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Inter-population variability of *Longidorus euonymus* Mali and Hooper, 1974 (Nematoda, Dorylaimida) and comment upon the number of juvenile developmental stages

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Summary

Longidorus euonymus is distributed widely in central and southern Europe. Nematodes typically have four pre-adult stages, however, there has been confusion in the scientific literature with regard to the number of juvenile developmental stages (JDS) of *L. euonymus* with three and four JDS being reported. A principal component analysis of published morphometric data from 33 *L. euonymus* populations unequivocally showed that there were four juvenile developmental stages. Furthermore, 18S rDNA sequence analysis indicated that *L. euonymus* was more closely related to *L. elongatus* and *L. attenuatus* than to *L. vineacola*. This was in discordance with the original description of the species; however, the maximum likelihood distances between the species were relatively small.

Key words: 18S rDNA; juvenile developmental stages; *Longidorus*; longidorids; nematode; variability

Introduction

Longidorus euonymus Mali and Hooper, 1974, was described from specimens recovered from the rhizosphere of *Euonymus* trees in the Czech Republic and subsequently, with one exception (Lamberti *et al.*, 1999), has been recorded only from mainland Europe: Bulgaria (Choleva-Abadzhieva, 1975; Lamberti *et al.*; 1983; Peneva & Choleva, 1992; Lamberti *et al.*, 1997); Greece (Roca *et al.*, 1986); Italy (Lamberti *et al.*, 1985; Roca *et al.*, 1985, 1987a,b, 1988, 1989, 1990, 1991; Roca & Lamberti, 1993); Lithuania (Stanelis, 2003); Poland (Brown & Taylor, 1987); Russia (Romanenko & Korchinsky, 1996); Slovakia (Lišková, 1995, 1997, 2001; Lišková *et al.*, 1995; Lišková & Brown, 1998, 1999; Lišková & Sturhan, 2000) and former Yugoslavia (Barsi, 1989, 1994; Barsi & Lamberti, 2003). Such a distribution is consistent with the species being restricted to the southern Europe, Holomediterranean, Apenino dinarical and Circum Adriatic transionical regions (Navas *et al.*, 1990, 1993). *Longidorus euonymus* has a wide host range having been associated with numerous annual, perennial, herbaceous and woody plants (Lamberti *et al.*, 1983; Lišková, 1995, 1997, 2001; Lišková & Brown, 1998).

Many of the above studies provide morphometric data of adult females but only four (Mali & Hooper, 1974; Lamberti *et al.*, 1997, 1999; Barsi & Lamberti, 2003), representing five populations, include data on juvenile developmental stages (JDS). The relationships between total body length and the lengths of odontostyle, replacement odontostyle and odontophore, used by Halbrendt and Brown (1992) to distinguish JDS of longidorid species, were inconclusive in these few studies. For example, it is unclear whether two Bulgarian populations of *L. euonymus* had three or four JDS (see Figs. 5 and 6, Lamberti *et al.*, 1997). Despite this inconclusive data, Lamberti *et al.* (1997) suggested that *L. euonymus* had three JDS (in Barsi and Lamberti (2003) this is now reported as four), whereas in the original species description Mali and Hooper (1974) presented data for four JDS. Also, Lamberti *et al.* (1999) published morphometric data for adult females and what was referred to as fourth stage juveniles. Robbins *et al.* (1995) and Halbrendt *et al.* (1997) reported that as with most longidorids, *L. euonymus* had four JDS, although Robbins *et al.* (1995) noted that a "substantial overlap" of odontostyle and replacement odontostyle lengths made identification of juvenile stages problematical.

Longidorus euonymus was reported as being morphologically most similar to *L. closelongatus* and *L. cohni* and

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also resembling *L. vineacola*, *L. elongatus* and *L. atenuatus* (Mali & Hooper, 1974). However, a hierarchical cluster analysis of all published *Longidorus* species (Ye, 2002) based on nine morphometric characters, only four of which were published in the original species description (Mali & Hooper, 1974) suggested that the most closely related species to *L. euonymus* were *L. apulus* Lamberti Bleve-Zacheo, 1977, *L. artemisiae* Rubtsova, Chizov and Subbotin, 1999 and *L. dunensis* Brinkman, Loof and Barbez, 1987. Comparison of 18S rDNA sequences has been successfully used to phylogenetically discriminate a range of animal, marine and soil nematodes at generic and species level (Blaxter *et al.*, 1998; Aleshin *et al.*, 1998; Floyd *et al.*, 2002) including longidorids (Hübschen *et al.*, 2002; Oliveira *et al.*, 2004a, b). Using such methodologies it should be possible to assess the taxonomic relationships between *L. euonymus* and other closely related European longidorid species.
The objectives of the present study were to a) make definitive comment on the number of juvenile developmental stages based on a morphological and morphometric study of numerous *L. euonymus* populations from Serbia, and b) identify the phylogenetic relationship of *L. euonymus* with selected European *Longidorus* species using 18S rDNA sequence data.

Material and Methods

Soil samples were taken from the rhizosphere of a range of plants from six localities in Serbia (Table 1). Nematodes were extracted by elutriation (Oostenbrink, 1960), fixed in hot 4:1 formalin, processed to glycerine and mounted on slides for identification and morphometric study. Measurements were made with the aid of an eyepiece micrometer and camera lucida using an Olympus HD-2 microscope.

L. euonymus paratypes
The majority of reports of *L. euonymus* contain extensive morphometric data, but the original species description (Mali & Hooper, 1974) did not contain data for the following common taxonomic characters: J; body diameters at the lip region, guide ring, base of oesophagus, mid-body/vulva, anus and the beginning of J. These data were obtained by measuring the holotype female and paratype females and juveniles (Slide nos. 168/26/1, 2, 13, 14, 15, 17, 18, 20, 26, 27, 28 and 36) made available from the Nematode Collection, Rothamsted Experimental Station, Harpenden, UK. This allowed us to include the type population in a principal component analysis based on a comparable set of morphometric data for all the populations used in the study.

DNA Extraction from Nematodes
DNA was extracted from adult females of *L. euonymus* and four closely related European *Longidorus* species occurring in Slovakia as described by Oliveira *et al.* (2004a). Furthermore, DNA was extracted from specimens of *L. litchii*, from China, and *Xiphinema index* (Germany) and included in the phylogenetic analysis as outgroups. A minimum of two nematodes from each population was sequenced to construct a consensus sequence.

PCR and Sequencing
18S rDNA sequences were obtained using the primers listed in Table 2 as described by Neilson *et al.* (2004). The following GenBank accession numbers were allocated to the nematode sequences, AY283169 and AY687992-AY687997.

Statistical and Sequence Analysis
To investigate inter-population variability of *L. euonymus* a principal component analysis, based on 18 morphological characters listed in Tables 3 – 5, for each of the 33 *L. euonymus* populations listed in Table 1, was done using multivariate analytical software (Community Analysis Package, Pisces Software, Lymington, UK).
Consensus 18S rDNA sequences for the six *Longidorus* and one *Xiphinema* species were obtained using Human Genome Mapping Project sequence analysis software (Devereaux *et al.*, 1984) from individual sequences obtained as described above. Sequences were aligned using Clustal X v 1.81 (Thompson *et al.*, 1994) with default gap penalty and gap extension settings. Thereafter, excess nucleotides at the 5' and 3' ends were trimmed to effect a common starting and end point using GeneDoc (Nicholas *et al.*, 1997).
The transition/transversion (Ts/Tv) ratio of the nucleotide substitution and α parameter of the gamma distribution for the F84 plus gamma+invariant rate heterogeneity models were estimated using TreePuzzle (Strimmer & von Haeseler, 1996). Phylip 3.6 (Felsenstein, 1993) software was used to perform a maximum likelihood analysis (Ts/Tv and α parameter set at 1.73 and 0.02, respectively) of the data and the level of statistical support for each branch of the phylogenetic tree was determined with a bootstrap analysis incorporating 100 replicates.

Results

Longidorus euonymus Mali and Hooper, 1974 was found at six sites in Serbia from a range of habitats. Adult females and four JDS were recorded from all sites, except Knjazevac, where the first JDS was not found (Tables 3 – 5; Figs 1a-f). Also, a male was present in specimens recovered from Loznica (Table 6). Morphometrically, none of the six Serbian populations in this study were distinct from each other, as the overall range of morphometric values for adult females and the different JDS for each of the 18 taxonomic characters overlapped, with only a few exceptions (Tables 3 – 5).
The single male *L. euonymus* from Loznica was morphologically similar to the single male reported by Roca (1991) and to two males recently reported by Barsi and Lamberti (2003).
Data from the Serbian populations presented in this study show a stepwise increase in total body length of JDS as a

Table 1. *Longidorus euonymus* populations used in the PCA analysis, with indication of the morphometric data available per population (note males were excluded from the PCA analysis)

Population	Country	Code	Host	Data	Reference
Type	Czechoslovakia	Typ	*Euonymus europaeus*	♀, J1-J4	Mali and Hooper, 1974
Kostinbrod	Bulgaria	BulKL	Poplar	♀	Lamberti *et al.*, 1983
Kalusha	Bulgaria	BulKa	*Pinus sylvestris*	♀	Peneva and Choleva, 1992
Zlatarevo	Bulgaria	BulZ	*Pinus nigra*	♀	Peneva and Choleva, 1992
Kostinbrod	Bulgaria	BulK	Poplar	♀, J1-J4	Lamberti *et al.*, 1997
Sandanski	Bulgaria	BulS	Olive	♀, J1-J4	Lamberti *et al.*, 1997
Squinzano	Italy	LpS	Grapevine	♀	Lamberti *et al.*, 1985
Otranto	Italy	LpO	Grapevine	♀	Lamberti *et al.*, 1985
San Severo	Italy	LpSS	Grapevine	♀	Lamberti *et al.*, 1985
Stigliano	Italy	LbS	Apricot	♀	Roca *et al.*, 1985
Cerveteri	Italy	LzC	Mixed herbs	♀	Roca *et al.*, 1987a
Santo Stefano di Magra	Italy	LIS	Grapevine	♀	Roca *et al.*, 1987b
Porto Garibaldi	Italy	ErPG	Poplar	♀	Roca *et al.*, 1988
Comiso	Italy	ScC	Grapevine	♀	Roca *et al.*, 1989
Vigliatore Terme	Italy	ScT	Grapevine	♀	Roca *et al.*, 1989
Vittoria	Italy	ScV	Grapevine	♀	Roca *et al.*, 1989
Spezzano	Italy	LaC	Grapevine	♀	Roca *et al.*, 1990
Avellino	Italy	LcA	Grapevine	♀, ♂	Roca *et al.*, 1991
Donoratico	Italy	TosD	Sugar beet	♀	Roca and Lamberti, 1993
Scansano	Italy	TosS	Cereals	♀	Roca and Lamberti, 1993
Palmanova	Italy	FvP	Grapevine	♀	Roca and Lamberti, 1994
Kanjiza	Serbia	K	Rose	♀, J1-J4	This study
Knjazevac	Serbia	Kn	Quince	♀, J1-J4	This study
Loznica	Serbia	L	Poplar	♀,♂, J1-J4	This study
Nis	Serbia	N	Cherry	♀, J1-J4	This study
Subotica	Serbia	S	Poplar	♀, J1-J4	This study
Vrtogos	Serbia	V	Apple	♀, J1-J4	This study
Idleb	Syria	Syr	Fig	♀, J4	Lamberti *et al.*, 1999
Novi Sad	Serbia	VP	Grass	♀	Barsi, 1989
Novi Becej	Serbia	Snb	Poplar	♀, J1-J4	Barsi and Lamberti, 2003
Novi Sad	Serbia	SnSe	Elder	♀, ♂	Barsi and Lamberti, 2003
Novi Sad	Serbia	SnSp	Poplar	♀	Barsi and Lamberti, 2003
Kanjiza	Serbia	Sk	Desert False Indigo	♀	Barsi and Lamberti, 2003

Table 2. Oligonucleotide primers used to obtain 18S rDNA sequences

Oligo name	Primer sequence (5' – 3')	Region	Direction
SSU_F_07	AAAGATTAAGCCATGCATG	18S rDNA	Forward
SSU_F_04	GCTTGTCTCAAAGATTAAGCC	18S rDNA	Forward
SSU_R_09	AGCTGGAATTACCGCGGCTG	18S rDNA	Reverse
SSU_F_22	TCCAAGGAAGGCAGCAGGC	18S rDNA	Forward
SSU_R_13	GGGCATCACAGACCTGTTA	18S rDNA	Reverse
SSU_F_02	GGAAGGGCACCACCAGGAGTGG	18S rDNA	Forward
SSU_R_18	TGATCCWKCYGCAGGTTCAC	18S rDNA	Reverse

All primers were sourced from the Blaxter Lab (*http://nema.cap.ed.ac.uk/biodiversity/sourhope/nemoprimers.html*)

percentage of the adult female length of approximately 20 %, 33 %, 50 % and 75 %. Odontostyle length in adult females is *c.* 1.7 times that of the first juvenile developmental stage. Tail length increases from J1 to J3 and then decreases through J4 to adult (Tables 3 – 5). The relative changes (ratios) in body volume between the different growth stages were as follows: 1 – 2 JDS (3.0), 2 – 3 JDS (2.3), 3 – 4 JDS (2.0), 4 JDS-adult female (2.0) and 1 JDS-adult female (25.6).

Scatter diagrams (Figs 1a-f) of total body length plotted against odontostyle, odontophore and replacement odontostyle lengths (after Halbrendt & Brown, 1992) for the six populations in this study clearly indicate that *L. euonymus* has four JDS (first JDS was not recorded at Knjazevac).

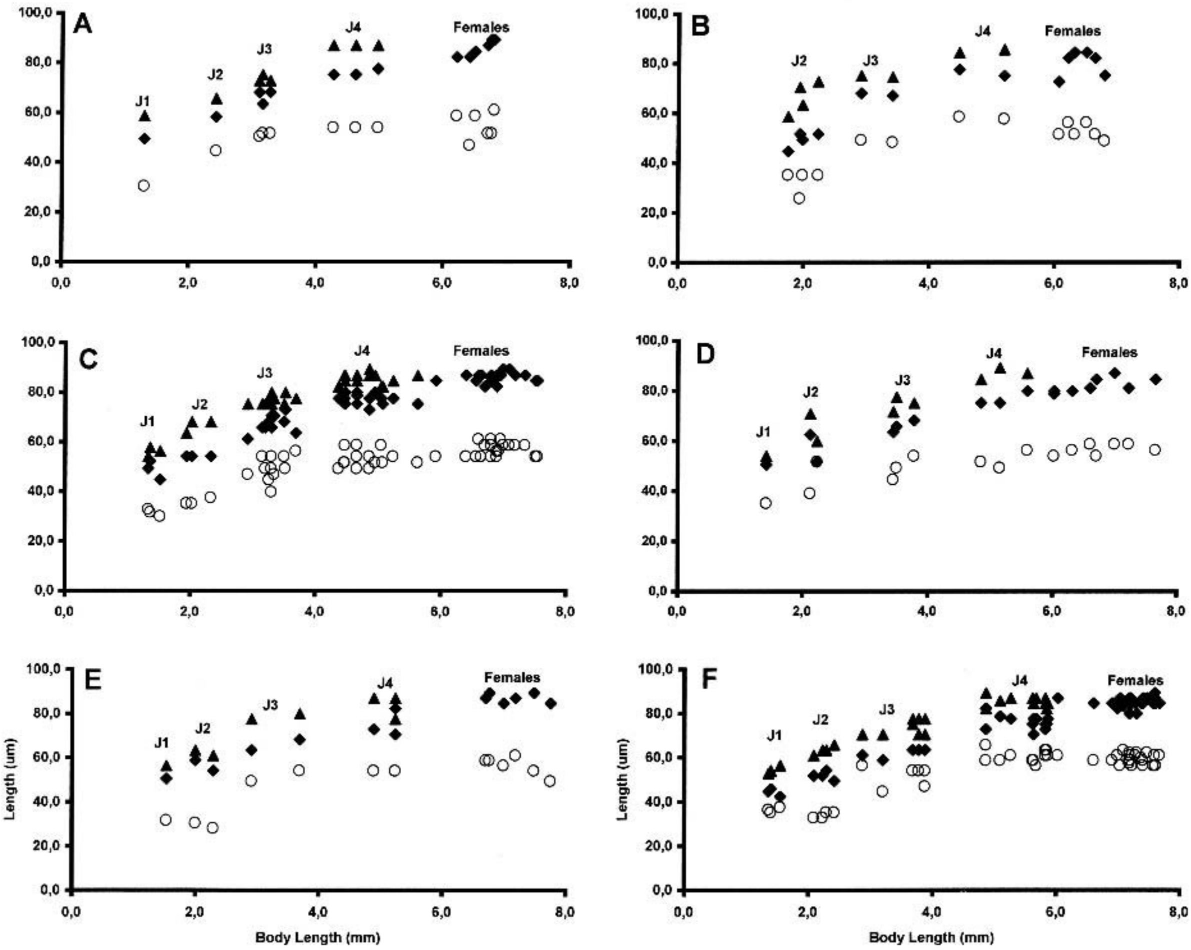

Fig. 1. Scatter diagrams separating juveniles and females of *L. euonymus* from the following Serbian populations: A) Kanjiza; B) Knjazavec; C) Loznica; D) Nis; E) Subotica and F) Vrtogos. J1 = 1st JDS; J2 = 2nd JDS; J3 = 3rd JDS and J4 = 4th JDS

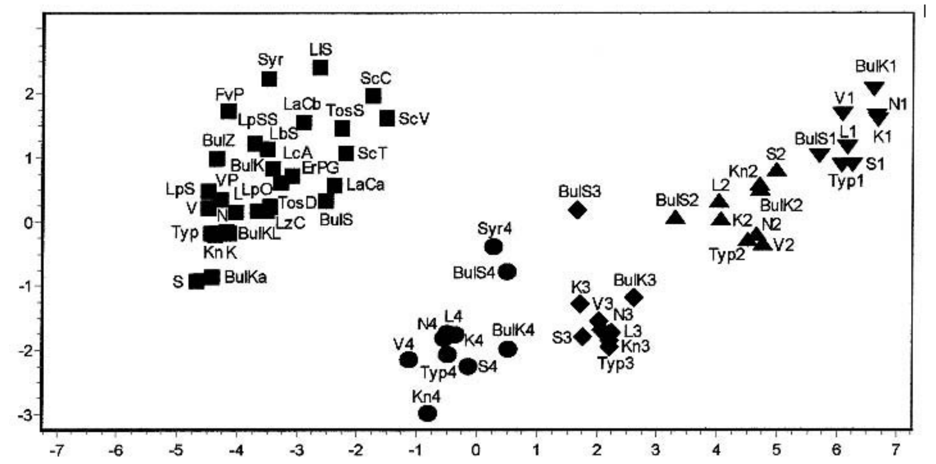

Fig. 2. Principal component analysis of 18 morphometric characters from 33 *L. euonymus* populations. Population codes are listed in Table 1.

■ Adult females; ● 4th JDS; ◆ 3rd JDS; ▲ 2nd JDS; ▼ 1st JDS

Table 3. Morphometrics of *L. euonymus* from Serbia (Kanjiza and Knjazevac populations). All measurements in µm except L.

Locality Host n	Kanjiza Rose 6♀	1 J_1	1 J_2	3 J_3	3 J_4	Knjazevac Quince 6♀	J_1	4 J_2	2 J_3	2 J_4
L (mm)	6.56 ± 0.23 (6.21 – 6.80)	1.30	2.43	3.18 ± 0.09 (3.11 – 3.28)	4.62 ± 0.35 (4.27 – 4.97)	6.42 ± 0.27 (6.06 – 6.79)	-	1.98 ± 0.20 (1.75 – 2.23)	2.91, 3.41	4.77, 5.19
a	144.7 ± 6.15 (137.8 – 151.5)	69.1	86.2	104.3 ± 2.89 (102.0 – 107.5)	131.7 ± 7.7 (124.4 – 139.8)	144.9 ± 9.56 (136.3 – 162.7)	-	74.9 ± 6.22 (67.7 – 82.3)	95.3, 101.6	122.7, 146.[illegible]
b	16.5 ± 1.40 (13.9 – 17.6)	7.9	8.3	10.3 ± 1.15 (9.1 – 11.3)	13.0 ± 0.22 (12.8 – 13.2)	15.1 ± 0.63 (13.9 – 15.5)	-	8.9 ± 0.93 (7.5 – 9.6)	9.6, 9.3	12.4, 12.8
c	154.9 ± 16.6 (129.9 – 170.2)	27.7	49.2	67.2 ± 2.14 (65.6 – 69.7)	98.3 ± 7.45 (90.9 – 105.7)	156.1 ± 18.6 (131.7 – 180.5)	-	45.1 ± 9.32 (35.5 – 55.8)	53.8, 63.1	82.7, 107.[illegible]
c'	1.13 ± 0.14 (1.00 – 1.40)	3.33	2.33	2.17 ± 0.26 (2.00 – 2.47)	1.50 ± 0.06 (1.43 – 1.54)	1.19 ± 0.13 (1.06 – 1.40)	-	3.00 ± 0.41 (2.62 – 3.57)	2.42, 2.07	1.62, 1.77
V %	50.7 ± 0.9 (49.3 – 51.6)	-	-	-	-	49.9 ± 1.24 (49.0 – 52.3)	-	-	-	-
Odontostyle	85.8 ± 3.24 (82.3 – 89.3)	49.3	47.0	66.6 ± 2.71 (63.5 – 68.1)	76.0 ± 1.36 (75.2 – 77.5)	80.3 ± 5.03 (72.8 – 84.6)	-	49.3 ± 3.30 (44.7 – 51.7)	68.1, 67.1	75.2, 77.5
Odontophore	54.8 ± 5.49 (47.0 – 61.1)	30.5	44.7	51.3 ± 0.68 (50.5 – 51.7)	54.1 ± 0.00	52.8 ± 2.97 (49.0 – 56.4)	-	32.9 ± 4.70 (25.9 – 35.3)	49.3, 48.5	57.9, 58.7
Replacement odontostyle	-	58.7	58.7	73.6 ± 1.36 (72.9 – 75.2)	86.9 ± 0.00	-	-	66.4 ± 6.50 (58.7 – 72.9)	75.2, 74.6	84.6, 85.8
Oral aperture to guide ring	25.7 ± 0.48 (24.7 – 25.9)	16.5	18.8	22.7 ± 0.68 (22.3 – 23.5)	24.3 ± 1.36 (23.5 – 25.9)	29.8 ± 3.21 (27.0 – 35.4)	-	21.4 ± 1.11 (20.0 – 22.3)	25.9, 22.4	22.3, 23.5
Tail	42.7 ± 3.76 (39.9 – 49.3)	47.0	49.3	47.7 ± 2.45 (44.7 – 49.3)	47.0 ± 0.00	41.8 ± 5.71 (33.6 – 49.3)	-	53.3 ± 4.64 (49.3 – 58.3)	54.1, 54.1	48.5, 54.1
J (hyaline portion of tail)	13.1 ± 1.56 (11.7 – 15.3)	7.1	7.1	11.7 ± 0.00	11.4 ± 0.68 (10.6 – 11.7)	14.0 ± 0.64 (12.9 – 14.9)	-	5.0 ± 1.51 (3.5 – 7.1)	7.1, 14.1	13.1, 15.3
Body diam. at lip region	14.4 ± 0.48 (14.1 – 15.3)	9.4	11.7	12.1 ± 0.68 (11.7 – 12.9)	13.6 ± 0.62 (12.9 – 14.1)	14.6 ± 0.94 (13.1 – 15.7)	-	10.9 ± 1.10 (9.4 – 11.7)	11.0, 11.0	14.1, 14.1
Body diam. at guide ring	18.2 ± 0.98 (16.5 – 18.8)	12.9	14.1	16.8 ± 0.68 (16.5 – 17.6)	16.1 ± 1.79 (14.1 – 17.6)	18.8 ± 1.40 (16.8 – 21.1)	-	15.6 ± 1.15 (14.1 – 16.5)	16.5, 17.2	16.5, 20.5
Body diam. at base of oesophagus	38.6 ± 1.88 (37.6 – 42.3)	14.1	25.9	29.0 ± 1.36 (28.2 – 30.5)	33.7 ± 3.59 (30.5 – 37.6)	37.7 ± 2.64 (34.5 – 41.1)	-	24.7 ± 2.35 (23.5 – 28.2)	29.4, 29.8	31.7, 32.9
Body diam. at mid-body or vulva	45.8 ± 2.46 (42.3 – 49.3)	18.8	28.2	30.5 ± 0.00	35.3 ± 4.70 (30.5 – 39.9)	44.5 ± 3.56 (39.9 – 49.0)	-	26.5 ± 2.93 (23.5 – 30.5)	30.5, 33.6	35.4, 36.4
Body diam. at anus	37.8 ± 2.16 (35.3 – 39.9)	14.1	21.1	21.9 ± 1.79 (20.0 – 23.5)	30.5 ± 2.35 (28.2 – 32.9)	34.9 ± 1.83 (31.7 – 36.7)	-	17.9 ± 1.19 (16.3 – 18.8)	22.3, 26.1	29.8, 30.5
Body diam. at beginning of J	19.6 ± 5.29 (14.1 – 25.9)	4.7	4.7	9.4 ± 0.00	12.5 ± 1.36 (11.7 – 14.1)	23.8 ± 2.26 (19.7 – 25.9)	-	3.8 ± 0.60 (3.5 – 4.7)	4.7, 7.1	14.1, 16.5

Table 4. Morphometrics of *L. euonymus* from Serbia (Loznica and Nis populations). All measurements in μm except L.

Locality	Loznica					Nis				
Host	Poplar					Cherry				
n	22♀	3 J_1	3 J_2	11 J_3	15 J_4	8♀	1 J_1	2 J_2	3 J_3	3 J_4
L (mm)	6.90 ± 0.37	1.40 ± 0.10	2.13 ± 0.26	3.31 ± 0.22	4.82 ± 0.34	6.69 ± 0.58	1.42	2.23, 2.12	3.58 ± 0.18	5.20 ± 0.37
	(5.92 – 7.57)	(1.33 – 1.52)	(1.94 – 2.43)	(2.82 – 3.69)	(4.37 – 5.63)	(6.02 – 7.65)			(3.45 – 3.78)	(4.85 – 5.59)
a	164.9 ± 6.12	71.3 ± 4.90	80.0 ± 4.30	104.1 ± 3.68	133.1 ± 6.52	148.9 ± 11.23	71.1	86.3, 73.0	113.0 ± 9.4	144.6 ± 8.3
	(152.3 – 173.5)	(66.3 – 76.1)	(75.1 – 82.7)	(99.1 – 109.9)	(124.0 – 147.7)	(134.8 – 171.3)			(106.4 – 123.7)	(137.6 – 153.5)
b	17.3 ± 1.09	5.5 ± 0.53	8.3 ± 1.46	8.8 ± 0.59	12.3 ± 0.85	16.1 ± 1.51	6.7	6.4, 7.1	9.9 ± 0.91	13.2 ± 1.06
	(15.1 – 19.1)	(5.2 – 6.1)	(6.7 – 9.5)	(7.6 – 9.5)	(11.2 – 14.5)	(14.8 – 19.2)			(9.0 – 10.8)	(12.5 – 14.4)
c	163.4 ± 10.5	29.9 ± 3.08	45.1 ± 3.44	63.9 ± 6.28	101.1 ± 7.41	156.7 ± 16.51	29.5	43.1, 39.2	64.9 ± 5.2	102.7 ± 8.2
	(143.3 – 183.7)	(28.1 – 33.5)	(41.1 – 47.2)	(52.2 – 74.8)	(90.6 – 117.4)	(140.4 – 180.9)			(60.0 – 69.9)	(95.3 – 111.5)
c’	1.22 ± 0.11	3.09 ± 0.30	2.42 ± 0.22	2.22 ± 0.24	1.61 ± 0.13	1.19 ± 0.15	2.93	2.75, 2.64	2.28 ± 0.21	1.60 ± 0.11
	(0.94 – 1.37)	(2.74 – 3.30)	(2.19 – 2.63)	(1.91 – 2.70)	(1.33 – 1.75)	(1.00 – 1.43)			(2.09 – 2.50)	(1.48 – 1.68)
V %	50.8 ± 1.3	–	–	–	–	50.8 ± 1.06	–	–	–	–
	(47.9 – 52.8)					(49.2 – 52.2)				
Odontostyle	85.7 ± 2.00	48.7 ± 3.82	54.1 ± 0.00	67.1 ± 3.38	77.2 ± 2.07	82.1 ± 2.89	50.5	51.7, 62.5	65.8 ± 2.35	76.8 ± 2.71
	(82.3 – 89.3)	(44.7 – 52.2)		(61.1 – 72.9)	(72.9 – 79.9)	(78.8 – 86.9)			(63.5 – 68.1)	(75.2 – 79.9)
Odontophore	56.8 ± 2.87	31.7 ± 1.18	36.0 ± 1.36	49.6 ± 4.87	53.3 ± 3.28	56.4 ± 2.18	35.3	51.7, 39.2	49.3 ± 4.70	52.5 ± 3.59
	(51.7 – 61.1)	(30.5 – 32.9)	(35.3 – 37.6)	(39.9 – 56.4)	(49.3 – 58.7)	(54.1 – 58.7)			(44.7 – 54.1)	(49.3 – 56.4)
Replacement odontostyle	–	56.1 ± 1.90	66.6 ± 2.71	76.7 ± 1.90	85.5 ± 2.14	–	54.1	59.9, 70.9	74.8 ± 2.95	86.9 ± 2.35
		(54.1 – 57.8)	(63.5 – 68.1)	(75.2 – 79.9)	(82.3 – 89.3)				(71.7 – 77.5)	(84.6 – 89.3)
Oral aperture to guide ring	26.0 ± 0.87	17.3 ± 1.27	19.3 ± 0.39	20.8 ± 0.55	23.9 ± 0.73	29.8 ± 3.01	16.5	21.1, 19.6	22.3 ± 1.18	25.1 ± 1.79
	(24.7 – 28.2)	(16.5 – 18.8)	(18.8 – 19.5)	(20.0 – 21.1)	(22.3 – 24.7)	(24.7 – 35.3)			(21.1 – 23.5)	(23.5 – 27.0)
Tail	42.5 ± 2.67	46.9 ± 1.57	47.4 ± 5.56	53.2 ± 2.41	47.8 ± 3.31	42.9 ± 3.72	48.2	51.7, 54.1	55.2 ± 3.11	50.9 ± 6.47
	(39.9 – 49.7)	(45.4 – 48.5)	(41.1 – 51.7)	(49.3 – 58.7)	(42.3 – 56.4)	(37.6 – 47.0)			(52.9 – 58.7)	(43.5 – 55.2)
J (hyaline portion of tail)	12.9 ± 1.17	8.2 ± 1.18	7.1 ± 2.35	11.6 ± 1.70	11.5 ± 1.25	12.5 ± 2.26	5.9	7.1, 7.5	10.2 ± 0.68	10.2 ± 1.36
	(10.6 – 14.1)	(7.1 – 9.4)	(4.7 – 9.4)	(9.4 – 14.1)	(9.4 – 15.3)	(9.4 – 15.3)			(9.4 – 10.6)	(9.4 – 11.7)
Body diam. at lip region	14.3 ± 0.46	9.3 ± 0.26	11.7 ± 0.00	12.0 ± 0.47	13.9 ± 0.41	14.7 ± 0.86	9.4	9.4, 9.5	11.7 ± 0.00	12.5 ± 0.68
	(14.1 – 15.3)	(8.9 – 9.4)		(11.7 – 12.9)	(12.9 – 14.1)	(14.1 – 16.5)				(11.7 – 12.9)
Body diam. at guide ring	18.7 ± 0.88	13.6 ± 0.82	16.1 ± 0.68	16.1 ± 0.92	18.0 ± 0.72	19.1 ± 1.04	11.7	15.3, 13.1	15.7 ± 1.36	17.6 ± 1.18
	(16.5 – 20.0)	(12.7 – 14.1)	(15.3 – 16.5)	(14.1 – 17.6)	(16.5 – 18.8)	(17.6 – 21.1)			(14.1 – 16.5)	(16.5 – 18.8)
Body diam. at base of oesophagus	34.7 ± 1.94	19.1 ± 0.73	26.5 ± 2.65	27.8 ± 1.90	31.5 ± 1.78	37.9 ± 2.33	18.8	25.9, 24.3	29.4 ± 1.18	33.3 ± 2.45
	(30.5 – 38.8)	(18.7 – 20.0)	(23.5 – 28.5)	(23.5 – 30.5)	(29.4 – 35.3)	(35.3 – 42.3)			(28.2 – 30.5)	(30.5 – 35.3)
Body diam. at mid-body or vulva	41.9 ± 2.58	19.7 ± 0.99	26.6 ± 2.45	31.8 ± 2.09	36.3 ± 3.17	43.9 ± 3.01	16.5	25.9, 29.0	31.7 ± 1.18	39.6 ± 6.47
	(37.6 – 47.0)	(18.6 – 20.5)	(24.7 – 29.4)	(28.2 – 35.3)	(32.9 – 44.7)	(38.8 – 49.3)			(30.5 – 32.9)	(35.3 – 47.0)
Body diam. at anus	33.9 ± 2.53	15.3 ± 2.09	19.6 ± 1.36	23.3 ± 1.47	30.5 ± 2.46	36.3 ± 2.91	9.4	18.8, 20.5	24.3 ± 1.36	31.7 ± 2.03
	(31.7 – 43.0)	(14.1 – 17.7)	(18.8 – 21.1)	(20.0 – 25.9)	(28.2 – 35.3)	(32.9 – 39.9)			(23.5 – 25.9)	(29.4 – 32.9)
Body diam. at beginning of J	17.6 ± 2.02	5.6 ± 1.59	6.3 ± 0.68	9.2 ± 0.88	12.4 ± 1.59	21.1 ± 2.18	5.9	3.5, 7.5	9.9 ± 2.53	13.3 ± 1.36
	(15.3 – 21.1)	(4.7 – 7.5)	(5.9 – 7.1)	(7.1 – 10.6)	(9.4 – 14.1)	(18.8 – 23.5)			(7.1 – 11.7)	(11.7 – 14.1)

Table 5. Morphometrics of *L. euonymus* from Serbia (Subotica and Vrtogos populations). All measurements in µm except L.

Locality	Subotica					Vrtogos				
Host	Poplar					Apple				
n	6♀	1 J_1	2 J_2	2 J_3	3 J_4	25♀	3 J_1	4 J_2	8 J_3	12 J_4
L (mm)	7.14 ± 0.41 (6.70 – 7.75)	1.53	2.29, 2.00	3.69, 2.91	5.12 ± 0.35 (4.89 – 5.24)	7.24 ± 0.36 (6.02 – 7.67)	1.44 ± 0.10 (1.36 – 1.55)	2.26 ± 0.14 (2.09 – 2.42)	3.59 ± 0.36 (2.87 – 3.88)	5.50 ± 0.38 (4.86 – 5.86)
a	148.1 ± 14.4 (131.9 – 167.5)	76.6	81.2, 85.1	87.2, 95.3	121.6 ± 7.3 (117.4 – 130.1)	163.9 ± 7.47 (145.5 – 172.2)	73.3 ± 3.85 (70.1 – 77.6)	84.3 ± 8.0 (73.6 – 92.8)	110.8 ± 4.46 (104.4 – 136.2)	138.7 ± 7.0 (127.4 – 155.3)
b	16.4 ± 1.57 (14.3 – 18.9)	6.9	10.7, 7.6	10.6, 10.0	13.4 ± 0.80 (12.6 – 14.2)	15.9 ± 1.52 (13.6 – 18.3)	7.1 ± 0.77 (6.5 – 8.0)	7.1 ± 0.52 (6.7 – 7.9)	9.8 ± 0.39 (9.2 – 10.3)	14.0 ± 1.24 (11.4 – 15.1)
c	155.1 ± 12.3 (138.9 – 173.6)	30.3	51.3, 37.0	68.3, 59.0	97.6 ± 3.44 (94.6 – 101.3)	171.4 ± 13.6 (144.0 – 197.8)	32.9 ± 1.50 (31.3 – 34.3)	41.7 ± 2.78 (39.5 – 45.3)	66.7 ± 3.34 (61.1 – 69.6)	109.5 ± 5.80 (96.3 – 115.2)
c'	1.26 ± 0.17 (1.12 – 1.58)	3.39	2.37, 3.07	3.28, 2.10	1.77 ± 0.38 (1.53 – 2.20)	1.18 ± 0.16 (0.96 – 1.72)	3.18 ± 0.02 (3.17 – 3.20)	2.87 ± 0.34 (2.40 – 3.21)	2.18 ± 0.11 (1.95 – 2.22)	1.52 ± 0.11 (1.33 – 1.79)
V %	49.4 ± 0.87 (48.6 – 50.7)	–	–	–	–	49.3 ± 1.60 (46.3 – 52.8)	–	–	–	–
Odontostyle	86.9 ± 2.10 (84.6 – 89.3)	50.5	54.1, 58.7	68.1, 63.5	75.2 ± 6.22 (70.5 – 82.3)	84.9 ± 2.13 (79.9 – 89.3)	44.3 ± 1.79 (42.3 – 45.8)	51.7 ± 1.92 (49.3 – 54.1)	62.6 ± 1.75 (58.7 – 63.5)	76.1 ± 1.30 (70.5 – 82.3)
Odontophore	56.4 ± 4.20 (49.3 – 61.1)	31.7	28.2, 30.5	54.1, 49.3	54.1 ± 0.00	59.5 ± 2.25 (56.4 – 63.5)	36.4 ± 1.18 (35.3 – 37.6)	34.1 ± 1.36 (32.9 – 35.3)	52.3 ± 4.1 (44.7 – 56.4)	60.5 ± 2.67 (56.4 – 65.8)
Replacement odontostyle	–	56.4	61.1, 63.5	79.9, 77.5	83.8 ± 5.43 (77.5 – 86.9)	–	54.4 ± 1.79 (52.9 – 56.4)	63.5 ± 1.92 (61.1 – 65.8)	73.7 ± 3.54 (70.5 – 77.5)	85.5 ± 2.07 (82.3 – 89.3)
Oral aperture to guide ring	27.4 ± 1.04 (25.6 – 28.2)	16.5	22.3, 17.6	25.9, 24.7	26.7 ± 2.44 (24.7 – 29.4)	27.3 ± 1.30 (25.9 – 30.1)	16.8 ± 0.68 (16.5 – 17.6)	20.0 ± 1.66 (18.8 – 22.3)	22.5 ± 1.17 (21.1 – 24.7)	25.0 ± 1.13 (23.5 – 27.0)
Tail	46.2 ± 3.54 (42.3 – 51.7)	50.5	44.7, 54.1	54.1, 49.3	52.5 ± 1.36 (51.7 – 54.1)	42.4 ± 2.87 (37.6 – 47.0)	43.6 ± 2.18 (41.1 – 45.1)	54.1 ± 2.88 (50.5 – 56.4)	53.8 ± 3.22 (47.0 – 56.6)	50.2 ± 2.12 (47.0 – 52.9)
J (hyaline portion of tail)	14.9 ± 1.21 (14.1 – 16.5)	8.2	7.1, 8.2	9.4, 10.6	12.1 ± 0.68 (11.7 – 12.9)	11.4 ± 0.99 (9.4 – 12.9)	7.2 ± 0.27 (7.1 – 7.5)	9.1 ± 2.01 (7.1 – 11.7)	9.5 ± 1.32 (8.2 – 11.7)	10.7 ± 1.84 (9.4 – 14.1)
Body diam. at lip region	14.5 ± 1.22 (12.9 – 16.5)	9.4	9.4, 9.4	14.1, 11.7	11.7 ± 0.00	14.3 ± 0.40 (14.1 – 15.3)	9.4 ± 0.00	10.0 ± 1.18 (9.4 – 11.7)	11.7 ± 0.63 (10.6 – 12.9)	13.2 ± 0.89 (11.7 – 14.1)
Body diam. at guide ring	19.2 ± 1.22 (17.6 – 21.1)	12.9	14.1, 14.1	18.8, 18.8	16.5 ± 0.00	18.7 ± 0.62 (17.6 – 20.0)	13.7 ± 1.80 (11.7 – 15.3)	15.0 ± 1.13 (14.1 – 16.5)	15.9 ± 0.89 (14.1 – 16.5)	16.8 ± 0.76 (16.5 – 18.8)
Body diam. at base of oesophagus	35.5 ± 4.05 (28.2 – 39.9)	18.8	27.0, 22.3	30.5, 28.2	29.8 ± 3.59 (25.9 – 32.9)	36.6 ± 2.28 (32.9 – 42.3)	18.8 ± 0.00	23.8 ± 3.88 (18.8 – 28.2)	28.8 ± 2.43 (24.7 – 31.7)	34.0 ± 2.03 (30.5 – 37.6)
Body diam. at mid-body or vulva	49.2 ± 6.66 (42.3 – 58.7)	20.0	28.2, 23.5	42.3, 30.5	42.3 ± 4.07 (37.6 – 44.7)	44.3 ± 2.6 (38.8 – 49.3)	19.6 ± 0.68 (18.8 – 20.0)	27.0 ± 3.95 (24.7 – 32.9)	32.5 ± 3.20 (25.9 – 35.3)	39.7 ± 2.41 (35.3 – 42.3)
Body diam. at anus	37.0 ± 4.80 (28.2 – 41.1)	14.9	18.8, 17.6	16.5, 23.5	30.5 ± 6.22 (23.5 – 35.3)	36.3 ± 3.34 (25.9 – 41.1)	14.0 ± 1.00 (12.9 – 14.9)	19.1 ± 3.09 (16.5 – 23.5)	24.7 ± 1.78 (21.1 – 25.9)	33.5 ± 1.77 (30.5 – 35.3)
Body diam. at beginning of J	24.3 ± 2.43 (21.1 – 28.2)	5.9	3.5, 5.9	9.4, 7.1	12.9 ± 3.53 (9.4 – 16.5)	23.2 ± 2.99 (16.5 – 28.2)	4.7 ± 0.00	7.1 ± 2.13 (4.7 – 9.4)	9.1 ± 1.51 (7.1 – 11.7)	16.3 ± 1.84 (14.1 – 18.8)

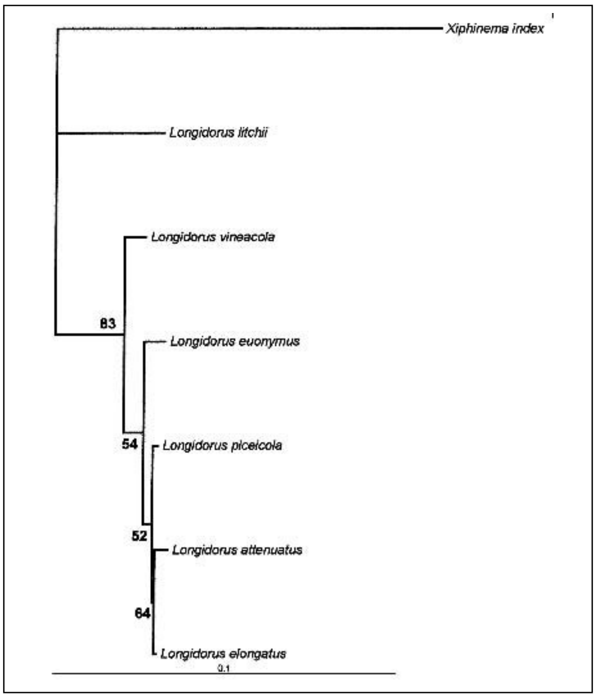

Fig. 3. A maximum likelihood tree based on sequences of 18S rDNA showing relationships between *Longidorus euonymus* and European *Longidorus* species known to be the most taxonomically similar (Mali and Hooper, 1974). Numbers indicate the bootstrap values > 50. Branch lengths are drawn proportional to the number of changes inferred

populations the second JDS of the Sandanski population has a longer body, odontostyle and odontophore, shorter hyaline portion of the tail (J) but is wider at both the anus and beginning of J. Also, the third JDS differs by having a shorter odontophore, tail and J, the body width at J is greater, the values of b, c are greater, but c' is less. The fourth JDS has a shorter body, odontostyle, odontophore, replacement odontostyle, distance anterior to guide ring and J, smaller a and c values and is narrower at J. The fourth JDS from the Idleb population has a shorter tail and J and is narrower at both the lip region and anus. However, adult females from the Sandanski and Idleb populations, although distinct from the Serbian populations, are not disparate from the other populations (Fig. 2).

Based on morphometrics available for females from the 33

Table 6. Morphometrics of male *L. euonymus* from Loznica (this study) and from the original description (Roca, 1991)

Locality	Avellino, Italy	Loznica, Serbia
n	1	1
L (mm)	5.50	6.23
a	145.4	165.7
b	14.7	14.3
c	129.5	132.5
c'	1.40	1.33
Odontostyle	88.8	86.9
Odontophore	50.5	54.1
Oral aperture to guide ring	27.0	27.0
Tail	43.0	47.0
J (hyaline portion of tail)	8.5	11.7
Body diam. at lip region	13.5	14.1
Body diam. at guide ring	19.0	18.8
Body diam. at base of oesophagus	33.5	35.3
Body diam. at mid-body or vulva	38.5	37.6
Body diam. at anus	31.5	35.3
Body diam. at beginning of J	12.5	14.1
Spicule length	56.5	50.4

populations minimal biogeographical clustering of populations is evident. For example, at a regional scale, the Italian and the Serbian populations of *L. euonymus* do not form population clusters (Fig. 2). At the Pan-European scale, populations from Serbia (K, Kn, L, N, S, V and VP; see Table 1 for explanation of codes) appear more closely associated with one another than with populations from either Bulgaria or Italy.

Analysis of 18S rDNA sequence data revealed that of the *Longidorus* species investigated, *L. euonymus* was most similar to the European species than the single Asian species, *L. litchii* (Fig. 3). Amongst the European species studied, *L. euonymus* was most similar to *L. piceicola* and *L. elongatus* and least similar to *L. vineacola* (Table 7).

Discussion

The principal morphometric characters recorded from female specimens of the six Serbian populations examined in this study: L, odontostyle length, odontophore length, V %, distance anterior to guide ring, and tail length, were each within the range reported for females from the type popu-

Table 7. Maximum likelihood distances of *Longidorus* species used in this study

	Lelong	Lpicei	Latten	Leuony	Lvinea	Llitch
Lelong	0.000					
Lpicei	0.0023	0.000				
Latten	0.0035	0.0046	0.000			
Leuony	0.0076	0.0075	0.0080	0.000		
Lvinea	0.0094	0.0100	0.0107	0.0112	0.000	
Llitch	0.0252	0.0251	0.0253	0.0245	0.0246	0.000

Codes used in table: Lelong = *L.elongatus*; Lpicei = *L. piceicola*; Latten = *L. attenuatus*; Leuony = *L. euonymus*; Lvinea = *L. vineacola*; Llitch = *L. litchii* lation by Mali and Hooper (1974). One minor exception was that females from the Knjazevac population had a slightly shorter odontostyle (72.8 – 84.6 vs. 81 – 90 µm). Similarly, with the four JDS from each of the Serbian populations examined in this study the morphometric values for lengths of total body, odontostyle, odontophore and replacement odontostyle were within the range recorded for the JDS of the type population (Mali & Hooper, 1974). Thus, the morphometrics of each of the six populations concurred with the amended polytomous species code (Chen *et al.*, 1997; Lamberti *et al.*, 1997).

Until recently, only one male *L. euonymus* specimen had been recorded (Roca, 1991). The male collected at the Loznica site in this study, as compared to the male reported from Avellino, Italy (Roca, 1991), was longer (6.23 v 5.50 mm), had a larger *a* value (166 v 145), a slightly longer tail (47.0 v 43.0 µm), longer hyaline portion of the tail (11.7 v 8.5 µm), was wider at the anus (35.3 v 31.5 µm), and had shorter spicules (50.4 v 56.5 µm). These minor differences concur with those reported by Barsi and Lamberti (2003) who collected two males from Novi Sad in northern Serbia.

Nematodes typically have four pre-adult stages (Maggenti, 1981), although a few longidorids have been reported with only three JDS (Halbrendt & Brown, 1992; Halbrendt *et al.*, 1997). Lamberti *et al.* (1997) reported that *L. euonymus* had only three JDS. This was based on the fact that in the two populations used in that study, scatter diagrams comparing the lengths of odontostyle, odontophore and replacement odontostyle with body length indicated that two sub-groups with similar odontostyle lengths occurred between the first and pre-adult JDS, but that these were distinguishable by body length. However, Barsi and Lamberti (2003) cite the Lamberti *et al.* (1997) dataset as having four JDS, even though the original article clearly states in the abstract that *L. euonymus* had three JDS. Conversely, our PCA analysis in which extensive morphometric data were used from 33 populations and that included both populations from Lamberti *et al.* (1997) (denoted here as BulK and BulS), clearly revealed that *L. euonymus*, as with the majority of *Longidorus* species (Robbins *et al.*, 1995), has four JDS. Thus, our data confirms the original description by Mali and Hooper (1974) and concurs with the single populations reported by Lamberti *et al.* (1999) and Barsi and Lamberti (2003). Although the data from this study and Lamberti *et al.* (1997) are apparently discordant, it may be due to morphological plasticity as a result of environmental factors (Brown, 1985; Arpin *et al.*, 1988; Doucet *et al.*, 1996; Arpin, 2001). This has been previously reported for predatory (Arpin, 2001) and plant-parasitic nematodes including *L. leptocephalus* (Yeates, 1978; Lišková and Brown, 1995). The relative change in body volume between each JDS averaged across all six Serbian populations of *L. euonymus* was similar to that reported for the type population by Yeates and Boag (2002), with the exception of the change between 1 and 2 JDS which was greater (3.0 *vs* 2.2). Similarly, the change between 1JDS and adult was greater (25.6 *vs* 19.0) to that reported by Yeates and Boag (2002).

18S rDNA sequences have been used to identify taxonomic relationships within Nematoda at the resolution of family and genus (Aleshin *et al.*, 1998; Blaxter *et al.*, 1998), molecular operating taxonomic unit (Floyd *et al.*, 2002) and more recently at the species level (De Ley *et al.*, 2002; Hübschen *et al.*, 2002; Oliveira *et al.*, 2004a,b). In this study, 18S rDNA sequence analysis indicated that *L. euonymus* was most similar to European than to the single Asian longidorid species studied. Also, that *L. euonymus* was more closely related to *L. elongatus* and *L. attenuatus* than to *L. vineacola*. This is in discordance with that suggested by Mali and Hooper (1974), however, the distances in the maximum likelihood tree between the species are relatively small. In the original species diagnosis, *L. euonymus* was described as being "most similar" to *L. closelongatus* and *L. cohni*, unfortunately, specimens of these species were not available to make a full comparison of the 18S rDNA sequence data. The sequence data presented here support Brown *et al.* (1997) who noted, using a canonical variate analysis of 46 populations based on five morphometric characters, that within the "*L. vineacola* species complex", *L. attenuatus* was most similar to *L. vineacola*. *Longidorus piceicola* that currently has only been recorded in Slovakia and Yugoslavia (Lišková *et al.*, 1997; Barsi & Lamberti, 2001), although morphologically and morphometrically distinct from *L. euonymus*, is closely related with regard to 18S sequence data.

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