

On the identity of *Labronema vulvapapillatum* (Meyl, 1954) Loof & Grootaert, 1981 (Dorylaimida, Qudsianematidae)

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Summary. Iranian populations of *Labronema vulvapapillatum* are studied in depth, including morphological and molecular data. They are characterised by having 1.76-2.50 mm long body in females and 1.55-2.19 mm in males, lip region offset by marked depression and 19-22 µm wide in females and 20-24 µm in males, odontostyle 22-26 µm long in females and 21-31 µm in males or 1.0-1.3 times the lip region width, neck 379-495 µm long in females and 366-484 µm in males, pharyngeal expansion 175-235 µm long in females and 164-226 µm in males or occupying 43-49% of total neck length, female genital system didelphic, uterus tripartite with a short intermediate section becoming a Z-organ and 199-254 µm long or 2.6-4.0 times the body diameter, *pars refringens vaginae* present, vulva longitudinal ($V = 49-59$), tail short and rounded, (17-37 µm, $c = 72-114$, $c' = 0.5-0.9$ in females; 19-28 µm, $c = 59-82$, $c' = 0.6-0.8$ in males), spicules 55-76 µm long and 16-25 contiguous ventromedian supplements. Iranian material is compared with other known populations, concluding that there are no relevant morphological differences among them but wide overlap in their main morphometrics. The 18S rRNA gene sequence of Iranian *L. vulvapapillatum* is very close to other *Labronema* sequences, especially to the Dutch population of the same species.

Key words: Description, Iran, *Labronema*, morphology, phylogeny, SEM, SSU-rDNA, taxonomy.

Meyl (1954) identified one Italian dorylaimid female as “*Dorylaimus obtusicaudatus* Bastian, 1865 var. *vulvapapillatus* n. var.”, provided a very short description and its Demanian ratios, and illustrated only the vulva region (original Fig. 39), in which one pre- and another post-vulval papillae were present. Andrassy (1959) classified this taxon under *Eudorylaimus* Andrassy, 1959 as *E. vulvapapillatus* (Meyl, 1954), but did not provide any further information about it. A few years later, however, the same author (Andrassy, 1962) studied and illustrated a couple of specimens of *E. vulvapapillatus* from Hungary. Loof and Grootaert (1981) re-described this species based on material collected in Scotland and maintained in culture in Belgium and Western Germany, and transferred it to *Labronema* Thorne, 1939. Subsequently, *L. vulvapapillatum* has been reported from The Netherlands (Bongers, 1988), Spain (Jiménez-Guirado, 1989; Murillo-Navarro & Jiménez-

Guirado, 2006), Korea (Choi *et al.*, 1997) and Hungary (Andrassy, 2002). Some authors (Andrassy, 2002; Murillo-Navarro & Jiménez-Guirado, 2006) noted significant morphometric differences between known populations of *L. vulvapapillatum* and suggested they might belong to more than one species.

Iranian populations of this species were collected during an extensive nematological survey. Their study revealed new relevant data on the identity of *L. vulvapapillatum*. The results obtained are presented here.

MATERIALS AND METHODS

Nematode materials. Nematodes were collected from natural and disturbed habitats in Iran, killed by heat, fixed in 4% formaldehyde, processed to anhydrous glycerol according to De Grisse (1969) and mounted on glass slides. Morphometrics included

Table 1. Nematode species and GenBank accession number used for phylogenetic study.

Origin	Reference	GenBank accession number	Species
The Netherlands	Holterman <i>et al.</i> , 2006	AY284801	<i>Allodorylaimus andrassyi</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284812	<i>Aporcelaimellus</i> cf. <i>paraobtusicaudatus</i>
UK	Blaxter & Eyualem-Abebe, unpubl.	DQ141212	<i>Aporcelaimellus obtusicaudatus</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284828	<i>Discolaimus major</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284821	<i>Dorylaimellus montenegricus</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284776	<i>Dorylaimus stagnalis</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284783	<i>Ecumenicus monohystera</i>
Iran	Pedram <i>et al.</i> , 2009	FJ042953	<i>Enchodelus macrodorus</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284803	<i>Epidorylaimus lugdunensis</i>
Antarctica	Raymond & Wharton, unpubl.	HQ270136	<i>Eudorylaimus</i> sp.
United States	Mullin <i>et al.</i> , 2005	AY552972	<i>Labronema ferox</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284807	<i>Labronema vulvapapillatum</i>
Iran	Present paper	KC574385	<i>Labronema vulvapapillatum</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284831	<i>Leptonchus granulosus</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284780	<i>Mesodorylaimus</i> sp.
The Netherlands	Holterman <i>et al.</i> , 2006	AY284806	<i>Microdorylaimus modestus</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284826	<i>Paractinolaimus macrolaimus</i>
Belgium	Meldal <i>et al.</i> , 2007	AY993978	<i>Paractinolaimus macrolaimus</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284788	<i>Pungentus silvestris</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284785	<i>Opisthodorylaimus sylphoides</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284786	<i>Opisthodorylaimus sylphoides</i>
The Netherlands	Holterman <i>et al.</i> , 2008	AY284824	<i>Oxydirus oxycephalus</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284835	<i>Tylencholaimus mirabilis</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284795	<i>Thonus circulifer</i>
The Netherlands	Holterman <i>et al.</i> , 2006	AY284794	<i>Thonus minutus</i>
India	Oliveira <i>et al.</i> , 2004	AY297821	<i>Mononchus aquaticus</i> (out group)

de Man's indices and most of the usual measurements. Some of the best preserved specimens were photographed with a Nikon Eclipse 80i microscope and a Nikon DS digital camera. Raw photographs were edited using Adobe® Photoshop® CS. After their examination and identification, a few specimens preserved in glycerin were re-processed for observation under SEM following the protocol by Abolafia and Peña-Santiago (2005). The nematodes were hydrated in distilled water, dehydrated in a graded ethanol and acetone series, critical point dried, coated with gold, and observed with a JEOL JSM-5800 microscope.

Phylogenetic analysis. The sequences of several species of the superfamily Dorylaimoidea used for phylogenetic analysis were obtained from the GenBank (Table 1). DNA extraction was done using an *AccuPrep* Genomic DNA extraction kit (Bioneer Corporation, Korea, <http://www.bioneer.com>) according to the manufacturer's instructions. Individual specimens (ten specimens belong to Khabr population) were picked

into 1.5 ml tubes containing 5 µl double distilled water. Each tube was frozen in liquid nitrogen and was crushed using a vortex. Tissue Lysis buffer (TL; 200 µl) and 20 µl Proteinase K (20 mg ml⁻¹) were added. The homogenate was incubated at 60°C for 2 h. The supernatant was extracted and stored at -20°C. The forward primer SSU_F_04 (5'-GCT TGT CTC AAA GAT TAA GCC-3') and the reverse primer SSU_R_26 (5'-CAT TCT TGG CAA ATG CTT TCG-3') (Blaxter *et al.*, 1998) were used in the PCR reactions for amplification of the partial 18S region (~900bp). PCR was conducted with 10 µl of the extracted DNA, 4 µl of PCR Master Mix (Kawsar Biotech company, Iran), 1 µl of each primers (10 pmol µl⁻¹) and ddH₂O to a final volume of 25 µl. The amplification was carried out using an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany), with the following parameters: 3 min at 94°C, 37 cycles of 45 s at 94°C, 45 s at 56°C and 1 min at 72°C, and finally one cycle of 6 min

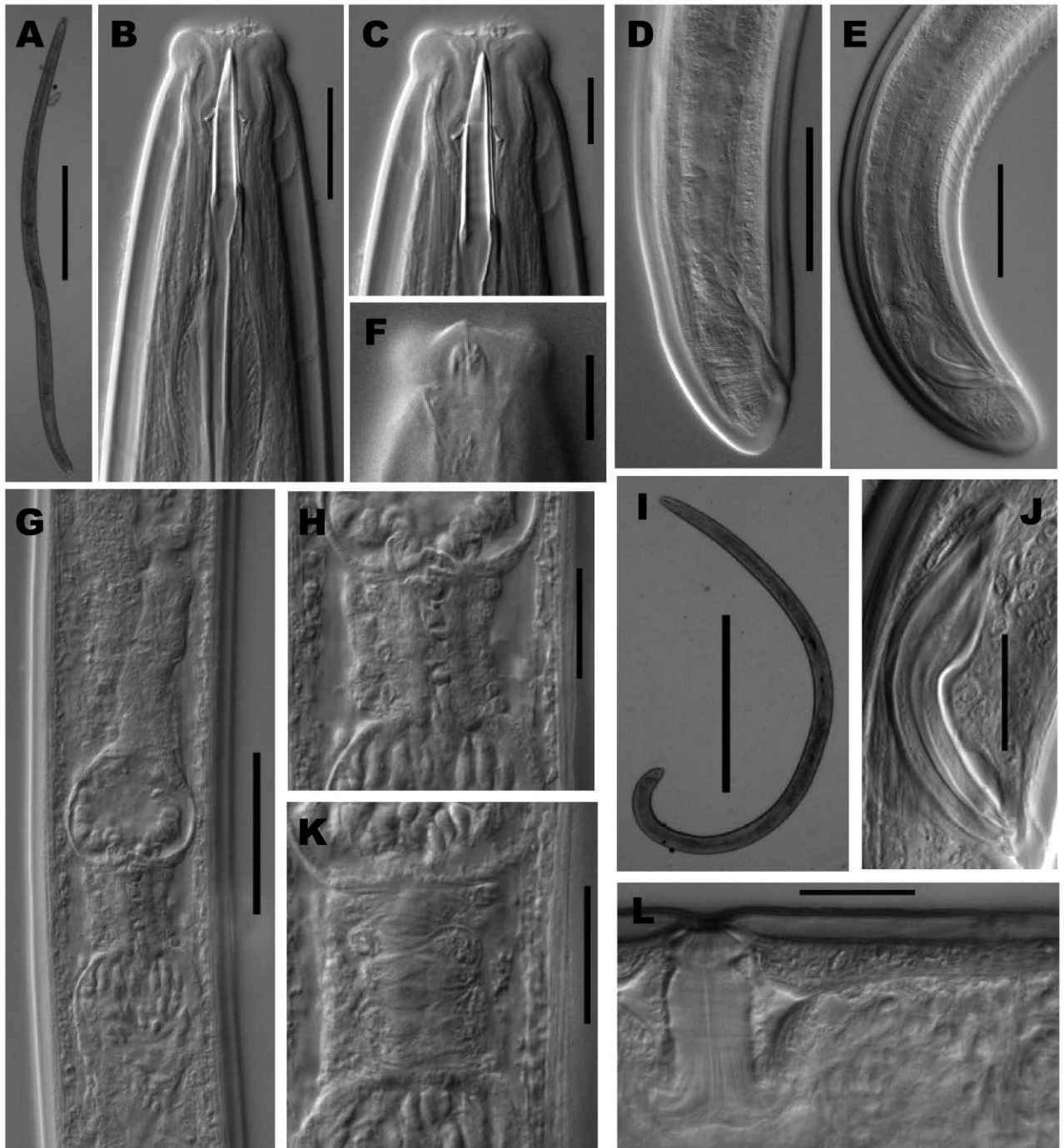
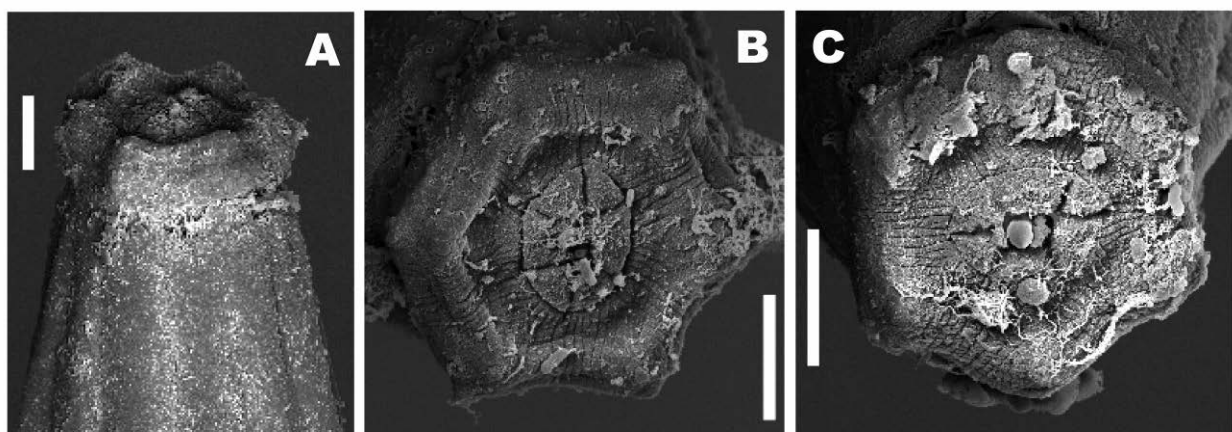


Fig. 1. *Labronema vulvapapillatum* (Meyl, 1954) Loof & Grootaert, 1981 (LM). A: Female, entire. B, C: Anterior region in median view. D: Female, posterior body region. E: Male, posterior body region. F: Lip region in lateral, surface view. G: Uterus, showing its three sections. H, K: Details of intermediate section (Z-organ) of uterus. I: Male, entire. J: Spicules. L: Vagina. (Scale bars: A, I = 500 μ m; B, H, K, J, L = 20 μ m; C, F = 10 μ m; D, E, G = 50 μ m).

Table 2. Morphometric data of *Labronema vulvapapillatum* (Meyl, 1954) Loof & Grootaert, 1981. Measurements in μm (except L, in mm) and in the form: mean \pm standard deviation (range).

Character	Habitat	Turf		Turf		Turf	
	Population	Khahr	Khahr	Kerman University Campus	Kerman University Campus	Kerman Baghe Melli	Kerman Baghe Melli
	n	5♀♀	7♂♂	2♀♀	2♂♂	3♀♀	3♂♂
L		2.12 \pm 0.26 (1.81-2.50)	1.83 \pm 0.19 (1.55-2.09)	2.08, 1.94	1.71, 2.19	1.76-1.83	1.83-1.96
a		30.2 \pm 3.6 (26-36)	29.0 \pm 3.9 (24-34)	33, 40	33, 35	25-30	22-31
b		5.0 \pm 0.1 (4.8-5.1)	4.4 \pm 0.3 (3.8-4.7)	4.5, 4.7	4.5, 4.5	4.4-4.5	4.5-4.8
c		99.7 \pm 13.2 (83-114)	76.7 \pm 8.6 (65-77)	72, 92	73, 59	77-90	74-82
c'		0.67 \pm 0.2 (0.5-0.9)	0.7 \pm 0.1 (0.6-0.8)	0.8, 0.6	0.7, 0.8	0.6-0.7	0.6-0.7
V		54.6 \pm 3.2 (52-59)	–	55, 51	–	49-52	–
Lip region diameter		21.4 \pm 0.5 (21-22)	21.4 \pm 1.4 (20-24)	21, 19	20, 22	19-21	20-21
Odontostyle length		26.8 \pm 1.6 (25-29)	26.9 \pm 2.2 (24-31)	26, 20	21, 24	21-25	24-26
Odontophore length		44.8 \pm 3.6 (41-49)	43.1 \pm 3.9 (39-49)	43, 41	37, 44	37-38	35-36
Neck length		424.0 \pm 44.4 (379-495)	417.6 \pm 35.9 (366-470)	463, 410	383, 484	386-411	388-427
Pharyngeal expansion length		204.4 \pm 21.2 (175-235)	204.0 \pm 17.2 (177-226)	198, 188	164, 224	178-200	186-195
Diameter at neck base		64.4 \pm 7.9 (56-76)	60.6 \pm 8.4 (51-75)	58, 58	51, 59	59-67	55-74
mid-body		70.6 \pm 9.7 (59-86)	63.6 \pm 8.5 (54-77)	64, 65	52, 63	65-73	63-82
anus		39.8 \pm 3.8 (34-43)	35.7 \pm 2.4 (33-39)	37, 35	40, 39	41-44	36-39
Prerectum length		87.4 \pm 6.9 (75-91)	124 \pm 24 (92-140)	67, 67	132, 132	71-109	165-189
Rectum/cloaca length		47.8 \pm 6.9 (40-56)	55.6 \pm 7.4 (45-66)	48, 50	53, 60	41-44	67-72
Tail length		21.6 \pm 4.5 (17-28)	25.0 \pm 3.1 (19-28)	37, 35	29, 30	20-23	24-26
Spicules length		–	62.0 \pm 6.5 (55-73)	–	62, 73	–	71-76
Ventromedian supplements		–	21-25	–	21	–	16-22

**Fig. 2.** *Labronema vulvapapillatum* (Meyl, 1954) Loof & Grootaert, 1981 (SEM). A: Anterior region in sublateral view; B, C: Lip region in face view. (Scale bars = 5 μm).

at 72°C followed by a holding temperature of 4°C. After DNA amplification, 5 µl of product was loaded on a 1% agarose gel (40 mM Tris, 40 mM boric acid, and 1 mM EDTA) to verify amplification. The bands were stained with 50 mM ethidium bromide and visualised and photographed on 1% agarose gel under a UV transilluminator. Product was stored at -20°C prior to sequencing. The PCR product was purified for sequencing and sequenced with primers that were used for the amplification step. Sequencing was performed in both directions. The DNA sequence was edited using Chromas version 1.45 (McCarthy, 1997). Sequencing reactions were performed by the Bioneer Company (South Korea, <http://eng.bioneer.com>). Additional sequences for the ingroups and outgroups were obtained from NCBI GenBanks (Table 1).

The ribosomal SSU sequences were analysed and aligned using BioEdit (Hall, 1999). Phylogenetic trees were generated using the Bayesian inference method as implemented in the program Mr Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The analysis under GTR model was initiated with a random starting tree and run with the Markov Chain Monte Carlo (MCMC) for 10⁶ generations. *Mononchus aquaticus* (AY297821) was used as outgroup for this phylogenetic analysis. This selection was based on a study by Álvarez-Ortega and Peña-Santiago (2012). The original partial 18S sequence of *Labronema vulvapapillatum* was deposited in GenBank under accession number KC574385. The Bayesian tree was visualised with the TreeView program (Page, 1996).

DESCRIPTION

Labronema vulvapapillatum (Meyl, 1954) Loof & Grootaert, 1981 (Figs 1 & 2)

Material examined. Ten females and 12 males from three localities, in good state of preservation.

Measurements. See Table 2.

Adult. Slender nematodes of medium size, 1.55-2.50 mm long. Habitus after fixation often curved ventrad, to an open 'C' in female, more curved ventrad in posterior body region in male, to G-shaped. Body cylindrical, tapering towards both ends, but more so towards the anterior extremity since the caudal region is rounded. Cuticle with very fine transverse striation, 3-5 µm thick in anterior region, 3.5-5.0 µm at mid-body and 3.0-8.0 µm on tail. Lateral chord 15-20 µm wide or occupying about one-fourth (19-29%) of mid-body diameter. Lateral pores small but visible throughout the body;

two to four dorsal and ventral, especially distinct, pores are present at level of odontostyle plus odontophore. Lip region weakly angular when observed in surface view but more rounded in median view, offset by marked depression (but a constriction is in no way perceptible), 2.8-3.8 times as broad as high, and about one-third (29-39%) of body diameter at neck base. SEM observations (Fig. 2): lip region hexagonal with straight sides and oral field perceptibly sunken (sucker-like) and bearing radial incisures; lips amalgamated, with a well demarcated perioral area, which is divided in six sectors (low liplets), the lateral ones trapezoidal, the others triangular; papillae located at the elevated margins of lip region and weakly protruding on surface of lips. Amphid fovea funnel-shaped, opening at level of the cephalic depression; its aperture 9-12 µm wide or about one-half (43-55%) of lip region diameter. Odontostyle strong, 5.2-6.9 times as long as wide, hardly longer (1.0-1.3 times) than lip region diameter and 1.03-1.35% of total body length; aperture occupying about two-fifths (38-46%) of its length. Odontophore rod-like, 1.6-2.1 times as long as the odontostyle. Guiding ring double, with fixed ring at 14-17 µm or about 0.6-0.8 times the lip region diameter from anterior end. Anterior slender region of the pharynx expanding very gradually; basal expansion 5.0-6.9 times as long as wide, 2.7-3.8 times as long as body diameter at neck base, and occupying up to one-half (43-49%) of total neck length. Pharyngeal gland nuclei and their outlets as follows (n = 4, females): DN = 59-61, S₁N₁ = 71-73, S₁N₂ = 76-78, S₂N = 88-89. Cardia rounded conoid, 15-25 × 11-19 µm, surrounded by intestinal tissue that forms a long conical projection bulging into intestinal lumen. A dorsal cell mass is present at the anterior end of intestine in some specimens. Caudal region short and rounded.

Female. Genital system didelphic-amphidelphic, with both genital branches equally and very well developed, the anterior 344-410 µm or 15-20% of body length, the posterior 329-407 µm or 16-20% of body length. Ovaries reflexed, 155-258 µm (anterior) and 61-233 µm (posterior) long, very often reaching and surpassing the sphincter level; oocytes arranged in a single row except near its tip. Oviduct 106-141 µm long or 1.4-2.1 times the body diameter, and consisting of a slender portion with prismatic cells joining the ovary subterminally and a well-developed *pars dilatata*, longer than wide, with distinct lumen and often containing sperm cells. A weak sphincter is present at oviduct-uterus junction. Uterus 194-254 µm long or 2.6-4.0 times the body diameter; tripartite, i.e. consisting of an intermediate and short section representing a Z-organ (with thick

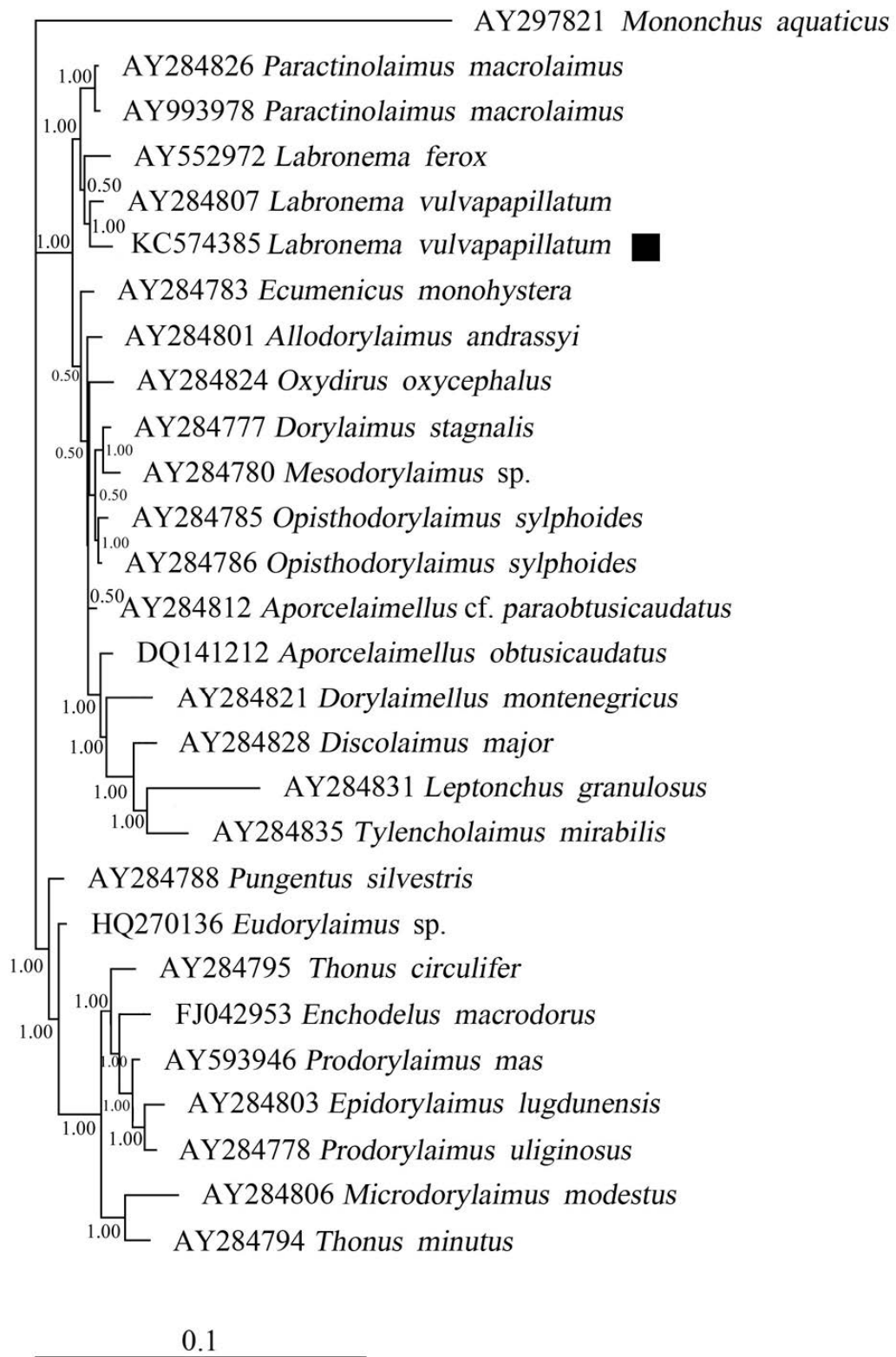


Fig. 3. The Bayesian Inference tree of newly sequenced *Labronema vulvapapillatum* from Iran and closely related species belonging to the superfamily Dorylaimoidea based on 18S rDNA region from GenBank.

walls surrounded by strong circular musculature, narrow lumen, and containing irregular, often triangular or trapezoidal, refractive elements), and proximal and distal regions wider than the intermediate one, which become visibly dilated close the Z-organ, nearly spherical when they contain sperm cells. Vagina extending inwards 34-37 μm or two-fifths to one-half (40-55%) of body diameter: *pars proximalis* 22-26 x 11-17 μm , with almost straight walls, and circled by weak musculature, *pars refringens* with two small, 5-7 x 2-3 μm , sclerotized pieces separated by a less sclerotized area and with a combined width of 13 μm , and *pars distalis* 3-4 μm long. Vulva an oval longitudinal slit. One female bearing one ventral papillae (paravulva) near the vulva. Prerectum 1.9-3.1 times, rectum 1.2-1.4 times the anal body diameter long. Caudal pores two pairs, one subdorsal and another sublateral, at the middle of tail.

Male. Prerectum 3.0-5.0, cloaca 1.3-2.0 times the anal body diameter long. Genital system diorchic, with opposite testes. In addition to the ad-cloacal pair, located at 7-10 μm from the cloacal aperture, there is a series of 16-24 contiguous ventromedian supplements, the most posterior of which situated at 70-86 μm from the ad-cloacal pair, out the range of spicules. Spicules dorylaimoid, 4-0-5.6 times as long as wide (length measured along the curve), and 1.6-1.9 times the anal body width long. Lateral guiding piece 12-18 μm long, 3.5-4.3 times as long as wide and furcate at the end. Caudal pores several pairs.

Molecular characterisation. One 18S rRNA gene sequence was obtained, consisting of 922 bps.

Diagnosis. Iranian populations of *L. vulvapapillatum* are distinguishable by having 1.76-2.50 mm long body in females and 1.55-2.19 mm in males, lip region offset by marked depression and 19-22 μm wide in females and 20-24 μm in males, odontostyle 22-26 μm long in females and 21-31 μm in males or 1.0-1.3 times the lip region width, neck 379-495 μm long in females and 366-484 μm in males, pharyngeal expansion 175-235 μm long in females and 164-226 μm in males or occupying 43-49% of total neck length, female genital system didelphic, uterus tripartite with a short intermediate section becoming a Z-organ and 199-254 μm long or 2.6-4.0 times the body diameter, *pars refringens vaginae* present, vulva longitudinal (V = 49-59), tail short and rounded, (17-37 μm , c = 72-114, c' = 0.5-0.9 in females; 19-28 μm , c = 59-82, c' = 0.6-0.8 in males), spicules 55-76 μm long and 16-25 contiguous ventromedian supplements.

Distribution. Three localities in Kerman province: i) Baghe Melli (N: 30°16'48.97"; S: 057°04'01.27"); ii) Campus of the Shahid Bahonar

University of Kerman, (N: 30°15'27.10"; E: 57°06'13.59"); and iii) Khabr National Park (N: 28°81'71.54"; S: 056°32'66.04"), all populations in association with a seed mixture of *Poa* sp., *Festuca* sp. and *Lolium* sp.

Remarks. The Iranian material examined is morphologically identical, with no significant difference in its diagnostic features. Morphometrically, it also confirms a rather homogeneous group since only small differences in main measurements and ratios are observed between the representatives of the three populations, whose ranges, however, often show coincidence or wide overlapping. These interpopulation differences are certainly explainable on the basis of geographical variability and/or the low number of nematodes available in each case.

The characterisation and the taxonomy of *L. vulvapapillatum* have been a matter of discussion and deserve special attention. Table 3 compiles the main morphometrics of the populations hitherto studied. Available data show that *L. vulvapapillatum* displays remarkable variation in general size and, especially important, in odontostyle length. For instance, in relation to the size, body length always exceeds 2.5 mm in specimens from Scotland/Belgium (originally collected in Scotland but maintained in culture in Belgium, *cf.* Loof and Grootaert, 1981), whereas nematodes from other locations only exceptionally reach 2.5 mm and are under 2.0 mm in several cases. Concerning the odontostyle length, a more reliable feature than body length for characterising dorylaims, the range (20-32 μm) is relatively large, with some overlapping when comparing specimens from distant locations, but also allowing their separation into two groups of populations whose respective ranges are 20-26 and 25-32 μm . Several authors (Andrássy, 2002; Murillo-Navarro & Jiménez-Guirado, 2006) noted such differences and suggested that they might correspond to two different species. Nevertheless, leaving aside the odontostyle length, it is not possible to find other morphological or morphometric differences to define two separate patterns. On the contrary, there are several remarkable morphological features (among others, lip region offset by marked depression but never a distinct constriction, uterus tripartite with Z-organ, longitudinal vulva and presence of ad-vulval papillae) that confer a great homogeneity to the group.

A Blastn search of *L. vulvapapillatum* SSU region revealed the highest match with a Dutch

population of the same species (GenBank accession number AY284807), differing from this in 12 bps (identity 99%), as well as American population of *L. ferox* (accession number AY552972), from which it differs in 14 bps (98%). Figure 3 shows the evolutionary relationships of the material examined as derived from molecular analysis. The sequence of Iranian *L. vulvapapillatum* is included, together with other two *Labronema* sequences, in a large clade dominated by long-tailed dorylaimid taxa, confirming other recent result, e.g. those by Álvarez-Ortega and Peña-Santiago (2012) based on D2D3 region sequences.

Labronema vulvapapillatum is probably a widely distributed taxon in Northern Hemisphere, hitherto restricted to the Palearctic range, since it was never recorded out of Eurasia.

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E. Shokoohi, J. Abolafia, A. Mehrabi-Nasab and R. Peña-Santiago. К идентификации вида *Labronema vulvapapillatum* (Meyl, 1954) Loof & Grootaert, 1981 (Dorylaimida, Qudsianematidae).

Резюме. Проведено всестороннее изучение популяций *Labronema vulvapapillatum* из Ирана и получены молекулярные и морфологические данные. Нематоды характеризуются длиной тела самок 1.76-2.50 мм и самцов 1.55-2.19 мм, обособленным заметным углублением губным отделом шириной 19-22 мкм у самок и 20-24 мкм у самцов, одонтостилем длиной 22-26 мкм у самок и 21-31 мкм у самцов, то есть равным или слегка превышающим (1.0-1.3 раза) ширину губного отдела, шейным отделом длиной 379-495 мкм у самок и 366-484 мкм у самцов, расширением пищевода длиной 175-235 мкм у самок и 164-226 мкм у самцов или занимающим 43-49% длины шейного отдела, дидельфной половой системой самок, состоящей из трех частей маткой с короткой промежуточной частью, функционирующей как орган Z длиной 199-254 мкм, что в 2.6-4.0 раза больше диаметра тела, наличием *pars refringens vaginae*, продольной вульвой (V = 49-59), коротким закругленным хвостом (17-37 мкм длиной, c = 72-114, c' = 0.5-0.9 у самок; 19-28 мкм длиной, c = 59-82, c' = 0.6-0.8 у самцов), спикулами длиной 55-76 мкм и присутствием 16-25 смежных вентромедианных супплементов. Материал из Ирана сравнили с данными по другим известным популяциям и пришли к заключению, что существенных морфологических отличий не имеется, а главные морфометрические показатели широко перекрываются. Последовательность участка 18S рДНК иранской *L. vulvapapillatum* оказывается очень близкой к другим последовательностям, известным для *Labronema*, особенно к голландской популяции того же вида.
