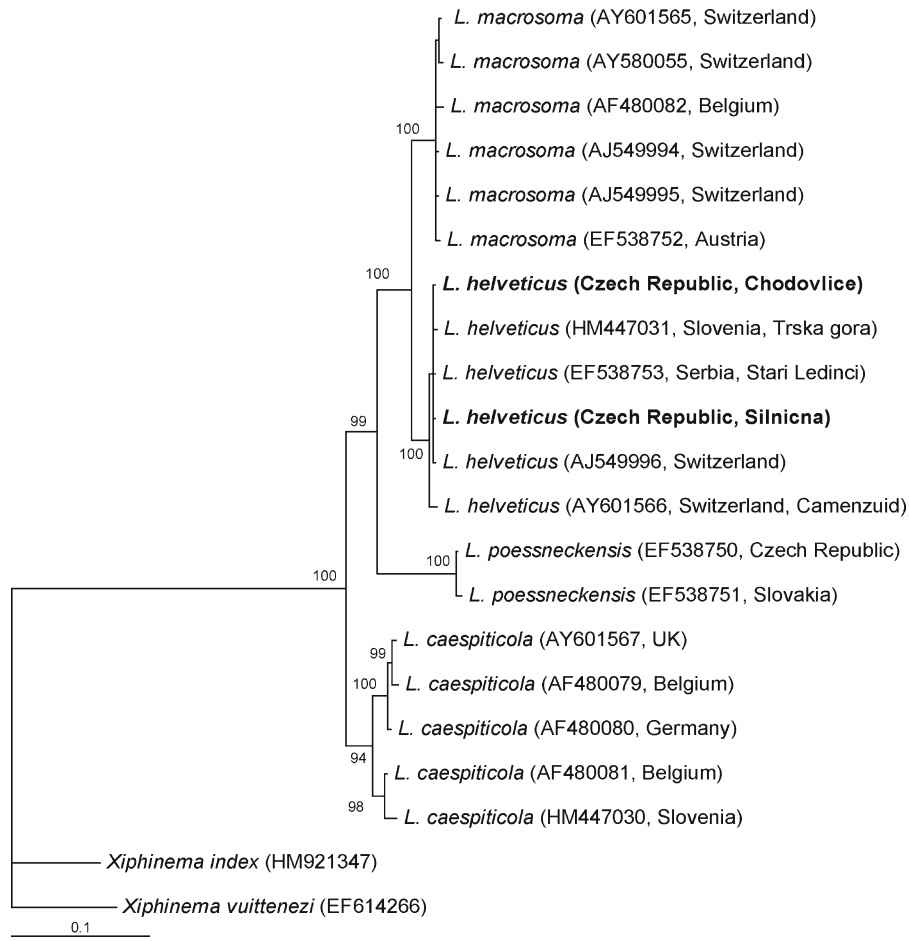

Fig. 3 Phylogenetic relationships of *L. helveticus* with closely related species as inferred from the Bayesian analysis of the D2-D3 of 28S rRNA gene sequences. Newly obtained sequences are indicated in bold type

Three different sequence variants of *cox1* were determined from these three populations. Within the 368 bp alignment (except for unequal ends) of these three variants there were 28 variable sites (24 transitions

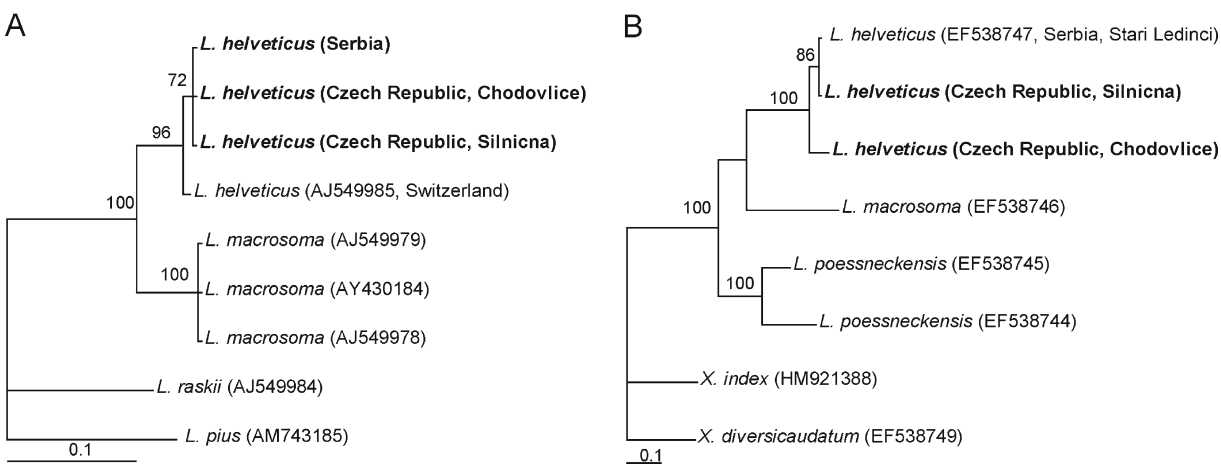

Fig. 4 Phylogenetic relationships of *L. helveticus* with closely related species as inferred from the Bayesian analysis of the ITS1 of rRNA (**a**) and *cox1* (**b**) gene sequences. Newly obtained sequences are indicated in bold type

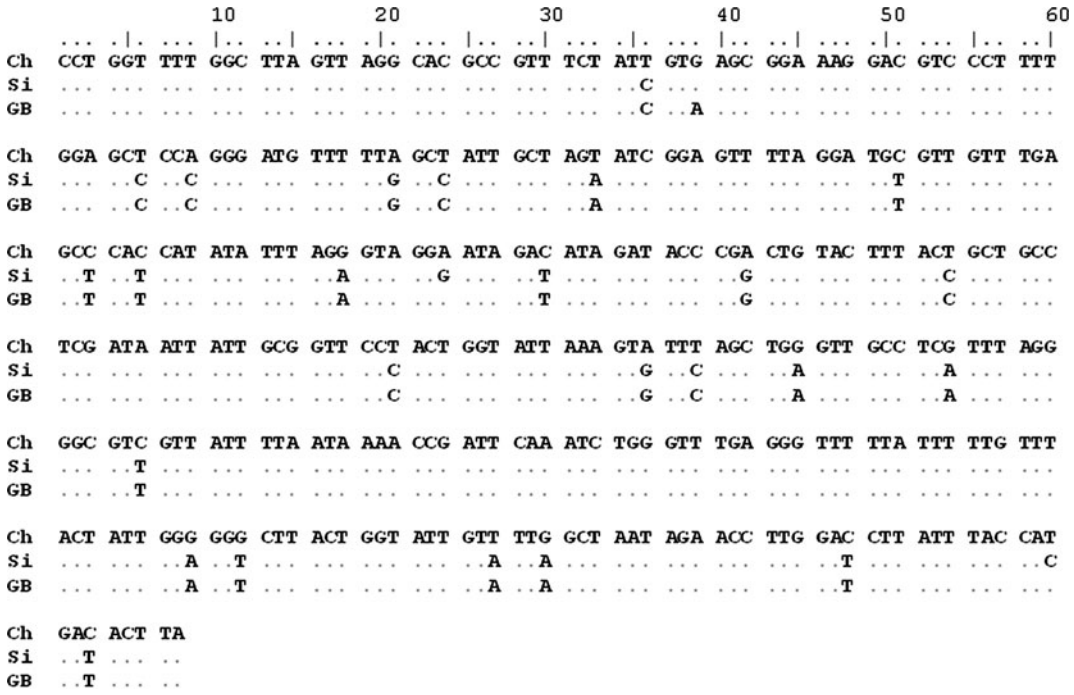

Fig. 5 Alignment of parital *cox1* gene of three variants of *L. helveticus*. Ch: population from the locality Chodovlice; Si: population from the locality Silničná; GB: accession number EF538747

and four transversions), each of which occurred at third codon position sites. The 28 variable nucleotide sites among all three *cox1* sequence variants represented transitions C<−>T (n=14) and A<−>G (n=10); and transversions A<−>C (n=1), A<−>T (n=2) and G<−>T (n=1) (Fig. 5). All variations occurred at silent sites. Pairwise comparisons among the three *cox1* sequence variants (excluding the unequal flanking sequence) revealed sequence variation ranging from 0.82 to 7.34% (Table 4). The A+T content was 53.3% at the first codon, 57.8% at second codon positions and the third codon position had a greater A+T bias (66%) compared with the other two positions.

The lengths of the partial *nad4* gene sequenced for *L. helveticus* were 360 bp for the population from Silničná and Serbia and 378 bp for the population from Chodovlice. Difference in length of the *nad4* gene is due to the different primers used (see "Materials and methods"). The population from Chodovlice was amplified using primers F400+R400 and Silničná and Serbia was amplified using primers RDF and RDR. Inter-population genetic variation was found among these three populations. Alignment of these three populations is given in Fig. 6. The *nad4* sequences were aligned unambiguously without gaps. Three different sequence variants of *nad4* were determined from these three populations. Within the 360 bp alignment (except for unequal ends) of these three variants there were 33 variable sites (26 transitions and seven transversions). Nucleotide variation was related mainly to changes at the third codon position (30 variations; 90.91%), while two changes were detected at the first codon (6.06%) and only one variation (3.03%) was detected at the second codon position. Inter-population variation represented transitions C<−>T (n=13) and A<−>G (n=13), and transversions A<−>C (n=6) and C<−>G (n=1) (Fig. 6). While most (n=28; 84.8%) nucleotide changes were synonymous, the transitions at position 66, 70 (C<−>T) and 201 (A<−>G) and transversions at 284 (A<−>C) and 331 (C<−>G) resulted in a change in the *nad4* amino acid

Table 4 Pairwise comparison of sequence differences among the three sequence samples of *Longidorus helveticus*

cox1	1	2	*nad4*	1	2
1 Chodovlice			1 Chodovlice		
2 Silničná	7.34		2 Silničná	8.33	
3 Serbia	7.07	0.82	3 Serbia	9.17	0.83

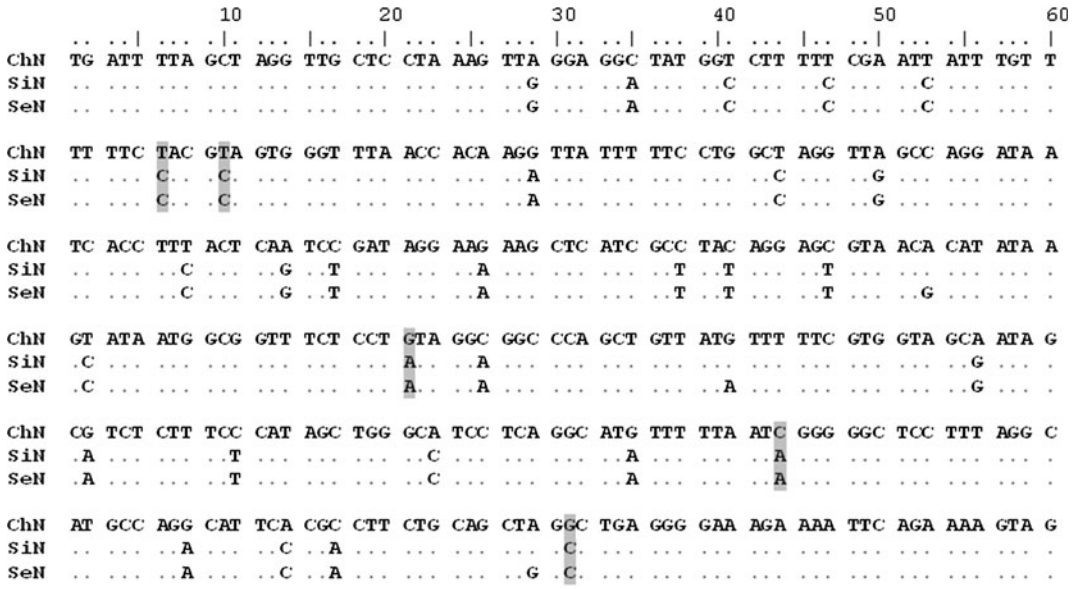

Fig. 6 Alignment of *nad4* gene of three variants of *L. helveticus*. ChN: populations from Chodovlice; SiN: Silničná; SeN: Serbia. Grey regions indicate nucleotides which resulted in a change in amino acid sequence

sequence (Fig. 6). Pairwise comparisons among the three *nad4* sequence variants (excluding the unequal flanking sequence) revealed sequence variation ranging from 0.83 to 9.17% (Table 4). There was a strong AT bias (T=31.1%, C=20.6%, A=24.3%, G=24.0%), but the A+T content was almost similar at each codon positions (56.6% at the first codon, 54.3% at the second codon positions, and 54.9% at the third codon position). There was no A+T bias toward the third codon as was found for the *cox1* gene.

Discussion

Morphometrical and morphological comparison

Morphometrics of the two Czech populations were in close agreement with the original description of the species (Lamberti et al. 2001). Main morphometrical characters (odontostyle, replacement odontostyle and tail lengths, distance from oral aperture to guide ring and body length) of adults were similar to the morphometric description of the species, except for the mean ratio ‘c’ in females from locality Silničná (179.7 *vs* 200.3 in the type population), female pharyngeal bulb length (132–151 and 142–151 *vs* 110–130 μm in the type population), the mean body length of males (7 mm and 6.8 mm *vs* 7.3 mm in the type population) and lip region width of females and males (19 and 20 *vs* 22 μm in the type population). Odontophore of females and males was shorter for both localities compared to type population (67 and 60 μm *vs* 89.5 μm females and 68 and 63 *vs* 90.5 μm males). The considerable difference in the length of odontophore might be caused by errors in measurement due to difficulties in locating accurately the base of the odontophore. Males with the posterior region were more coiled than in those of females due to presence of copulatory muscles. Means of morphometrical characters of males were lower than females. Spicules were robust, ventrally curved. Number of supplements varied from 13 to16.

Juveniles clearly morphometrically separated into four groups. The first developmental stage possessed a digitate tail with 12–21 μm long mucro. Second, third and fourth juvenile stages had a bluntly rounded tail (Fig. 1). Measurements of all juvenile stages corresponded well with the morphometrics of the type specimens from Switzerland, with the exception of the ratio ‘b’ in the second stage juvenile. The second stage juvenile from the Czech Republic had a higher ratio ‘b’ than in the type population (8.6 *vs* 7.1).

The identification codes according to the polytomous key for *Longidorus* (Chen et al. 1997) for the Czech population from Chodovlice are A-5, B-4, C-4, D-1, E-4, F-34, G-1, H-1, I-2 and Silničná A-56, B-4,

C-34, D-1, E-4, F-34, G-1, H-1, I-2. These codes are in agreement with the codes of the type population (Lamberti et al. 2001).

Molecular analysis

Molecular diagnostic PCR with species specific primers (Hübschen et al. 2004) reliably discriminated two populations of *L. helveticus* from the Czech Republic and one population from Serbia. Accurate analysis of genetic variation in nematodes has important implications for studying population biology, epidemiology, and genetic structure of nematodes, and the study of different markers provides a benchmark for comparison. Therefore in our work six markers of ribosomal and mitochondrial DNA were analyzed and compared. Analysis of 18S gene of Czech and Serbian populations did not reveal any variation between samples, whereas ITS1 showed polymorphism at three sites and D2-D3 expansion segments of 28S rRNA gene revealed difference only in two nucleotides between two populations from the Czech Republic.

It has been known that mitochondrial DNA evolves faster than ribosomal DNA and has more discriminative power in the separation of closely related species (Morgan and Blair 1998). Among mitochondrial genes, *cox1* assessed the nucleotide variability among three populations of *L. helveticus*, therefore primers were designed to study another marker *nad4*. This gene exhibits less conservation sites than *cox1* and it is often suggested for prospecting closely related species (Blouin 2002). In this study *nad4* also showed a slightly higher level of nucleotide substitutions (3 to 30 nucleotides) than *cox1* (3 to 25 nucleotides). As is it commonly found for mitochondrial DNA sequences in other parasitic nematodes (Blouin et al. 1998), there was a higher AT bias for *cox1* and *nad4* gene; however, for the *nad4* gene, AT bias was not found toward the third codon position for *L. helveticus*. The reasons for this G and C ending shift are presently unknown and require detailed research. Understanding the cases of biases in codon usage and nucleotide composition is essential to future study of the phylogeny of longidorids.

Surprisingly, highest genetic variation (7.34% for *cox1*) was observed among individuals of two populations from the Czech Republic, whereas low variation (0.82% for *cox1* and 0.83% for *nad4*) was found between populations from Silničná (Czech Republic) and Serbia. The highest genetic variation (9.17%) for the *nad4* gene was found between the population from Chodovlice and Serbia. The two markers (*cox1* and *nad4*) produced patterns of genetic differentiation that were consistent with each other and the degree of diversity that observed here is in agreement with earlier published data on other nematodes (Blouin et al. 1995; Fisher and Viney 1998; Braisher et al. 2004; Höglund et al. 2006).

Phylogenetic analyses of ribosomal (D2-D3, ITS1) and mitochondrial (*cox1*) DNA supported the morphological identification and molecular PCR diagnostic of *L. helveticus*. The phylogenetic trees obtained by Bayesian analyses of D2-D3, ITS1 and *cox1* sequences of *L. helveticus* from the Czech Republic formed highly supported clades (96 to 100 posterior probability) with other populations of the species (see Figs. 3 and 4). In two phylogenetic trees *L. macrosoma* was placed as the sister taxon to *L. helveticus* with consistently strong support (100 posterior probability).

Morphological and molecular analysis confirmed that longidorid samples found in the Czech Republic belongs to *L. helveticus*. In this study we present sequences of mitochondrial *nad4* gene data from individuals of *L. helveticus* for the first time for longidorid nematodes.

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