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ORIGINAL RESEARCH

Molecular characterisation and diagnostics of some *Longidorus* species (Nematoda: Longidoridae) from Russia and other countries using rRNA genes

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Abstract The needle nematodes of the genus *Longidorus* can cause diseases of various crops and trees, and are comprised of more than 150 valid species. Eleven valid and six unidentified species of the genus *Longidorus* collected in different regions of Russia, two states of USA, Germany, New Zealand and Ukraine were molecularly characterized using analysis of the partial 18S rRNA and the D2–D3 expansion segments of the 28S rRNA gene sequences. Fifty-four partial 28S rRNA and fifteen partial 18S rRNA gene sequences were obtained for the present study. Using molecular criteria, we confirmed the morphological identification and distinguished between the following species: *L. aetnaeus, L. africanus*, *L. andalusicus*, *L. artemisiae*, *L. caespiticola*, *L. distinctus*, *L. elongatus*, *L. euonymus*, *L. intermedius*, *L. leptocephalus* and *L. lignosus.* Two longidorid populations from Russia and four from California were not identified to a species level. We obtained the full length D2–D3 of 28S rRNA gene sequence from several freshly-collected *L. artemisiae* samples. We confirmed the identity of the D2 region of 28S rRNA gene sequence with a short D2 of 28S rRNA gene fragment sequence previously obtained from formalin-fixed nematodes embedded in the *L. artemisiae* paratype slides. *Longidorus lignosus* was molecularly characterized and *L. aetnaeus* was reported from Russia for the first time. PCR-D2-D3-RFLP diagnostic profiles generated by five restriction enzymes: *Alu*I, *Hinf*I, *Bsp*143I, *Tru*1I and *Rsa*I are presented for sixteen *Longidorus* species.

Keywords Needle nematodes · PCR-RFLP · Phylogeny · 18S rRNA gene · 28S rRNA gene

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The needle nematodes of the genus *Longidorus* Micoletzky, 1922 are comprised of more than 150 valid species. These parasitic nematodes feed on roots of many plants and can cause significant yield losses in various crops by directly damaging roots or by transmitting nepoviruses. Nine *Longidorus* species have been known as vectors of seven nepoviruses (Taylor and Brown 1997; Decraemer and Robbins 2007; Gutiérrez-Gutiérrez et al. 2013). Rapid and accurate identification of longidorids to the species level is important as a first step in selecting appropriate control measures against these pests.

Morphological and morphometric characters traditionally are used for identification of longidorids. Species determination of longidorids is complex, difficult and time-consuming even for experts. Recently, DNA-

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Table 1 *Longidorus* species and populations used in the present study

Species	Location	Host	Sample code	Genbank number		Collector/Identifier
				D2–D3 of 28S rRNA	18S rRNA	
L. aetnaeus	Varenikovskaya, Krymsk district, Krasnodar Territory, Russia	*Populus alba*	CD1138	KF242324	KF242287	Khusainov R.V.
L. aetnaeus	Kholmovka, Krym, Ukraine	*Populus* sp.	CD1121	KF242323	–	Rogozhin E.A.
L. aetnaeus	Kolomna, Kolomna district, Moscow region, Russia	*Salix fragilis*	CD1111	KF242318	–	Khusainov R.V.
L. aetnaeus	Lugovoy, Lunino district, Penza region, Russia	*Acer tataricum*	CD1129	KF242321	–	Khusainov R.V.
L. aetnaeus	Ramon, Ramon district, Voronezh region, Russia	*Salix alba*	CD1142	KF242322	–	Khusainov R.V.
L. aetnaeus	Kantaurovo, Bor district, Nizhny Novgorod region, Russia	*Alnus glutinosa*	CD1108	KF242320	–	Khusainov R.V.
L. aetnaeus	Lugovoy, Lunino district, Penza region, Russia	*Salix alba*	CD1143	KF242319	–	Khusainov R.V.
L. africanus	Westmorland, Imperial county, California, USA	*Gossypium* sp.	CD83	KF242340, KF242339	–	Subbotin S.A.
L. africanus	Calipatria, Imperial county, California, USA	*Citrus* sp.	CD151	KF242337, KF242341	–	Subbotin S.A.
L. africanus	El Centro, Imperial county, California, USA	Grasses	CD1301	KF242338	KF242279	Dong K., Subbotin S.A.
L. andalusicus	Island Hallig Hooge, North Sea, Germany	*Halimione portulacoides*	868	KF242336	–	Sturhan (2013)
L. artemisiae	Shestikhino, Myshkin district, Yaroslavl region, Russia	*Poa* sp. and *Trifolium* sp.	CD1115, CD1146	KF242313, KF242315	KF242286	Chizhov V.N.
L. artemisiae	Troitsk, Troitsk district, Chelyabinsk region, Russia	*Poa* sp.	CD1127	KF242314	–	Pridannikov M., Khusainov R.V.
L. artemisiae	Shestikhino, Myshkin district, Yaroslavl region, Russia	*Elytrigia* sp.	CD1144	KF242316	–	Chizhov V.N.
L. caespiticola	Gostagaevskaya, Anapa district, Krasnodar Territory, Russia	*Rubus caesius*	CD1109	KF242344	KF242280	Khusainov R.V.
L. distinctus	Pyatigorsk, Stavropol Territory, Russia	*Salix* sp.	CD1128	KF242317	KF242290	Chizhov V.N.
L. elongatus	Moscow, Russia	*Malus domestica*	CD1145	KF242310	–	Khusainov R.V.
L. elongatus	Yagodnaya, Ulyanovsk district, Kaluga region, Russia	*Urtica dioica*	CD1134	KF242300	–	Khusainov R.V.
L. elongatus	Yagodnaya, Ulyanovsk district, Kaluga region, Russia	*Rubus coesius*	CD1116	KF242301	–	Khusainov R.V.
L. elongatus	Nikulki, Taldom district, Moscow region, Russia	*Alnus incana*	CD1132	KF242304	–	Khusainov R.V.
L. elongatus	Moscow, Russia	*Acer platanoides*	CD1140	KF242303	–	Chizhov V.N.

Table 1 (continued)

Species	Location	Host	Sample code	Genbank number		Collector/Identifier
				D2–D3 of 28S rRNA	18S rRNA	
L. elongatus	Moscow, Russia	*Populus* sp.	CD1139	KF242308	–	Chizhov V.N.
L. elongatus	Serpukhov, Serpukhov district, Moscow region, Russia	*Salix alba*	CD1135	KF242305	–	Khusainov R.V.
L. elongatus	Klyazma, Pushkin district, Moscow region, Russia	*Poa annua*	CD1114	KF242306	KF242285	Khusainov R.V.
L. elongatus	Moscow, Russia	*Urtica dioica*	CD1126	KF242297	–	Khusainov R.V.
L. elongatus	Vladimir, Vladimir region, Russia	*Acer negundo*	CD1123	KF242296	–	Khusainov R.V.
L. elongatus	Shurovo, Kolomna district, Moscow region, Russia	*Salix fragilis*	CD1141	KF242307	–	Khusainov R.V.
L. elongatus	Engels, Engels district, Saratov region, Russia	*Elaeagnus angustifolia*	CD1125	KF242298	–	Khusainov R.V.
L. elongatus	Salt Lake, Utah, USA	Unknown	CD854	KF242295	–	Chitambar J., Subbotin S.A.
L. elongatus	Pyatigorsk, Stavropol Territory, Russia	*Salix* sp.	CD1205	KF242299	–	Chizhov V.N.
L. elongatus	Pyatigorsk, Stavropol Territory, Kislovodsk, Russia	Unknown	CD1345	KF242294	–	Chizhov V.N.
L. elongatus	Marin county, California, USA	Grasses	CD1290	KF242302	KF242283	Chitambar J., Subbotin S.A.
L. elongatus	Christchurch, New Zealand	Grasses	591	KF242309	–	Sturhan D.
L. euonymus	Krivoborye, Ramon district, Voronezh region, Russia	*Artemisia* sp.	CD1086	KF242331	KF242291	Khusainov R.V.
L. euonymus	Bolshoy Vyas, Lunino district, Penza region, Russia	*Asparagus cicer*	CD1118	KF242333	–	Khusainov R.V.
L. euonymus	Anapa, Anapa district, Krasnodar Territory, Russia	*Juglans regia*	CD1130	KF242332	–	Khusainov R.V.
L. intermedius	Kamennomostsky, Adygeya, Russia	*Fagus orientalis*	CD1122	KF242312	–	Khusainov R.V.
L. intermedius	Abrau–Dyurso, Novorossiysk district, Krasnodar Territory, Russia	*Cornus mus*	CD1085	KF242311	KF242284	Khusainov R.V.
L. leptocephalus	Potrosovo, Kozelsk district, Kaluga region, Russia	*Urtica dioica*	CD1136	KF242326	–	Khusainov R.V.
L. leptocephalus	Kantaurovo, Bor district, Nizhny Novgorod region, Russia	*Poa annua*	CD1119	KF242327	–	Khusainov R.V.
L. leptocephalus	Tselinny, Troitsk district, Chelyabinsk region, Russia	*Poa* sp.	CD1131	KF242325	–	Pridannikov M., Khusainov R.V.
L. lignosus	Sukko, Anapa district, Krasnodar Territory, Russia	*Acer campestre*	CD1124	KF242345	KF242281	Khusainov R.V.
L. lignosus		*Fagus orientalis*	CD1133	KF242346	–	Khusainov R.V.

Table 1 (continued)

Species	Location	Host	Sample code	Genbank number		Collector/Identifier
				D2–D3 of 28S rRNA	18S rRNA	
	Sochi, Sochi district, Krasnodar Territory, Russia					
L. lignosus	Lazarevskoe, Sochi district, Krasnodar Territory, Russia	*Fagus orientalis*	CD1120	KF242347	–	Khusainov R.V.
Longidorus sp.1	Folsom, California, USA	Unknown	CD833	KF242330	KF242288	Subbotin S.A.
Longidorus sp.2	Napa county, California, USA	Unknown	CD897	KF242329	–	Subbotin S.A.
Longidorus sp.3	Natukhaevskaya, Novorossiysk district, Krasnodar Territory, Russia	*Prunus divaricata*	CD1112	KF242335	KF242292	Khusainov R.V.
Longidorus sp.4	Proletarka, Krasnosulinsk district, Rostov region, Russia	*Salix babylonica*	CD1117	KF242334	KF242293	Khusainov R.V.
Longidorus sp.5	Butte, Butte county, California, USA	*Juglans* sp.	CD506	KF242342, KF242343	KF242282	Subbotin S.A.
Longidorus sp.6	Point Reyes, Marin county, California, USA	Unknown	CD876	KF242328	KF242289	Subbotin S.A.

based approaches have been successfully applied for molecular characterization, diagnostics and study of phylogeny of the genus *Longidorus.* Several genes were used for molecular characterization of these nematodes: partial 28S rRNA gene (Rubtsova et al. 2001; De Luca et al. 2004a; Handoo et al. 2005; He et al. 2005; Gutiérrez-Gutiérrez et al. 2010; 2013; Kumari and Subbotin 2012; Amrei et al. 2013), 18S rRNA gene (Neilson et al. 2004; Oro et al. 2005; Gutiérrez-Gutiérrez et al. 2010; 2013; Niknam et al. 2010; Kumari and Subbotin 2012 and others), ITS rRNA gene (De Luca et al. 2004a; Ye et al. 2004; Kumari et al. 2009; Niknam et al. 2010; Kumari and Subbotin 2012) and *cox1* of mtDNA (Kumari et al. 2009; Kumari and Subbotin 2012) and *nad4* of mtDNA (Kumari and Subbotin 2012). Lamberti et al. (2001) and De Luca et al. (2004a, 2009) designed a PCR-ITS-RFLP assay with six restriction enzymes to distinguish some *Longidorus* species. A similar approach was developed by Širca and Urek (2009), who used the D2–D3 of 28S rRNA gene and five restriction enzymes to distinguish six *Longidorus* species. After analysis of the ITS rRNA gene sequences, Hübschen et al. (2004) developed species-specific primers for the identification of six longidorid nematodes: *L. attenuatus*, *L. elongatus*, *L. macrosoma*, *L. helveticus*, *L. profundorum* and *L. sturhani*.

Needle nematodes of the genus *Longidorus* are found in all inhabited continents, with the highest number of species in Europe (more than 75 species) followed by Asia, North America and Africa (Decraemer and Robbins 2007). Fourteen valid species of this genus are reported in Russia: *L. artemisiae*, *L. attenuatus*, *L. caespiticola*, *L. cylindricaudatus*, *L. distinctus*, *L. elongatus*, *L. euonymus*, *L. leptocephalus*, *L. macrosoma*, *L. martini*, *L. olegi*, *L. profundorum*, *L. rubi* and *L. vineacola* (Brown et al. 1990; Romanenko 1993, 1998; Rubtsova et al. 1999; Khusainov 2012). Except for *L. artemisiae*, these nematode species were identified using only morphological characters. Rubtsova et al. (2005) amplified and sequenced a short fragment of the D2 of 28S rRNA gene obtained from glycerine-embedded nematodes recovered from *L. artemisiae* paratype slides. Sequences of short fragments of 28S rRNA gene for *L. artemisiae* and several other *Longidorus* obtained from fixed materials species were published by Rubtsova et al. (2005).

The main objectives of this study were to: *i*) verify morphological identification of some *Longidorus*

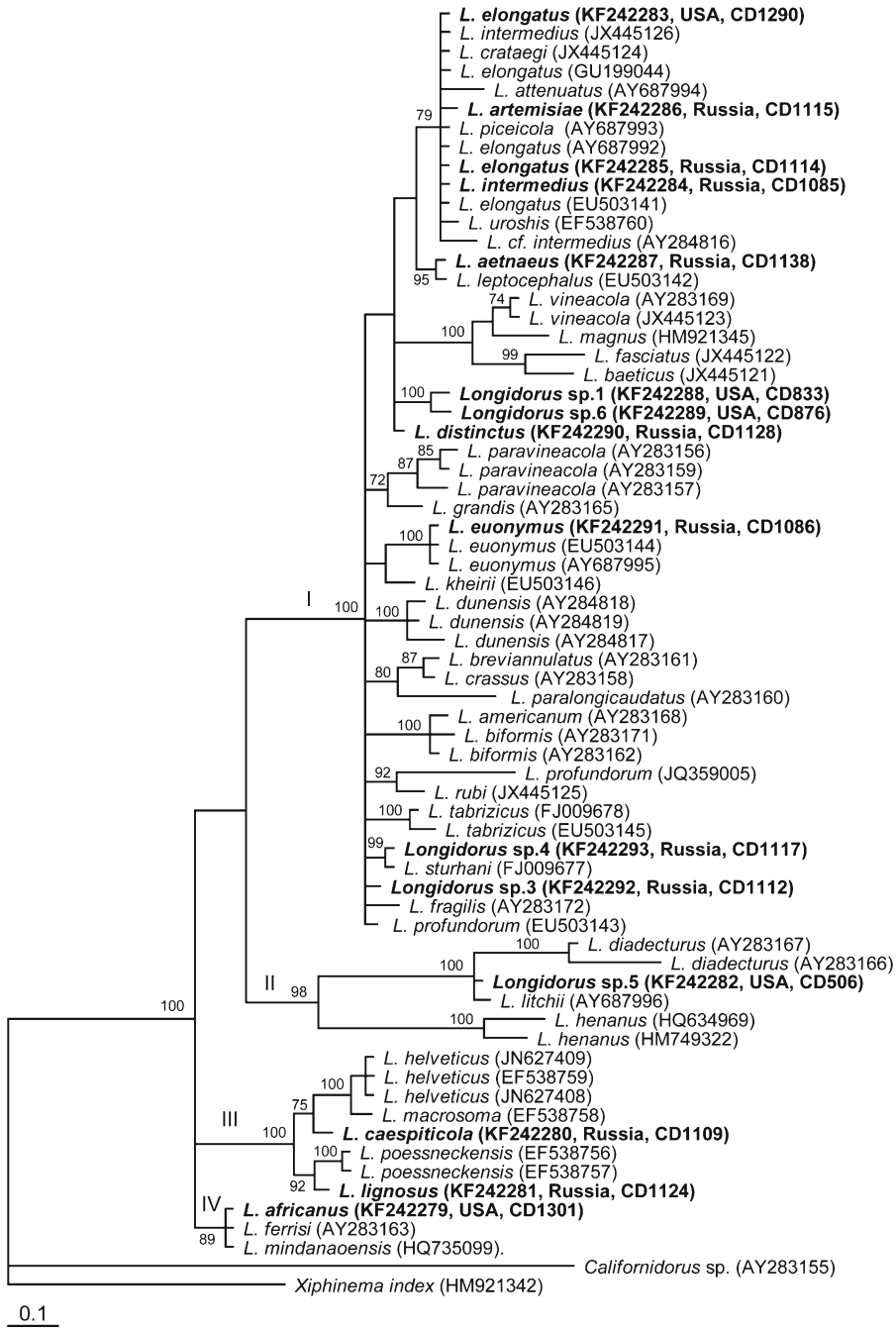


Fig. 1 Phylogenetic relationships within the genus *Longidorus*: Bayesian 50 % majority rule consensus tree from two runs as inferred from partial 18S rRNA gene sequence alignments under the GTR+I+G model. Posterior probabilities more than 70 % are given for appropriate clades. Newly obtained sequences are indicated by bold letters

species collected in Russia, USA and other countries using molecular approaches; *ii*) provide molecular characterization of studied species using sequence analysis of partial 18S rRNA and 28S rRNA genes; *iii*) reconstruct phylogenetic relationships within the genus *Longidorus* using partial 18S rRNA and 28S rRNA gene sequences using Bayesian inference; *iv*) provide a PCR-RFLP assay using D2–D3 of 28S rRNA gene for rapid diagnostics of longidorid species.

Materials and methods

Nematode populations and morphological studies

Nematode populations for this study were obtained from several sources. Most nematode specimens were obtained from soil samples collected in different regions of Russia (37 samples), USA (nine samples) (states of California and Utah) and Ukraine (one sample). Nematodes from Germany (one sample) and New Zealand (one sample) were kindly provided by Dr. D. Sturhan (Table 1). The D2–D3 of 28S rRNA gene sequence of an unidentified *Longidorus* from Utah, USA under the accession number KF292307 was provided by Dr. L. Poiras. The sample of *L. americanum* (Handoo et al. 2005) was included in the PCR-RFLP study.

The nematodes were extracted from soil by a sieving and decanting method (Brown and Boag 1988). Specimens from some populations were killed by gentle heat fixed in 4 % formalin or TAF and mounted in anhydrous glycerin for morphological examination. Preliminary morphological identifications of specimens were done by R.V. Khusainov and V.N. Chizhov using Chen et al. (1997)'s key and corresponding species descriptions (Khusainov 2012). Some Russian populations of longidorid species used in this study will be described morphologically and morphometrically in separate publications by R.V. Khusainov (pers. comm.).

In this study, most species were defined and delimited based on an integrated approach that considered morphological evaluation, molecular based phylogenetic inference (tree based methods) and sequence analyses (genetic distance methods) (Sites and Marshall 2004).

DNA extraction, PCR assays and sequencing

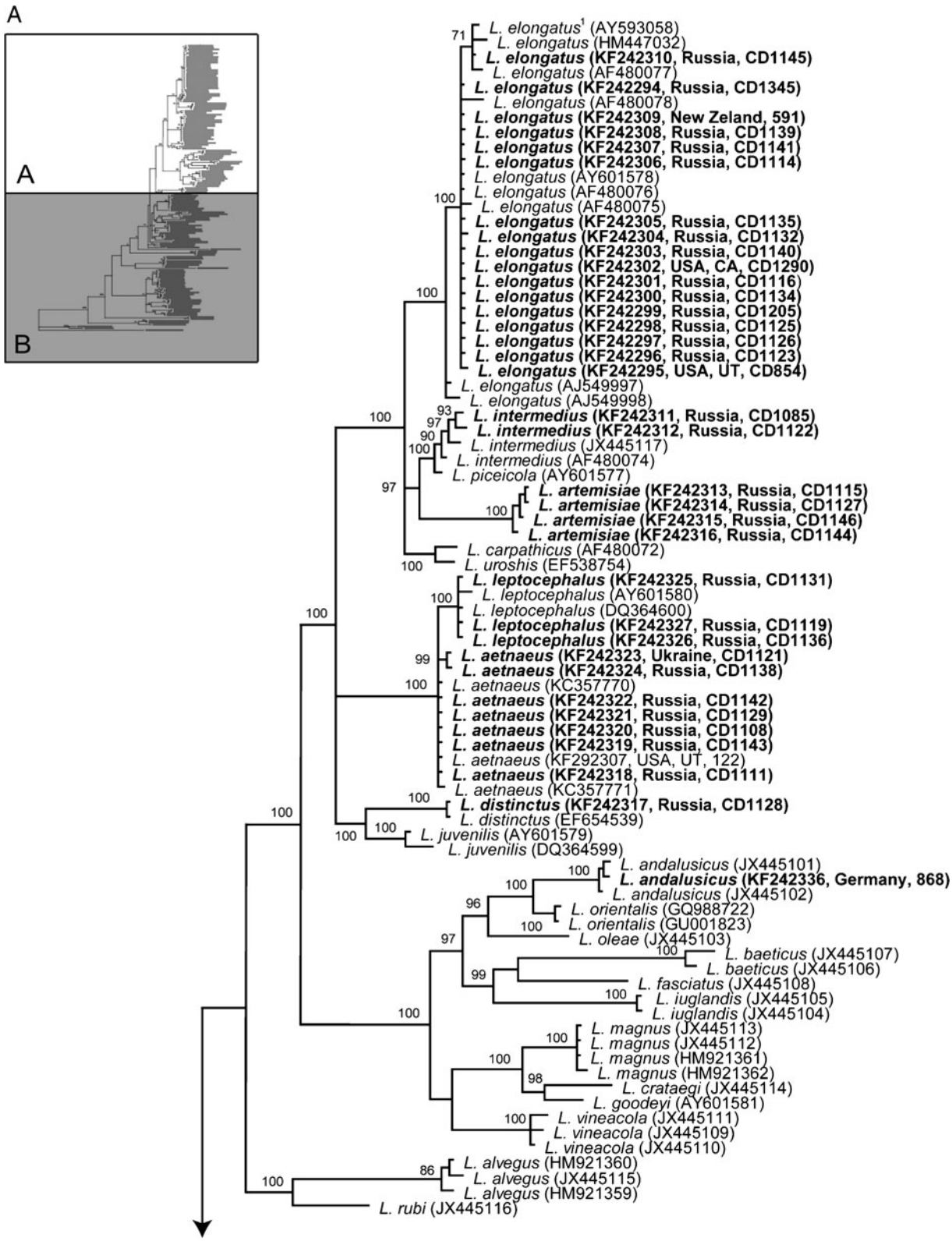
Nematode DNA from nematodes was extracted from a single individual. Several specimens were analysed for some populations. Protocols for DNA extraction, PCR and sequencing were described by Tanha Maafi et al. (2003). The following primers were used for amplification of two rRNA gene fragments: partial 18S rRNA – the forward G18SU (5′-GCT TGT CTC AAA GAT TAA GCC-3′) and the reverse R18Tyl1 (5′-GGT CCA AGA ATT TCA CCT CTC-3′) (Chizhov et al. 2006) primers and D2–D3 expansion segments of 28S rRNA gene - the forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and the reverse D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) (Rubtsova et al. 2001) primers.

Fig. 2 **a** and **b** Phylogenetic relationships within the genus *Longidorus*: Bayesian 50 % majority rule consensus tree from two runs as inferred from D2–D3 of 28S rRNA gene sequence alignments under the GTR+I+G model. Posterior probabilities more than 70 % are given for appropriate clades. Newly obtained sequences are indicated by bold letters. Originally identified as: [1]*L. intermedius*; [2]*L. profundorum*; [3]*L. raskii*; [4]*L. latocephalus* (synonym of *L. pisi*). The D1–D2 of 28S rRNA gene sequences of *L. profundorum* and *L. raskii* provided by De Luca et al. (2004b) do not likely belong to those species and named here as *Longidorus* sp. These sequences are very similar with each other and with those for *L. arthensis*

PCR products were purified after with QIAquick (Qiagen, USA) gel extraction kit. The same primers were used for direct sequencing. Several PCR products were cloned into the pGEM-T vector and transformed into JM109 High Efficiency Competent Cells (Promega, USA). Several clones of each sample were isolated using blue/white selection and submitted to PCR with same primers. PCR products from each clone were sequenced. Sequences were submitted to the GenBank database under accessions numbers: KF242279–KF242347 (Table 1).

Sequence and phylogenetic analysis

Sequences of the partial 18S rRNA and D2–D3 of 28S rRNA genes were aligned using ClustalX 1.83 (Thompson et al. 1997) with default parameters with corresponding published gene sequences, respectively (Rubtsova et al. 2001; He et al. 2005; Kumari et al. 2009; Širca and Urek 2009; Gutiérrez-Gutiérrez et al. 2010; 2013; Niknam et al. 2010; Palomares-Rius et al. 2010; Guo et al. 2011; Coomans et al. 2012; Pedram et al. 2012; Amrei et al. 2013; Peneva et al. 2013 and others). Outgroup taxa for each dataset were chosen according to the results of previous publications (He et al. 2005). The best fit model of DNA evolution was obtained using the program JModeltest ver. 0.1.1 (Posada 2008) with the Akaike Information Criterion. Sequence datasets were analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). BI analysis for each aligned dataset was initiated with a random starting tree and was run with four chains for 2.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. 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. Trees were visualised using TreeView


A
A
B
L. elongatus[1] (AY593058)
L. elongatus (HM447032)
L. elongatus (KF242310, Russia, CD1145)
L. elongatus (AF480077)
L. elongatus (KF242294, Russia, CD1345)
L. elongatus (AF480078)
L. elongatus (KF242309, New Zeland, 591)
L. elongatus (KF242308, Russia, CD1139)
L. elongatus (KF242307, Russia, CD1141)
L. elongatus (KF242306, Russia, CD1114)
L. elongatus (AY601578)
L. elongatus (AF480076)
L. elongatus (AF480075)
L. elongatus (KF242305, Russia, CD1135)
L. elongatus (KF242304, Russia, CD1132)
L. elongatus (KF242303, Russia, CD1140)
L. elongatus (KF242302, USA, CA, CD1290)
L. elongatus (KF242301, Russia, CD1116)
L. elongatus (KF242300, Russia, CD1134)
L. elongatus (KF242299, Russia, CD1205)
L. elongatus (KF242298, Russia, CD1125)
L. elongatus (KF242297, Russia, CD1126)
L. elongatus (KF242296, Russia, CD1123)
L. elongatus (KF242295, USA, UT, CD854)
L. elongatus (AJ549997)
L. elongatus (AJ549998)
L. intermedius (KF242311, Russia, CD1085)
L. intermedius (KF242312, Russia, CD1122)
L. intermedius (JX445117)
L. intermedius (AF480074)
L. piceicola (AY601577)
L. artemisiae (KF242313, Russia, CD1115)
L. artemisiae (KF242314, Russia, CD1127)
L. artemisiae (KF242315, Russia, CD1146)
L. artemisiae (KF242316, Russia, CD1144)
L. carpathicus (AF480072)
L. uroshis (EF538754)
L. leptocephalus (KF242325, Russia, CD1131)
L. leptocephalus (AY601580)
L. leptocephalus (DQ364600)
L. leptocephalus (KF242327, Russia, CD1119)
L. leptocephalus (KF242326, Russia, CD1136)
L. aetnaeus (KF242323, Ukraine, CD1121)
L. aetnaeus (KF242324, Russia, CD1138)
L. aetnaeus (KC357770)
L. aetnaeus (KF242322, Russia, CD1142)
L. aetnaeus (KF242321, Russia, CD1129)
L. aetnaeus (KF242320, Russia, CD1108)
L. aetnaeus (KF242319, Russia, CD1143)
L. aetnaeus (KF292307, USA, UT, 122)
L. aetnaeus (KF242318, Russia, CD1111)
L. aetnaeus (KC357771)
L. distinctus (KF242317, Russia, CD1128)
L. distinctus (EF654539)
L. juvenilis (AY601579)
L. juvenilis (DQ364599)
L. andalusicus (JX445101)
L. andalusicus (KF242336, Germany, 868)
L. andalusicus (JX445102)
L. orientalis (GQ988722)
L. orientalis (GU001823)
L. oleae (JX445103)
L. baeticus (JX445107)
L. baeticus (JX445106)
L. fasciatus (JX445108)
L. iuglandis (JX445105)
L. iuglandis (JX445104)
L. magnus (JX445113)
L. magnus (JX445112)
L. magnus (HM921361)
L. magnus (HM921362)
L. crataegi (JX445114)
L. goodeyi (AY601581)
L. vineacola (JX445111)
L. vineacola (JX445109)
L. vineacola (JX445110)
L. alvegus (HM921360)
L. alvegus (JX445115)
L. alvegus (HM921359)
L. rubi (JX445116)

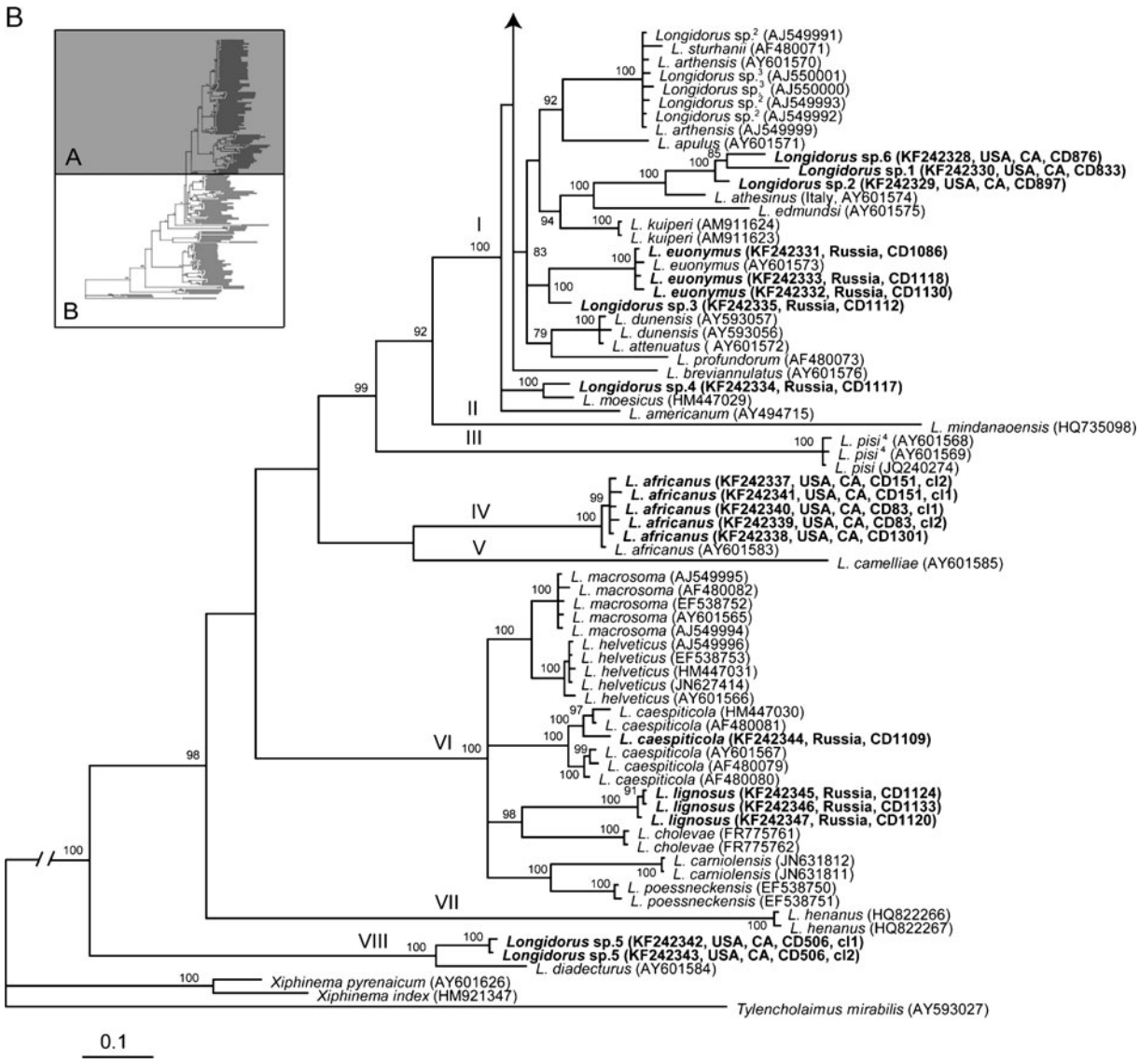


Fig. 2 continued.

program (Page 1996). Further 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.

PCR-D2-D3-RFLP

Five to seven μl of the purified PCR product of D2–D3 of rDNA was digested by one of the following restriction enzymes: *Alu*I, *Hinf*I, *Bsp*143I (*Mbo*I), *Tru*1I (*Mse*I) or *Rsa*I (Fermentas International, Inc) in the buffer stipulated by the manufacturer. Digested DNA was run on a 1.4 % TAE buffered agarose gel, stained with ethidium bromide, visualised on UV transilluminator and photographed. The length of each restriction fragment from the PCR products was predicted by a virtual digestion of the sequences using WebCutter 2.0 (www.firstmarket.com/cutter/cut2.html) or estimated from a gel.

Results

Species identification and delimiting

We distinguished the following species in our samples: *L. aetnaeus*, *L. africanus*, *L. andalusicus*, *L. artemisiae*, *L. caespiticola*, *L. distinctus*, *L. elongatus*, *L. euonymus*, *L. intermedius*, *L. leptocephalus* and *L. lignosus*. Samples of *L. caespiticola*, *L. distinctus* and some samples of *L. aetnaeus* were identified using only molecular criteria. Two populations from Russia and four samples from California were not identified to species level because of the lack of sufficient numbers of females and named here as representatives of six unidentified *Longidorus* species. More detailed morphological and molecular analyses are required to further evaluate and identify these samples.

Sequence and phylogenetic analysis

Partial 18S rRNA gene The alignment for the partial 18S rRNA gene including 66 *Longidorus* sequences and two outgroup sequences was 836 positions in length. New fifteen sequences of *Longidorus* were obtained in the present study. Forty-six *Longidorus* species were included in the analysis. Intraspecific sequence divergence for some species were: *L. dunensis* – 1–4 bp (0.2–0.6 %); *L. paravineacola* – 1–4 bp (0.1–0.5 %), *L. henanus* – 10 bp (1.2 %) and *L. diadecturus* – 10 bp (1.2 %). There were no differences in the partial 18S rRNA gene sequences between *L. elongatus*, *L. intermedius*, *L. crataegi* and *L. piceicola*; *L. sturhani* and *Longidorus* sp.4; *L. africanus*, *L. ferrisi* and *L. mindanaoensis*.

The 18S rRNA gene BI tree revealed four major supported clades (Fig. 1), three of them were highly supported (PP=98–100) and one moderate supported clade (PP=89). The first highly supported clade included majority species and more than 70 % of studied sequences. The relationships between species within this clade were poorly or not resolved. The clade II included *L. diadecturus*, *Longidorus* sp. 5, *L. litchi* and *L. henanus*. The clade III contained *L. helveticus*, *L. macrosoma*, *L. caespiticola*, *L. poessneckensis* and *L. lignosus*. The clade IV included *L. africanus*, *L. ferrisi* and *L. mindanaoensis*.

D2–D3 expansion segments of 28S rRNA gene The alignment for the D2–D3 of 28S rRNA gene included 150 *Longidorus* sequences and three outgroup sequences and was 847 positions in length. New fifty-four sequences of *Longidorus* were obtained in the present study. Forty-eight known and six unidentified *Longidorus* species were included in this analysis. Our sequences of *L. aetnaeus*, *L. africanus*, *L. andalusicus*, *L. caespiticola*, *L. distinctus*, *L. elongatus*, *L. euonymus*, *L. intermedius* and *L. leptocephalus* clustered and showed high similarity with sequences of corresponding species from GenBank. Sequences of *L. artemisiae* were identical to the D2 of 28S rRNA gene sequence fragment, which was 75 bp in length and obtained from the paratype slides of this species and published by Rubtsova et al. (2005).

Intraspecific sequence dissimilarities for some species were: *L. elongatus* – 0–10 bp (0–2.1 %), *L. intermedius* – 2–5 bp (0.3–0.7 %), *L. aetnaeus* – 0–3 bp (0–0.4 %), *L. caespiticola* – 3–20 bp (0.4–2.7 %), *L. euonymus* – 1–4 bp (0.1–0.3 %), *L. africanus* – 1–5 bp (0.1–0.7 %), *L. juvenilis* – 8 bp (1.1 %), *L. leptocephalus* – 0–3 bp (0–0.4 %) and *L. artemisiae* – 0–1 bp (0.2 %). Interspecific divergence between *L. aetnaeus* and *L. leptocephalus* was 5–9 bp (0.7–1.4 %) and between *L. carpathicus* and *L. uroshis* – 9 bp (1.2 %). The nucleotide dissimilarity between *Longidorus* sp.1 and *Longidorus* sp.2 was 23 bp (3.0 %) and between *Longidorus* sp.2 and *Longidorus* sp.6 was 17 bp (2.3.%).

Longidorus species were distributed among eight highly supported major clades in the D2–D3 of 28S rRNA gene BI tree (Fig. 2a, b). Clade I included the majority of *Longidorus* species, clade VI contained seven species, clade VIII - two species, other clades (II, III, IV, V, VII) represented by single species lineages.

PCR-RFLP study

PCR-D2-D3-RFLP profiles generated by five enzymes for 16 species of *Longidorus* are given in Fig. 3. Lengths of restriction fragments from RFLP for the D2–D3 of the 28S rRNA gene obtained with using WebCutter 2.0 are presented in Table 2. Five restriction enzymes: *Alu*I, *Hinf*I, *Bsp*143I, *Tru*1I or *Rsa*I distinguished all species with an exception of two unidentified species (*Longidorus* sp.1 and *Longidorus* sp.2). Restriction enzyme *Hinf*I generated nine distinct profiles, whereas *Alu*I and *Rsa*I – seven, *Tru*1I – six and *Bsp*143I - four

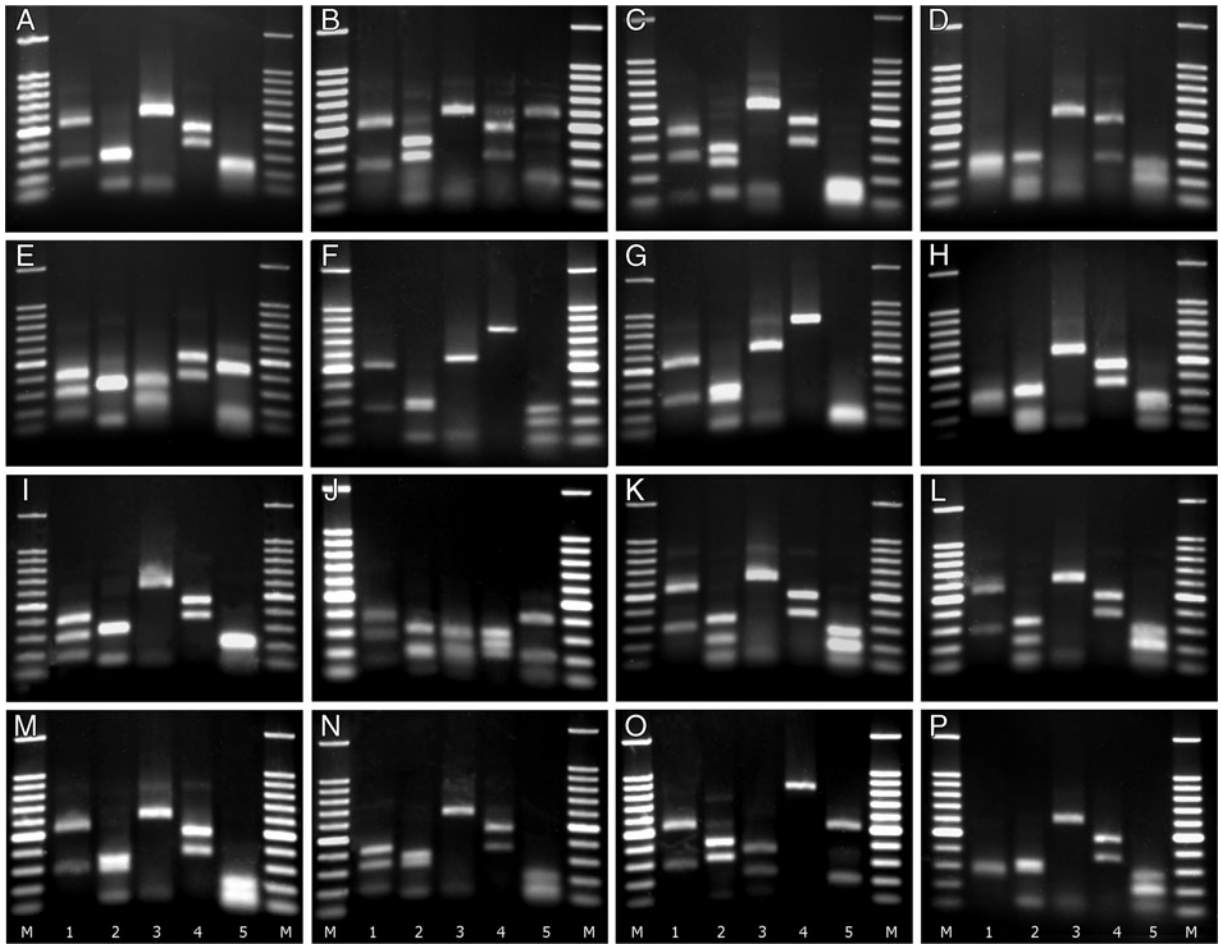


Fig. 3 PCR- D2-D3-28S-RFLP diagnostic profiles for some *Longidorus* species. **a** *L. aetnaeus* (CD1142); **b** *L. africanus* (CD1301); **c** *L. americanum* (720); **d** *L. artemisiae* (CD1115); **e** *L. caespiticola* type B (CD1109); **f** *L. elongatus* (CD1145); **g** *L. euonymus* (CD1130); **h** *L. intermedius* (CD1122); **i**: *L. leptocephalus* (CD1136); **j** *L. lignosus* (CD1124); **k** *Longidorus* sp.1 (CD833); **l** *Longidorus* sp.2 (CD897); **m** *Longidorus* sp.3 (CD1112); **n** *Longidorus* sp.4 (CD1117); **o** *Longidorus* sp.5 (CD506); **p** *Longidorus* sp.6 (CD876). *M* 100 bp DNA marker (Promega, USA), *1* - *Alu*I, *2* - *Hinf*I, *3* - *Bsp*143I, *4* - *Tru*1I, *5*- *Rsa*I

restriction profiles. Lengths of restriction fragments visible on the gels after PCR–RFLPs for all studied enzymes were similar to those expected from *in silico* analysis.

Discussion

Identification of longidorids is difficult because of high intra- and interspecific variability of morphological and morphometrical characters. Identification using morphological and molecular methods agreed for most our samples and we were able to identify the majority of samples in our study. However, the identification of some of them remains uncertain because of a lack of morphological data for some isolates and the absence of similar sequences in the GenBank database. Final identification of these populations will be possible after a more thorough morphological characterization of these isolates.

PCR-RFLP remains the effective method of identification of different nematode groups comprising many species. This technique is a simple, rapid and cost-effective in comparison with other approaches. Širca and Urek (2009) provided PCR-RFLP diagnostic profiles for identification of 6 species of *Longidorus* using a fragment of the D2–D3 of 28S rRNA gene. Restriction of D2–D3 amplicons by five enzymes produces species-specific restriction patterns for those species. In the present study we confirmed the value of this technique for identification and providing diagnostic profiles for

Table 2 Length (bp) of restriction fragments after digestion of PCR products obtained from the D2–D3 of 28S rRNA gene for some *Longidorus* species generated using WebCutter 2.0

Species	Unrestricted PCR	Restriction enzymes				
		*Alu*I	*Hinf*I	*Bsp*143I (*Mbo*I)	*Tru*1I (*Mse*I)	*Rsa*I
L. aetnaeus	811	530, 267, 14	300, 279, 129, 103	574, 106, 63, 46, 22	456, 355	223, 204, 186, 107, 50, 35, 6
L. africanus	822	523, 285, 14	401, 318, 103	586, 128, 63, 45	472, 305, 45	578, 204, 21, 13, 6
L. americanum	828	418, 286, 110, 14	319, 257, 129, 103, 20	591, 128, 63, 46	474, 354	160, 157, 134, 112, 107, 105, 47, 6
L. artemisiae	806	330, 267, 225, 14	300, 172, 128, 103, 103	569, 128, 63, 46	515, 291	269, 204, 185, 127, 35, 6
L. caespiticola type B	842	387, 286, 155, 14	319, 311, 109, 103	315, 225, 174, 65, 63	482, 360	420, 157, 107, 70, 47, 35, 6
L. elongatus	804	523, 267, 14	300, 273, 128, 103	567, 128, 63, 46	804	269, 204, 132, 107, 51, 35, 6
L. euonymus	827	527, 286, 14	319, 276, 129, 103	590, 128, 63, 46	827	204, 160, 133, 112, 107, 70, 35, 6
L. intermedius	799	300, 267, 218, 14	300, 172, 121, 103, 103	562, 128, 63, 42	455, 344	262, 204, 134, 107, 51, 35, 6
L. leptocephalus	810	369, 266, 161, 14	299, 279, 129, 103	574, 106, 63, 45, 22	455, 355	223, 204, 186, 107, 50, 34, 6
L. lignosus	838	384, 285, 155, 14	318, 223, 197, 103,	301, 223, 173, 78, 63	312, 265, 214, 47	400, 204, 124, 70, 34, 6
Longidorus sp.1	832	531, 287, 14	320, 207, 129, 103, 73	594, 128, 63, 47	475, 357	269, 204, 190, 113, 36, 14, 6
Longidorus sp.2	833	535, 284, 14	317, 210, 129, 103, 74	598, 128, 63, 44	473, 360	269, 205, 204, 116, 33, 6
Longidorus sp.3	829	529, 286, 14	319, 278, 129, 103	592, 128, 63, 46	474, 355	204, 161, 134, 112, 107, 70, 35, 6
Longidorus sp.4	828	375, 286, 153, 14	319, 277, 129, 103	591, 128, 63, 46	474, 354	204, 160, 134, 112, 107, 70, 35, 6
Longidorus sp.5	839	536, 289, 14	414, 322, 103	385, 236, 128, 63, 47	839	521, 204, 72, 36, 6
Longidorus sp.6	831	286, 280, 251, 14	319, 280, 129, 103	594, 128, 63, 46	474, 357	269, 204, 204, 113, 35, 6

10 known, and six unidentified longidorid species. The D2–D3 of 28S rRNA gene exhibited intraspecific polymorphism for *L. caespiticola*, which caused variation in restriction profiles for the Slovenian population (type A) (Širca and Urek 2009) and the Russian population (type B) (present study) by the restriction enzyme *Rsa*I. Moreover, *in silico* analysis predicted the third profile (type C) generated by this enzyme for *L. caespiticola* from UK, Germany and Belgium. The high level of rRNA gene sequence divergence indicates that *L. caespiticola* might represent a species complex. Further studies of the morphological characters in these three groups will be needed to support or confirm this hypothesis.

Phylogenetic relationships within longidorids using 28S and 18S rRNA gene sequences were recently reconstructed by several researchers. Our present study with the highest included numbers of species and sequences showed that relationships between taxa and clades are in general agreement with those presented in other publications (He et al. 2005; Gutiérrez-Gutiérrez et al. 2010; 2013; Amrei et al. 2013). *Longidorus lignosus* clustered and shared pouch-shaped amphids with other members of clade VI. *Longidorus* sp. 5 from California clustered with *L. diadecturus* as well as shares an expanded lip region with rounded contour and a tail dorsally convex with bluntly rounded terminus.

In this study *L. aetnaeus* was molecularly identified from Russia and USA for the first time. R.V. Khusainov noticed (pers. comm.) that some Russian *L. aetnaeus* populations do not fit original description of this species and confirmation of molecular identification using morphology still needs to be done. Amrei et al. (2013) showed that *L. aetnaeus* is closely related to *L. leptocephalus*. Although these species differ in body and odontostyle lengths, position of the vulva, spicule length and supplements number, they are very similar in general morphology, especially, in shape of the lip and tail regions and could be easily confused. Overlapping geographical distribution of these species makes their correct identification more problematic, for example, in this study both species were found from samples collected in the same location: Kantaurovo, Nizhny Novgorod region, Russia, but from different host-plants.

Molecular characterization of nematode materials from the type localities and defining a reference sequence for a certain species is important for correct identification of samples. One of the remarkable results of our study is that we confirmed complete sequence identity of a fragment of rRNA gene obtained from DNA recovered from the paratype slides of *L artemisiae* after a long storage with those obtained from freshly extracted DNA of this species. We showed that, at least in this case, there were no artificial modifications and mutations in DNA recovered from formalin-fixed materials and, thus, the method of extraction and amplification of DNA described by Rubtsova et al. (2005) allows using archived longidorid collections for obtaining reference sequences.

Thus, the present study with PCR-RFLP and sequencing of D2–D3 of 28S rRNA gene markers confirmed that these methods are useful and appropriate for characterization and accurate identification of longidorids. Larger numbers of species and populations from diverse origins should be included in future studies to confirm the findings made in the present work.

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