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# Morphological and molecular characterization of six *Longidorus* species (Nematoda: Longidoridae) from Slovenia

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**Summary.** Six species of *Longidorus* were recovered from the rhizosphere of vineyards and orchards from Slovenia between 2003 and 2008. These species were: *Longidorus juvenilis, L. leptocephalus, L. caespiticola, L. elongatus, L. moesicus* and *L. helveticus*. The occurrence of *L. moesicus* and *L. helveticus* is reported in Slovenia for the first time. Nematodes were identified using morphological characters of females. Slovenian populations of six *Longidorus* species were morphometrically identified. In addition, the morphometric data obtained were compared with data from the literature using software Scramble 2.2. Species identification was further confirmed by molecular analyses of the 28S rRNA gene D2/D3 expansion region sequences. Sequences were compared with the sequences deposited in the NCBI database. Phylogenetic analysis was used to confirm and support identification of the reported species. The sequences of *Longidorus* species from Slovenia and sequences of the same species from the databank clustered together with high bootstrap support. In addition, RFLP method was applied to distinguish *Longidorus* species found in Slovenia. The importance of publishing morphometric data of various nematode populations from different countries is discussed.

**Key words:** D2D3 rDNA, DNA sequencing, multivariate morphometrical analysis, Nematoda, vineyards, orchards, identification, morphology, RFLP.

Needle nematodes, *Longidorus* spp. Micoletzky (Filipjev), are an important group of plant parasites that cause serious damage to many host plant species. They are easily recognized from most other Dorylaims by their long (2 – 12 mm) slender body and their elongated needle-like spear (odontostyle) plus a non-flanged extension (odontophore) about half the odontostyle length (Hooper, 1974). Symptoms associated with *Longidorus* generally are non specific. Infection by *Longidorus* generally results in greatly reduced root systems with severely stunted lateral and, sometimes, tap roots (Taylor & Brown, 1997). Besides causing direct feeding damage, some species also transmit plant viruses (Taylor, 1962; Harrison, 1964; Lamberti & Roca, 1987; De Waele & Coomans, 1990). This ability places them in the group of very important plant parasites; therefore, unambiguous identification of *Longidorus* species is essential.

The genus *Longidorus* currently comprises 144 valid species. Species identification is based primarily on morphometrics (body length, tail length, length of odontostyle etc.), but a high degree of variability within morphometrics leads to considerable overlap among species and increases the potential for mis-identification (Ye & Robbins, 2004). In our study, we comprehensively examined morphometrical characters of six *Longidorus* species from Slovenia. The occurrence and geographical distribution of *Longidorus* species in Slovenia was examined between 2003 and 2008. The occurrence of *L. moesicus* and *L. helveticus* is recorded for the first time in Slovenia. Morphometrical data of *Longidorus* species from Slovenia were compared (multivariate morphometrical analysis) with the data from the literature displaying certain intra-specific morphometrical variability, which rendered morphometrical identification difficult.

Molecular techniques and phylogenetic studies can potentially overcome this problem and facilitate species identification. Recently, molecular sequence data of ribosomal DNA (rDNA) of various *Longidorus* species have become available to complement morphological identification (Rubtsova *et al.*, 2001; De Luca *et al.* 2004; Neilson *et al.* 2004; He *et al.* 2005; Kumari *et al.* 2009). Sequences of the D2/D3 expansion region of the large subunit rRNA nuclear gene have been shown to be very useful for Longidoridae phylogenetic

analyses and their species differentiation (He *et al.*, 2005). rDNA sequences of *Longidorus* species found in Slovenia and sequences of the same and closely related species from NCBI databank were used for phylogenetic analysis in order to confirm morphometrical identification. Additionally, a PCR-RFLP approach was adopted to separate all *Lonogidorus* species found in Slovenia. Restriction of the D2/D3 rDNA amplified fragments successfully separated analysed species using five restriction enzymes. The method has a potential in aiding the diagnostics of analysed species.

## MATERIAL AND METHODS

**Nematodes.** Soil samples were taken from the rhizosphere of *Vitis vinifera* L. and *Malus domestica* Borkh from intensively cultivated ground all over Slovenia. The sampling was performed by digging holes beneath host plants and carefully collecting soil around grapevine or apple tree roots at 40-50 cm depth. Nematodes were extracted from the soil samples by whirling motion described by Hržič (1973). Longidorid nematodes were hand picked, killed and fixed in TAF (triethanolamine formaline) solution, examined and measured under a light microscope with image analysing system (Lucia; Nikon, Japan). Species identification based on average morphometric characters of females, including body length, distance from vulva opening to head end, lip width, odontostyle length, body diameter, tail length and diameter etc., was performed using *Longidorus* polytomous key (Chen *et al.*, 1997; Loof & Chen, 1999).

**Multivariate morphometrical analysis.** Measured characters of six *Longidorus* species from Slovenia were compared with data from the literature. Distance matrix based multivariate analysis with nonlinear presentation of the morphometrical data was done with Scramble 2.2 (Tiefenbrunner *et al.*, 2002). The data of 137 *Longidorus* species (Ye & Robbins, 2004) and five *Longidorus* species described after 2004 were included in this analysis, namely *L. kheiri* Pedram *et al.*, 2008, *L. danuvii* Barsi *et al.*, 2007, *L. dimorphicaudatus* Baniyamuddin *et al.*, 2006, *L. cylindricapitatus* Krnjaic *et al.*, 2005 and *L. americanum* Handoo *et al.*, 2005. Nine measured characters were analysed including body length (L), distance of vulva from the anterior end (VL), lip width, odontostyle length, the distance of guide ring from anterior end, oesophagus length, body width, tail length and anal body width.

**DNA extraction and amplification.** DNA was extracted from a single female nematode of each species. Extracted females were transferred into 1.5 ml tube in a 1 µl drop of sterile water. A mixture of 10 µl 1M EDTA pH 8 and 50 µl nucleic lysis solution (Promega Wizard DNA purification kit) was added to each tube and homogenised with a micropestle. Isolation of DNA was done according to the manufacturer's instructions. Isolated DNA was re-suspended in 10 µl of distilled water of which 2 µl was used in each PCR reaction. A fragment of the D2 and D3 expansion region of the 28S rDNA gene was amplified using the primers D2A (5'-ACA AGT ACC GTG AGG GAA AGT TG-3') and D3B (5'-TCG GAA GGA ACC AGC TAC TA-3') (He *et al.*, 2005) as described earlier (Širca *et al.*, 2007).

**RFLP and sequence analyses.** A quantity (8 µl) of PCR product was treated with 5 restriction enzymes: *Alu*I (Fermentas), *Hinf*I (Invitrogen), *Mbo*I (Fermentas), *Mse*I (Invitrogen) and *Rsa*I (Fermentas). The reactions were incubated at 37°C for 2 hours and left overnight in order to obtain complete restriction of PCR fragments. Restricted fragments were separated on 1.5 % agarose gel stained with ethidium bromide.

ABI PRISM 310 DNA Sequencer with BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used for direct sequencing of the PCR products. PCR products were purified using the Quick Jet PCR purification kit (Genomed) and sequenced using D2A and D3B primers (He *et al.*, 2005). Sequences were aligned using ClustalW (Larkin *et al.*, 2007) and clustered with neighbour joining method using MEGA3 software (Kumar *et al.*, 2004). Other sequences from the NCBI database were included in phylogenetic analyses (see species and accession number in Fig. 5).

## RESULTS

The population level of extracted *Longidorus* nematodes was rather low, ranging from 1 – 20 per 100 ml of soil. Four species of *Longidorus* were extracted from vineyard soil samples and two species from apple orchards. They include *L. caespiticola* Hooper, 1961, *L. elongatus* (de Man) Thorne & Swagger (Hooper, 1961), *L. leptocephalus* Hooper, 1961, *L. juvenilis* Dalmasso, 1969, *L. moesicus* Lamberti *et al.*, 1983 and *L. helveticus* Lamberti *et al.*, 2001. Specimens of *L. juvenilis* and *L. leptocephalus* were recovered from soil samples taken from vineyards near Svetinje and Jursinci, respectively (north-eastern part of Slovenia). *Longidorus caespiticola* and *L. elongatus* were extracted from apple orchard soil samples from Brdo near Lukovica (central part of Slovenia) and Maribor (north-eastern part of Slovenia), respectively.

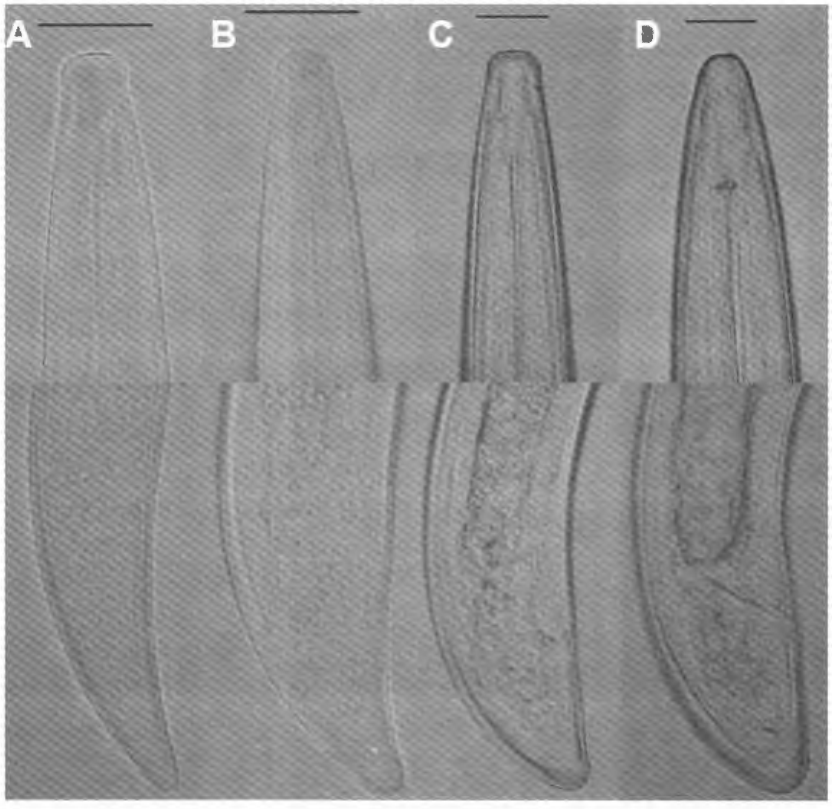


**Fig. 1:** Head and tail region of *L. juvenilis* (A), *L. leptocephalus* (B), *L. elongatus* (C) and *L. moesicus* (D) females from Svetinje, Juršinci, Maribor and Vrhpolje, respectively. Bars = 20µm.

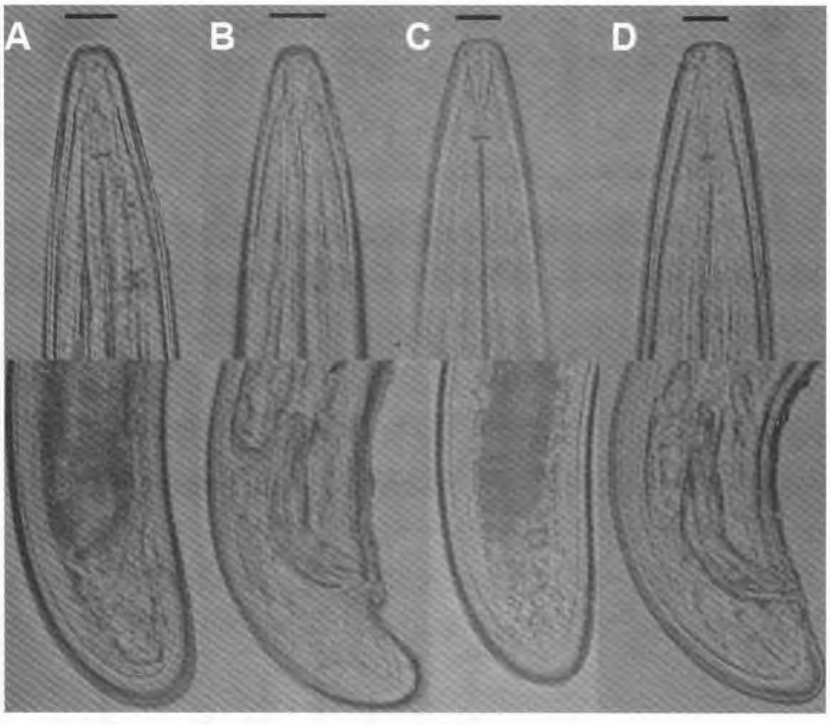


**Fig. 2.** Head and tail region of a female (A) and a male (B) of *L. caespiticola* from Brdo and a female (C) and a male (D) of *L. helveticus* from Trška gora. Bars = 20µm.

**Table 1.** Morphometric data of the six *Longidorus* species from Slovenia. Measurements are in µm.

| Species | *L. caespiticola* | | *L. elongatus* | *L. juvenilis* | *L. leptocephalus* | *L. helveticus* | | *L. moesicus* |
|---|---|---|---|---|---|---|---|---|
| Specimens | ♀ | ♂ | ♀ | ♀ | ♀ | ♀ | ♂ | ♀ |
| n | 16 | 8 | 10 | 16 | 9 | 3 | 3 | 17 |
| L | 7840.7 ± 488.5 (7016.0 – 8642.0) | 7238.3 ± 766.4 (6239.0 - 8455.0) | 4908.5 ± 216.2 (4536.9 - 5246.0) | 3964.2±362.7 3521.4-4718.9 | 4581.9 ± 283.3 (4029.9 – 4878.9) | 7141.7 ± 511.1 (6663.0 - 7680.0) | 7640.0 ± 757.9 (6765.0 – 8090.0) | 6902.4 ± 730.9 (5951.0 – 8397.0) |
| a | 96.0 ± 4.8 (89.4 - 103.6) | 101.0 ± 7.0 (90.4 - 109.8) | 88.9 ± 5.3 (83.7 - 96.4) | 113.6 ± 11.4 (89.6 – 128.8) | 109.5 ± 5.4 (98.8 – 117.1) | 73.2 ± 5.0 (68.7 - 78.5) | 80.4 ± 1.3 (79.6 – 81.9) | 128.6 ± 7.4 (117.0 – 145.3) |
| b | 15.5 ± 1.3 (13.1 - 18.5) | 13.5 ± 1.5 (11.2 - 15.5) | 12.1 ± 0.3 (11.7 - 12.5) | 12.3 ± 1.1 (11.1 – 14.4) | 12.4 ± 0.9 (11.0 – 13.8) | 13.4 ± 0.9 (12.4 - 14.1) | 14.0 ± 1.2 (12.7 – 15.0) | 15.8 ± 1.4 (13.1 – 18.3) |
| c | 176.0 ± 11.9 (159.5 - 205.2) | 152.2 ± 18.9 (131.6 - 195.0) | 113.1 ± 11.3 (94.7 - 126.6) | 74.5 ± 7.5 (63.2 – 90.1) | 101.8 ± 8.6 (88.5 – 116.1) | 176.7 ± 9.7 (167.0 - 186.4) | 159.3 ± 12.2 (147.1 – 171.4) | 164.3 ± 18 (129.0 – 200.8) |
| c' | 0.74 ± 0.03 (0.71 - 0.78) | 1.00 ± 0.16 (0.80 - 1.20) | 1.09 ± 0.07 (1.02 - 1.19) | 2.18 ± 0.19 (1.80 – 2.47) | 1.54 ± 0.15 (1.35 – 1.90) | 0.64 ± 0.05 (0.60 - 0.69) | 0.77 ± 0.06 (0.70 – 0.81) | 1.05 ± 0.08 (0.90 – 1.23) |
| V% | 49.8 ± 1.3 (46.2 - 51.9) | – | 48.7 ± 0.9 (47.2 - 49.9) | 48.1 ± 1.0 (46.9 – 51.0) | 50.7 ± 2.8 (47.8 – 54.6) | 50.4 ± 0.8 (49.6 - 51.2) | – | 52.7 ± 1.4 (49.6 – 54.6) |
| Total stylet length | 165.0 ± 4.8 (157.6 - 176.9) | 165.3 ± 6.2 (157.1 - 175.2) | 141.1 ± 6.3 (132.8 - 147.4) | 99.4 ± 4.3 (03.6 - 110.4) | 107.0 ± 5.0 (100.5 – 117.1) | 198.9 ±5 .2 (193.0 - 202.9) | 214.3 ± 10.0 (204.9 – 224.8) | 155.8 ± 5.9 (143.1 – 161.0) |
| Odontostyle | 102.5 ± 3 .4 (96.1 - 109.3) | 103.8 ± 5.8 (94.5 - 108.7) | 89.9 ± 1 .4 (88.0 - 91.6) | 64.3 ± 1.7 61.1 – 67.7 | 66.2 ± 3.7 (60.9 – 70.9) | 132.7 ± 3.9 (128.5 - 136.2) | 138.9 ± 5.9 (134.6 – 145.6) | 107.1 ± 4.4 (96.3 – 114.6) |
| Odontophore | 62.5 ± 2.4 (58.8 - 67.6) | 61.5 ± 3.6 (55.8 - 67.3) | 50.9 ± 4.8 (44.1 - 56.5) | 35.1 ±3.3 29.8 – 42.7 | 39.8 ± 2.3 (36.8 – 44.2) | 66.2 ± 2.9 (64.5 - 69.5) | 75.4 ± 4.6 (70.3 – 79.2) | 48.7 ± 4.0 (42.0 – 56.4) |
| Oral aperture to guide ring | 39.0 ± 1.6 (36.8 - 42.5) | 38.7 ± 1.5 (36.7 - 40.5) | 30.7 ± 1.4 (28.3 – 32.1) | 24.8 ± 1.0 23.4 – 26.9 | 29.2 ± 1.7 (26.9 – 32.2) | 45.6 ± 0.9 (44.8 - 46.5) | 47.9 ± 2.6 (45.0 – 50.0) | 35.6 ± 1.6 (32.8 – 39.1) |
| Spicule length | – | 87.4 ± 5.1 (77.9 - 94.6) | – | – | – | – | 113.5 ± 3.5 (110.7 – 117.4) | – |
| Body diameter at lip region | 16.8 ± 1.2 (14.5 - 19.4) | 16.5 ± 0.9 (15.1 - 18.2) | 14.2 ± 0.8 (12.9 - 15.3) | 11.6 ± 0.4 (11.2 – 12.5) | 10.2 ± 0.6 (9.8 – 11.6) | 19.5 ± 1.8 (18.4 - 21.5) | 20.4 ± 1.3 (19.5 – 21.9) | 12.4 ± 1.0 (10.5 – 13.9) |
| Body diameter at guide ring | 34.3 ± 2.0 (31.4 - 38.3) | 32.8 ± 1.6 (29.8 - 34.6) | 22.9 ± 0.3 (22.3 - 23.3) | 17.0 ± 0.8 (16.0 – 18.7) | 18.3 ± 0.4 (17.7 – 18.9) | 43.1 ± 0.7 (42.4 - 43.8) | 43.6 ± 2.0 (41.4 – 45.3) | 26.6 ± 1.1 (24.2 – 28.4) |
| Body diameter at base of pharynx | 66.2 ± 4.2 (57.4 - 72.8) | 62.6 ± 3.3 (57.8 - 66.7) | 46.6 ± 1.6 (43.9 - 48.8) | 30.9 ± 1.7 (28.4 - 34.7) | 34.7 ± 0.9 (33.6 – 36.3) | 78.7 ± 6.4 (71.7 - 84.2) | 78.3 ± 5.5 (72.2 – 82.9) | 46.0 ± 2.9 (41.9 – 50.8) |
| Body diameter at vulva | 81.8 ± 5.0 (72.2 - 90.6) | 71.1 ± 6.0 (64.7 - 79.7) | 54.5 ± 2.3 (51.5 - 58.2) | 35.1 ± 3.5 (29.8 – 41.4) | 41.9 ± 1.5 (40.4 – 44.7) | 97.8 ± 8.0 (90.2 - 106.1) | 95.0 ± 8.9 (84.8 – 101.6) | 53.6 ± 3.7 (48.4 – 59.9) |
| Distance from oral aperture to the base of pharynx | 506.8 ± 28.7 (443.6 - 547.0) | 536.7 ± 18.1 (495.9 - 557.0) | 413.0 ± 19.4 (388.4 - 441.2) | 324.4 ± 11.3 (342.4 – 305.6) | 371.1 ± 20.1 (348 – 416.9) | 534.0 ± 14.4 (518.0 - 546.0) | 545.3 ± 19.7 (532.0 – 568.0) | 437.6 ± 27.5 (381.0 – 466.0) |
| Tail length | 44.6 ± 2.4 (39.3 - 50.3) | 48.0 ± 6.2 (39.2 - 56.1) | 43.3 ± 2.5 (40.0 - 47.9) | 53.3 ± 2.6 (47.6 – 56.8) | 45.2 ± 4.0 (39.9 – 54.6) | 40.4 ± 0.7 (39.9 - 41.2) | 47.9 ± 2.4 (46.0 – 50.6) | 42.2 ± 3.8 (36.2 – 49.4) |
| Body diameter at anus | 60.1 ± 3.3 (55.3 - 67.0) | 48.2 ± 5.1 (40.7 - 55.4) | 39.5 ± 1.1 (38.1 - 41.4) | 24.6 ± 1.8 (21.4 – 28.8) | 29.3 ± 0.8 (28.4 – 31.3) | 63.2 ± 5.2 (57.9 - 68.2) | 62.6 ± 5.2 (56.9 – 67.1) | 40.2 ± 3.0 (36.3 – 46.1) |

**Table 2.** Rank list of the neighbour species in relation to analysed *Longidorus* species from Slovenia.
"Next neighbour" species represent the species with the greatest morphometric similarity to the analysed one.

| Analysed species* / RCP** | *L. juvenilis* | *L. leptocephalus* | *L. elongatus* | *L. moesicus* | *L. helveticus* | *L. caespiticola* | *L. caespiticola*** |
|---|---|---|---|---|---|---|---|
| 1 | *L. capetanensis* | *L. bernardi* | *L. athesinus* | *L. apulus* | *L. nevesi* | *L. raskii* | ***L. caespiticola-BE*** |
| 2 | *L. bernardi* | *L. carpetanensis* | *L. sturhani* | *L. dunensis* | ***L. helveticus*** | *L. crataegi* | |
| 3 | ***L. juvenilis*** | *L. sylphus* | *L. indicus* | *L. trapezoides* | | *L. iuglandis* | |
| 4 | | *L. danuvii* | *L. igoris* | *L. euonymus* | | *L. olegi* | |
| 5 | | ***L. leptocephalus*** | *L. artemisiae* | ***L. moesicus*** | | *L. sylvae* | |
| 6 | | | *L. intermedius* | | | *L. picenus* | |
| 7 | | | *L. crassus* | | | *L. nevesi* | |
| 8 | | | ***L. elongatus*** | | | *L. israelenensis* | |
| 9 | | | | | | *L. poessneckensis* | |
| 10 | | | | | | *L. juglandicola* | |
| ↓ | | | | | | ↓ | |
| 19 | | | | | | ***L. caespiticola*** | |
| ↓ | | | | | | | ↓ |
| 30 | | | | | | | ***L. caespiticola*** |
| 31 | | | | | | | ***L. caespiticola-GE*** |

*Analysed species = Slovenian *Longidorus* populations.
**RCP = Rank of comparative *Longidorus* population from reference file.
*** Multivariate morphometrical analysis of *L. caespiticola* with additional two populations (BE and GE).

*Longidorus moesicus* and *L. helveticus* were discovered for the first time in Slovenia. Both species were extracted from vineyard soil samples. Specimens of *L. moesicus* were found near Vrhpolje (western part of Slovenia), while *L. helveticus* was recovered from Trška gora (southern part of Slovenia).

**Morphometrics.** Morphometric data of all analysed *Longidorus* species from Slovenia are presented in Table 1. *Longidorus juvenilis* from Svetinje was characterised by medium sized (L = 3.52 - 4.71 mm) and slender (a = 89 - 128) body, slightly expanded, anteriorly flat and laterally rounded head (Fig. 1), offset from neck contour by a slight depression, symmetrically bilobed amphideal pouches, medium odontostyle length (61.1 - 67.7 µm) and elongate, conoid tail (47.6 - 56.8 µm; c' = 1.8 - 2.5). The codes for identifying *L. juvenilis* when using identification key of Chen *et al.* (1997) are: A-2, B-12, C-2, D-3, E-2, F-2, G-2, H-6, I-1 and the same codes were determined for *L. juvenilis* from Svetinje. Males were not found. In addition to females, many juveniles of different developmental stages (3 juvenile stages) and a specimen of the bivulval *L. juvenilis* female were also found (Širca *et al.,* 2007).

*Longidorus leptocephalus* from Juršinci was characterised by medium sized (L = 4.00 - 4.90 mm) and slender (a = 99 - 117) body. The lip region was narrow, about half the neck width at the guide ring, very slightly offset from the body contour; amphids somewhat bilobed at the base, medium odontostyle length (61.0 - 70.0 µm). The tail is roundly conoid with a narrow conoid terminus (41.7 - 44.9 µm; c' = 1.43 - 1.55). The codes for identifying *L. leptocephalus* after the key of Chen *et al.* (1997) are: A-2, B-1, C-23, D-3, E-12, F-2, G-2, H-45, I-1 and the same codes were determined for *L. leptocephalus* from Jursinci. In addition to females, juveniles were also found. The species had four juvenile developmental stages (Širca *et al.,* 2007). Males were not found.

*Longidorus caespiticola* from Brdo near Lukovica was characterised by a robust body (L = 6.20 - 8.50 mm), usually forming an open "c" when killed by heat. Lip region smoothly rounded, continuous with neck contour, which is convex in the amphid region; amphids large, flask shaped, not bilobed; moderate odontostyle length (96.1 - 109.3 µm). Tail dorsally convex, bluntly conoid to rounded (Fig. 2), 39.3 - 50.3 µm long (c' = 0.71-0.78). In addition to female and juveniles, eight males were also found. Anterior end of males similar to that of female; posterior third more ventrally curved when heat killed. Spicules large, 77.9 - 94.6 µm long. Tail about one anal-body-width long, dorsally convex, ventrally concave with bluntly rounded terminus. The codes for identifying *L. caespiticola* after the key of Chen *et al.* (1997) are: A-34, B-3, C-34, D-1, E-4, F-34, G-12, H-1, I-2 and the same codes were determined for *L. caespiticola* from Brdo near Lukovica.

*Longidorus elongatus* from Maribor was characterised by moderate sized (L = 4.50 - 5.20 mm), ventrally curved in an open "c" shape body when killed by heat. Lip region anteriorly flattened, continuous or very slightly offset from neck contour; amphids large, pouch like, slightly bilobed; medium odontostyle length (88.0 - 91.6 µm). The tail is dorsally convex, ventrally flattened or very slightly concave, 40.0 - 47.9 µm long (c' = 1.02-1.19) with a roundly conoid terminus. The identification codes for Maribor population of *L. elongatus* were: A-3, B-2, C-23, D-3, E-2, F-2, G-2, H-2, I-1, which fit the codes proposed by Chen *et al.* (1997). Males were not found.

*Longidorus moesicus* from Vrhpolje was characterised by 5.9-8.4 mm long, robust body tapering gradually toward the anterior end, ventrally curved in an open "c" shape body when killed by heat. Lip region subacute, continuous with the rest of the body; amphids more or less asymemetrically bilobed; odontostyle 96.3 – 114.6 µm long. Tail conoid, with narrowly rounded terminus, 36.2-49.4 µm long (c' = 0.90-1.23). Males were not found. The identification codes for Vrhpolje population of *L. moesicus* were: A-4, B-2, C-3, D-1, E-3, F-3, G-3, H-2, I-1, which fits the code proposed by Chen *et al.* (1997).

*Longidorus helveticus* from Trška gora near Novo mesto was characterised by 6.6-7.7 mm long, ventrally curved in an open "c" shape body when killed by heat. Lip region hemi-eliptical, continuous with the rest of the body (Fig. 2); amphids pocket-like, not bilobed; odontostyle 128.5 – 136.2 µm long. Tail bluntly rounded, 40-41 µm long (c' = 0.60-0.69). In addition to female and juveniles, three males were also found. Posterior region of males more ventrally curved than in female when heat killed; spicules robust, ventrally curved, 110.7 – 117.4 µm long. The codes for identifying *L. helveticus* after Lamberti *et al.* (2001) are: A-56, B-45, C-34, D-1, E-4, F-34, G-1, H-1, I-2 and the same codes were determined for *L. helveticus* from Trška gora.

**Multivariate morphometrical analysis.** The similarity of *L. caespiticola, L. elongatus, L. leptocephalus, L. juvenilis, L. moesicus* and *L. helveticus* from Slovenia to the *Longidorus* species of the reference file (a single value for each species

obtained from paratype means or the holotype of the original descriptions (Ye & Robbins, 2004)) is presented in Table 2.

The result for *L. juvenilis* showed that the nearest neighbours of the selected object (*L. juvenilis* from Svetinje) of the analysis file are *L. carpetanensis, L. bernardi* and *L. juvenilis.* Specimens of *L. juvenilis* described from Svetinje are generally somewhat longer than the specimens in other regions (Širca *et al.*, 2007).

The nearest neighbours of *L. leptocephalus* from Juršinci are *L. bernardi, L. carpetanensis, L. sylphus, L. danuvii* and *L. leptocephalus* from the reference file. Specimens of *L. leptocephalus* from Jursinci, when compared with data from the literature, have larger c' (1.5 (1.43-1.57) *vs* 1.3 (1.2-1.4)). All other characteristics are very close to the morphometrics described by Hooper, 1961.

*Longidorus elongatus* from Maribor was found to be closest to *L. athesinus, L. sturhani, L. indicus, L. igoris, L. artemisiae, L. intermedius, L. crassus* and *L. elongatus* from the reference file. Specimens of *L. elongatus* from Maribor are somewhat shorter, have shorter odontostyle (89.9 *vs* 94 μm) and tail length (43.3 *vs* 56 μm) than the specimens used in the reference file.

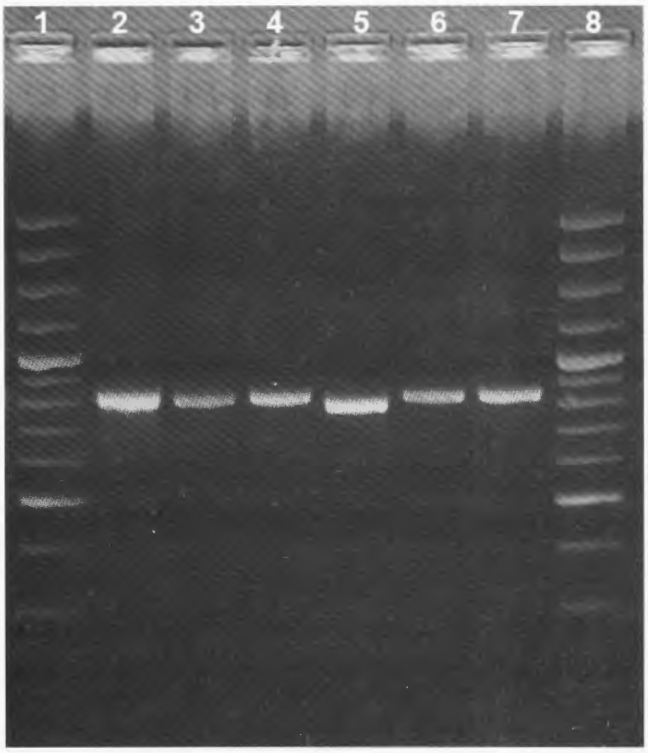


**Fig. 3.** PCR products of amplified D2/D3 rDNA region of *L. juvenilis* (lane 2), *L. leptocephalus* (lane 3), *L. caespiticola* (lane 4), *L. elongatus* (lane 5), *L. moesicus* (lane 6) and *L. helveticus* (lane 7) loaded on 1 % agarose gel. 100 bp Marker (Fermentas) on lanes 1 and 8.

*Longidorus moesicus* from Vrhpolje is slightly more slender and has somewhat shorter odontostyle (89.9 *vs* 94 μm) than *L. moesicus* from the reference file. According to multivariate morphometrical analysis, its nearest neighbours are *L. apulus, L. dunensis, L. trapezoids, L. euonymus* and *L. moesicus* from the reference file.

*Longidorus helveticus* was extracted from the soil samples from vineyards in Trška gora together with a dagger nematode *Xiphinema diversicaudatum.* Its characteristics are very close to the morphometrics described by Lamberti *et al.* (2001). The result of multivariate morphometrical analysis shows that the nearest neighbours of Slovenian population of *L. helveticus* are *L. nevesi* and *L. helveticus* from the reference file.

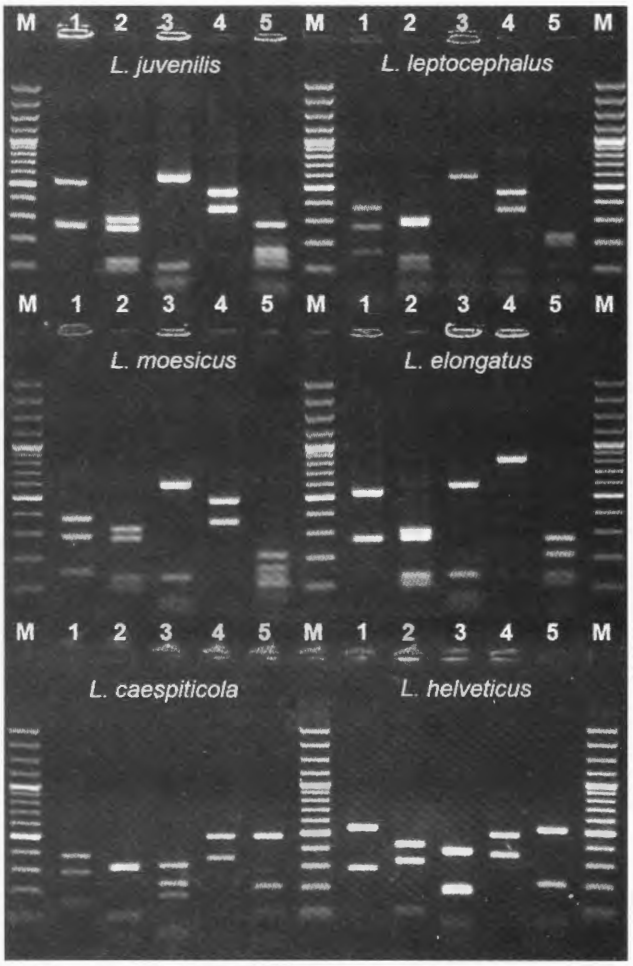


**Fig. 4.** Restricted fragments of D2/D3 rDNA PCR products of *L. juvenilis, L. leptocephalus, L. caespiticola, L. elongatus, L. moesicus* and *L. helveticus* on 1.5 % agarose gel. *Alu*I (lanes 1), *Hinf*I (lanes 2), *Mbo*I (lanes 3), *Mse*I (lanes 4) and *Rsa*I (lanes 5), 100 bp Marker (Fermentas) on lanes M.

**Table 3:** Restriction fragments approximate lengths (bp) of six *Longidorus* species from Slovenia. Amplified fragments of D2/D3 rDNA region were digested using five restriction enzymes. The sizes of restricted fragments were deducted from the gel (compare with Figure 4).

| | Undigested | *Alu*I | *Hinf*I | *Mbo*I | *Mse*I | *Rsa*I |
|---|---|---|---|---|---|---|
| *L. juvenilis* | 810 | 530, 280 | 300, 260, 130, 100, 20 | 570, 100, 70, 50, 20 | 460, 350 | 290, 170, 140, 110, 50 |
| *L. leptocephalus* | 810 | 380, 270, 160 | 300, 280, 130, 100 | 590, 110, 50 | 460, 350 | 230, 190, 110, 50 |
| *L. moesicus* | 830 | 380, 290, 160 | 320, 280, 130, 100 | 600, 130, 60, 40 | 480, 350 | 210, 160, 130, 110, 70 |
| *L. elongatus* | 800 | 520, 280 | 300, 270, 130, 100 | 560, 130, 60, 50 | 800 | 280, 210, 150, 110, 50 |
| *L. caespiticola* | 820 | 380, 290, 150 | 310, 300, 110, 100 | 310, 230, 170, 60, 50 | 480, 340 | 480, 210, 110, 20 |
| *L. helveticus* | 830 | 540, 390 | 410,320, 100 | 380, 190, 170, 50, 40 | 480, 350 | 520, 210, 80, 20 |

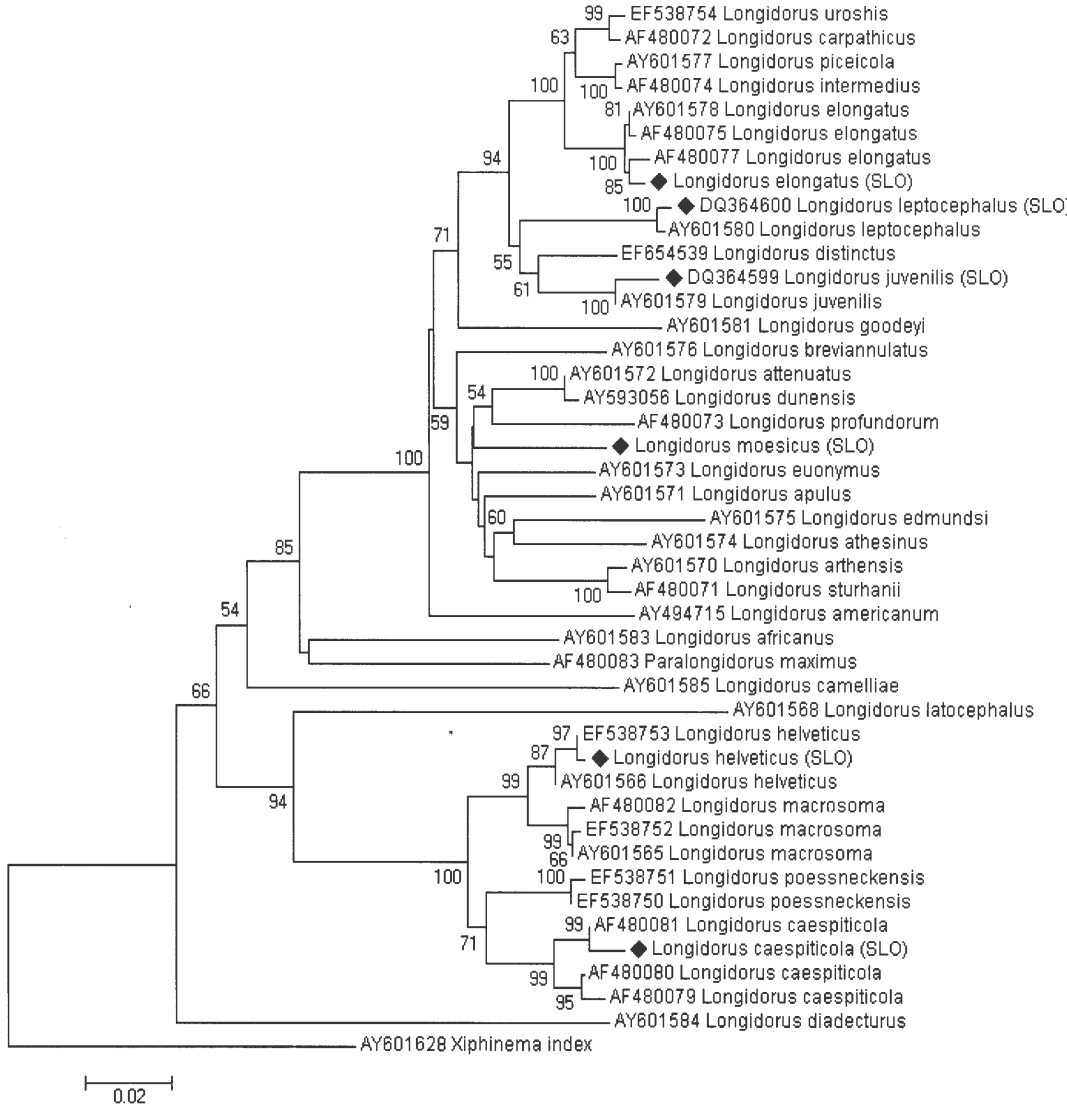


**Fig. 5.** Phylogenetic tree of rDNA D2/D3 expansion region sequences of *L. juvenilis, L. leptocephalus, L. caespiticola, L. elongatus, L. moesicus* and *L. helveticus* from Slovenia and sequences of closely related *Longidorus* species (NCBI GenBank). Sequences were analysed using neighbour joining method. Bootstrap support values more than 50 % are shown.

Specimens of *L. caespiticola* were discovered from the rhizosphere of apple orchard in Brdo near Lukovica, central Slovenia. They are somewhat longer (7.8 *vs* 6.7 µm) and have shorter odontostyle (102.5 *vs* 110 µm) and tail (44.6 *vs* 65 µm) than specimens described by Hooper, 1961. The similarity test shows that the nearest neighbour of *L. caespiticola* from Brdo near Lukovica is *L. raskii* from the reference file. It is followed by *L. crataegi, L. iuglandis, L. olegi* etc.; *L. caespiticola* from the reference file is in 19th place. Due to expressed dissimilarity between *L. caespiticola* population from Brdo and the population described by Hooper (1961), two other populations including *L. caespiticola* from Grossheubach/Main, Germany and from Belgium (Sturhan, 1963) were included for further multivariate morphometrical analysis. Unfortunately, only seven measured characters were analysed because lip width and anal body width were not available from Sturhan (1963). The Belgium population was placed right next to the Brdo population (Table 2), whereas population of Hooper (1961) and Sturhan's German population (Sturhan, 1963) were placed further away, in 30th and 31st place, respectively (Table 2).

**RFLP and sequence analyses.** The amplification of D2/D3 region of 28S rRNA gene gave one fragment ranging approximately from 800 bp to 830 bp depending on nematode species (Figure 3, Table 3). The patterns of restriction fragments were sometimes species-specific, which all together allowed the separation of the analysed *Longidorus* species. The lengths of restricted fragments were determined from the gel (Table 3) and represent actual bands which can be seen on the gel. RFLP patterns for each analysed species are presented on Figure 4. Sequences of D2 and D3 expansion region of the 28S rDNA nuclear gene obtained in this study were analysed together with the sequences of the same and closely related species from the NCBI database. Sequence of *L. juvenilis* from Svetinje clustered together with the sequence AY601579 of *L. juvenilis* (He *et al.*, 2005) and formed a separate clade with high bootstrap support (Fig. 5). Similar results were observed for *L. leptocephalus*, *L. elongatus*, *L. helveticus* and *L. caespiticola* species. *Longidorus moesicus* from Vrhpolje was sequenced for the first time and it is unique. No group pattern was resolved with high support.

## DISCUSSION

Correct identification of *Longidorus* species is economically very important. Therefore, the development of proper diagnostic tools is crucial. The genus now comprises 144 nominal species. In the past, many approaches attempted to establish a good identification key for this genus, beginning with a dichotomous key by Lamberti (1975), which is now considered outdated and difficult to update to include new species descriptions (Rey *et al.*, 1988). A few years later, the first polytomous key was published by Romanenko in 1978 (Chen *et al.*, 1997) and later revised by Chen *et al.* (1997) and Loof & Chen (1999), which permits a range of characters to be used simultaneously and made it more effective for identifying closely related species with overlapping features (Ye & Robbins, 2004). Polytomous key is better than dichotomous key, but species identification could still be unreliable because morphometrical characterisation displayed high intra-specific variability and relatively small interspecific differences. Moreover, some qualitative parameters e.g. amphid shape, are sometimes hardly distinguishable and difficult to classify in a proper group, so that unambiguous identification is additionally compromised.

A computer method (Rey *et al.*, 1988) and a computer software (Tiefenbrunner *et al.*, 2002) and, more recently, hierarchical cluster analysis (Ye & Robbins, 2004) were developed to aid the species identification in *Longidorus* based on female morphometric character means. Because single values (means) of paratype and holotype of each species were used as a reference file using multivariate morphometrical analysis, the similarity of two different populations of the same species were not expressed well in some cases e.g. *L. caespiticola*.

Different molecular approaches have been developed to aid morphometrical identification. Sequence data of various *Longidorus* species based on ribosomal DNA can be used for phylogenetic studies as well as species identification. However, at the moment only a limited number of rDNA sequences of *Longidorus* species is available at the NCBI database. PCR-RFLP approach proved to be useful for differentiating six *Longidorus* species from Slovenia. We believe that the method has potential to become a diagnostic tool for *Longidorus* species identification because it enabled differentiation of phylogenetically closely related species such as *L. juvenilis* and *L. leptocephalus*. *L. juvenilis* and *L. leptocephalus*. D2/D3 rDNA sequences clustered in adjacent clades in several studies (He *et al.*, 2005; Širca *et al.*, 2007; this study), which demonstrates high sequence similarity. Using PCR-RFLP method of D2/D3 rDNA region we were able to differentiate these closely related species with four out of five

restriction enzymes used. However, the capacity of the method should be tested on several *Longidorus* species and populations from different parts of the world.

Our results support the fact that conclusive identification of *Longidorus* species must, besides available morphometrical data also refer to qualitative morphology (head shape, amphid shape, tail shape, etc.), presence or absence of males, geographical distribution, DNA sequences, comparison with type specimens, if possible (Ye & Robbins, 2004), and other DNA analyses (e.g. PCR-RFLP). Because of high intra-specific variability and minor inter-specific differences of *Longidorus* species, it is very important to build up the data base of the genus *Longidorus* (morphometrical and DNA characteristics) continuously. Therefore, specialists from different parts of the world should be encouraged to analyse *Longidorus* populations morphometrically and genetically more intensively, publish their findings and improve the quality of existing data bases.

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**S. Širca, G. Urek.** Морфологическая и молекулярная характеристика шести видов *Longidorus* (Nematoda: Longidoridae) из Словении.

**Резюме.** Из ризосферы виноградников и садов Словении в период между 2003 и 2008 годами было выделено шесть видов *Longidorus*: *Longidorus juvenilis, L. leptocephalus, L. caespiticola, L. elongatus, L. moesicus* и *L. helveticus*. Виды *L. moesicus* и *L. helveticus* отмечены для Словении впервые. Выявленные шесть видов *Longidorus* были определены по морфологическим особенностям самок и морфометрическим показателям. Кроме того, было проведено сравнение морфометрических показателей обнаруженных нематод и литературных данных с использованием программы Scramble .2.2. Видовое определение было подтверждено молекулярным анализом D2D3 сегмента последовательности большой субъединицы рибосомы (28S rRNA). Были проведены сравнение полученных последовательностей с данными из Генбанка NCBI и филогенетический анализ для подтверждения определения выявленных видов. Последовательности видов *Longidorus* из Словении демонстрировали родство с высоким уровнем поддержки с последовательностями тех же видов из Генбанка. Был также проведен RFLP-анализ, позволяющий отличать друг от друга виды *Longidorus*, найденные в Словении. Приводится обсуждение необходимости публикации морфометрических данных по популяциям лонгидорид из различных стран.