MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *LONGIDORUS HELVETICUS* (NEMATODA: DORYLAIMIDA) FROM SERBIA

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Summary. *Longidorus helveticus* Lamberti, Kunz, Grunder, Molinari, De Luca, Agostinelli *et* Radicci, 2001 is reported from Serbia. Morphometric variability of females, males and four juvenile stages are presented. PCR-RFLP analysis of the ITS region was carried out.

During a preliminary survey of longidorid nematodes undertaken during the period 1984-1987, several female and male specimens of a long and robust *Longidorus* species were found in the rhizosphere of blackberry (Iriški venac, Fruška gora Mountain), hazelnut and *Picea* sp. (Ledinci, Fruška gora Mountain) in Serbia. They were identified and reported as *Longidorus macrosoma* Hooper, 1961 (Barsi, 1989). Morphometrically and morphologically the population from Iriški venac was most similar to a German population (Brown and Boag, 1975) taking into consideration the smaller length of body, slightly rounded labial region in both sexes and smaller number of ventro-median supplements in males [10-12 *vs* 15-19 in type population (Hooper, 1961)]. In the last decade, specimens of this species have been found in several other localities in Serbia and in the last few years a sufficient number of them have been collected with which to make detailed study. Lamberti *et al.* (2001) published a description of *Longidorus helveticus* from Switzerland, which was very similar to *L. macrosoma*. This publication has made it necessary to re-examine the findings of *L. macrosoma* in Serbia.

A detailed study of two populations of a *Longidorus* species, similar to *L. macrosoma*, resulted in their identification as *L. helveticus* Lamberti, Kunz, Grunder, Molinari, De Luca, Agostinelli *et* Radicci, which was confirmed by molecular analysis. Results of this study are presented here.

MATERIALS AND METHODS

Soil samples were collected in July 2001 and August 2004 from the rhizosphere of *Ruscus aculeatus* L. at Stari Ledinci (UTM: DR00), Fruška gora Mountain, and in May 2002 from the rhizosphere of *Carpinus betulus* L. at Obrež (UTM: DQ15).

Nematodes were extracted by Cobb's wet sieving technique. Specimens were killed by hot FP 4-1 and transferred to glycerin by a slow evaporation method and mounted on permanent microscope slides. Measurements were made with an ocular micrometer, except for body length, which was measured with the aid of a drawing tube and map measurer.

A comparison of populations of *L. helveticus* and *L. macrosoma* (females and juveniles; males were omitted) was made by means of cluster analysis. Data used are original (presented in this paper) or published (Barsi, 1989; Lamberti *et al.*, 2001).

Cluster analysis was performed on non-transformed data using the average population values of a set of 17 characters (body length, ratios a, b, c and c′, V, odontostyle length, odontophore length, distance of the guide ring from the anterior extremity, tail length, length of the hyaline portion of tail, and body diameter at: lip region, guide ring, the base of the oesophagus, mid-body or vulva, anus, and the hyaline portion). The unweighted pair group average as a clustering method and percent similarity as a coefficient were used.

Genomic DNA was isolated from ten single individuals, as described by De Luca *et al.* (2004). The crude DNA extracted from each individual nematode was directly amplified by using the forward primer 18S (5' TGATTACGTCCCTGCCTTT 3') and the reverse primer 26S (5' TTTCACTCGCCGTTACTAAGG 3') spanning from the 3' end of 18S rDNA to the 5' end of the 26S rDNA and including the ITS1, ITS2 and 5.8S rDNA. PCR conditions were 5 min at 94 °C and then 35 cycles of 50 seconds/94 °C, 50 seconds/55 °C and 1 min/72 °C, followed by a post-amplification extension at 72 °C for 7 min. After DNA amplification, 1/10 of each product was run on a 1% agarose gel. Ten µl of each PCR product was digested with the following restriction enzymes: *Eco* RI, *Dde* I, *Rsa* I, *Alu* I, *Ava* II and *Hinf* I (Lamberti *et al.*, 2001). The digested DNA was loaded onto a 2% agarose gel, separated by electrophoresis, stained with ethidium bromide, visualized on a UV transilluminator and recorded by photography.

DESCRIPTION

LONGIDORUS HELVETICUS
Lamberti, Kunz, Grunder, Molinari, De Luca, Agostinelli & Radicci, 2001
(Tables I-III; Figs 1-6)

Female. (Stari Ledinci, Fruška gora Mountain, *Ruscus aculeatus*, n = 16). Female *habitus* curved in an open "C" when killed. Body of large size, robust and cylindrical, tapering towards the extremities. Cuticle smooth in appearance, with very fine transverse striations visible in the tail region. Lip region continuous with the rest of the body, anteriorly hemi-elliptical or almost flattened, laterally broadly rounded. Amphidial pouches large, pocket-like, not lobed at the base. Odontostyle, odontophore and guide ring typical of the genus. Oesophagus dorylaimoid, with the basal bulb occupying 22-28% of the total oesophagus length and measuring 133-155 µm long and 28-36 µm wide. Location of oesophageal gland nuclei typical for genus. Nuclei of dorsal and subventral glands situated at 29 (27-34) (n = 12) and 54 (50-59)% (n = 13), respectively. Vulva a transverse slit, vagina occupying 49-60% of the corresponding body diameter; *pars distalis vaginae* and thick walled *pars proximalis vaginae* 21-29 µm and 20-26 µm long, respectively. Gonads amphidelphic, of approximately the same length and structure; uteri large, well muscularized, 216-355 µm long. Pre-rectum 405-572 µm long or 6.5-9 times the anal body width; rectum 59-91% of body diameter at anus. Tail short, bluntly rounded with two pairs of caudal pores.

Male. (Stari Ledinci, Fruška gora Mountain, *R. aculeatus*, n = 15). Body C-shaped, more strongly curved at posterior end. Spicules robust, ventrally curved; lateral guiding piece 28 (26-29) µm long. Adanal pair of supplements preceded by a row of 10-14 ventro-median supplements. Tail bluntly rounded, dorsally convex and ventrally concave, bearing two pairs of caudal pores.

Juveniles. (Stari Ledinci, Fruška gora Mountain, *R. aculeatus*, n = 77). Separated into four developmental stages (Fig. 3). The first stage has a digitate tail with a 12.1 (9.7-14.4) µm long mucro; second, third and fourth juvenile stages have bluntly rounded tails.

All juvenile developmental stages generally correspond well with those from Switzerland described by Lamberti *et al.* (2001), with the exception of body length, which is slightly shorter in the population from Stari Ledinci.

Compared to the type population (Lamberti *et al.*, 2001), adults of *L. helveticus* from Stari Ledinci and Obrež have slightly shorter body lengths (5.88-8.33 *vs* 6.2-9.3 mm in females and 5.83-8.30 *vs* 6.0-8.6 mm in males, respectively), slightly lower c ratio value (146.8-253.8 *vs* 150.4-260 in females and 119.3-204.9 *vs* 144.8-216.4 in males, respectively), slightly longer odontostyle (129.5-151.1 *vs* 127-145.5 µm in females and 127.5-152.4 *vs* 125-146.7 µm in males, respectively), slightly shorter odontophore (73.8-92.5 *vs* 76-98.2 µm in females and 73.8-91.3 *vs* 85-97.5 µm in males, respectively), and males have slightly shorter spicules (98.5-111.4 *vs* 104-118 µm) and fewer ventro-median supplements (10-14 *vs* 15-17).

Intersex. (Stari Ledinci, Fruška gora Mountain, *R. aculeatus*, n = 1). Similar to female with incompletely developed female and male genital system. Vulva and vagina as in female. Vulva a transverse slit, vagina occupying 49% of the corresponding body diameter; *pars distalis vaginae* and thick walled *pars proximalis vaginae* 21 µm and 22.5 µm long, respectively. Female gonads reduced with only uteri present. Anterior uterus 281 µm long, ending with a constriction, followed by a very short part of the *pars dilatata oviductus*. Posterior uterus 256 µm long, ending blindly. Rectum 51 µm long, with three bodies, two longer (30 and 22 µm long, respectively) and one shorter (about 13 µm long), which probably correspond to unformed spicules and lateral guiding piece, respectively. A scarcely developed adanal pair of supplements preceded by a row of 7 small ventro-median supplements. Tail short, bluntly rounded with three caudal pores on each side.

The identification code, according to the polytomous key for *Longidorus* (Chen *et al.*, 1997; Loof and Chen, 1999), for the Serbian populations of *L. helveticus* is: A 5/6, B 4/5, C 3/4, D 1, E 4, F 3/4, G 1/2, H 1, I 2, which fits the code proposed by Lamberti *et al.* (2001).

DISCUSSION

According to the original description (Lamberti *et al.*, 2001), *L. helveticus* is very similar to *L. macrosoma* Hooper, 1961, which makes their correct identification difficult. It was differentiated from *L. macrosoma* by its shorter body (*L. macrosoma* generally longer than 9 mm), much lower a ratio value (105-115 in *L. macrosoma*) and frontally rounded lip region (flat/slightly depressed in *L. macrosoma*). Also, the hyaline portion of the tail of the first stage juveniles of *L. helveticus* is much longer than in the corresponding juveniles of *L. macrosoma* [ca. 12 µm in *L. macrosoma* from Liestal, Switzerland (Lamberti *et al.*, 2001)].

Figure 5 shows the dendrogram obtained from the cluster analysis. CA indicated the occurrence of two main clusters with five sub-clusters. Each sub-cluster represents a different developmental stage (J1, J2, J3, J4 and females, respectively). On the sub-cluster level *L. helveticus* is clearly separated from *L. macrosoma*.

Taking into consideration the results of this study, the earlier report of *L. macrosoma* from Serbia (Barsi, 1989) should be attributed to *L. helveticus*.

The PCR product of the ITS region of *L. helveticus* from Serbia was almost 2.0 kb and the restriction patterns obtained are shown in Fig. 6. The RFLP results revealed that the Serbia population of *L. helveticus* showed the same patterns as *L. helveticus* from Switzerland (Lamberti *et al.*, 2001), even though two enzymes

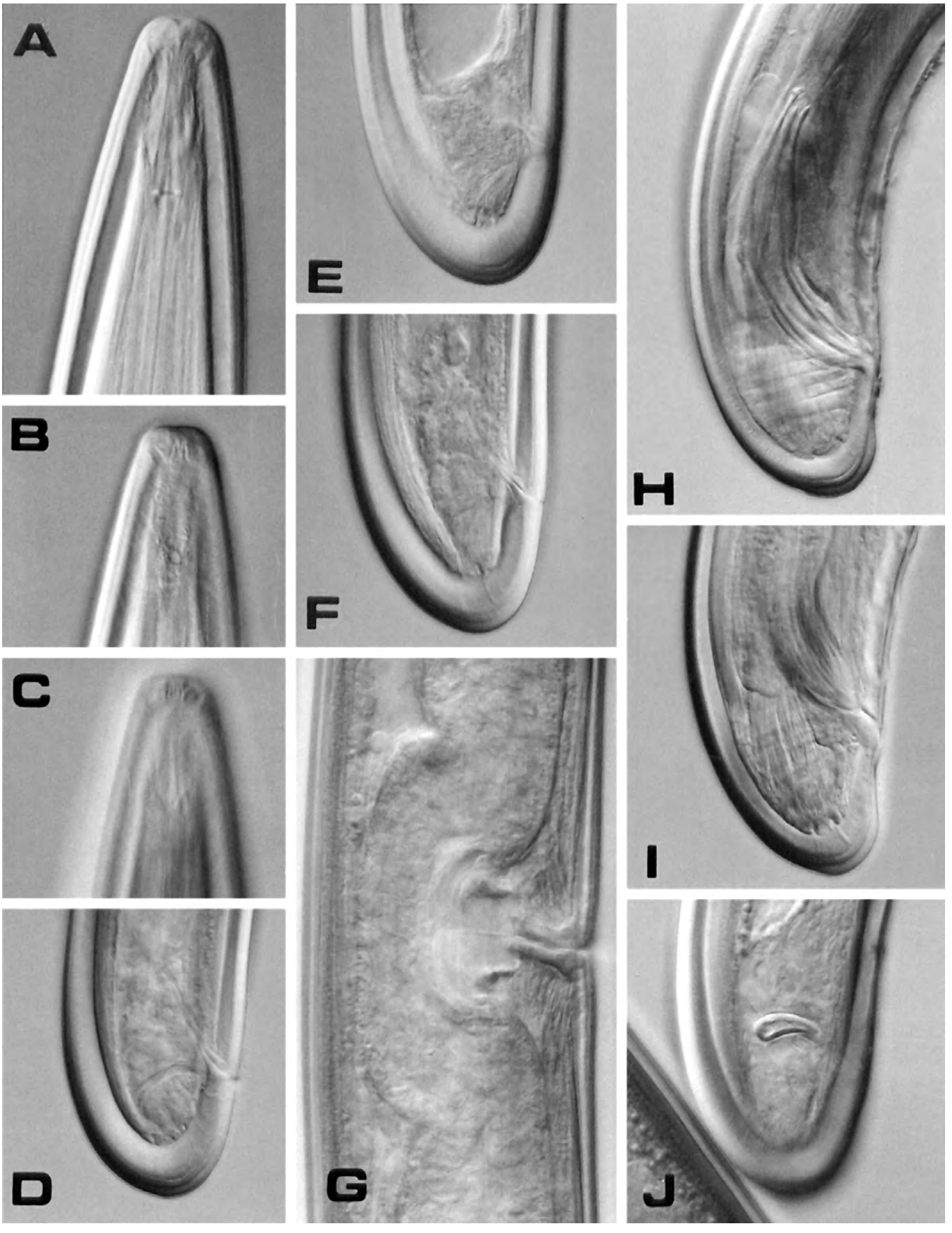

Fig. 1. Photomicrographs of *Longidorus helveticus*. A: female anterior region; B-C: female amphidial pouch; D-F: female tail; G: vulva region; H-I: male tail; J: intersex tail.

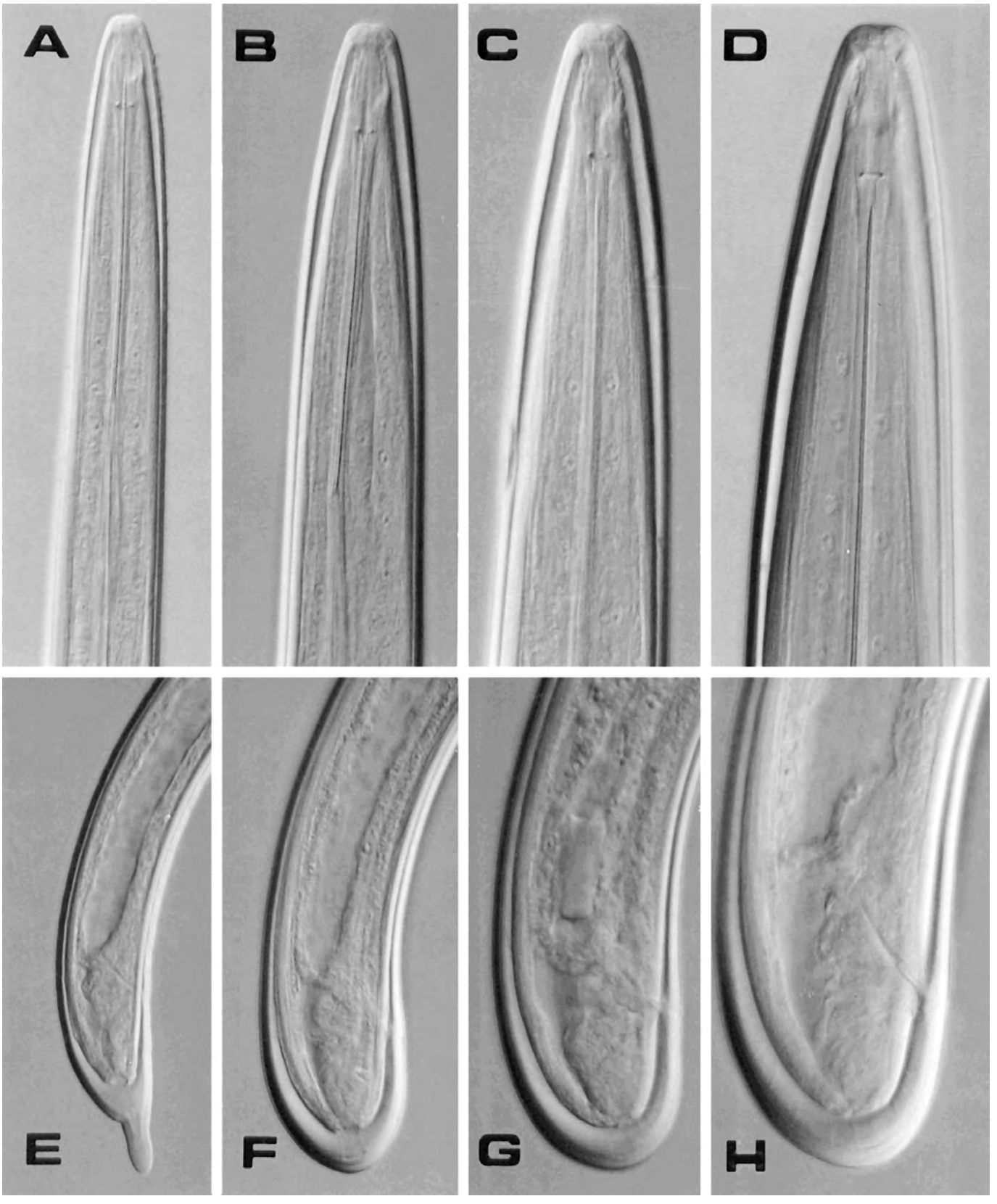

Fig. 2. Photomicrographs of juvenile stages of *L. helveticus*. A-D: anterior region of J1, J2, J3 and J4 stage; E-H: tail of J1, J2, J3 and J4 stage.

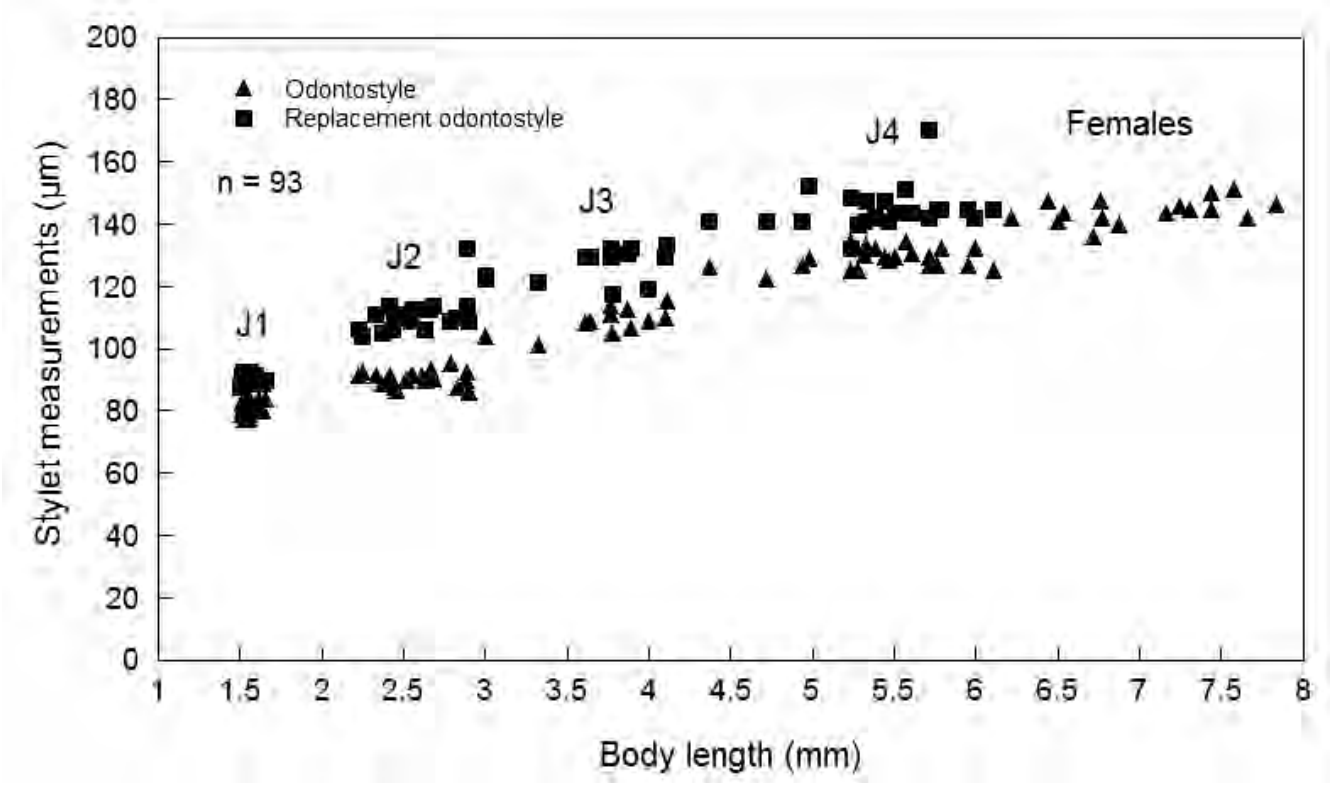

Fig. 3. Scatter diagram separating juveniles and females of *L. helveticus*.

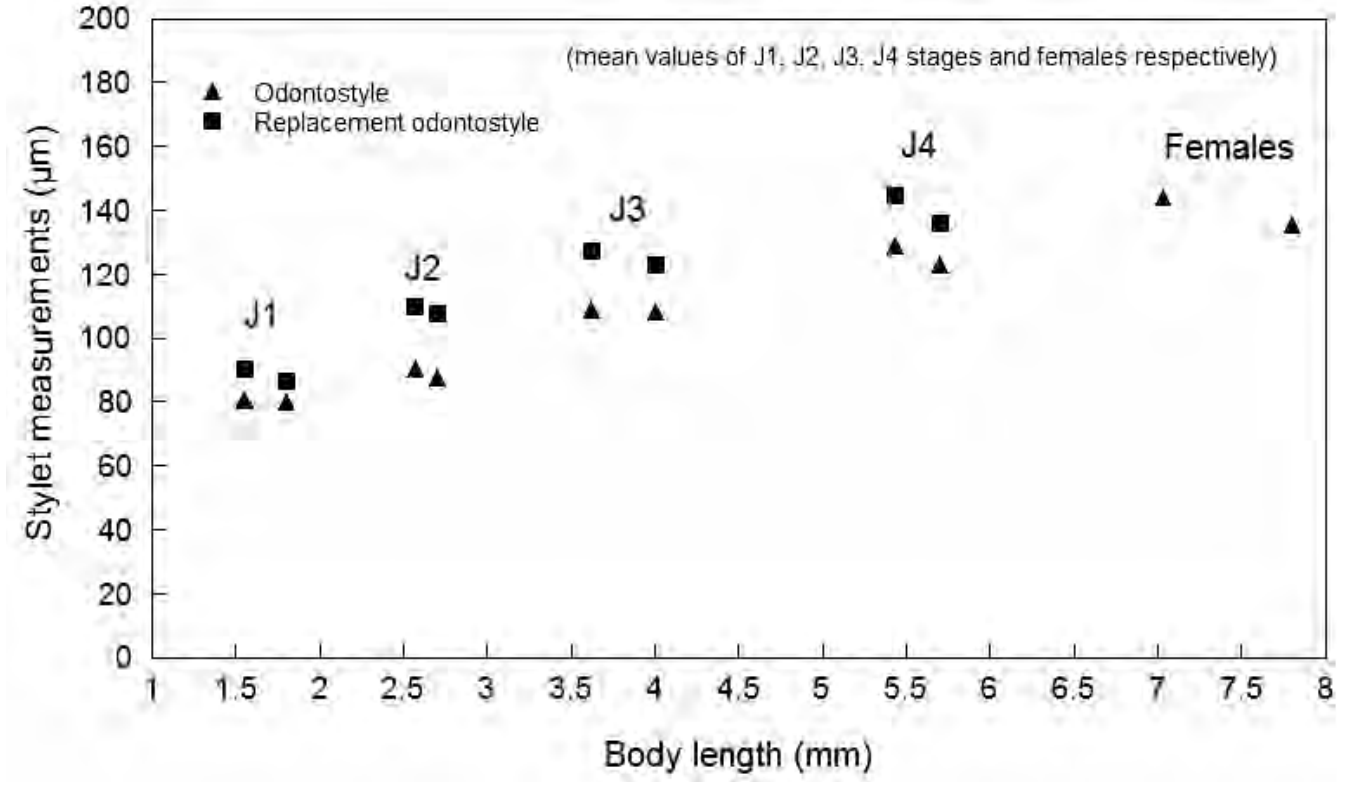

Fig. 4. Scatter diagram separating juveniles and females of *L. helveticus* from Switzerland and Serbia (for details see Table III).

Table I. Morphometric characters of adult populations of *Longidorus helveticus* from Serbia.

Locality:	Stari Ledinci, Fruška gora Mountain			Obedska bara, Obrež	
Host:	*Ruscus aculeatus*			*Carpinus betulus*	
n :	16 females	15 males	1 intersex	16 females	14 males
L (mm)	7.03±0.49 (6.22-7.84)	6.94±0.66 (5.83-8.10)	6.23	7.33±0.67 (5.88-8.33)	6.89±0.77 (5.88-8.30)
a	74.8±4.26 (69.0-82.8)	83.4±4.82 (72.6-92.6)	70.1	81.0±5.57 (73.5-89.8)	87.0±8.87 (73.2-103.1)
b	11.8±0.91 (9.9-13.6)	11.9±1.22 (9.8-13.7)	10.8	12.7±1.42 (9.8-15.2)	12.6±1.08 (11.3-14.2)
c	170.7±18.05 (146.8-215.6)	152.7±23.49 (120.9-204.9)	150.4	192.0±22.68 (157.4-253.8)	163.0±20.27 (119.3-190.5)
c′	0.67±0.04 (0.60-0.74)	0.76±0.06 (0.63-0.84)	0.67	0.64±0.05 (0.55-0.74)	0.72±0.05 (0.66-0.84)
d	2.0±0.12 (1.8-2.3)	2.0±0.08 (1.8-2.1)	1.9	2.0±0.09 (1.8-2.1)	2.0±0.12 (1.7-2.2)
d′	1.9±0.09 (1.7-2.0)	1.8±0.05 (1.7-1.9)	1.8	1.8±0.08 (1.6-1.9)	1.8±0.08 (1.7-1.9)
J′	0.3±0.02 (0.2-0.3)	0.3±0.02 (0.3-0.4)	0.3	0.3±0.02 (0.2-0.3)	0.4±0.03 (0.3-0.4)
V	51.7±1.53 (49.4-54.5)	–	51.7	53.2±1.86 (48.7-56.1)	–
Odontostyle µm	144.3±3.79 (136.2-151.1)	141.2±6.63 (129.5-152.4)	134.6	137.8±4.12 (129.5-143.7)	136.3±6.17 (127.5-146.0)
Odontophore µm	84.6±3.56 (73.8-88.8)	86.7±2.48 (81.3-91.3)	78.8	84.9±4.61 (76.3-92.5)	84.4±4.91 (73.8-91.3)
Total stylet µm	228.9±5.17 (218.7-237.4)	227.9±7.95 (214.5-243.7)	213.4	222.7±7.07 (205.8-232.5)	220.7±5.82 (213.0-230.0)
Replacement odontostyle µm	–	–	–	132.1, 149.9*	–
Oral aperture to guide ring µm	42.8±2.16 (37.5-46.3)	42.3±2.08 (37.5-45.0)	40.0	42.8±1.68 (40.6-48.1)	41.7±1.99 (38.8-45.0)
Tail µm	41.4±3.29 (33.6-45.0)	45.9±4.05 (38.6-52.8)	41.4	38.4±2.95 (32.8-42.5)	42.5±3.31 (37.1-49.3)
J (hyaline portion of tail) µm	15.7±1.46 (11.3-17.5)	12.7±0.59 (11.3-13.8)	14.4	16.3±1.60 (13.8-20.0)	14.1±1.08 (12.5-16.3)
Body diam. at lip region µm	21.3±0.75 (20.0-22.8)	21.4±0.80 (20.3-23.4)	21.3	21.9±0.82 (21.3-23.8)	21.2±0.80 (20.0-22.5)
Body diam. at guide ring µm	39.7±1.93 (35.0-42.1)	38.6±1.45 (35.9-41.3)	37.5	39.5±1.59 (37.2-42.2)	38.4±1.49 (36.3-41.3)
Body diam. at base of oesophagus µm	72.7±3.68 (67.1-81.3)	70.6±3.08 (66.9-77.2)	71.3	71.5±3.84 (66.3-80.6)	67.6±3.66 (61.3-75.4)
Body diam. at mid-body or vulva µm	94.1±6.39 (82.2-103.8)	83.1±4.25 (76.3-90.0)	88.8	90.6±7.34 (80.0-105.6)	79.5±7.48 (68.1-91.3)
Body diam. at anus µm	62.2±4.39 (55.0-70.0)	60.7±2.16 (57.5-64.1)	61.3	60.0±2.68 (54.1-64.7)	58.9±2.93 (54.2-64.2)
Body diam. at beginning of J µm	43.6±3.75 (36.3-48.1)	38.5±2.27 (35.0-43.1)	43.8	43.8±3.04 (36.3-47.9)	38.4±2.49 (35.0-43.8)
Spicules µm	–	103.7±3.03 (98.5-111.4)	–	–	105.7±3.82 (95.0-110.0)

d, anterior to guide-ring/body width at lip region (Brown *et al.*, 1994)
d′, body width at guiding-ring/body width at lip region (Brown *et al.*, 1994)
J′, length of the hyaline region of the tail/hyaline width (Lišková *et al.*, 1997)
*Two females possess an additional odontostyle, both longer than the functional one.

Table II. Morphometric characters of juvenile stages of *L. helveticus* from Serbia.

Locality:	Stari Ledinci, Fruška gora Mountain			
Host:	*R. aculeatus*			
n	21 J1	20 J2	14 J3	22 J4
L (mm)	1.55±0.04 (1.50-1.65)	2.57±0.21 (2.22-2.90)	3.62±0.41 (2.89-4.11)	5.43±0.42 (4.37-6.10)
a	51.7±1.72 (48.6-55.1)	56.4±2.67 (51.4-60.8)	62.3±3.82 (56.7-71.9)	72.9±3.70 (65.5-81.4)
b	4.4±0.16 (4.1-4.7)	6.2±0.68 (5.1-8.0)	7.6±1.01 (6.3-10.2)	9.7±0.95 (7.9-12.5)
c	34.8±2.00 (31.2-37.9)	70.9±7.61 (60.6-88.8)	98.5±12.14 (74.2-116.5)	134.5±11.87 (108.4-157.2)
c′	1.86±0.11 (1.67-2.04)	0.93±0.09 (0.74-1.07)	0.76±0.05 (0.65-0.82)	0.68±0.04 (0.60-0.75)
d	2.2±0.09 (2.0-2.3)	2.1±0.06 (1.9-2.2)	2.0±0.09 (1.8-2.1)	2.0±0.09 (1.9-2.2)
d′	1.8±0.06 (1.7-1.9)	1.8±0.05 (1.6-1.9)	1.8±0.07 (1.6-1.9)	1.9±0.06 (1.7-2.0)
J′	1.2±0.08 (1.1-1.4)	0.4±0.03 (0.3-0.5)	0.3±0.02 (0.3-0.4)	0.3±0.02 (0.3-0.3)
Odontostyle µm	80.8±2.18 (77.5-85.0)	90.4±2.29 (86.2-95.0)	108.7±4.23 (101.2-115.6)	128.9±3.31 (122.5-134.6)
Odontophore µm	48.8±2.65 (43.8-52.5)	63.6±2.39 (60.0-68.8)	72.2±2.86 (66.3-75.0)	79.0±3.90 (71.3-86.3)
Total stylet µm	128.6±5.72 (106.2-133.7)	154.0±3.58 (148.7-162.5)	180.9±5.48 (170.0-188.1)	207.9±5.78 (193.8-218.4)
Replacement odontostyle µm	90.3±1.53 (87.5-92.5)	109.7±3.03 (103.7-113.7)	127.3±5.32 (117.5-133.3)	144.8±7.08 (132.1-170.2)
Oral aperture to guide ring µm	23.1±0.60 (21.9-23.8)	28.6±0.92 (26.9-30.0)	33.0±1.48 (30.0-35.0)	38.2±1.34 (35.6-40.6)
Tail µm	44.8±2.34 (40.0-48.6)	36.4±2.36 (32.5-41.1)	36.9±2.68 (32.1-42.1)	40.5±2.46 (34.6-44.6)
J (hyaline portion of tail) µm	20.3±1.51 (17.5-22.5)	11.1±0.81 (9.4-12.2)	11.7±1.31 (8.8-13.8)	13.3±0.91 (10.6-15.0)
Body diam. at lip region µm	10.6±0.36 (10.0-11.3)	13.9±0.25 (13.4-14.4)	16.8±0.58 (15.9-17.5)	19.0±0.54 (18.1-20.0)
Body diam. at guide ring µm	18.8±0.24 (18.1-19.4)	24.6±0.90 (22.5-26.3)	30.0±1.18 (27.8-31.7)	35.7±1.01 (34.2-37.5)
Body diam. at base of oesophagus µm	30.0±0.33 (29.4-30.9)	42.8±2.92 (36.9-47.2)	53.3±4.15 (47.5-59.4)	65.2±2.94 (57.5-69.4)
Body diam. at mid-body µm	30.0±0.47 (29.4-31.3)	45.6±4.38 (37.5-52.5)	58.2±6.88 (49.2-69.1)	74.5±5.11 (65.9-85.0)
Body diam. at anus µm	24.2±0.51 (23.4-25.0)	39.5±2.60 (34.1-43.8)	48.9±3.96 (41.6-57.5)	59.7±2.05 (55.4-63.8)
Body diam. at beginning of J µm	16.7±0.77 (15.3-18.1)	28.4±2.43 (23.8-32.5)	35.6±3.26 (28.4-40.9)	42.1±1.95 (37.5-44.7)

Table III. Morphometrics of juvenile stages and females of the *L. helveticus* populations from Switzerland and Serbia.

Developmental stages and populations	Body length (mm) (mean)	Odontostyle (µm) (mean)	Replacement odontostyle (µm) (mean)
J1			
Gersau, Camenzind, Switzerland (Lamberti *et al.*, 2001)	1.80	80.2	86.8
Stari Ledinci, Serbia (original)	1.55	80.8	90.3
J2			
Gersau, Camenzind, Switzerland	2.70	87.7	108.0
Stari Ledinci, Serbia	2.57	90.4	109.7
J3			
Gersau, Camenzind, Switzerland	4.00	108.2	123.1
Stari Ledinci, Serbia	3.62	108.7	127.3
J4			
Gersau, Camenzind, Switzerland	5.70	123.2	136.0
Stari Ledinci, Serbia	5.43	128.9	144.8
Females			
Gersau, Camenzind, Switzerland	7.80	135.4	–
Stari Ledinci, Serbia	7.03	144.3	–

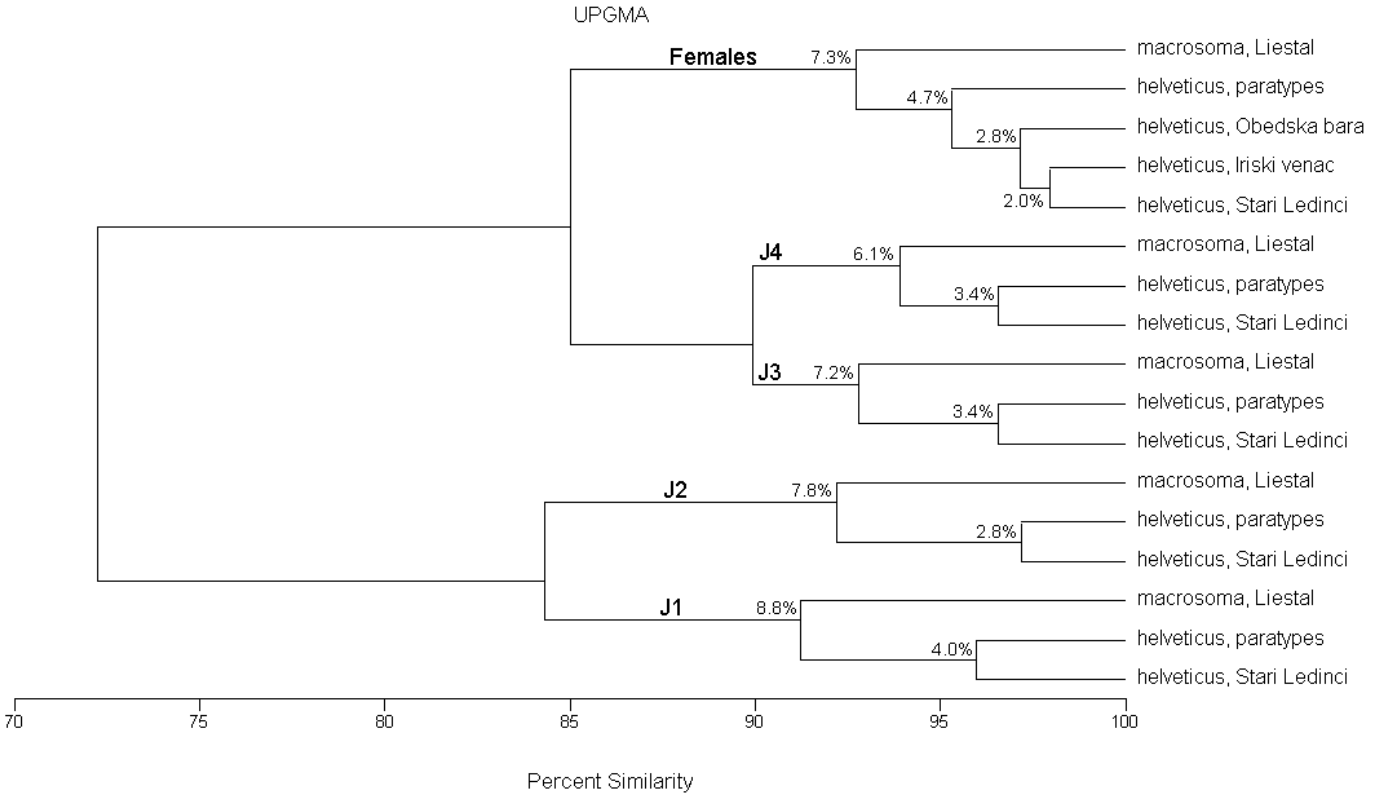

Fig. 5. Dendrogram showing similarity between populations of *L. helveticus* and *L. macrosoma*.

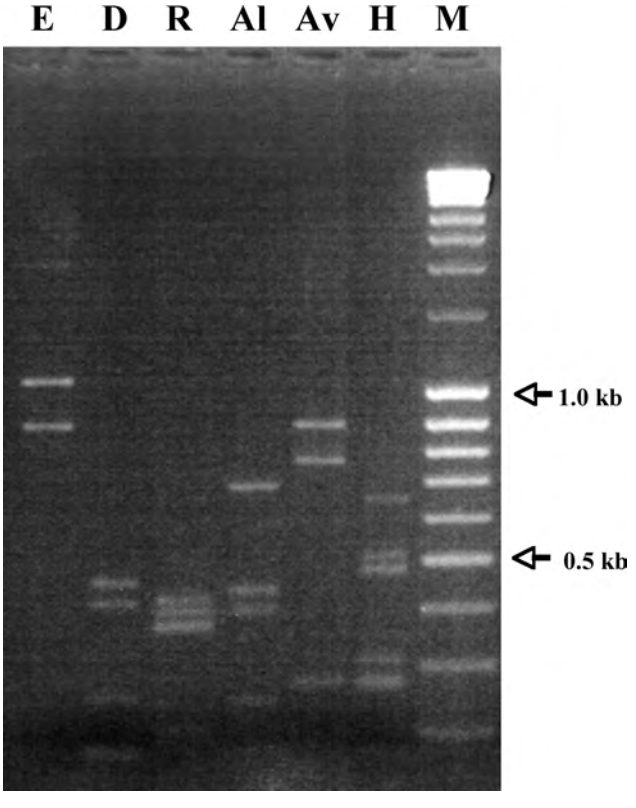

Fig. 6. Restriction patterns of the amplified ITS region of *L. helveticus*. A: *Alu* I; Av: *Ava* II; D: *Dde* I; E: *Eco* RI; R: *Rsa* I; H: *Hinf* I and M: 100 bp DNA ladder.

Rsa I and *Hinf* I produced polymorphic bands. The Switzerland population produced, with *Rsa* I, an extra band of 670 bp that is absent from the Serbia population; on the other hand, the *Hinf* I enzyme produced an extra band (520 bp) just in the Serbia population. Repeated digestions with an extended digestion period suggested heterogeneity of the ITS region in the *L. helveticus* from Serbia. Differences in RFLP between populations can be explained by the existence of differences in restriction sites in ITS sequences and/or the appearance of additional ITS haplotypes with different sequences. Heterogeneity in ITS regions is widely reported among free-living and parasitic nematodes (Powers *et al.*, 1997; Hugall *et al.*, 1999; Hung *et al.*, 1999; Subbotin *et al.*, 2000; Morales-Hojas *et al.*, 2001; Elbadri *et al.*, 2002; Otranto *et al.*, 2003).

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