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Molecular and morphological delineation of *Longidorus poessneckensis* Altherr, 1974 (Nematoda: Dorylaimida)

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Abstract The plant–parasitic nematode *Longidorus poessneckensis* from the Czech Republic was morphologically and molecularly characterised. Molecular analyses were carried out using mitochondrial DNA (cytochrome *c* oxidase subunit 1—*cox1*) and ribosomal DNA (ITS2—second internal transcribed spacer, 18S gene and D2/D3 expansion segments of the 28S gene), which were amplified and sequenced. Phylogenetic relationship of *L. poessneckensis* with three morphologically closely related species, i.e. *L. macrosoma*, *L. helveticus* and *L. uroshis*, was inferred by using maximum likelihood and maximum parsimony methods, with a female of *Xiphinema diversicaudatum* and a bivulval female of *X. vuittenezi* as outgroups. All multiple alignments yielded similar basic trees supporting the uniqueness of *L. poessneckensis* and the validity of the four *Longidorus* species identified using morphological characters. Phylogenetic analyses revealed that *L. poessneckensis* is more closely related to *L. macrosoma* and *L. helveticus* than to *L. uroshis*. High inter-population diversity (19%) was observed across the *cox1* gene between two populations of *L. poessneckensis*.

Keywords Mitochondrial DNA · Morphology · Phylogeny · Ribosomal DNA

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Introduction

Plant parasitic nematodes belonging to the genus *Longidorus* (Nematoda: Dorylaimida) are of great economic importance due to their ability to act as vectors of *Nepoviruses* (Taylor and Brown 1997). Hence, given a possible vector role, the genus *Longidorus* has gradually received greater taxonomical attention. Species identification of *Longidorus* is mainly based on morphological characters and morphometric data of females (Chen et al. 1997). Recently, molecular sequence data of various *Long-*

idorus species based on ribosomal DNA (rDNA) have become available to complement morphological identification (Luca et al. 2004; Neilson et al. 2004; Ye et al. 2004; He et al. 2005b; Oro et al. 2005). Research in plant parasitic nematodes has also turned to exploit the usefulness of mitochondrial DNA (mtDNA) for analysing phylogeny of economically important taxa e.g. *Meloidogyne* and *Xiphinema americanum*-group (Blok et al. 2002; Lazarova et al. 2006). However, despite the agricultural importance of *Longidorus* species, there is no information available on the mtDNA genes of species ranked within this genus. For instance, *Longidorus poessneckensis* Altherr 1974 is among those species of *Longidorus* that needs to be studied in depth more from a molecular approach. *Longidorus poessneckensis* was originally described from the periphery of a well in Germany (Altherr 1974). Later, it was recorded from several localities in Germany and from one site in Slovakia, mostly from moist woodland (Sturhan and Loof 2001). Additionally, it was recorded from several additional localities in Slovakia from floodplain forests, river meadow and river banks (Lišková and Sturhan 2000; Lišková and Brown 2003). Recently, *L. poessneckensis* was also recorded from Austria from the rhizosphere of the wild grape in the riparian woods of the rivers Danube and March (Tiefenbrunner and Tiefenbrunner 2004).

Species delimitation in several nematode taxa remains problematic due to high phenotypic plasticity. Molecular techniques and phylogenetic analyses can potentially overcome this problem. However, deciding when individuals are sufficiently differentiated to discern them as different species based on sequence information is also problematic in a number of taxa. The problem of either morphological or molecular species delimitation can be resolved by applying an holistic approach, in which analyses of several independently evolving molecular markers circumvent flaws of the molecular methods (Derycke et al. 2008).

Hence, given the lack of knowledge on molecular features of *L. poessneckensis*, the present study provides comprehensive information on molecular characters of *L. poessneckensis* from the Czech Republic by using four independently evolving molecular markers, allowing a better supported delineation of the species. The evolutionary relationship of *L. poessneckensis* with three closely resembling *Longidorus* species was inferred from the characterisation of sequences within mtDNA (i.e. gene encoding for the cytochrome *c* oxidase subunit 1—*cox1*) and rDNA (i.e. second internal transcribed spacer—ITS2, 18S gene and D2/D3 expansion segments of the 28S gene). Moreover, additional morphological and morphometrical characters of *L. poessneckensis* from the Czech Republic are also used to complement the molecular study and previous studies of the species from Austria, Germany and Slovakia.

Materials and methods

Sampling and morphological observations

Soil samples were collected from a forest under the rhizosphere of *Quercus* in October, 2002 and in September, 2005 at Černé Voděrady (Bohemia, Czech Republic) from a small surface area of 10 m^2. Bulk samples consisted of four to six sub-samples taken at a depth of 0–40 cm. Nematodes for morphological and molecular study were extracted from soil by the sieving and decanting method. For morphological study, specimens were heat-killed, fixed in triethanolamine formalin, processed and mounted in anhydrous glycerin. Nematode identification was made using an Olympus BX51 light microscope, equipped with a digital camera C4040 and differential interference contrast (DIC, Nomarski). Measurements were made with the aid of imaging software (Olympus DP-soft).

DNA extraction, PCR protocols and sequencing

Two specimens from each population were examined molecularly to assess both inter- and intraspecific variation for all four studied loci (Fig. 1, Table 1). Live individual specimens of *L. poessneckensis* from the Czech Republic were mounted in distilled water with glass beads on temporary glass slides and relaxed using gentle heat. Measurements and photomicrographs were taken and slides were dismantled immediately and the specimens were placed in 0.5 ml Eppendorf tubes containing 20 µl of 0.25 M NaOH under a binocular microscope. Specimens of *L. poessneckensis* and *Longidorus uroshis* Krnjaić, Lamberti, Krnjaić, Agostinelli & Radicci, 2000 from Slovakia, *Longidorus macrosoma* Hooper 1961 from Austria and *Longidorus helveticus* Lamberti, Kunz, Grunder, Molinari, Luca, Agostinelli & Radicci, 2001 from Serbia were mailed to the Czech Republic in 1 M NaCl. Temporary mounts of these nematodes were made in a drop of IM NaCl

Fig. 1 Specimens used for molecular study. *Longidorus poessneckensis* (Czech)—**a**, **b** anterior and posterior regions of female 1; **c**, **d** anterior and posterior regions of female 2; *L. poessneckensis* (Slovakia)—**e**, **f** anterior and posterior regions of female 1; **g**, **h** anterior and posterior regions of female 2; *L. macrosoma*—**i**, **j** anterior and posterior regions of juvenile; **k**, **l** anterior and posterior regions of female; *L. helveticus*—**m**, **n** anterior and posterior regions of female; **o**, **p** anterior and posterior regions of male; *L. uroshis*—**q**, **r** anterior and posterior regions of female; **s**, **t** anterior and posterior regions of male

containing glass beads and after taking measurements and photomicrographs the slides were dismantled, individual nematodes removed, and added to digest in 0.25 M NaOH overnight. Morphological and molecular data were thus obtained for all *Longidorus* species from the same specimens (Table 1, Fig. 1). A single female specimen of *Xiphinema diversicaudatum* (Micoletzsky 1927) Thorne, 1939 from Slovakia and a single bivulval specimen of *Xiphinema vuittenezi* Luc, Lima, Weischer & Flegg, 1964 from the Czech Republic were also examined molecularly and used as outgroups for phylogenetic analyses.

Total genomic DNA was extracted from single nematode individuals with slight modification to the protocols previously described (Klimyuk et al. 1993; Stanton et al. 1998). Briefly, the tubes containing nematodes in 20 µl of 0.25 M NaOH were incubated overnight at room temperature, and then heated to 99°C for 3 min. Subsequently, 10 µl of 0.25 M HCl, 5 µl each of 0.5 M Tris-HCl (pH 8) and 2% Triton X-100

Table 1 Morphometrics of specimens of *L. poessneckensis*, *L. macrosoma*, *L. helveticus* and *L. uroshis* used for molecular study

Species	*L. poessneckensis*		*L. poessneckensis*		*L. macrosoma*		*L. helvetiucs*		*L. uroshis*	
Population	Czech		Slovakian		Austrian		Serbian		Slovakian	
Locality	Černé Voděrady		Velké Kapušany		Orth		Stari Ledinci		Velké Pole	
Host	*Quercus*		*Quercus*		unknown		*Ruscus hypoglossum; R. aculeatus*		*Fagus; Quercus; Acer*	
Specimen	♀ (1)	♀ (2)	♀ (1)	♀ (2)	Juvenile	♀	♀	♂	♀	♂
L	7419	5504	6724	8095	4541	10411	7354	7170	6818	6162
a	96.3	77.5	98.9	115.6	79.7	126.9	77.4	95.6	103.3	106.2
b	13.6	10.4	10.6	13.7	6.1	16.4	–	13.2	11.9	12.1
c	154.6	153.0	160.1	161.9	106.3	359.0	204.3	170.7	158.6	134.0
c′	0.83	0.67	1.05	1.04	0.88	0.54	0.61	0.81	0.86	1.00
V/spicule	55.6	56.3	46.9	52.8	–	52	50	97	53.7	70
Replacement odontostyle	–	–	–	–	120	–	–	–	–	–
Odontostyle	122	125	139	142	100	140	142	–	148	143
Odontophore	65	57	58	63	55	58	66	–	60	60
Total stylet length	187	182	197	205	155	198	208	–	208	203
Oral aperture to guide ring	36	36	39	41	33	42	41	45	46	41
Pharyngeal bulb length	166	157	168	161	121	143	–	154	139	139
Pharyngeal bulb diam.	27	26	30	30	29	36	–	32	32	29
Tail length	48	36	42	50	28	29	36	42	43	46
Length of hyaline tip	18	12	18	18	18	19	16	15	16	19
Number of supplements	–	–	–	–	–	–	–	12	–	10
Body diam. at lip region	17	16	15	15	14	19	18	19	19	18
At guiding ring	31	31	33	34	33	38	39	39	33	29
At base of pharynx	62	59	58	65	68	74	73	67	56	54
At mid body/at vulva	77	71	68	70	71	82	95	75	66	58
At anus/cloacal opening	58	54	52	58	51	54	59	53	50	46
At beginning of hyaline tip	42	37	40	48	41	48	44	38	39	32

All measurements in μm

were added and the mixture incubated again for 3 min at 99°C. Finally, the DNA suspension was cooled and the DNA was either used directly for PCR or stored at −20°C until template was needed for PCR reactions. The number of specimens of *L. poessneckensis* examined molecularly was increased for the *cox1* gene sequencing (Table 2). The increased numbers of specimens were not documented prior to DNA extraction. The DNA was extracted as described above.

Primers used for the amplification of mtDNA and rDNA are given in Table 3. Primer WDF was designed using the online PRIMER 3 software (http://frodo.wi.mit.edu/) after aligning *Longidorus* sequences of the 5.8S gene available in GenBank. An informative region within the *cox1* gene was amplified for all studied species by COIF and COIR primers, with the exception of *L. urhosis*, for which COIF and XIPHI

Table 2 Number of amplicons for *cox1* sequenced in both direction for *Longidorus poessneckensis*

Population	Amplicon	Number of specimens per tube
Czech	1st	1 female[a]
	2nd	1 female[a]
	3rd	1 female
	4th	18 first stage juvenile
	5th	18 first stage juvenile
Slovakian	1st	1 female[a]
	2nd	1 female[a]
	3rd	1 female
	4th	4 juveniles of mixed stages

[a] Shown in Fig. 1 and morphometrics are given in Table 1

Table 3 Primers used to amplify *cox1*-mtDNA, ITS2-rDNA, D2/D3 expansion segments of 28S-rDNA and 18S-rDNA

Gene	Primer name	Direction	Primer sequence 5′–3′	Reference
cox1-mtDNA	COIF	Forward	GAT TTT TTG GKC ATC CWG ARG	He et al. (2005a)
cox1-mtDNA	COIR	Reverse	CWA CAT AAT AAG TAT CAT G	He et al. (2005a)
cox1-mtDNA	XIPH1	Reverse	ACA ATT CCA GTT AAT CCT CCT ACC	Lazarova et al. (2006)
28S-rDNA	D2A	Forward	ACA AGT ACC GTG AGG GAA AGT TG	De Ley et al. (1999)
28S-rDNA	D3B	Reverse	TCG GAA GGA ACC AGC TAC TA	De Ley et al. (1999)
ITS2-rDNA	WDF	Forward	AGA CAC AAA GAG CAT CGA CT	This study
ITS2-rDNA	VRAIN 2R	Reverse	TTT CAC TCG CCG TTA CTA AGG GAA TC	Derycke et al. (2005)
18S-rDNA	SSU_F_04	Forward	GCT TGT CTC AAA GAT TAA GCC	–[a]
18S-rDNA	SSU_R_09	Reverse	AGC TGG AAT TAC CGC GGC TG	–[a]
18S-rDNA	SSU_F_22	Forward	TCC AAG GAA GGC AGC AGG C	–[a]
18S-rDNA	SSU_R_13	Reverse	GGG CAT CAC AGA CCT GTT A	–[a]
18S-rDNA	SSU_F_23	Forward	ATT CCG ATA ACG AGC GAG A	–[a]
18S-rDNA	SSU_R_81	Reverse	TGA TCC WKC YGC AGG TTC AC	–[a]

[a] http://www.nematodes.org/biodiversity/sourhope/nemoprimers.html

primers were used. The PCR was performed in a 25 µl total volume containing 2.5 µl 10× PCR buffer, 2.5 µl of dNTP (2.5 mM each), 0.2 µl TaKaRA Taq™ (5 units μl^{-1}) (TaKaRa, Shiga, Japan) and 1.0 µl of each primer (10 pmol μl^{-1}) (synthesised by Generi Biotech, Hradec Králové, Czech Republic); sterile water was adjusted to 24.5/24.0 µl and to this 0.5/1.0 µl of DNA was added as a template for PCR. A negative control (sterilised water) was included in all PCR experiments. All PCR reactions were performed on a DNA Engine PTC-1148 thermal cycler (Bio-Rad). The cycling profile for mtDNA was as described by He et al. (2005a): 95°C for 10 min, five cycles at 94°C for 30 s, 45°C for 40 s, and 72°C for 1 min, and a 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.

The 18S gene was amplified in three fragments. Primer combination was as follows: first fragment SSU_F_04+SSU_R_09; second fragment SSU_F_22+SSU_R_13, and third fragment SSU_F_23+SSU_R_81. The cycling profile for 18S and 28S was as follows: first denaturation for 2 min at 94°C, 40 cycles for 30 s at 94°C, 1 min at 58°C and 2 min at 69°C. A final elongation step was run at 72°C for 10 min, and the cycling profile for ITS2, as described by Shannon et al. (2005), was 94°C for 3 min, 35 cycles at 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min, followed by an extension at 72°C for 10 min.

The PCR reaction was separated on 1.5% agarose gel in Tris-Acetate-EDTA, stained with syber-safe and visualised with UV illumination (312 nm). Amplicons were separated on agarose gels, recovered from the gel by excision, and purified with a QIAGEN gel extraction kit (Qiagen, Hilden, Germany). Purified DNA fragments were sequenced directly in both directions using the Big Dye Terminator v 3.1 cycle sequencing ready reaction kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The final sequences were determined by a 3130×1 Genetic Analyser (Applied Biosystems). Sequences were edited using Sequencher software (Genes codes Corporation, Ann Arbor, Michigan, USA).The nucleotide sequences of four loci (*cox1*, ITS2, D2/D3 and 18S) were sequenced from the same DNA of a single individual for each population (Fig. 1, Table 1).

Phylogeny inference

Longidorus sequences were aligned using the Clustal W algorithm as implemented in Mega 3.1 (Kumar et al. 2004). The corresponding sequences of *X. diversicaudatum* and *X. vuittenezi* were used as outgroups and added to the *Longidorus* alignment profile. Pairwise and multiple alignment parameters included a gap opening penalty=15; gap extension penalty=6.66; DNA weight matrix = Clustal W (1.6); transition weight=0.5; divergent sequence delay=30%. Unambiguous alignments were obtained from the *cox1* and 18S sequences, while indels were observed in D2/D3 and ITS2. Alignments of *cox1*, D2/D3 and 18S produced by Clustal W were used (except trimming of unequal 5′ and 3′ ends to consider an equal amount of data for all sequences) for further analyses. No further refinement of the alignments was done.

Table 4 Morphometrics of *Longidorus poessneckensis*

Locality	Černé Voděrady				
Host	*Quercus*				
Specimens	J1	J2	J3	J4	Females
n	19	10	18	12	43
L	1,523±70 (1,385–1,676)	2,397±316 (1,948–2,994)	3,510±497 (2,679–4,390)	4,786±399 (4,118–5,583)	6,851±884 (5,315–9,012)
a	47.3±3.5 (43.2–55.4)	56.8±5.6 (47.1–64.9)	62.0±7.7 (49.3–78.4)	72.3±7.6 (60.6–83.2)	85.5±8.2 (70.9–104.6)
b	4.6±0.5 (4.2–6.0)	6.1±0.7 (5.0–7.6)	7.5±1.2 (5.8–9.7)	8.7±0.6 (7.7–9.9)	11.4±1.5 (8.8–15.9)
c	42.2±2.7 (37.8–48.7)	62.9±9.9 (48.7–80.6)	87.6±13.5 (68.7–125.4)	125.9±16.4 (95.5–155.1)	177.3±21.2 (134.0–222.8)
c′	1.66±0.19 (1.17–1.91)	1.21±0.27 (0.89–1.67)	0.86±0.11 (0.6–1.1)	0.68±0.06 (0.60–0.79)	0.67±0.07 (0.53–0.83)
V	–	–	–	–	55.6±1.0 (54.0–58.0)
Replacement odontostyle	81±3.5 (75–87)	98±3.5 (92–103)	113±2.9 (108–118)	129±4.4 (124–137)	–
Odontostyle	76±2.3 (71–79)	80±1.4 (78–82)	99±3.4 (93–106)	114±5.2 (106–125)	128±5.1 (116–136)
Odontophore[a]	37±2.6 (34–40)	39±3.2 (36–45)	45±5.2 (37–53)	53±4.6 (49–58)	59±3.9 (52–68)
Total stylet length[a]	110±4.7 (105–114)	118±2.5 (116–123)	143±7.4 (130–151)	163±6.9 (155–167)	185±7.3 (168–199)
Oral aperture to guide ring	21.5±0.7 (21–23)	25±1.0 (23–26)	28±1.9 (25–33)	33±1.4 (31–35)	37±1.9 (32–41)
Pharyngeal bulb length	79±4.0 (72–86)	101±6.8 (88–111)	118±9.2 (103–140)	138±10.1 (123–155)	156±10.6 (137–178)
Pharyngeal bulb diameter	16±1.1 (13–18)	18±1.6 (16–21)	22±3.6 (17–33)	25±3.2 (21–32)	26±2.0 (23–32)
Tail length	36±2.8 (32–42)	39±3.2 (33–45)	39±3.2 (32–45)	37±2.4 (34–43)	39±3.9 (32–54)
Length of hyaline tip	8±1.0 (6–9)	10±1.3 (7–11)	13±2.0 (10–17)	14±3.0 (8–20)	15±1.2 (13–17)
Body diam. at lip region	8±0.9 (6–9)	9±0.8 (8–10)	11±1.0 (10–13)	13±0.7 (12–14)	15±3.1 (11–21)
At guiding ring	17±1.0 (15–18)	20±2.0 (17–23)	25±2.4 (21–31)	29±1.9 (27–33)	32±1.6 (28–35)
At base of pharynx	32±2.7 (27–35)	41±6.9 (30–53)	54±5.9 (44–68)	64±7.6 (53–78)	67±8.4 (55–91)
At mid body/at vulva	31±2.8 (25–35)	43±8.8 (29–59)	57±6.6 (45–69)	67±7.8 (58–81)	80±9.6 (66–104)
At anus	21±1.9 (18–24)	33±6.4 (24–44)	47±5.4 (36–56)	56±5.3 (50–67)	58±4.9 (51–70)
At beginning of hyaline tip	12±0.9 (10–13)	19±3.3 (15–26)	32±3.6 (26–39)	38±5.6 (27–49)	42±6.2 (33–55)

Measurements in μm (in form): mean ± standard deviation (range)

[a] *n* J1=4; J2=6; J3=6; J4=3; Females=26

Sequences of ITS2 including flanking regions were aligned. Subsequently, flanking regions and columns with 50% gaps were deleted manually. (Alignments are available from the corresponding author on request).

An appropriate model of evolution was chosen by the implementation of the maximum likelihood ratio test employing Modeltest (Posada and Crandall 1998). Phylogenetic reconstruction, bootstrap re-sampling and sequence statistics were evaluated using PAUP*4.0b10 (Swofford 1998). Maximum likelihood (ML) and most parsimonious (MP) trees were calculated using heuristic searches and the tree-bisection–reconnection branch swapping algorithm (10,000 rearrangements), and a random stepwise addition of sequences in 100 replicates for ML and ten replicate trials of sequence addition. One tree held at each step. Gaps were treated as missing data. The robustness of the trees was evaluated using the bootstrap (1,000 replicates).

Results

Morphological and morphometrical study

In almost all specimens of a population of *L. poessneckensis* collected in October, 2002 the odontophore was rather obscure and its base difficult to locate with certainty from the permanent slides. Therefore repeat sampling was done in September, 2005. All juvenile stages and female specimens were found in both sampling periods, but males were absent. Morphometrics of females and juveniles are given in Table 4, and photomicrographs of the Czech population are presented in Fig. 2. Morphometrics of two specimens from each of the two populations (Czech and Slovakian) of *L. poessneckensis* and single population each of *L. macrosoma*, *L. helveticus* and *L. uroshis* used for the molecular study are given

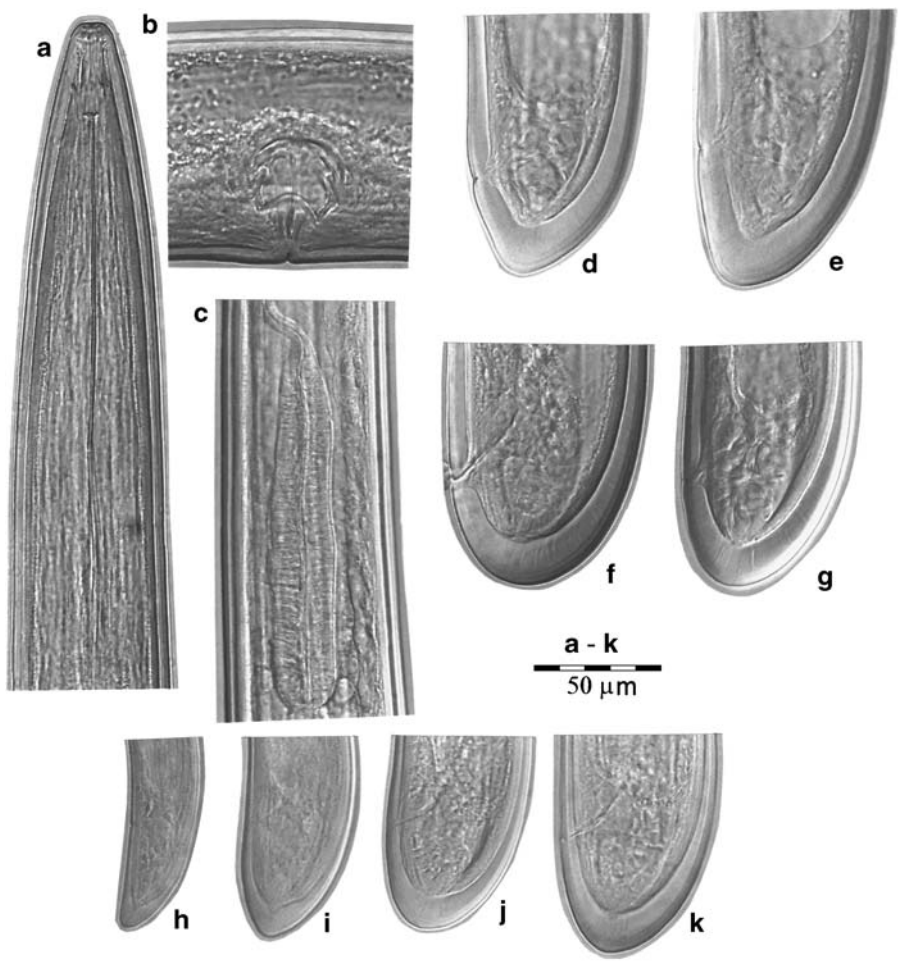

Fig. 2 *Longidorus poessneckensis* from the Czech Republic. **a** Female, anterior region; **b** vagina region; **c** female, pharyngeal bulb; **d**–**g** female, tail region; **h**–**k** tail regions of J1, J2, J3 and J4 respectively

Table 5 NCBI accession numbers for *cox1*-mtDNA, ITS2-rDNA, D2/D3 expansion segments of 28S-rDNA and 18S-rDNA sequences

Species	Population	NCBI accession numbers			
		cox1	ITS2	D2/D3	18S
L. poessneckensis	Czech	EF538744	EU444012	EF538750	EF538756
L. poessneckensis	Slovakian	EF538745	EU444013	EF538751	EF538757
L. macrosoma	Austrian	EF538746	EU444014	EF538752	EF538758
L. helveticus	Serbian	EF538747	EU444015	EF538753	EF538759
L. uroshis	Slovakian	EF614264	EU444016	EF538754	EF538760
X. diversicaudatum	Slovakian	EF538749	–	EF538755	EF538761
X. vuittenezi	Czech	EF614265	EU444017	EF614266	EF614267

in Table 1, and their photomicrographs presented in Fig. 1.

Molecular analysis

For the molecular study, two populations (Czech and Slovakian) of *L. poessneckensis* and a single population each of *L. macrosoma*, *L. helveticus*, *L. uroshis*, *X. diversicaudatum* and *X. vuittenezi* were considered. The sequences of representative individuals for each population were deposited at the National Centre for Biotechnology Information (NCBI) database and their accession numbers are listed in Table 5. The length of the four studied loci for *Longidorus*, including the two outgroups, ranged from 342 to 416 bp for *cox1*-mtDNA; 378 to 499 bp for ITS2-rDNA (excluding the 3′ end of the 5.8S-rDNA gene and the 5′ end of the 28S gene), 774 to 796 bp for D2/D3 expansion segments of 28S-rDNA and 1,760 to 1,786 bp for 18S-rDNA. For ITS2 analyses only one outgroup was used because sequences of *X. diversicaudatum* were repeatedly of low quality. Multiple alignment length, constant and variable sites are given in Table 6.

Identical sequences were obtained for two individuals from each population for all studied loci. Inter-population sequence divergence of *cox1*, ITS2 and D2/D3 expansion segments was observed for *L. poessneckensis*. Inter-population sequence divergence of ITS2 and D2/D3 expansion segments was only one and two substitutions, respectively. On the other hand, very high inter-population sequence divergence (19%) for *cox1* was detected between the Czech and Slovakian populations. Because the numbers of specimens sequenced were minimum (two specimens per population), the numbers of specimens for sequencing the *cox1* gene of *L. poessneckensis* were increased to re-confirm the diversity. In total, five amplicons for Czech and four amplicons for Slovakian populations were sequenced in both directions (Table 2). All amplicons produced identical sequences for the same population. The conserved small subunit 18S gene gave identical sequences for the Slovakian and Czech populations of *L. poessneckensis*; therefore only one sequence was used for phylogeny to avoid redundancy.

The substitution model based on likelihood ratio tests chosen was HKY+G, HKY, TrNef+G, TrN+I+G for *cox1*, ITS2, D2/D3 and 18S respectively. The following parameters were estimated: base frequencies A=0.27, C=0.17, G=0.20, T=0.36; transition/transversion ratio=4.3687, gamma (G) rate shape parameter alpha α=0.1946 for *cox1*; base frequencies A=0.29, C=0.19, G=0.26, T=0.26, transition/transversion ratio=1.4028 for ITS2; base frequencies equal; the substitution rate matrix A–C=1.0, A–G=2.3, A–T=1.0, C–G=1.0,

Table 6 Alignment parameters of *cox1*-mtDNA, ITS2-rDNA, D2/D3 expansion segments of 28S-rDNA and 18S-rDNA

Character	*cox1*	ITS2	D2/D3	18S
Length	343	443	800	1,763
Constant sites	192	76	556	1,668
Variable sites	151	367	244	95
Parsimony informative sites	112	172	129	61

C–T=4.8, G–T=1.0, α=0.3947 for D2/D3; base frequencies A=0.28, C=0.21, G=0.26, T=0.25, the substitution rate matrix A–C=1.0, A–G=1.7, A–T=1.0, C–G=1.0, C–T=5.2, G–T=1.0, the proportion of invariable sites=0.7763, α=0.7330 for 18S. The genetic distance with ML and MP methods yielded very similar topologies for the phylogenetic relationship of *L. poessneckensis* with *L. macrosoma*, *L. helveticus* and *L. urohsis* using *X. diversicaudatum* and *X. vuittenezi* as outgroups for four observed loci (Fig. 3). However, ML and MP trees inferred from sequences of *cox1* are supported by low bootstrap values. MP analysis yielded only a single most parsimonious tree for *cox1* (tree length (TL): 336; parsimony informative consistency index (iCI):0.6585; retention index (RI): 0.3533); ITS2 (TL: 560; iCI: 0.8487; RI: 0.7819); D2/D3 expansion segments (TL: 331; iCI: 0.8929; RI: 0.8772) and 18S (TL: 108; iCI: 0.9315; RI: 0.9296).

The inter-species sequence divergence among four *Longidorus* species ranged from 24–32% across *cox1*, 28% to 302% across ITS2, 2% to 19% across D2/D3 and 0.2% to 2% across 18S gene, while sequence divergence among three *L. poessneckensis*, *L. macrosoma* and *L. helveticus* (excluding *L. uroshis*) species ranged from 24% to 31% across *cox1* gene, 28% to 39% across ITS2, 2% to 7% across D2/D3 and 0.2% to 0.6% across the 18S gene. The inter-population sequence divergence for *L. poessneckensis* was 19%, 0.24%, 0.26% and 0% for *cox1*, ITS2, D2/D3 and 18S, respectively.

Discussion

Morphometrical and morphological comparison

Morphometrics and morphological characters of the Czech population of *L. poessneckensis* were compared with other European populations. Morphometrics of the Czech population appeared similar to Slovakian (Lišková and Sturhan 2000), German (Sturhan and Loof 2001) and Austrian (Tiefenbrunner and Tiefenbrunner 2004) populations apart from some minor differences. Comparison with a Slovakian population revealed a lower ratio 'c' (154 *vs* 177.3) and shorter odontophore (59 *vs* 69 μm) in the Czech population. Compared to German populations, Czech females had a lower ratio 'a' (85.5 *vs* 104) and a shorter mean odontophore length (59 *vs* 70 μm). In general, morphometrics of Czech females were similar to those of the Austrian population except for the upper range of tail length, which was much higher than in the Czech population (35–74 *vs* 33–54 μm). However, the differences observed between different populations of *L. poessneckensis* could be influenced by the fixation precision of measuring. Differences in odontophore length might be a result of

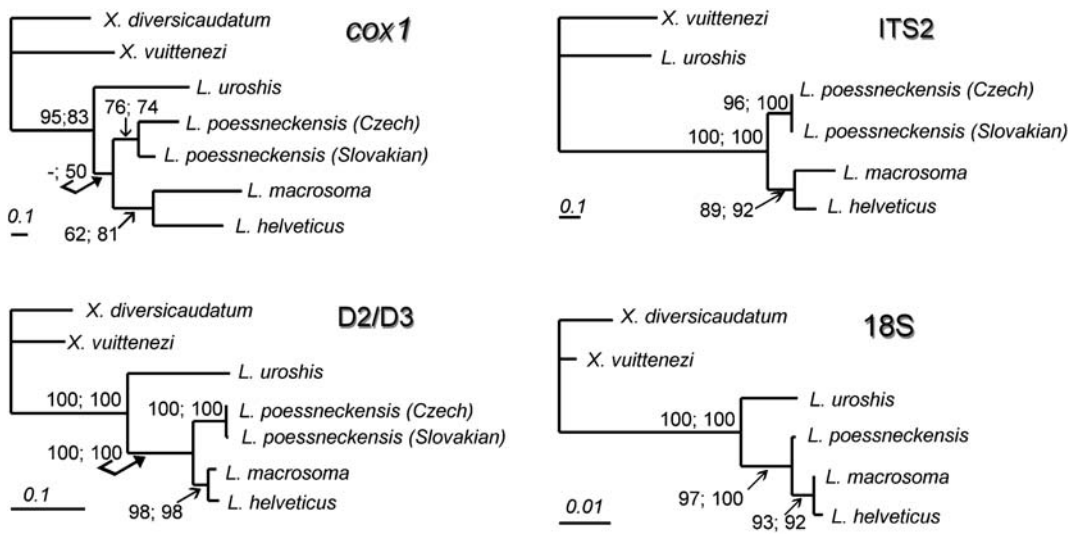

Fig. 3 Maximum likelihood heuristic trees for *cox1*, *ITS2*, *D2/D3* and *18S* sequences. The two sets of bootstrap values on branches (or indicated by *arrow*) represent maximum likelihood and maximum parsimony respectively. Only bootstrap values above 50 are indicated

the difficulty of accurately locating the base of the odontophore from permanent slides. In agreement with Tiefenbrunner and Tiefenbrunner (2004), nearly 50% of specimens examined had the 'a' ratio <80, i.e. outside the range given by Chen et al. (1997) and Sturhan and Loof (2001). The codes for *L. poessneckensis* of the Czech population: A5 – B1234 – C34 – D3 – E4 – F34 – G12 – H1 – I1 largely agree with the adapted codes for the species by Sturhan and Loof (2001) : A5(6) – B3(4) – C34 – D3 – E4 – F345 – G2 – H1 – I1 except for characters B (lip region) and G (ratio a).

Morphometrics and morphological characters of all juvenile stages were also in agreement with those described by Sturhan and Loof (2001) except for ratio 'a' and lip region. Ratio 'a' for all juvenile stages of German populations was higher than for the Czech population, and mean values for body length of J4 were longer in German populations. Sturhan and Loof (2001) observed that tail length of juveniles increased slightly by each moult, but in the Czech population tail length of all juvenile stages and females were about the same. According to Sturhan and Loof (2001), the most closely related species to *L. poessneckensis* is *L. macrosoma*, discriminated by the shape of the lip region (rounded *vs* truncate), cuticle structure on the tail-end (in *L. macrosoma*, thick, distinct median layers), shape of J1 tail (conoid *vs* subdigitate) and absence of males.

Krnjaić et al. (2000) described a new species, *L. uroshis* from Montenegro. These authors described it as being close to *L. saginus* Khan, Seshadri, Weischer & Mathen, 1971 and *L. apulus* Lamberti and Bleve-Zacheo 1977 (Khan et al. 1971; Lamberti and Bleve-Zacheo 1977). However, Lišková and Sturhan (2002) considered *L. uroshis* to be also close to *L. poessneckensis*, and differentiated *L. poessneckensis* from *L. uroshis* by the absence of males and by several morphological characters (not specified). Lamberti et al. (2001) described a new species, *L. helveticus*, from Switzerland, resembling *L. poessneckensis* (based on Sturhan and Loof 2001). *Longidorus helveticus* differs from *L. poessneckensis* (Sturhan and Loof 2001) by a lower 'a' ratio (71 *vs* 104); a higher 'c' ratio (200 *vs* 179); the lip region rounded *vs* flat or depressed; an anterior vulva (52% *vs* 55%) and the first stage juvenile with a digitate *vs* rounded tail shape.

Morphometrics of *Longidorus* specimens (see Table 1) used for the molecular study were compared with populations described from the same country. Morphometrics of juveniles of *L. macrosoma* are not described from Austria; therefore they are compared with data from the literature (Hooper 1961). Morphometrics of the female specimens of *L. macrosoma* are within the minimum–maximum range of specimens described by Tiefenbrunner and Tiefenbrunner (2004), except ratio 'a' and 'c' and the main morphometrics of juveniles are close to the J3 development stage described by Hooper (1961). Morphometrics of two female specimens of *L. poessneckensis* from Slovakia were compared with four females described from another population from Slovakia by Lišková and Sturhan (2000). They show some minor morphometrical differences: higher ratio 'a' (98.9;115.6 μm *vs* 71–84 μm); higher ratio 'c' (1.04;1.06 μm *vs* 0.6–0.8 μm) and a longer distance from the oral aperture to the guide ring (39;41 μm *vs* 32–36 μm). Morphometrics of *L. helveticus* female and male specimens were similar to the morphometrics of females and male specimens described by Barsi and Luca (2005). Morphometrics of *L. uroshis* female and male specimens from Slovakia were similar to the morphometrics of another population described from Slovakia (Lišková and Sturhan 2002).

Molecular analyses

Molecularly *L. poessneckensis* compared closely to three *Longidorus* species (*L. macrosoma*, *L. helveticus* and *L. uroshis*) which were described as being closely related to *L. poessneckensis* in the literature (Sturhan and Loof 2001; Lamberti et al. 2001; Lišková and Sturhan 2002). To date *L. poessneckensis* has been recorded only from central Europe (Austria, Czech Republic, Germany and Slovakia); unfortunately, specimens were not available from Germany and Austria.

Mitochondrial DNA contains powerful genetic markers for phylogenetic studies of closely related species of nematodes at the population and species levels since it shows a comparatively high rate of evolution because of the speed of evolution (Simon et al. 1994; Hu and Gasser 2006). The *cox1* sequences varied both among four *Longidorus* species and among two populations of *L. poessneckensis*. Inter-population sequence divergence was high between the two populations of *L. poessneckensis* (19%). Whilst most (n=63) nucleotide changes were silent, the transitions at positions 91 (A↔G) and 151 (A↔G)

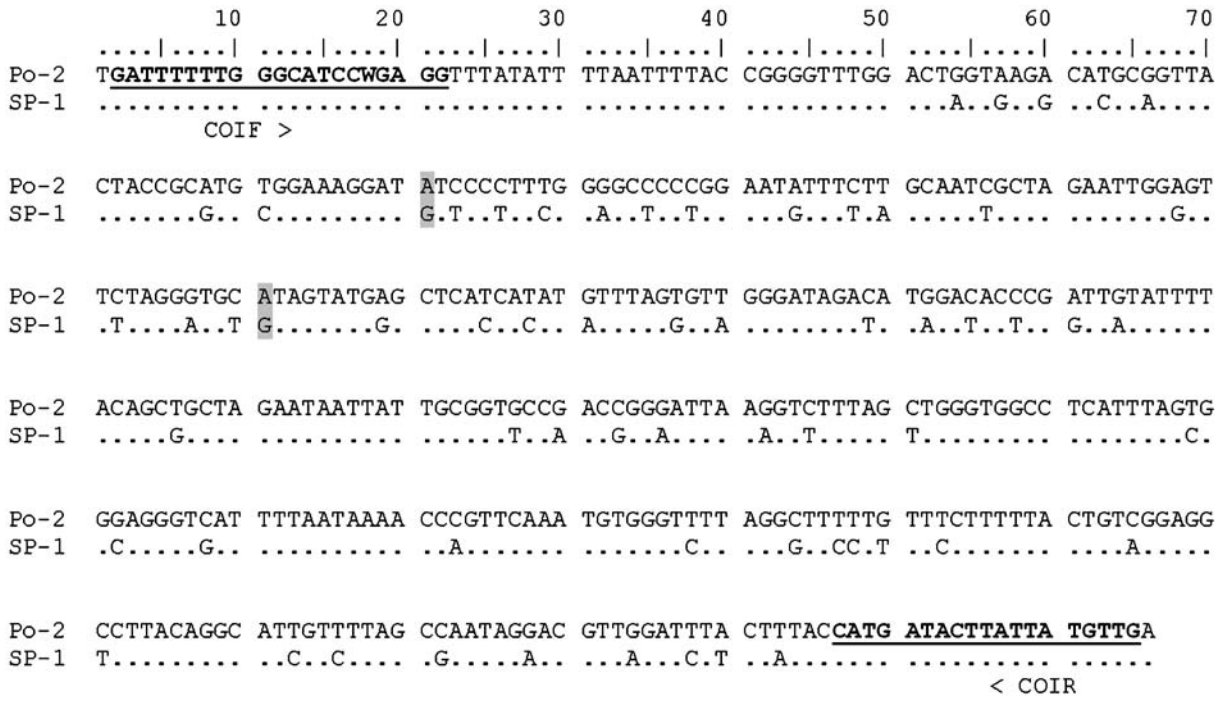

Fig. 4 Alignment (5′–3′) of *cox1* sequence of Czech (Po-2) and Slovakian (SP-1) populations of *Longidorus poessneckensis*. Identical bases are indicated by a *dot*. *Grey regions* indicate nucleotides which resulted in a change in amino acid sequence. *Bold and underlined* are the sequences of the oligonucleotide primers used in the PCR

resulted in a change in the *cox1* amino acid sequence for an isoleucine to a valine and a methionine to a valine, respectively. Both transitions were at the first codon position (Fig. 4). This level of intraspecific variation is high compared with other plant–parasitic nematodes but similar to that recorded for the marine nematode *Geomonhystera disjuncta* (Bastian 1865) Jacobs, 1987 (13.8–25.7%) (Derycke et al. 2007). In all phylogentic trees (inferred from all four loci), three *Longidorus* species grouped together while *L. uroshis* did not group with them (Fig. 3). On the basis of phylogenetic trees the three species are considered closely related while *L. uroshis* is distantly related. According to Blouin et al. (1998) the mtDNA sequence difference between closely related species is typically in the 10–20% range. The results of the analysis of *cox1* showed 24–31% divergence between three closely related species, *L. poessneckensis*, *L. macrosoma* and *L. helveticus*. Phylogenetic analyses of ML and MP trees inferred from sequences of *cox1* were supported by low bootstrap values. However, the molecular marker appeared inappropriate to support the relationship of four *Longidorus* species though it appeared to be a good marker to study population genetics within species.

Because the *cox1* fragment showed high divergences between the two populations of *L. poesseneckensis*, we analysed a second internal transcribed spacer for comparison. ITS2 evolves faster than the other two studied regions (D2/D3 and 18S) of ribosomal DNA. ITS2 is likely to show less sequence conservation and thus enables the discrimination of closely related species on this basis. Previous studies (Ye et al. 2004; Luca et al. 2004) have characterised and compared the

Table 7 Nucleotide variations among two populations of *L. poessneckensis* and two closely related species *L. macrosoma* and *L. helveticus*

Region	LP (Czech) vs LP (Slovakian)	*L. macrosoma vs L. helveticus*
cox1	63 nt (2 aa)	78 nt (11 aa)
ITS2	1 nt	142 nt
D2/D3	2 nt	4 nt
18S	0 nt	2 nt

LP L. poessneckensis, *nt* nucleotide, *aa* amino acid

ITS1 and ITS2 rDNA sequences for different *Longidorus* species. The general finding has been that ITS rDNA provides valuable genetic markers for species delineation. Here, interspecific differences in ITS2 sequences have been exploited successfully for the discrimination of four *Longidous* species. Almost identical sequences (a difference of one substitution) of ITS2 provide support that two populations of *L. poessneckensis* belong to a single species though highly divergent across *cox1*. Derycke et al. (2007) also observed lower divergence across ITS and high divergence across *cox1* for the marine nematode *Geomonhystera disjuncta*. Nucleotide comparison of *L. macrosoma vs L. helveticus* and the Czech *vs* Slovakian population of *L. poessneckensis* presented in Table 7 also reveals that Czech and Slovakian populations of *L. poessneckenis* belong to a single species.

From the extensive large subunit, the D2/D3 region was chosen because it consists of a tandem of two variable regions alternating with more conserved parts of the gene. However, the potential usefulness of the D2/D3 sequence data clearly depends on how quickly or slowly this region evolved within the investigated taxa. Nonetheless, these variable regions were distinctive enough to differentiate four species ranked within the genus *Longidorus*, since all of them consistently discriminated between these species with high resolution (>98%) using both ML and MP analyses.

The sequence of *L. poessneckensis*, obtained from two populations (Slovakian and Czech), shared the same 18S sequence, thus suggesting the absence of inter-population variability. However, the absence of different geographical populations does not allow any conclusion on the homogeneity of these sequences. 18S rDNA sequences have proven useful in resolving phylogenetic relationships among longidorid nematodes (Neilson et al. 2004). The region is among the slowest evolving in a living organism but is long enough to yield statistically valid information, which allows the inference of phylogenetic history. The present analyses also support the phylogenetic relationship inferred from 18S with high (>92%) statistical support for ML and MP trees.

Analysis of mtDNA and rDNA sequence data revealed that of the *Longidorus* species investigated, *L. poessneckensis* was more similar to *L. macrosoma* and *L. helveticus* than to *L. uroshis*. ML and MP analyses yielded similar basic trees for all four studied loci, supporting the uniqueness of *L. poessneckensis* and the validity of the four *Longidorus* species identified using morphological characters. However, molecular markers (ITS2, D2/D3 and 18S) appeared appropriate for resolving the relationships of the four *Longidorus* species. Due to its low resolution, the *cox1* gene appears more appropriate to study population diversity within the genus *Longidorus*.

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References

Altherr, E. (1974). Nématodes de la nappe phréatique du réseau fluvial de la Saale (Thuringe) II. *Limnologica*, *9*, 81–132.

Barsi, L., & Luca, F. D. (2005). Morphological and molecular characterization of *Longidorus helveticus* (Nematoda: Dorylaimida) from Serbia. *Nematologia Mediterranea*, *33*, 41–49.

Blok, V. C., Wishart, J., Fargette, M., Berthier, K., & Phillips, M. S. (2002). Mitochondrial DNA differences distinguishing *Meloidogyne mayaguensis* from the major species of tropical root-knot nematodes. *Nematology*, *4*, 773–781. doi:10.1163/156854102760402559.

Blouin, M. S., Yowell, C. A., Courtney, C. H., & Dame, J. B. (1998). Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. *Molecular Biology and Evolution*, *15*, 1719–1727.

Chen, Q. W., Hooper, D. J., Loof, P. A. A., & Xu, J. (1997). A revised polytomous key for the identification of species of the genus *Longidorus* Micoletzky, 1922 (Nematoda: Dorylaimoidea). *Fundamental and Applied Nematology*, *20*, 15–28.

De Ley, P., Félix, M. A., Frisse, L. M., Nadler, S. A., Sternberg, P. W., & Thomas, W. K. (1999). Molecular and morphological characterisation of two reproductively isolated species with mirror-image anatomy (Nematoda: Cephalobidae). *Nematology*, *1*, 591–612. doi:10.1163/156854199508559.

Derycke, S., Backeljau, T., Vlaeminck, C., Vierstraete, A., Vanfleteren, J., Vincx, M., et al. (2007). Spatiotemporal analysis of population genetic structure in *Geomonhystera disjuncta* (Nematoda, Monhysteridae) reveals high levels of molecular diversity. *Marine Biology*, *151*, 1799–1812. doi:10.1007/s00227-007-0609-0.

Derycke, S., Fonseca, G., Vierstraete, A., Vanflenteren, J., Vincx, M., & Moens, T. (2008). Disentangling taxonomy within the *Rhabditis* (*Pellioditis*) *marina* (Nematoda, Rhabditidae) species complex using molecular and morphological tools. *Zoological Journal of the Linnean Society*, *152*, 1–15.

Derycke, S., Remerie, T., Vierstraete, A., Backeljau, T., Vanfleteren, J., Vincx, M., et al. (2005). Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. *Marine Ecology Progress Series*, *300*, 91–103. doi:10.3354/meps300091.

He, Y., Jones, J., Armstrong, M., Lamberti, F., & Moens, M. (2005a). The mitochondrial genome of *Xiphinema americanum sensu stricto* (Nematoda: Enoplea): considerable economization in the length and structural features of encoded genes. *Journal of Molecular Evolution*, *61*, 819–833. doi:10.1007/s00239-005-0102-7.

He, Y., Subbotin, S. A., Rubtsova, T. V., Lamberti, F., Brown, D. J. F., & Moens, M. (2005b). A molecular phylogenetic approach to Longidoridae (Nematoda: Dorylaimida). *Nematology*, *7*, 111–124. doi:10.1163/1568541054192108.

Hooper, D. J. (1961). A redescription of *Longidorus elongatus* (De Man, 1876) Thorne & Swanger, 1936, (Nematoda, Dorylimidae) and descriptions of five new species of *Longidorus* from Great Britain. *Nematologica*, *6*, 237–257.

Hu, M., & Gasser, R. B. (2006). Mitochondrial genomes of parasitic nematodes—progress and perspectives. *Trends in Parasitology*, *22*, 78–84. doi:10.1016/j.pt.2005.12.003.

Khan, E., Seshadri, A. R., Weischer, B., & Mathen, K. (1971). Five new nematode species associated with coconut in Kerala, India. *Indian Journal of Nematology*, *1*, 116–127.

Klimyuk, V. I., Carroll, B. J., Thomas, C. M., & Jones, J. D. G. (1993). Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *The Plant Journal*, *3*, 493–494. doi:10.1046/j.1365-313X.1993.t01-26-00999.x.

Krnjaić, D., Lamberti, F., Krnjaić, S., Agostinelli, A., & Radicci, V. (2000). Three new longidorids (Nematoda: Dorylaimida) from Montenegro. *Nematologia Mediterranea*, *28*, 235–248.

Kumar, S., Tamura, K., & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings in Bioinformatics*, *5*, 150–163. doi:10.1093/bib/5.2.150.

Lamberti, F., & Bleve-Zacheo, T. (1977). Two new species of *Longidorus* (Nematoda: Longidoridae) from Italy. *Nematologia Mediterranea*, *5*, 73–83.

Lamberti, F., Kunz, P., Grunder, J., Molinari, S., Luca, F. D., Agostinelli, A., et al. (2001). Molecular characterization of six *Longidorus* species from Switzerland with the description of *Longidorus helveticus* sp.n. (Nematoda, Dorylaimida). *Nematologia Mediterranea*, *29*, 181–205.

Lazarova, S. S., Malloch, G., Oliveira, C. M. G., Hübschen, J., & Neilson, R. (2006). Ribosomal and mitochondrial DNA analyses of *Xiphinema americanum*-group populations. *Journal of Nematology*, *38*, 404–410.

Lišková, M., & Brown, D. J. F. (2003). Longidoridae (Nematoda: Dorlyaimida) in the Slovak Republic. *Helminthologia*, *40*, 165–172.

Lišková, M., & Sturhan, D. (2000). Occurrence and ecology of *Longidoridae* (Nematoda: *Dorylaimida*) in floodplain forests in the Slovak Republic. *Helminthologia*, *37*, 113–117.

Lišková, M., & Sturhan, D. (2002). *Longidorus uroshis* Krnjaić et al., 2000 (Nematoda, Dorylaimida) in the Slovak Republic. *Helminthologia*, *39*, 233–236.

Luca, F. D., Reyes, A., Grunder, J., Kunz, P., Agostinelli, A., Giorgi, C. D., et al. (2004). Characterization and sequence variation in the rDNA region of six nematode species of the Genus *Longidorus* (Nematoda). *Journal of Nematology*, *36*, 147–152.

Neilson, R., Ye, W., Oliveira, C. M. G., Hübschen, J., Robbins, R. T., Brown, D. J. F., et al. (2004). Phylogenetic relationships of Longidoridae species (Nematoda: Dorylaimida) from North America inferred from 18S rDNA sequence data. *Helminthologia*, *41*, 209–215.

Oro, V., Hübschen, J., Karanastasi, E., Krnjaic, S., Krnjaic, D., Brown, D. J. F., et al. (2005). Inter-population variability of *Longidorus euonymus* Mali and Hooper, 1974 (Nematoda, Dorylaimida) and comment upon the number of juvenile developmental stages. *Helminthologia*, *42*, 155–165.

Posada, D., & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics*, *14*, 817–818. doi:10.1093/bioinformatics/14.9.817.

Shannon, A. J., Browne, J. A., Boyd, J., Fitzpatrick, D. A., & Burnell, A. M. (2005). The anhydrobiotic potential and molecular phylogenetics of species and strains of *Panagrolaimus* (Nematoda, Panagrolaimidae). *The Journal of Experimental Biology*, *208*, 2433–2445. doi:10.1242/jeb.01629.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook, P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, *87*, 651–701.

Stanton, J. M., McNicol, C. D., & Steele, V. (1998). Non-manual lysis of second-stage *Meloidogyne* juveniles for identification of pure and mixed samples based on the polymerase chain reaction. *Australasian Plant Pathology*, *27*, 112–115. doi:10.1071/AP98014.

Sturhan, D., & Loof, P. A. A. (2001). Redescription of *Longidorus poessneckensis* Altherr, 1974 (Nematoda: Dorylaimida). *Russian Journal of Nematology*, *9*, 43–49.

Swofford, D. L. (1998). *PAUP* phylogenetic analysis using parsimony (*and other methods). Version 4*. Sunderland, MA: Sinauer.

Taylor, C. E., & Brown, D. J. F. (1997). *Nematode vectors of plant viruses*. UK: CABI.

Tiefenbrunner, A., & Tiefenbrunner, W. (2004). Longidoridae (Nematoda: Dorylaimida) from the rhizosphere of the wild growing grape (*Vitis vinifera* ssp. *silvestris*) in the riparian woods of the rivers Danube and March in Austria. *Helminthologia*, *41*, 45–53.

Ye, W., Szalanski, A. L., & Robbins, R. T. (2004). Phylogenetic relationships and genetic variation in *Longidorus* and *Xiphinema* species (Nematoda: Longidoridae) using ITSl sequences of nuclear ribosomal DNA. *Journal of Nematology*, *36*, 14–19.