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Molecular characterisation of plant parasitic nematode *Longidorus poessneckensis* Altherr, 1974 (Nematoda: Longidoridae)

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Abstract The plant parasitic nematode *Longidorus poessneckensis* found in Austria, the Czech Republic, Germany, Poland and the Slovak Republic was molecularly characterized. Mitochondrial genes encoding cytochrome c oxidase subunit I (*COI*) and nicotinamide dehydrogenase subunit 4 (*nad*4), the D2 and D3 expansion segments of 28S rRNA and Internal transcribed spacer 1 (ITS1) rRNA were sequenced for 16 *L. poessneckensis* populations. Six haplotypes of *COI* and five haplotypes of *nad*4 were determined. Nucleotide intraspecific variation was up to 17.1% for the partial sequenced *COI* gene and up to 17.7% for the partial sequenced *nad*4 gene, the latter being the highest up to date known intraspecific variation in this genus. The analyses of multiple amino acid sequence alignments of mitochondrial genes revealed low variability (0–2.4%) for *COI* gene and high divergence (0–7.6%) for *nad*4 gene. Intraspecific sequence diversity for the D2-D3 of 28S rRNA gene was up to 1.2% and for ITS1 rRNA gene was up to 1.6%. It has been hypothesized, that during the Last Glacial Maximum, *L. poessneckensis* populations probably persisted in refuge areas in the Carpathian Mountains and subsequently expanding from these areas and colonizing other European regions.

Keywords *COI* gene · Mitochondrial DNA · *nad*4 gene · Nematode · Ribosomal RNA

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Introduction

Plant parasitic nematodes of genus *Longidorus* (Nematoda, Dorylaimida, Longidoridae) are polyphagous root-ectoparasites. They are polyphagous root-ectoparasites and cause damage to a wide range of plants. Some of the species are of economic importance as pests of agricultural crops. They cause damage to plant either by feeding on roots or by the ability of some species to transmit plant nepoviruses (Taylor and Brown 1997).

The genetic characterization of plant parasitic nematodes is important for diagnosis, studying population biology and genetic structure of nematodes. Molecular approaches have been used to characterize and explore the population genetic

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structures in Longidoridae (Gutiérrez-Gutiérrez et al. 2011; Kumari and Subbotin 2012; Kumari 2014). Ribosomal RNA genes are used to identify *Longidorus* species in routine practice (He et al. 2005a; Subbotin et al. 2014). However, intra- and inter-population genetic variability using mitochondrial DNA is still largely unknown for plant parasitic nematodes of genus *Longidorus* (Palomares-Rius et al. 2017a). Only several studies have been carried out using cytochrome c oxidase subunit I (*COI*) and nicotinamide dehydrogenase subunit 4 (*nad*4) for characterisation of species from this genus (Kumari et al. 2009; Kumari and Subbotin 2012; Kumari 2014; Subbotin et al. 2015; Palomares-Rius et al. 2017b).

The longidorid species *Longidorus poessneckensis* Altherr 1974 occurs in permanent or seasonal moist soils with various water courses, soils with woodland, along river banks and occasionally in hilltops. Up to date, this species was recorded only from Central Europe, e.g. Austria (Tiefenbrunner and Tiefenbrunner 2004), the Czech Republic (Kumari et al. 2009), Germany (Altherr 1974; Sturhan and Loof 2001), Poland (Kornobis and Peneva 2011), Slovakia (Lišková and Sturhan 2000) and Ukraine (Susulovska et al. 2017).

To study population variability of *L. poessneckensis*, we focused our attention on mitochondrial DNA. This is due to the fact that it provides a rich source of genetic markers for populations genetic studies of parasitic nematodes due to its high mutation rate and maternal inheritance, which also enables better discrimination of a closely related species (Jex et al. 2010; Derycke et al. 2013). The aim of the present study was to expand the results of initial research which has demonstrated the existence of nucleotides variation for *COI* gene among two Czech and Slovakian populations (Kumari et al. 2009). The sequences of the genes encoding for *COI* and *nad*4 genes have been used for studying genetic variation both within and among populations of key parasitic nematodes of veterinary and human concern and marine nematodes (Blouin et al. 1998; Jex et al. 2010; Derycke et al. 2013). The objective of this study was to analyze the genetic variation of 16 populations of *L. poessneckensis* by using *COI* and *nad*4 genes of mitochondrial DNA and support these results by sequencing the Internal transcribed spacer 1 (ITS1) and the D2-D3 expansion segments of 28S gene of ribosomal rRNA genes.

Materials and methods

Nematode samples

Total 16 populations were analyzed for *L. poessneckensis* (Table 1 and Fig. 1). One population from Austria, two populations from the Czech Republic, nine populations from Poland and four populations from the Slovak Republic. Individual nematodes from all localities were stored in 1 M NaCl. These nematodes were used to extract DNA. Total genomic DNA was prepared by a rapid technique of Stanton et al. (1998). The specimens were added into 0.5 ml Eppendorf microtubes containing 20 μl of 0.25 M NaOH under a binocular microscope, incubated overnight at room temperature and thereafter heated to 99 °C for 3 min. Afterwards 10 μl of 0.25 M HCl, and 5 μl each of 0.5 M Tris-HCl (pH 8) and 2% Triton X-100 were added and the mixture was incubated for another 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. DNA of one population (Černé Voděrady) from the Czech population and one population (Velké Kapušany) from the Slovak Republic were same as used by Kumari et al. (2009).

PCR and sequencing

New primer sonaF and sonaR (Table 2) were designed by using the online software PRIMER3 from the sequence of *X. americanum* accession number AY382608. Primer sequences and reference are given in Table 2. The PCR for *COI*, *nad*4, ITS1 and partial 28S rRNA genes was performed in a 25 μl total volume containing one 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 0.5 μl of DNA lysis. A negative control (sterilized water) was included in all PCR experiments. All PCR reactions were performed on a DNA Engine PTC–1148 thermal cycler (Bio-Rad). The cycling profile for *nad*4 and *COI* was as described by He et al. (2005b): 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. Cycling profile for ITS1 and D2-D3 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

Table 1 Populations of *L. poessneckensis* used in the present study and NCBI accession numbers of representative individual specimen. Numbers of specimens sequenced are given in brackets

Country	Locality	Isolate code	*COI*	*nad4*	D2-D3 of 28S rRNA	ITS1
Austria	Orth	51LAO	MF503906 (2)	MF503921 (2)	MF503937 (1)	MF503951 (1)
Czech Republic	Křivoklát	48LK	MF503907 (2)	MF503922 (2)	MF503938 (1)	MF503952 (1)
	Černé Voděrady	LPC	EF538744*	MF503923 (5)	EF538750*	MF503953 (1)
Germany	Wuhden	A89A	MF503908 (3)	MF503924 (4)	MF503939 (1)	MF503954 (1)
	Wuhden	A89B	MF503909 (1)	-	-	-
Poland	Ustjanowa Dolna	Long607	MF503910 (4)	MF503925 (4)	MF503940 (1)	MF503955 (1)
	Gaj Koniemłocki	A28	MF503911 (4)	MF503926 (4)	MF503941 (1)	MF503956 (1)
	Pawłowiczki	A65	MF503912 (4)	MF503927 (4)	MF503942 (1)	MF503957 (1)
	Owczary1	Long471	MF503913 (4)	MF503928 (4)	MF503943 (1)	MF503958 (1)
	Stara Wieś Pierwsza	A33	MF503914 (4)	MF503929 (4)	MF503944 (1)	MF503959 (1)
	Chorowice	Long859	MF503915 (4)	MF503930 (4)	MF503945 (1)	MF503960 (1)
	Krzeszowice	Long868	MF503916 (4)	MF503931 (4)	MF503946 (1)	MF503961 (1)
	Owczary2	Long468	MF503917 (4)	MF503932 (4)	MF503947 (1)	MF503962 (1)
Slovak Republic	Kopčany	61 L	MF503918 (4)	MF503933 (2)	MF503948 (1)	MF503963 (1)
	Podčičva	35 L	MF503919 (2)	MF503934 (1)	MF503949 (1)	MF503964 (1)
	Tahanovce	59 L	MF503920 (2)	MF503935 (2)	MF503950 (1)	MF503965 (1)
	Velké Kapušany	LPSK	EF538745*	MF503936 (4)	EF538751*	MF503966 (1)

*after Kumari et al. 2009

72 °C for 10 min. Aliquots (5 μl) of PCR products were analyzed by 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, The Netherlands). Sequencher™ 4.8 (Genes Codes Corp., Ann Arbor, MI, USA) was used to assemble and view each sequences and check for base-calling errors. The sequences of representative individual specimens were deposited at National Centre for Biotechnology Information (NCBI) database and their accession numbers are listed in Table 2. The total numbers of nematodes sequenced per populations are also given in Table 2.

Sequence and phylogenetic analysis

The newly obtained sequences for the D2-D3 of 28S rRNA, ITS rRNA, *nad*4 and *COI* genes were aligned using ClustalX 1.83 (Thompson et al. 1997) with default parameters with corresponding published gene sequences of *L. poessneckensis* (Kumari et al. 2009; Susulovska et al. 2017) and several outgroup taxa (Palomares-Rius et al. 2008). Sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001) under the GTR + G + I model. BI analysis for each gene was initiated with a random starting tree and was run with four chains for 1×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 1000 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. Sequence analyses of alignments were performed with PAUP∗ 4b10 (Swofford 2003). Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on whole alignment, with adjustment for missing data. The *COI* and *nad*4 sequence alignments were also used to construct Minimum Spanning Networks using POPARTsofware (http://popart.otago.ac.nz) (Bandelt et al. 1999).

Results

Cytochrome c oxidase subunit I gene

Total 48 specimens were sequenced and two sequences (accession numbers EF538744, EF538745) from

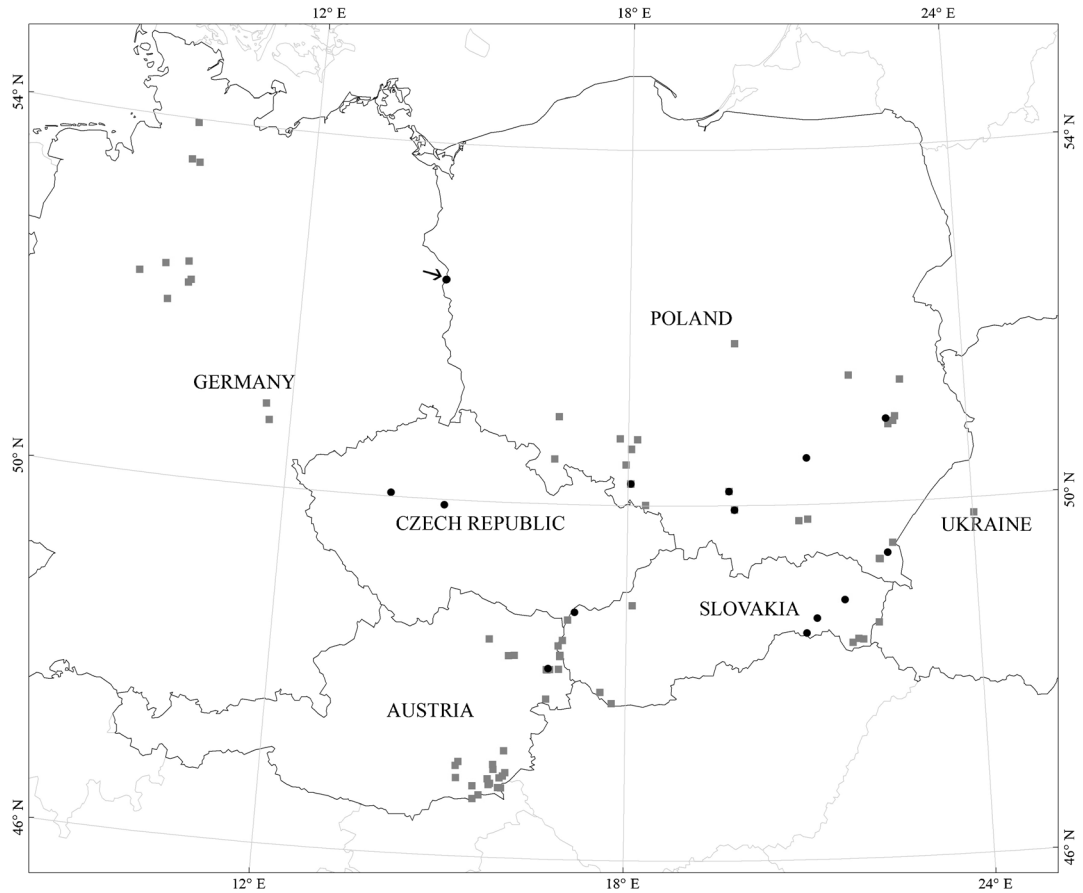

Fig. 1 Geographical distribution of *Longidorus poessneckensis*. Sixteen populations which were used for sequencing are in dark black dots. Arrow indicates where three localities (Wuhden from Germany, Owczary1 and Owczary2 from Poland) in which populations were in close geographic proximity

previous study were included in the analysis. Fifteen new original sequences were obtained in the present study. The Minimum Spanning Network is given in Fig. 2a. Gaps were not observed at any alignment positions. Identical sequences were obtained for all individuals studied for the same population except one population from Germany in which two haplotypes were found. A total of six different variants were observed within the 372 bp *COI* gene amplicon (except primers sequences). Isolates from Orth, Černé Voděrady (EF538745), Křivoklát, Wuhden (A89B), Pawłowiczki, Chorowice, Krzeszowice represent the first variant (V1); isolates from Ustjanowa Dolna, Gaj Koniemłocki, Kopčany represent the second variant (V2); an isolate

Table 2 Primers used in this study

Gene	Primer name	Direction	Primer sequence 5′ - 3′	Reference
COI	COIF	forward	GAT TTT TTG GKC ATC CWG ARG	He et al. (2005b)
COI	XIPHR2	reverse	GTA CAT AAT GAA AAT GTG CCA C	Lazarova et al. (2006)
ITS1	TW81	forward	GTT TCC GTA GGT GAA CCT GC	Joyce et al. (1994)
ITS1	ChR	reverse	ACG AGC CGA GTG ATC CAC CG	Cherry et al. (1997)
D2-D3 of 28S rRNA	D2A	forward	ACA AGT ACC GTG AGG GAA AGT TG	Nunn (1992)
D2-D3 of 28S rRNA	D3B	reverse	TCG GAA GGA ACC AGC TAC TA	Nunn (1992)
nad4	sonaF	forward	GTT AGT ACT TCA GGA AAA A	This study
nad4	sonaR	reverse	GTA AAG CTA CCA GTA TTT G	This study

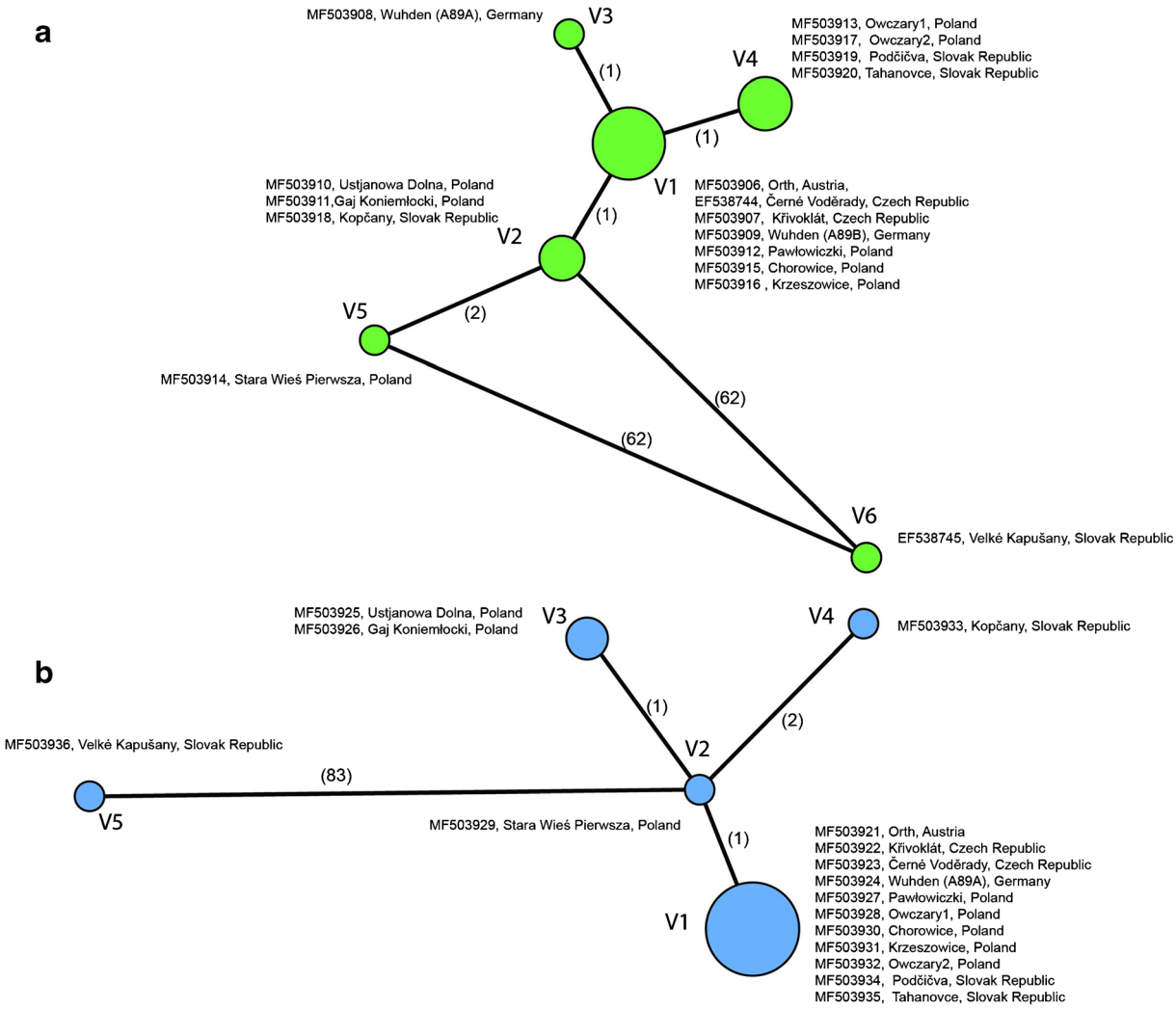

Fig. 2 Minimum spanning network showing the relationships between haplotypes of *Longidorus poessneckensis*. A: *COI;* B: *nad*4. Small black cycles represent missing haplotypes. Pie chart sizes are proportional to the number of samples with a particular haplotype

from Wuhden (A89A) represents the third variant (V3); isolates from Owczary1, Owczary2, Podčičva, Tahanovce represent the fourth variants (V4); an isolate from Stara Wieś Pierwsza represent the fifth variant (V5) and an isolate from Velké Kapušany (EF538745) represents the sixth variant (V6) (Figs. 2a, 3).

Maximal intraspecific differences with *L. poessneckensis* sequences were 17.1% (66 bp) with sequences obtained from Slovakia, Velké Kapušany. Intraspecific variation after exclusion of this population was up to only 1% (4 bp). The nucleotide composition and variable sites are given in Table 3. Nucleotide variation was found at 66 positions within the 372 bp alignment of *L. poessneckensis* of *COI* gene (Fig. 3; Table 3). Translation of the sequences into amino acids revealed 3 out of 124 amino acids (0–2.4%) were variable for *L. poessneckensis*. While most ($n = 62$; 93.6%) nucleotide changes were synonymous, the transition at position 69 (A↔G) and transversion at position 71 (C↔T) resulted in a change in the *COI* amino acid sequence for an isolecuine to a valine, transitions at position 129 (A↔G) resulted in a change in the *COI* amino acid sequence for a methionine to a valine and transversion at position 283 (T↔C) for a valine to an alanine (Fig. 4).

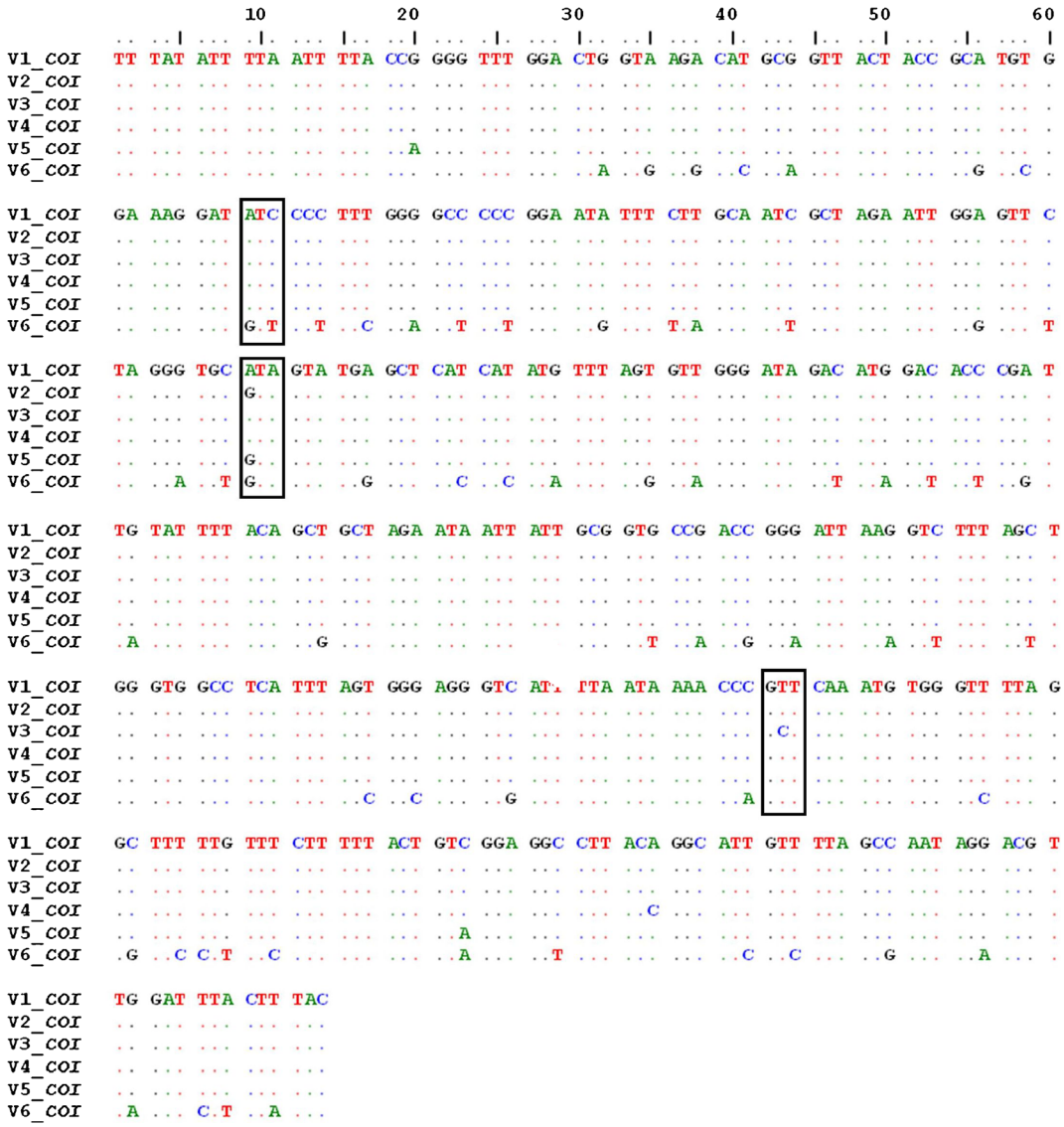

Fig. 3 Alignment of the six haplotypes of *COI* gene of *L. poessneckensis*. Rectangles indicate nucleotides which resulted in a change in amino acid sequence

Nicotinamide dehydrogenase subunit 4 gene

A total of 54 specimens were sequenced and analyzed (Table 2), with 16 new original sequences obtained in the present study. The Minimum Spanning Network is given in Fig. 2b. Identical sequences were obtained for all individuals studied for the same population. Gaps were not observed at any alignment positions. Total five different variants were observed within the 471 bp *nad*4 gene amplicon (except primers sequences). Isolates from Orth, Křivoklát and Černé Voděrady, Wuhden (A89A), Pawłowiczki, Owczary1, Chorowice, Krzeszowice, Owczary2, Podčičva, Tahanovce represent first variant (V1); an isolate from Stara Wieś Pierwsza represent second variant (V2); isolates from Ustjanowa Dolna and Gaj Koniemłocki represent third variant (V3); an isolate from Kopčany represent fourth variant (V4) and an isolate from Velké Kapušany (EF538745) represent fifth variant (Figs. 2b and 5).

Maximal intraspecific differences with *L. poessneckensis* sequences were 17.7% (84 bp) with sequences obtained from Slovakia, Velké Kapušany. Intraspecific variation with deletion of this population was up to only 0.6% (3 bp). The nucleotide composition and

Table 3 Nucleotides comparison of partial *COI* and *nad*4 genes of *Longidorus poessneckensis*

Character	*COI*	*nad4*
Total sites compared	372	471
Total variables sites (%)	66 (17.1)	84 (17.7)
at 1st codon position	6	14
at 2nd codon position	1	3
at 3rd codon position	59	67
Total transitions	49	65
A ↔ G	22	25
T ↔ C	27	40
Total transversions	17	18
C ↔ A	3	7
G ↔ T	5	1
A ↔ T	4	8
C ↔ G	5	2
Multiple substitution	0	1
A + T content at 1st codon position	53.1%	50.4%
A + T content at 2nd codon position	57.1%	54.4%
A + T content at 3rd codon position	62.3%	62.1%
Nucleotide composition		
A	22.8%	24.6%
T	34.6%	31.3%
C	16.7%	24.1%
G	25.9%	20.0%

variable sites are given in Table 3. Nucleotide variation was found at 84 positions within the 471 bp alignment of *L. poessneckensis* of *nad*4 gene (Fig. 5; Table 3). Translation of the sequences to amino acids revealed 12 out of 157 amino acids were variable for *nad*4 (0–7.6%). While most ($n = 69$; 82.1%) nucleotide changes were synonymous the following nucleotide position resulted in 12 amino acid changes: 142 (C↔T, alanine ↔ valine), 146 (C↔A, phenylalanine↔leucine), 171 (G↔A, glycine ↔serine), 179 (A↔T, methionine↔isoleucine), 264 and 266 (G↔A, A↔G, glycine↔serine), 280 and 281 (C↔T, A↔T, proline↔lecine), 288 (A↔G, isoleucine↔valine), 293 (G↔A, glycine↔serine), 408 (A↔G, serine↔glycine), 428 (A↔C, lecine↔phenylalanine), 441(G↔A, valine↔isoleucine) and at position 259 (A↔G; A↔T, threonine-serine, methionine) multiple substitution (Fig. 6).

D2-D3 of 28S ribosomal RNA gene

The D2-D3 of 28S rRNA gene alignment included 18 sequences of *L. poessneckensis* and three sequences of *L. helveticus*, *L. caespiticola* and *L. carniolensis* selected as outgroup taxa and was 781 bp in length. Fourteen new sequences were obtained in the present study. Four sequences (EF538750, EF538751, MF716962, MF716963) were from GenBank. Intraspecific sequence diversity for *L. poessneckensis* was up to 1.2% (8 bp). Phylogenetic analysis resulted in a majority consensus BI tree given in Fig. 7a. The D2-D3 sequence of the population from Velké Kapušany clustered with the sequence obtained from the Lviv population from Ukraine.

Internal transcribed spacer 1 of ribosomal RNA gene

The ITS1 rRNA gene alignment included 16 sequences of *L. poessneckensis* and sequences of *L. helveticus* and *L. macrosoma* selected as outgroups and was 1143 bp in length. 16 new sequences were obtained in the present study. Maximal intraspecific

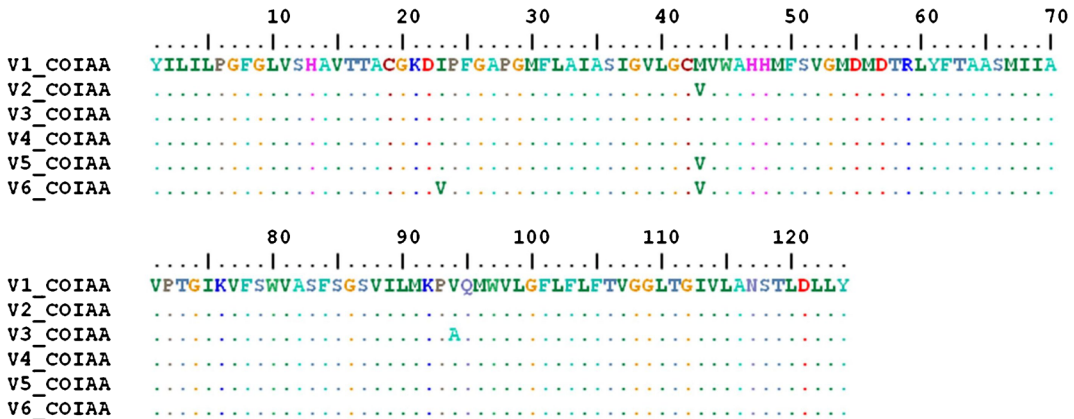

Fig. 4 Alignment of the six haplotypes of *COI* amino acids of *L. poessneckensis*

Fig. 5 Alignment of the five haplotypes of *nad*4 of *L. poessneckensis*. Rectangles indicate nucleotides which resulted in a change in amino acid sequence

differences with *L. poessneckensis* sequences were 1.6% (15 bp). Majority consensus BI phylogenetic tree generated under the GTR + G + I model is given in Fig. 7b.

Discussion

In the previous study of *L. poessneckensis* (Kumari et al. 2009) nucleotide variability of one mitochondrial marker, partial *COI* gene, was assessed between two populations from the Czech Republic and the Slovak Republic (Kumari et al. 2009). Here, 16 populations of *L. poessneckensis* were characterized with *COI* gene and *nad*4 gene, for which original designed primers have been proposed. The present results revealed high nucleotide variability for both mtDNA genes. Comparison of two mitochondrial genes (*COI* and *nad*4) has shown that although they have similar percentages of variable sites and similar level of intra-specific pairwise sequence variations, *nad*4 gene revealed higher variation in amino acid level with 12 out of 157 amino acids comparing with *COI* where three out of 124 amino acids were variable. Most of the variable substitution rates for *COI*

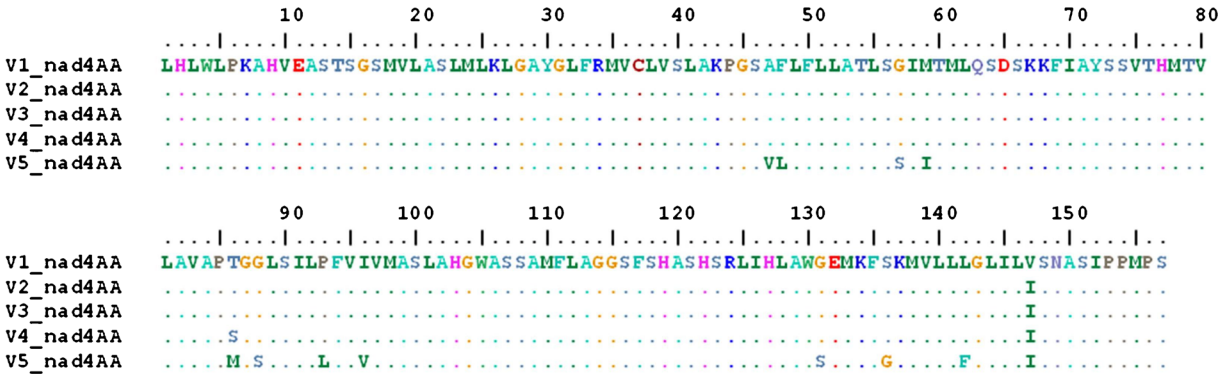

Fig. 6 Alignment of the five haplotypes of *nad*4 amino acids of *L. poessneckensis*

and *nad*4 partial sequences were lowest in second codon sites and highest in third codon sites which is typical for protein coding regions.

L. poessneckensis intraspecific sequence variation for *COI* observed in this study was lower than *L. magnus* (21.30%), *L. orientalis* (22.22%) and *L. vineacola* (31.09%) and higher than *L. aetnaeus* (0.63%), *L. helveticus* (7.34%), *L. crataegi* (1.59%), *L. caespiticola* (3.14%) and *L. macrodorus* (0.32%) (Kumari and Subbotin 2012; Kumari 2014; Subbotin et al. 2015; Palomares-Rius et al. 2017b). Comparison among the four *nad*4 sequence variants of *L. helveticus* revealed sequence variation ranging from 0.83 to 9.17% (Kumari and Subbotin 2012) which is lower than *L. poessneckensis* (17.7%). Notably, in this study much of the genetic variation for both genes *nad*4 and *COI* was

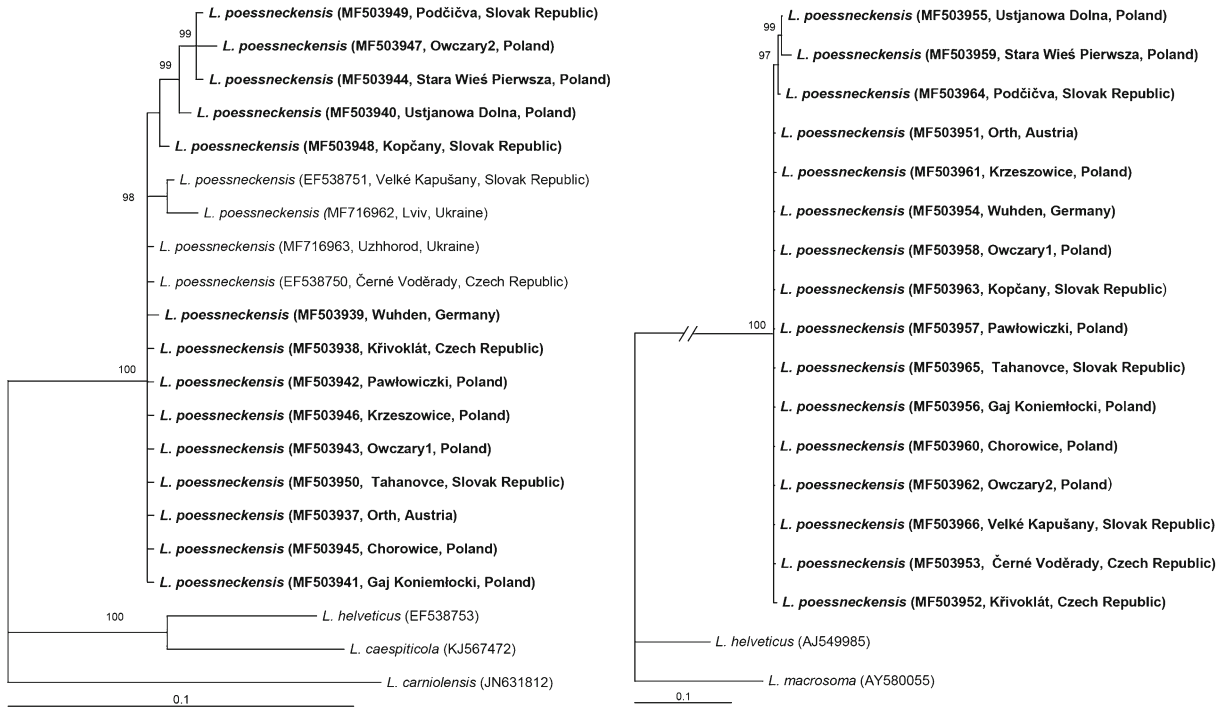

Fig. 7 Phylogenetic relationships within populations of *Longidorus poessneckensis* as inferred from Bayesian analysis using the sequence datasets under the GTR + I + G model. A: D2-D3 of 28S rRNA; B: ITS1 rRNA. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. New sequences are indicated by bold font

found in the population from Velké Kapušany, Slovak Republic, which morphology and morphometrics are not different from other populations (Kumari et al. 2009).

In all *L. poessneckensis* populations, except one population from Germany, only one *COI* variant was present. In German populations two variants were present. Partial *nad*4 sequences of all populations contained a single variant. High AT mutational bias is commonly found for mitochondrial DNA sequence in parasitic nematodes (Blouin et al. 1998; Derycke et al. 2010). In this study there was A + T bias for both *COI* and *nad*4 toward the third codon compared with the other two positions (Table 3). The degree of diversity for *COI* that observed here is in agreement with *L. orientalis* and marine nematodes (Derycke et al. 2010; Subbotin et al. 2015). The AT composition of *COI* gene at all three codon positions is less as compared with *L. vineacola* (Palomares-Rius et al. 2017b) but the AT composition of *nad*4 gene at the 1st and 2nd codon positions is comparable to *L. helveticus* (Kumari and Subbotin 2012).

Genetic diversity for *L. poessneckensis* for the D2-D3 was up to 1.2%. Which is within the range of diversity found for other species of *Longidorus* e.g. Archidona-Yuste et al. (2016) found three nucleotides differing for the D2-D3 among eight populations of *L. indalus* and 4.03% for *L. caespiticola* (Palomares-Rius et al. 2017b). Sequence variation for ITS1 was 1.6%. This degree of variability is within the intraspecific variability reported for other longidorid populations, e.g. *L. bioformis* (0.6–15%, Ye et al. 2004; Palomares-Rius et al. 2017b).

Presently, *L. poessneckensis* is known to occur in the Central Europe and is not reported from Western Europe or Mediterranean region (Fig. 1). There are several records in Germany, close to Hamburg and up to the Weser River, however, this species has not been found in southern Germany (Dr. D. Sturhan, per. Comm.). It could be argued that this species was simply not yet recorded from these areas, however, considering the fact, that the Longidoridae, at least compared to other groups of small, soil-inhabiting invertebrates, are relatively intensively sampled in Europe, presently reported distribution of *L. poessneckensis* reflects its actual distribution. Such a range of occurrence raises an intriguing question about origin and survival of this species during the Last Glacial Maximum and its subsequent dispersal to a current distribution pattern. According to Schmitt (2007) in Europe three possible patterns of species survival during glaciations can be distinguished: first, "Mediterranean" one in which almost all of species had their ice-age refugia in one of the three major European peninsulas (i.e. Iberia, Italy and Balkans); second includes "Continental" species with centers of origin located outside of Mediterranean and third; "Alpin" and/or "Arctic" species which are found in alpine and/or arctic centers of retreat today. It seems, that *L. poessenckensis* represents "continental" mode of survival. In this scenario, *L. poessenckensis* could survive in refuges, probably, in the Carpathians, and subsequently be dispersed along rivers acting as corridors in other regions. The hypothesis of origin and survival in the Carpathians has been yet proposed by Chizhov et al. (2014) for *Xiphinema diversicaudatum* and it could be applied for several other longidorids species. It is also possible that *L. poessenckensis* survived glaciations in several refuge areas, on which may indicate substantial differences in mtDNA sequences between its haplotypes.

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Compliance with ethical standards

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Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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