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MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF *ECUMENICUS MONOHYSTERA* (NEMATODA DORYLAIMIDA QUDSIANEMATIDAE) AND ITS PHYLOGENETIC RELATIONS FROM IRAN

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Azimi S. – Morphological and molecular characterisation of *Ecumenicus monohystera* (Nematoda Dorylaimida Qudsiianematidae) and its phylogenetic relations from Iran.

Ecumenicus monohystera was collected from the rhizosphere of faba bean (*Vicia faba* L.) fields in Khuzestan province, south-western Iran. Morphological and morphometric data are provided for this species. Additionally, sequence of the D2-D3 expansion segments of 28S rRNA gene for this species was also used for molecular phylogenetic analysis. The phylogenetic relationships of *E. monohystera* in relation to representatives of the order Dorylaimida, obtained from Bayesian inference (BI) analysis of the D2-D3 sequences, are presented and discussed.

KEY WORDS: 28S rRNA gene, *Ecumenicus*, morphology, morphometric, phylogeny.

INTRODUCTION

More than 80% of the most environmental stress-sensitive nematode families belong to the orders Mononchida Jairajpuri, 1969 and Dorylaimida Pearse, 1942 (HOLTERMAN *et al.*, 2008). Dorylaims, the representatives of the nematode order Dorylaimida, are probably the most diverse taxon within the phylum Nematoda. The families Dorylaimidae de Man, 1876 (with 24 valid genera and 336 valid species) and Qudsiianematidae Jairajpuri 1965 (with 31 valid genera and 402 valid species) are two important, highly diverse, free-living, dorylaimid taxa (PEÑA-SANTIAGO and ALVAREZ-ORTEGA, 2014).

The genus *Ecumenicus* was proposed by Thorne in 1974 to accommodate a cosmopolitan species, *E. monohystera* (de Man, 1880) Thorne, 1974, transferred from *Eudorylaimus* Andrassy, 1959. The new taxon was characterized by its mono-opisthodelphic female genital system, a rather rare feature among members of Dorylaimoidea (PEÑA-SANTIAGO and ABOLAFIA, 2007). DAREKAR & KHAN (1979) established a genus *Indokochinema*, with a single species, *I. conicauda*. This genus corresponds perfectly to *Ecumenicus* with ovary single, no prevulval uterin sac, oesophagus expanded posteriorly, amphids on lateral lips, spear small, tail short, conoid, so ANDRÁSSY (1991) synonymized *Indokochinema* with *Ecumenicus*. The second species, *Indokochinema ekramullahi* Jana & Baqri (1983), has been already synonymized by BAQRI & COOMANS (1985) with *Ecumenicus monohystera* (ANDRÁSSY, 1991).

PEÑA-SANTIAGO & ABOLAFIA (2007) studied the location of amphid aperture in *E. monohystera* with SEM and confirmed ANDRÁSSY's (1991) action, regarding *Indokochinema* as a junior synonym of *Ecumenicus*. According to ANDRÁSSY (1991), the genus *Ecumenicus* includes four species. *E. monohystera* is a cosmopolitan species and has also been reported from many countries (PEÑA-SANTIAGO and ABOLAFIA, 2007).

The present study aims to characterize *E. monohystera* from the rhizosphere of faba bean in Iran using morphological and molecular data. Additionally, the phylogenetic relationships of this species is evaluated on the basis of the D2-D3 expansion segments of the 28S rRNA gene.

MATERIALS AND METHODS

NEMATODE SAMPLES

Soil samples were collected from faba bean (*Vicia faba* L.) fields in Khuzestan province, south-western Iran. The JENKINS's (1964) method was used to extract the nematodes from soil samples. The collected specimens were killed by adding boiling formaldehyde solution (4%), transferred to anhydrous glycerin according to DE GRISSE's (1969) method. Nematodes were mounted in a small drop of glycerin on permanent slides. Observations and measurements were done using a Leitz SM-LUX light microscope equipped with drawing tube. Some of the best-preserved specimens were photographed using an Olympus DP12 digital camera attached to an Olympus BX51 light microscope. Nematode species were identified based on morphological, morphometric and molecular characters. SIDDIQI, 2000 used for the abbreviations and ratios used in the morphological description.

DNA EXTRACTING, PCR AND SEQUENCING

For molecular analyses, a single female was picked out from samples, examined in drop of distilled water on a temporary slide under the light microscope, transferred to 7 µl of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) on a clean slide, and then crushed using a cover slip. The suspension was collected by adding 20 µl AE buffer. Each DNA sample was stored at -20°C until used as a PCR template (PEDRAM *et al.*, 2011). The D2-D3 expansion

segments of the 28S rDNA was amplified using the forward D2A (5'-ACAAGTACCGTGAGGGAAAGTTG-3') and reverse D3B (5'-TCGGAAGGAACCAGCTACTA-3') primers (NUNN, 1992). PCR reactions of 25 µl were made with 14 µl of distilled water, 2.5 µl of 10 × PCR buffer, 0.5 µl of dNTP mixture, 1.5 µl of 50 mM MgCl₂, 1 µl of each primer (10 pmol/µl), 0.5 µl of Taq polymerase (CinnaGen, Tehran, Iran, c. 5 U/µl), and 4 µl of DNA template. The thermal cycling program was as follows: initial denaturation at 95°C for 6 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min (PEDRAM *et al.*, 2011). Amplification success was evaluated electrophoretically on 1% agarose gel. The PCR products were purified using the QIAquick PCR purification kit (Qiagen®) following the manufacturer's protocol and sequenced directly using the PCR primers with an ABI 3730XL sequencer (Bioneer Corporation, South Korea). The newly obtained sequence was deposited into the GenBank database (accession number MF667960).

PHYLOGENETIC ANALYSES

The newly obtained sequence of the D2D3 fragments of 28S rDNA and additional sequences of relevant taxa selected after a BlastN search, were aligned by Clustal X2 (<http://www.clustal.org/>) using the default parameters. The outgroup taxa were chosen according to a previous study (HOLTERMAN *et al.*, 2008). Model of base substitution was selected using Mr Model test 2 (NYLANDER, 2004), and

based on the Akaike criteria. A general time reversible model, including among-site rate heterogeneity and estimates of invariant sites (GTR + G + I), was selected for the phylogenetic analyses. Bayesian analysis was used to infer the phylogenetic tree on MrBayes v3.1.2 (RONQUIST & HUELSENBECK, 2003), running the chain for one million 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 estimate the posterior probabilities of the phylogenetic tree (LARGET & SIMON, 1999) using the 50% majority rule. The Bayesian posterior probabilities (BPP) higher than 50% were given on appropriate clades. The output file of the phylogenetic program was visualized using Dendroscope V.3.2.8 (HUSON & SCORNAVACCA 2012) and re-drawn in CorelDRAW software version 12.

RESULTS AND DISCUSSION

Ecumenicus monohystera (de Man, 1880) Thorne, 1974 (Figs I and II)

Measurements: Table 1.

DESCRIPTION

FEMALE: Body straight to slightly ventrally curved upon fixation; Medium-sized, 0.9-1.2 mm long. Cuticle very

Fig. 1 – *Ecumenicus monohystera* - 1. Pharynx; 2. Lip region; 3. Pharyngeal cardia; 4. Vulval region; 5. Prerectum region; 6-8. Posterior region.

Fig. II – *Ecumenicus monohystera* – 1, 2. Lip region; 3. Pharyngeal cardia; 4, 5. Vulval region; 6. Prerectum region; 7-9. Posterior region. (Scale bars = 20 µm).

finely striated. Lip region set off by a slight depression, lips distinct. Odontostyle with aperture about one-third length, guiding ring single. Odontophore simple, rod-like. Pharynx enlarges more gradually and basal expansion part of pharynx occupying less than one-half of pharynx length. Dorsal oesophageal gland nucleus located a little bit behind to its orifice. Female genital system monodelphic-opistodelphic, Vulva a transverse slit, with slightly sclerotised labia. Vagina oblique. The length of rectum is slightly less than the anal body width. Tail conoid with straight ventral site and digitated rounded tip.

MALE: not found.

REMARKS: Iranian population of *E. monohystera* resembled more those described by PEÑA-SANTIAGO & ABOLAFIA (2007) than some other populations. Compared to Indian population (MUSHTAQ & AHMAD, 2007), the ratio *c* is slightly higher (30.6-40 vs 27.3-30.2), rectum and tail

lengths are shorter (15.5-17.5 vs 22-29 µm and 29.0-34.5 vs 34-39 µm, respectively). Compared to Bulgarian population (Ilieva *et al.*, 2017), the range of ratio *V* is higher (34-39.1 vs 29-34) and rectum length is shorter (15.5-17.5 vs 18-33 µm). These differences can be attributed to the intraspecific variation due to geographical differences.

E. monohystera is widely distributed in the world and has been reported from Iran by FADAEI-TEHRANI, 2008 (grapevine, Chaharmahal va Bakhtiari province), KASHI NAHANGI & KAREGAR BIDEH, 2010 (sugar beet, Hamadan) and HADI-ALIJEVAND & FADAEI-TEHRANI, 2013 (wheat, barley and clover, Chaharmahal va Bakhtiari province). In present study, this species was recovered from the rhizosphere of faba bean fields in the vicinity of Shushtar (GPS coordinates: 32°02'44''N, 48°51'24''E) city, Khuzestan province, south-western Iran. This is new record of *E. monohystera* for nematodes fauna in Khuzestan province.

Table 1 – Morphometrics of *Ecumenicus monohystera* collected from south-western Iran and their comparison with some other populations. All measurements are in μm and in the form: mean $\pm$ s.d. (range).

Character	Present study	Mushtaq& Ahmad (2007)	Peña-Santiago & Abolafia (2007)	Ilieva <i>et al.</i> (2017)
n	14	9	15	11
L	1136.9 $\pm$ 98.7 (949-1224)	1050 $\pm$ 50.0 (980-1120)	1003 $\pm$ 60.0 (910-1140)	1120 $\pm$ 70.0 (990-1180)
a	37.2 $\pm$ 3.4 (31.6-42.2)	33.4 $\pm$ 1.4 (31.6-35.0)	36.1 $\pm$ 4.4 (31.8-48.0)	37.0 $\pm$ 2.0 (34.0-39.0)
b	4.8 $\pm$ 0.1 (4.6-5.0)	4.6 $\pm$ 0.1 (4.3-4.8)	4.7 $\pm$ 0.2 (4.3-5.0)	4.6 $\pm$ 0.5 (3.9-5.1)
c	36.1 $\pm$ 3.0 (30.6-40.0)	29.1 $\pm$ 0.8 (27.3-30.2)	32.5 $\pm$ 2.6 (29.4-39.8)	32.0 $\pm$ 2.0 (31.0-36.0)
c'	1.5 $\pm$ 0.1 (1.4-1.7)	1.6 $\pm$ 0.1 (1.5-1.8)	1.6 $\pm$ 0.1 (1.3-1.8)	1.6 $\pm$ 0.1 (1.5-1.7)
V	36.1 $\pm$ 2.0 (34.0-39.1)	35.3 $\pm$ 0.5 (35.0-36.0)	35.4 $\pm$ 2.2 (32.0-39.0)	32.0 $\pm$ 2 (29-34)
Lip region width	10.9 $\pm$ 0.5 (10.0-11.5)	10.7 $\pm$ 0.4 (10.0-11.0)	10.3 $\pm$ 0.5 (10.0-11.0)	9.9 $\pm$ 0.2 (9.5- 10.0)
Odontostyle length	10.9 $\pm$ 0.0 (10.5-11.0)	12 $\pm$ 0.5 (11.0-13.0)	10.1 $\pm$ 0.5 (9.0-11.0)	10.0 $\pm$ 0.5 (10.0-11.0)
Neck length	236.5 $\pm$ 19.9 (198 -251)	228 $\pm$ 11.8 (210-246)	220.0 $\pm$ 12.0 (200-240)	245 $\pm$ 17.0 (225-261)
Body width at neck base	28.7 $\pm$ 1.7 (27.0-32.0)	30 $\pm$ 1.0 (28.0-32.0)	-	30.0 $\pm$ 2.0 (28.0-32.0)
Pharyngeal expansion length	87.5 $\pm$ 10.2 (71.0-95.0)	84.1 $\pm$ 3.7 (75.0-87.0)	82.2 $\pm$ 7.0 (70.0-90.0)	-
Body width at mid body	30.5 $\pm$ 1.7 (29.0-34.0)	31.5 $\pm$ 1.2 (29.0-33.0)	28.9 $\pm$ 3.3 (22.0-33.0)	31.0 $\pm$ 1.0 (29.0- 32.0)
Anal body width	19.9 $\pm$ 0.8 (19.0-21.0)	20.8 $\pm$ 0.7 (20.0-22.0)	20.3 $\pm$ 1.3 (18.0-22.0)	22.0 $\pm$ 2.0 (20.0- 24.0)
Prerectum length	46.9 $\pm$ 10.8 (24.3-58.0)	40.8 $\pm$ 6.7 (35.0-50.0)	35.8 $\pm$ 6.0 (21.0-45.0)	41.0 $\pm$ 9.0 (24.0-47.0)
Rectum length	16.5 $\pm$ 0.9 (15.5-17.5)	23.1 $\pm$ 1.5 (22.0-29.0)	-	24.0 $\pm$ 6.0 (18.0-33.0)
Tail length	31.5 $\pm$ 2.0 (29.0-34.5)	36.1 $\pm$ 1.4 (34.0-39.0)	31.9 $\pm$ 2.1 (27.0-35.0)	35.0 $\pm$ 2.4 (32.0-38.0)

MOLECULAR PHYLOGENETIC STATUS

The alignment of the D2D3 expansion fragments of 28S rRNA gene sequences of 32 taxa (including two out group taxa), yielded a data set with 1194 characters. The phylogenetic relationships between the Iranian population of *E. monohystera* and representatives of Dorylaimida, as inferred from the BI analysis, are presented in Figure III. Phylogenetic relationships among dorylaimid nematodes in this present are mostly congruent with those published by HOLTERMAN *et al.* (2008) and PEÑA -SANTIAGO & ALVAREZ-ORTEGA (2014). The BI analysis showed that Qudsianematidae are a polyphyletic taxon and some of its genera are closer to members of other families. This result is congruent with previous study focusing on the dorylaimid nematodes (PEÑA-SANTIAGO and ALVAREZ-ORTEGA, 2014). Three *Ecumenicus* sequences were grouped in a clade with high support (BI = 100). This is the first molecular study of *E. monohystera* in Iran. There were only two records in GenBank for sequences of D2-D3 expansion segments of 28S rRNA gene for the genus *Ecumenicus*.

In a study by HOLTERMAN *et al.* (2006) based on small subunit ribosomal DNA (SSU rDNA) gene, the phylogenetic relationships within the order Dorylaimida were fully unclear. Because of low diversity of the SSU rDNA within the order Dorylaimida, HOLTERMAN *et al.* (2008)

used the large subunit ribosomal DNA (LSU rDNA) gene for this purpose. The LSU trees show a better resolution within the Dorylaimida, although a large basal polytomy still remained. The relatively high degree of variability of the LSU rDNA genes among nematodes reduces the chances of unwanted cross reactivity considerably (HOLTERMAN *et al.*, 2008).

PEÑA-SANTIAGO and ALVAREZ-ORTEGA (2014) studied the evolutionary relationships of rounded-tailed dorylaimid genera traditionally classified under the family Qudsianematidae. Evidences were provided that support a closer relationship of these genera with members of Dorylaimidae rather than with other representatives of Qudsianematidae. They believe that the rounded-tailed forms may evolutionary derive from long-tailed forms and transferred eight genera from Qudsianematidae to Dorylaimidae including *Labronema* Thorne, 1939, *Cras-solabium* Yeates, 1967 (syn. *Thonus* Thorne, 1974) and *Labronemella* Andrassy, 1985. Postembryonic development and molecular data of most of these genera has not been studied yet.

Currently, a limited number of the genera among dorylaimid families have been sequenced. As a result, in order to clarify the relationships among genera of these families, a more comprehensive phylogenetic study is

Fig. III – Bayesian 50% majority rule consensus tree inferred from analysis of the D2-D3 domains of the 28S rRNA gene under the GTR + G + I model. Bayesian posterior probability values more than 50% are given for appropriate clades. New sequence is indicated in bold.

needed, in particular on all available species of these genera from different geographical origins.

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