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Research Article

Morphological and molecular characterization of *Leptonchus granulosus* Cobb, 1920 (Dorylaimida: Leptonchidae) from Iran

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Abstract: *Leptonchus granulosus*, recovered from Lorestan province, is described and illustrated based on morphological, morphometric and molecular data. The Iranian population of the species is characterized by its body length of 1091 - 1374 µm, cuticle distinctly two layered, outer layer finely annulated, inner layer distinctly annulated, being partly separated from the body and shriveled after fixation, cap-like lip region separated from the rest of body by constriction, distinctly sclerotised walls of prestoma and stoma, delicate needle-like odontostyle with distinct narrow lumen, 8.0-9.5 µm long, slightly arcuate odontophore, 17-21 µm long, with arms slightly thickened at base, small pear-shaped pharyngeal bulb, occupying 16.6-24.3% of pharynx length, simple intestine, very long prerectum (617-663 µm long), its junction with intestine having three distinct guard cells located between anterior ovary and cardia, didelphic female reproductive system, composed of equally sized less developed tracts, but with distinct parts (tubular uterus, simple oviduct and ovary), conoid to hemispheroid tail and absence of males. In comparison with the available reports of the species, no remarkable variation in morphometric data ranges was observed.

This is the first representative of the genus for Iran's nematode fauna found so far. Molecular phylogenetic studies of Iranian population of *L. granulosus* using 1669 nt partial sequences of 18S rDNA revealed it forming a clade with another isolate of the species in Bayesian inference (BI) with 0.95 Bayesian posterior probability (BPP).

Keywords: 18S rDNA, Bayesian, Iran, *Leptonchus*, taxonomy

Introduction

The genus *Leptonchus* was erected by Cobb (1920) and according to Andrássy (2009) is mainly characterized by having tylencholaimoid cuticle finely striated in outer layer and distinctly annulated in inner one, cap like lip region lacking a disc, delicate short odontostyle with fine aperture and lumen, rod-like (simple) odontophore with usually arcuate and slightly thickened arms at base, narrow slender anterior part of pharynx, prerectum long and didelphic female reproductive system. It belongs to the family Leptonchidae Thorne, 1935, superfamily Tylencholaimoidea Filipjev, 1934. The genus *Leptonchus*, which is not one of the most widespread dorylaim genera, according to Andrássy (2009) currently contains 11 known species. The species *L. caudatus* Baqri and Jairajpuri, 1968 was enclosed within the valid species of the genus listed by Jairajpuri and Ahmad (1992) and reported by Andrássy (2009); however, a species with this name has never been described and its report is clearly the result of a *lapsus calami* by Jairajpuri and

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Ahmad, 1992, probably instead of *L. capitatus* Baqri and Jairajpuri, 1968 which in the same list was lacking; therefore the specific name *L. caudatus* is regarded here as *nomen nudum*.

Based on our knowledge, there is only one report dealing with occurrence of Leptonchidae members in Iran (Pedram *et al*., 2012). The species *L. granulosus* Cobb, 1920 was recovered from sandy soil samples collected around the rhizosphere of unknown grasses near Kashkan River in city of Kouhdasht, Lorestan province and studied based on morphological and molecular characters as the first representative of the genus occurring in Iran.

Materials and Methods

Soil samples were collected from city of Kouhdasht, Lorestan province, southwestern Iran, during September 2014. The nematodes were extracted from soil using the tray method (Whitehead and Hemming, 1965). Nematodes of interest were handpicked under a Nikon SMZ1000 stereomicroscope, heat-killed by adding boiling 4% formalin solution and transferred to anhydrous glycerin according to De Grisse (1969). To see details of body structure and make microphotographs, temporary slides were made using live females (sharp body females were selected and killed by gentle heat in a drop of water. A support was used to prevent pressure on the nematode while putting the slide cover). Drawings were made using permanent slides and a drawing tube attached to a Nikon E600 light microscope. The handmade drawings were redrawn using CorelDRAW® software version 16. Light microphotographs were provided using an Olympus BX51 light microscope powered with differential interference contrast (DIC) and a DP72 digital camera.

For molecular phylogenetic study, a single live nematode specimen was selected, observed in a drop of clean water (a temporary slide was made for checking of correct selection), transferred to a small drop of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0, QIAGEN Inc., Valencia CA, USA) on a clean slide and squashed using a clean cover glass. The suspension (DNA sample) was recollected by adding 30 µl AE buffer and stored at -20 °C until used as DNA template. Primers for SSU amplification were forward primer 988F (5′-CT CAAAGATTAAGCCATGC-3′) and reverse primer 1912R (5′-TTTACGGTCAGAACTAG GG-3′), forward primer 1813F (5′-CTGCGTG AGAGGTGAAAT-3′) and reverse primer 2646R (5′-GCTACCTTGTTACGACTTTT-3′) (Holterman *et al*., 2006). The 30 µl PCR mixture contained: 18.5 µl distilled water, 3 µl 10 × PCR buffer, 0.6 µl dNTP mixture, 1.2 µl 50 mM $MgCl_2$, 0.6 µl of each primer (10 pmoles/µl), 0.5 µl of *Taq* DNA polymerase (CinnaGen, Tehran, Iran, 5 u/µl) and 5 µl of DNA template. The thermal cycling program was as follows: an initial denaturation at 94 ºC for 4 min, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 54 ºC for 40 s, and extension at 72 ºC for 80 s. A final extension was performed at 72 ºC for 10 min. The PCR products were sequenced in both directions using the same PCR primers using an ABI 3730XL sequencer (Bioneer Corporation, South Korea). The newly obtained partial SSU sequence was deposited in GenBank database under accession number KR184128. For reconstructing of phylogenetic relationships, the partial sequence of the Iranian isolate of *L. granulosus* and DNA sequences obtained from GenBank, using the BLAST homology search program, were aligned by the software Clustal X2 (http://www.clustal.org/). The model of base substitution was selected using MrModeltest 2 (Nylander, 2004). The Akaike-supported model, a general time reversible model, including among-site rate heterogeneity and estimates of invariant sites (GTR+G+I) was used in phylogenetic analysis. Bayesian analysis was performed to infer a phylogenetic tree using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) running the chains for 10^6 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analyses. The Markov chain Monte Carlo (MCMC) method within a Bayesian framework was used to determine equilibrium distribution and help

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estimate the posterior probabilities of the phylogenetic tree (Larget and Simon, 1999) using the 50% majority rule. The BPP values higher than 0.50 are given on appropriate clades. The output file of the pylogenetic program was visualized using Dendroscope V.3.2.8 (Huson and Scornavacca, 2012) and re-drawn in CorelDRAW software version 16. The species *Nygolaimus brachyuris* (de Man. 1880) Thorne, 1930 (accession number AY284771) was used as outgroup taxon. To calculate the similarity matrix of selected species (species belonging to *Leptonchus*), the program MatGat (Campanella *et al*., 2003) was used.

Results and Discussion

Iranian population of *Leptonchus granulosus* Cobb, 1920

(Figs. 1 and 2)

Measurements

See Table 1.

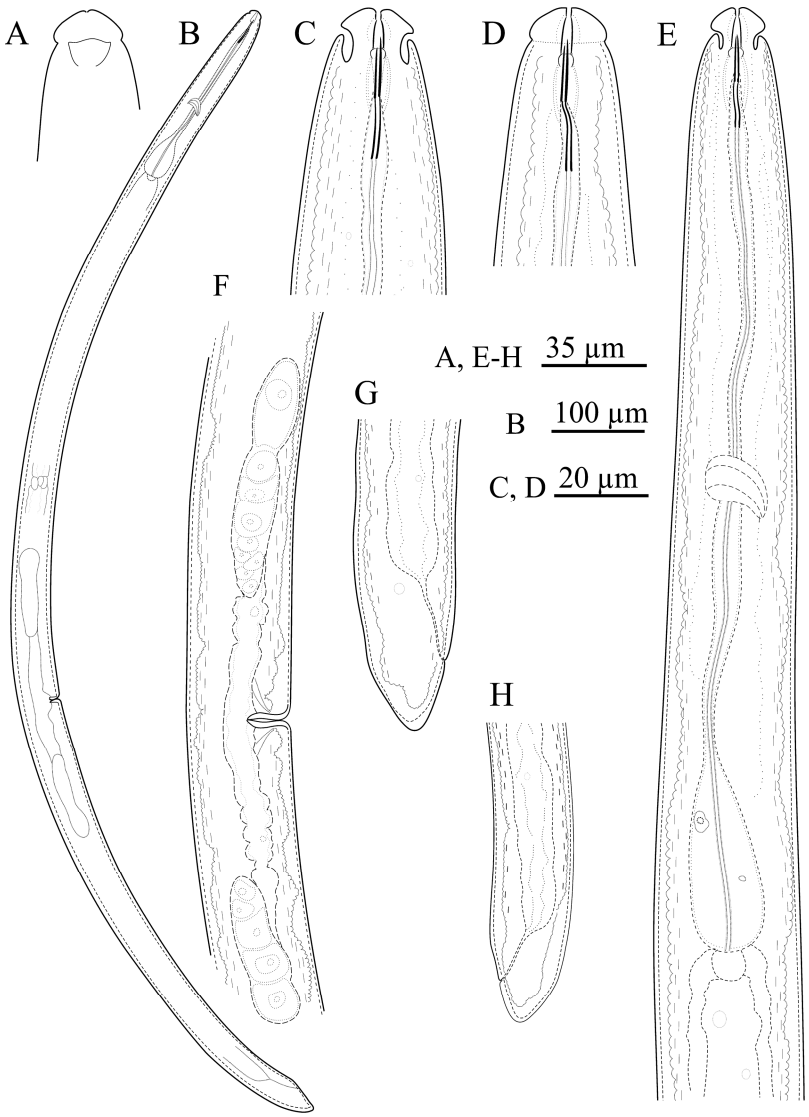

Figure 1 Iranian population of *Leptonchus granulosus*, female. A: Anterior end showing amphidial fovea, lateral view; B: Entire body; C and D: Anterior region in detail (C, Dorso-ventral view, D, Lateral view); E: Pharyngeal region; F: Reproductive system; G and H: Posterior body region and tail morphology.

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Figure 2 Iranian population of *Leptonchus granulosus*, female. A-D: Anterior region; E and F: Details of anterior end; G: Terminal bulb; H: Vulva region; I: Anterior tract of reproductive system; J: Nature of inner layer of the cuticle; K: Guard cells of prerectum and their position related to anterior ovary; L and M: Variation in tail morphology. All scale bars = 10 µm.

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Table 1 Morphometric data of *Leptonchus granulosus* Cobb, 1920. All measurements are in µm and in the form mean ± sd (range).

Origin	Iranian population	Peralta and Peña-Santiago, 1996	Andrássy, 2009
Characters	Female	Female	Female
n	9	4	?
L	1210.8 ± 83.7 (1091-1374)	1100 (1030 - 1140)	(800 - 1300)
a	35.1 ± 1.3 (33.2 - 37.5)	26.5 (20.1 - 31.4)	(26 - 34)
b	5.1 ± 0.6 (4.4 - 6.1)	5.1 (4.3 - 5.8)	(4.3 - 5.2)
c	77.2 ± 8.6 (54.5 - 91.2)	56.3 (55.5 - 57.0)	(64 - 100)
c'	0.7 ± 0.1(0.5 - 0.8)	0.86 (0.83 - 0.90)	(0.7 - 0.8)
V or T	60.1 ± 1.6 (57.7 - 61.9)	56.6 (51.8 - 62.2)	(56 - 64)
Lip region diameter	9.6 ± 0.8 (9 - 11)	-	(10 - 11)
Odontostyle length	8.5 ± 0.3 (8.0 - 9.5)	9	(10 - 12)
Odontophore length	19.1 ± 1.3 (17 - 21)	17	-
Guiding ring from anterior end	8	7 - 8	-
Neck length	237.3 ± 21.6 (210 - 270)	-	(170 - 220)
Pharyngeal bulb length	49.0 ± 5.5 (42 - 59)	-	-
Body diameter at neck base	30.6 ± 1.8 (27 - 33)	-	-
at mid - body	34.4 ± 2.1 (32 - 38)	-	-
at anus / cloaca	21.4 ± 1.7 (18 - 24)	-	-
Prerectum length	633 ± 21 (617 - 663)	-	-
Prerectum / anal body diam.	28.0 ± 0.5 (27.4 - 28.8)	-	20 - 26
Tail length	19.6 ± 1.1 (18 - 21)	-	-

Description

Female

Slender nematodes with medium size. Body slightly curved ventrad to open 'C' upon fixation, gently tapering towards both extremities. Cuticle tylencholaimoid, *ca.* 1.5 µm thick at anterior body region (odontostyle level) and *ca.* 2 thick all over the body, typical of the genus, with two distinct layers, outer layer thin, with constant thickness throughout the body and finely annulated, the inner one with very irregular outline, distinctly annulated, being partly separated from the body and shriveled after fixation. Lip region cap-like, separated from the rest of body by sharp constriction. Prestoma short, its wall sclerotised. Stoma, a truncate cone, with thickened sclerotised walls, confined with a simple and refractive guiding ring at base. Amphidial fovea cup-shaped, its opening a wide slit, slightly less than the corresponding body width long. Odontostyle delicate, needle-like, with distinct narrow lumen and aperture. Odontophore slightly arcuate, with arms slightly thickened at base. Nerve ring located at 80-92 µm from anterior end. Pharynx composed of an anterior slender and weakly muscular part, ending to a small and pear-shaped bulb, occupying about one-fifth or 21.1% (16.6-24.3%) of total neck. Cardia short and rounded, intestine simple, pre-

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rectum very long, 26-28 times longer than anal body width, its junction with intestine having three distinct guard cells located at 120-167 µm distance from vulva toward anterior end, between anterior ovary and cardia. Rectum simple, *ca.* 26 µm long (n = 2). Reproductive system didelphic, amphidelphic, composed of equal size and less developed tracts, 84-110 µm long, each composed of a reflexed ovary *ca.* 62 µm long including single row of oocytes, simple oviduct *ca.* 45 µm long and lacking a *pars dilatata oviducti*, a hard to see but present sphincter, a tubular uterus *ca.* 40 µm long, vagina perpendicular to body axis, extending into the body slightly less than half body width, composed of *pars distalis vaginae ca.* 4.5 µm long, *pars refringens vaginae* apparently lacking, and *pars proximalis vaginae* about as high as wide, *ca.* 6.5×6.5 µm (n = 2). Tail short, shorter than anal body width, with slight variation in morphology from rounded to bluntly conoid.

Male

Not found.

Remark

Morphological and morphometric characters of Iranian population of *Leptonchus granulosus* are in accordance with the data given in two other reports of the species (Peralta and Peña-Santiago, 1996, Andrássy, 2009). As far as we know, this is the first report on the occurrence of this genus in Iran.

Molecular characterization and phylogenetic relationships

For molecular phylogenetic analysis, a 1669 nt partial 18S rDNA sequence of Iranian population of *Letponchus granulosus* (accession no. KR184128) was sequenced. A BlastN search of this sequence revealed the highest similarity to isolate LeOnGra of the species (accession number AY284831) with 99% identity and 100% coverage. On the other hand, three other unpublished isolates identified as *L. granulosus* with accession numbers AY919148, AY919149 and AY146538 are deposited in GenBank. Another isolate, identified as *L. microdens* Thorne 1974 (accession number AY146539) and deposited in Genbank database was also selected for reconstructing of phylogenetic relationships. All of the aforementioned isolates together with recently sequenced population, 21 species/isolates of different representatives of Dorylaimida Pearse, 1942 families and one nygolaim species as outgroup taxon were included in phylogenetic analysis. The dataset was composed of 1767 total characters of which1433 characters were conserved.

Figure 3 presents a phylogenetic tree reconstructed using the above mentioned dataset. With lacking of enough molecular sequences for SSU rDNA of tylencholaim members, depicting reliable phylogenetic relationships between species and genera is impossible. Using current data, the species of the genus *Leptonchus* form a fully supported (1.00 BPP) clade. The Iranian population of *L. granulosus* is enclosed in a fully supported major clade (clade A) which also includes one isolate of the species (accession number AY284831) in closer phylogenetic position, three other isolates with accession numbers AY284831, AY919149 and AY146538, whose separate position could be due to short size of the corresponding sequences or misidentification of the species, as well as another isolate (AY146539) were identified as *L. microdens*. Unfortunately, morphological data of the aforementioned species of *Leptonchus* are not available, which makes judgment on correct identification of those species impossible, but, according to similarity matrix of SSU data of the species of the genus used for reconstructing the tree (Table 2), it seems that the species identified as *L. microdens* (AY146539) might also belong to *L. granulosus* (its similarity percent with the five isolates of *L. granulosus* falls within the range 98.7-99.7%).

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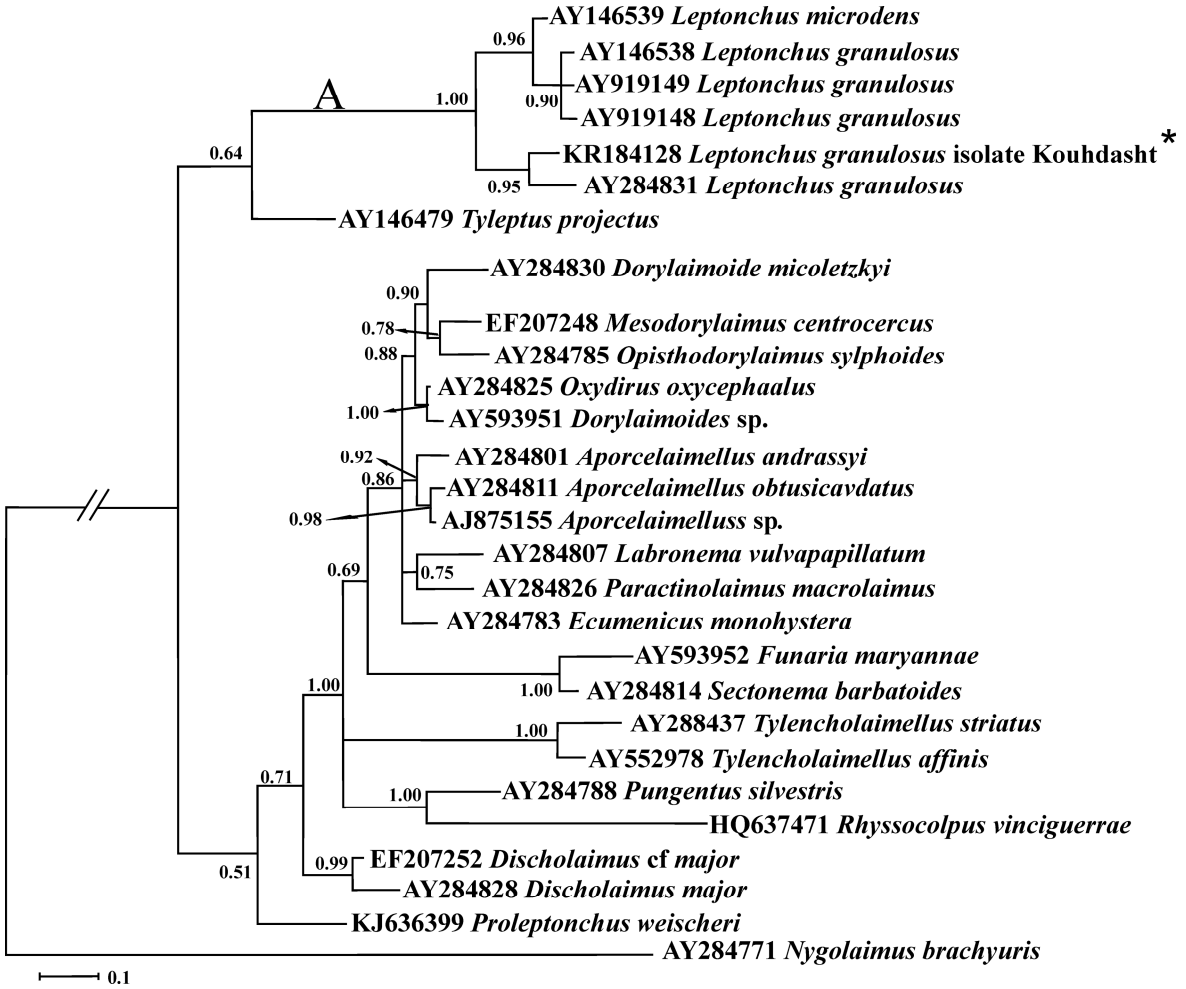

Figure 3 Bayesian 50% majority rule consensus tree inferred from SSU rDNA under the GTR + G + I model and using *Nygolaimus brachyuris* as outgroup. Posterior probabilities more than 50% are given for appropriate clades. The newly sequenced isolate is marked with asterisk.

Table 2 Similarity matrix for SSU sequences for the species of *Leptonchus* used in the reconstruction of the phylogenetic tree.

No.	Species (Accession number)	1	2	3	4	5	6
1	*Leptonchus granulosus* (AY284831)	-					
2	*Leptonchus granulosus* (AY919148)	98.4	-				
3	*Leptonchus granulosus* (AY146538)	98.4	100	-			
4	*Leptonchus granulosus* (AY919149)	98.4	100	100	-		
5	*Leptonchus granulosus* (KR184128)	99.5	98.9	98.9	98.9	-	
6	*Leptonchus microdens* (AY146539)	98.7	99.7	99.7	99.7	99.2	-

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مطالعه خصوصیات ریخت‌شناسی و مولکولی گونه *Leptonchus granulosus* Cobb, 1920 (Dorylaimida, Tylencholaimoidea) از ایران

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چکیـــده: گونـه *Leptonchus granulosus*، از بالاخـانواده Tylencholaimoidea، یافـت شـده از اسـتان لرستان با شاخص‌های ریخت‌سنجی، ریخت‌شناسی و مولکولی مورد مطالعه قرار گرفت. جمعیـت ایرانـی این‌گونه با طول بدن ۱۰۹۱-۱۳۷۴ میکرومتر، کوتیکول بـا دو لایـه مجـزا متـشکل از لایـه بیرونـی بـا شیارهای ریز، لایه داخلی با شیارهای مشخص که در بخش‌هـایی از بـدن جـدا شـده و پـس از تثبیـت چروکیده می‌شود، ناحیه لب کلاهک ماننـد متمـایز از بقیـه بـدن، دیـواره دهـان و پـیش دهـان کـاملاً اسکلروتیزه، اودنتوستایلت سـوزنی ظریـف بـا مجـرای باریـک مـشخص، بـه‌طـول ۸/۰-۹/۵ میکرومتـر، اودنتوفور تقریبا کمانی، به‌طول ۱۷-۲۱ میکرومتر، در قاعـده دارای بازوهـای تـا حـدی ضـخیم، حبـاب انتهایی مری کوچک گلابی شکل که تقریباً ۱۶/۶-۲۴/۳ درصد از طول مـری را اشـغال مـی‌کنـد، روده ساده، پری‌رکتوم بسیار طویل(۵۶۹-۶۲۰ میکرومتر)، در محل اتصال با روده دارای سه سـلول مـشخص قرار گرفته بین کاردیا و تخمدان جلویی، سیستم تولید مثل ماده متشکل از دو لولـه هـم‌انـدازه کـم‌تـر توسعه یافته، اما با بخش‌های مجزا (رحم لوله‌ای، مجرای عبور تخمک و تخمدان)، دم مخروطی شکل تا گرد و عدم وجود جنس نر در جمعیت شناخته می‌شود. در مقایسه با داده‌هـای موجـود از گونـه، تنـوع درون گونه‌ای قابل ملاحظه‌ای بین داده‌های ریخت‌شناسی جمعیت ایرانی و گزارش‌های موجود مشاهده نشد. این اولین گزارش از وقوع گونه‌ای از جنس *Leptonchus* برای فون نماتدهای ایران اسـت. مطالعـه فیلوژنی جمعیت ایرانی گونه *L. granulosus* با استفاده از توالی ۱۶۶۹ نوکلئوتیدی ناحیـه ژنـومی SSU rDNA نشان داد که جمعیت ایرانی یک گروه تک تبار با یکی از ایزوله‌های این گونه در درخت بیس بـا احتمال پسین ۰/۹۵ تشکیل می‌دهد.

واژگان کلیدی: استان لرستان، بیس، تاکسونومی، ناحیه ژنومی 18S rDNA

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