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Description of *Enchodorus yeatsi* n. sp. (Dorylaimida, Nordiidae) from Southern Iran and Its Molecular Phylogenetic Study

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Abstract: Enchodorus yeatsi n. sp., a new species of the rare genus *Enchodorus* is described and illustrated based on its morphological and molecular characters. It was recovered from southern Iran. Females of the new species are characterized by having 1,511.3- to 1,792.5-µm long slender body, angular lip region having prominent papillae, 12- to 13-µm long odontostyle, double guiding ring, simple rod-like odontophore, didelphic–amphidelphic reproductive system, and 102- to 160-µm long elongate-conoid tail with rounded tip. Males of the new species are abundant and functional, characterized by 1,484- to 1,576-µm long body, 40- to 43-µm long spicules, 5 to 6 ventromedian supplements, and ventrally bent elongate conical tail. Compared to the type species, *Enchodorus dolichurus*, the new species has differences in its tail morphology and *V* value. These morphological differences and the separation of two species was further supported with basic differences in sequences of 28S rDNA D2/D3 and internal transcribed spacer 1 (ITS1) fragments. Compared to *Enchodorus neodolichurus*, it has basic differences in tail characters and spicule lengths. Molecular phylogenetic studies using partial sequences of 28S rDNA D2/D3 fragment of the new species and available sequences of Nordiidae members and several other dorylaim species/genera, revealed *E. yeatsi* n. sp. and *E. dolichurus* forming a clade with 0.81 Bayesian posterior probability (BPP). This clade forms a sister clade to the clade of *Heterodorus* sp. and *Rhyssocolpus vinciguerrae*, again with 0.81 BPP. In ITS1 tree, reconstructed using few available sequences, the new species and *E. dolichurus* formed a clade with 0.98 BPP.

Key words: 28S rDNA D2/D3, Bayesian, *Enchodorus dolichurus*, *E. neodolichurus*, ITS1, Khuzestan province, new species, taxonomy.

The genus *Enchodorus* Vinciguerra, 1976, belongs to one of the rarest dorylaim soil and freshwater nematode taxa. The type species, *Enchodorus dolichurus* Vinciguerra, 1976, and the species *Enchodorus neodolichurus* Ahmad and Wu, 1999, are currently the only two known species under the genus. Molecular data currently are available for an Iranian population of the type species (redescribed by Pourjam et al., 2010, its molecular phylogenetic characters given in Pedram et al., 2015), and no molecular data are available for the second species, described by Ahmad and Wu (1999). Molecular data are also not available for the type population of *E. dolichurus*. In present paper, a new species of this rare genus, recovered in southern Iran, is described and illustrated using morphological and molecular data. The most recent nordiid species being originally described from Iran is *Heterodorus youbertghostai* Pedram, Pourjam, Atighi and Panahandeh, 2015, and the history of studies on the members of the family in the country is given by Pedram et al. (2009a, 2009b, 2011).

Materials and Methods

Sampling, extracting, mounting, and drawing: Several soil and moss samples were collected from southern parts of Iran. To obtain a cleaner suspension of nematodes, the tray method (Whitehead and Hemming, 1965) was used. The described new species was recovered from thin soil layer on rhizosphere of mosses collected from Khuzestan province. Nematodes of interest were handpicked under a Nikon SMZ1000 stereomicroscope, heat-killed by adding boiling 4% formalin solution, transferred to anhydrous glycerin according to De Grisse (1969), mounted in permanent slides, and examined using a Nikon Eclipse E600 light microscope. Photographs were taken using an Olympus DP72 digital camera attached to an Olympus BX51 microscope powered with differential interference contrast. Drawings were made using a drawing tube attached to the microscope and were redrawn using CorelDRAW® software version 16. The terminology used for naming of the various spicule parts follows Peña-Santiago et al. (2014).

DNA extraction, PCR, and sequencing: DNA was extracted from four single individuals. Each juvenile or female was handpicked, examined one by one by light microscopy, and transferred to 2.0 µl AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) on separate clean slides and squashed using a clean slide cover. Each suspension was collected separately by adding 23 µl AE buffer. DNA samples were stored at −20°C until used as a PCR template. PCR was carried out in a total volume of 30 µl (19.2 µl distilled water, 3 µl 10× PCR buffer, 0.6 µl 10 mM dNTP mixture, 1.2 µl 50 mM $MgCl_2$, 1.2 µl of each primer [10 pmol/µl], 0.6 µl of Taq DNA polymerase [5 unit/µl, CinnaGen, Tehran, Iran], and 3 µl of DNA template). The thermal cycling program for amplifying of 28S rDNA D2/D3 and internal transcribed spacer 1 (ITS1) rDNA fragments was as follows: denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec, and extension at 72°C for 80 sec. A final extension was performed at 72°C for 10 min. Primers for 28S rDNA D2/D3 amplification were forward primer D2A (5′-ACAAGT ACCGTGAGGGAAAGT-3′) and reverse primer D3B (5′-TGCGAAGGAACCAGCTACTA-3′) (Nunn, 1992). Primers for amplifying of ITS1 fragment were forward primer rDNA1 (5′-TTGATTACGTCCCTGCCCTTT-3′) and reverse primer rDNA 1.58S (5′-ACGAGCCGAGTGATCC ACCG-3′) as listed in Subbotin et al. (2000). The PCR

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products were sequenced in both directions using the same primers with an ABI 3730XL sequencer. After several PCR, and several sequencing experiments, only one 28S sequence of the new species had the highest quality and was deposited in GenBank under the accession number KX691911 for the new species. Two ITS1 sequences belonging to different individuals of *E. dolichurus* (KY366267 and KY366268) and the new species (KY366265 and KY366266) were also generated herein and deposited in the database.

Phylogenetic analyses: Almost all available 28S rDNA D2/D3 sequences of nordiid taxa in GenBank were downloaded. Sequences of other dorylaim species and genera were also selected. The DNA sequences were aligned using MUSCLE (Edgar, 2004) as implemented in MEGA6 (Tamura et al., 2013). To eliminate the ambiguously aligned parts, the online version of Gblocks 0.91b (Castresana, 2000) with all the three less stringent parameters was used (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). 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 analyses of 28S and a general time reversible model, including among-site rate heterogeneity (GTR+G) was used in analysis of ITS1 datasets. Bayesian analysis was performed using MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003) running the chains for one million generations. After discarding burn-in samples, the remaining samples were retained for further analyses. The Markov chain Monte Carlo method within a Bayesian framework was used to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule. Convergence and congruence across runs were assessed using AWTY (Nylander et al., 2008). Tracer v1.5 software (Rambaut and Drummond, 2009) was used to visualize the results of each run in order to check the effective sample size of each parameter. A maximum likelihood (ML) tree was reconstructed by using RaxmlGUI 1.1 (Silvestro and Michalak, 2011) software using the same nucleotide substitution model as in the Bayesian analysis in 1,000 bootstrap (BS) replicates for 28S dataset. Two species belonging to the genus *Tylencholaimus* de Man, 1876 (accession numbers EF207243 and AY593027), were used as outgroup taxa. The output file of the used phylogenetic programs was visualized using Dendroscope V.3.2.8 (Huson and Scornavacca, 2012) and redrawn in CorelDRAW software version 16. The Bayesian posterior probability (BPP) and ML BS values exceeding 0.50 and 50%, respectively, are given on appropriate clades in the shape BPP/ML BS.

For reconstructing of the ITS1 tree, with paucity of the sequences of this genomic fragment for members of Nordiidae Jairajpuri and Siddiqi, 1964, few available sequences were selected from the public database and used according to the method already described.

Description

Enchodorus yeatsi n. sp.
(Figs. 1,2)

Measurements: See Table 1.

Females (type population): Body slightly curved ventrally after heat relaxation, tapering toward both ends gradually, more toward distal end, due to having a long conical tail. Cuticle marked with slight transverse striae, ca. 3 µm thick all over the body. Lip region cap-like (as described for the genus), separated from the rest body by a sharp constriction, ca. 2 times as wide as high, labial and cephalic papillae prominent, giving lips an angular aspect. Amphidial fovea cup-shaped, opening at lips base, occupying 65% to 70% of corresponding body diameter. Odontostyle needle-like, 1.5 to 2.0 times longer than lip region width, its lumen fine, distinct, with very small aperture, its base furcate. Odontophore rod-like, 1.8 to 2.3 times longer than odontostyle, lacking knobs or flanges at base. Guiding ring double, with very short guiding sheath. Pharynx consisting of a slender anterior part, expanding gradually into the basal pharyngeal bulb, the latter occupies 33% to 37 % of pharynx. Pharyngeal gland nuclei located as follows: DN: 71% to 73.0%, AS1 and AS2 almost at the same level (n = 2): 26 to 32; PS: 50% to 55%. Cardia cylindrical, 9 to 18 µm long, 9 to 10 µm wide. Intestine simple, containing green material in almost all examined specimens. Reproductive system didelphic-amphidelphic, genital branches 270 to 290 µm long,

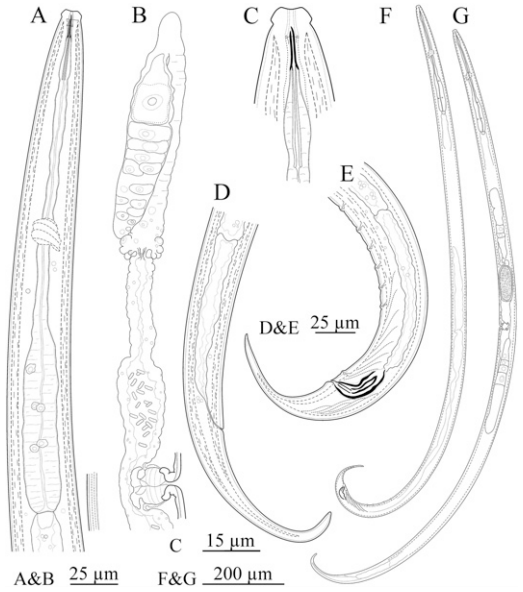

Fig. 1. Line drawings of *Enchodorus yeatsi* n. sp. A. Pharynx. B. Anterior genital tract of female. C. Details of anterior end. D, E. Details of female and male posterior body region. F, G. Male and female entire body.

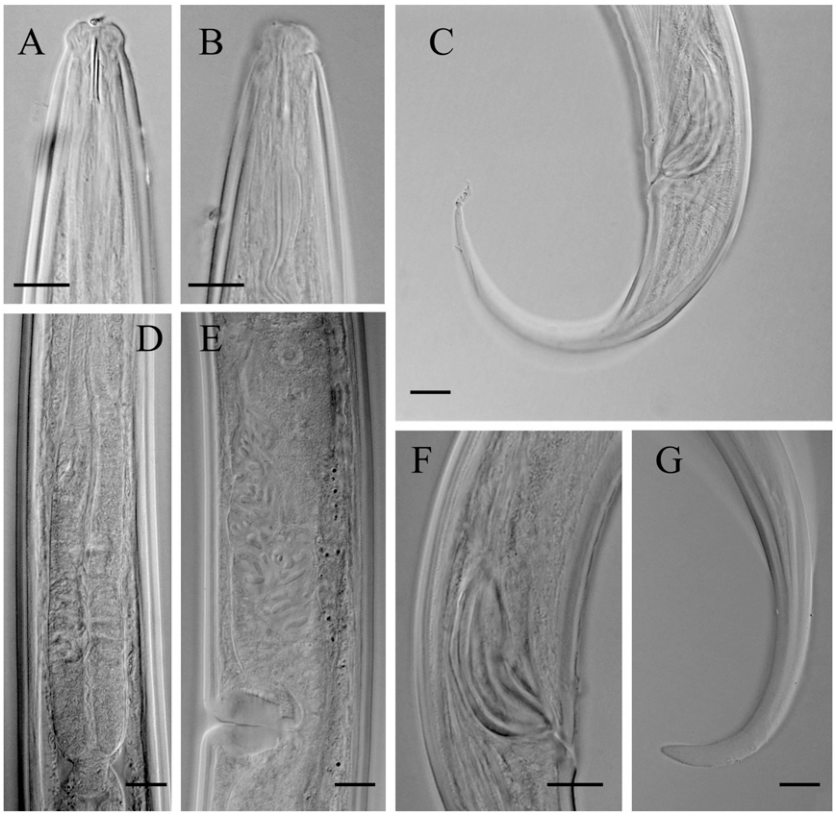

Fig. 2. Microphotographs of *Enchodorus yeatsi* n. sp. A, B. Details of anterior end. C. Male posterior body region. D. Pharyngeal bulb. E. Part of female reproductive system and sperm inside the wide proximal part of the uterus. F. Spicules. G. Female tail end (all scale bars = 10 μm).

each composed of a reflexed ovary 106 to 225 μm long, 140 to 170 μm long oviduct having a distinct *pars dilatata oviductus*, a sphincter, a two-partite uterus, composed of a slender distal part and tubular proximal part containing rod-like (bacilliform) sperm cells, 6.0 × 1.5 μm sized, vagina perpendicular to body axis with 30 to 60 % ingrowth, composed of *pars distalis vaginae* ca. 8.5 μm long, *pars refringens vaginae* apparently lacking and *pars proximalis vaginae* about as long as wide, 14 to 24 μm sized, and vulva a small transverse slit. Prerectum 3.7 to 4.1 and rectum 1.0 to 1.5 times anal body width long. Tail elongate-conoid, gradually and uniformly narrowing toward distal end, ventrally bent at distal part and having a rounded tip.

Males: General morphology similar to that of female, except for sexual dimorphism and posterior body end more ventrally bent after fixation. Genital system diorchic, with opposed testes. An adanal pair of copulatory supplements located at 8 to 13 μm from cloacal opening and a series of 5 to 6 well-spaced ventromedian supplements ending at 45 to 59 μm distance from cloacal pair. Spicules dorylaimoid, massive, 10 to 11 μm wide with wide head (*capitulum*), occupying ca. 20% of total length, distinct hump, shallow hollow, and median piece about 70% of total spicules length. Lateral guiding pieces 9 to 11 μm long. Tail as in the female.

Type habitat and locality: Recovered from thin layer of soil at rhizosphere of mosses collected in a natural region, close to city of Andimeshk, Khuzestan province, southern Iran.

Type material: Holotype female, one paratype female and two paratype males were deposited in the Nematode Collection at the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran. One female and two male paratypes deposited in each of the following collections: USDA Nematode Collection, Beltsville, MD, and WANECO collection, Wageningen, The Netherlands (http://www.waneco.eu/).

Diagnosis and relationships: Enchodorus yeatsi n. sp. is characterized by having females with 1,511.3- to 1,792.5-μm long slender body, angular lip region having prominent papillae, 12- to 13-μm long odontostyle, 23- to 28-μm long rod-like odontophore lacking knobs or flanges at base, double guiding ring, dorylaimoid pharynx, pharyngeal bulb with five nuclei, didelphic-amphidelphic reproductive system, intestine containing green material, and 102- to 160-μm long elongate-conoid tail, gradually and uniformly narrowing toward distal end, ventrally bent at distal part and having a rounded tip. Males of the new species are abundant and functional, characterized by their 1,484- to 1,576-μm long body, 40- to 43-μm long spicules, 5 to 6 ventromedian supplements, and ventrally bent elongate conical tail, similar to that in females. Compared to *E. dolichurus*, the type species of the genus *Enchodorus*, the new species has differences in its tail morphology (elongate conoid, uniformly attenuating

TABLE 1. Morphometric data for *Enchodorus yeatsi* n. sp.

		Paratypes	
	Holotype female	Female	Male
n	-	4	6
L	1,748.8	1,655 ± 136 (1,511.3–1,792.5)	1,514.5 ± 36.0 (1,484–1,576)
a	40.2	36.8 ± 2.4 (34.5–40.2)	41 ± 2 (39.5–44.5)
b	6.5	6.0 ± 0.6 (5.3–6.6)	6.0 ± 0.5 (5.7–7.0)
c	13.8	13.4 ± 1.6 (11.2–14.8)	16.0 ± 1.5 (13.5–18.3)
c′	5.3	5.0 ± 0.5 (4.6–5.7)	3.5 ± 0.5 (3.0–4.2)
V	44.2	44.3 ± 0.5 (43.5–45.0)	-
Anterior end, vulva	772.5	732 ± 51 (679–780)	-
Lip region width	11	10 ± 0 (10–11)	10.8 ± 0.4 (10–11)
Lip region height	5	5 ± 0 (5–5)	5.3 ± 0.5 (4.5–6.0)
Odontostyle length	12.5	12.5 ± 0.5 (12–13)	12.8 ± 0.7 (12 -14)
Odontophore length	28	25.5 ± 2.5 (23–28)	22.3 ± 2.0 (23–25)
Stylet total length	40.5	38 ± 2 (35.5–40.5)	35.2 ± 2.2 (35.5–39.0)
Guiding ring from anterior end	8	8 ± 1 (8–10)	7.5 ± 0.5 (7–8)
Neck length	270	274.5 ± 7.5 (270–285)	250 ± 16 (224–264)
Pharyngeal expansion length	92	94 ± 4 (90–100)	83.0 ± 3.5 (78–87)
Pharyngeal expansion width	20	21 ± 2 (19–25)	16.8 ± 0.8 (16–18)
Diameter at neck base	38	41 ± 6 (38–50)	36.0 ± 1.5 (35–38)
At mid-body	43.5	45 ± 4 (42–52)	37 ± 1 (35.5–38.0)
At anus	24	24.5 ± 2.5 (22–28)	27.0 ± 1.2 (25–28)
At guiding ring level	14	13 ± 0 (13–14)	13.3 ± 0.8 (12–14)
Prerectum length	97.5	96.5 ± 11.5 (84–107)	-
Rectum length	35	29.5 ± 4.5 (26–35)	-
Tail length	127.0	125 ± 25 (102–160)	95.0 ± 8.5 (86–110)
Spicules	-	-	42.0 ± 1.3 (40–43)
Lateral accessory pieces	-	-	10 ± 1 (9–11)

All measurements are in μm and in the form: mean ± standard deviation (range).

toward distal end *vs* conical, tapering rapidly in first third) and smaller V (43.5-45.0 *vs* 46-47 in type population). These morphological differences were supported with differences in sequences of 28S rDNA D2/D3 (21 mismatches and 6 indels) and basic differences in sequences of ITS1 rDAN. Compared to *E. neodolichurus*, it has basic differences in female tail length (tail = 102–160 μm, c′ = 4.6–5.7 *vs* 49–73 μm, c′ = 2.1–3.5), and longer spicules (40–43 *vs* 31–34 μm).

Etymology: The new species named in honor of late, Dr. Gregor Yeates, a pioneer in taxonomy of dorylaims.

Molecular phylogenetic status: The partial sequencing of 28S rDNA D2/D3 fragment of the new species yielded a single fragment of 704 nt. The Blast search showed this is a unique sequence, and has 95% identity with Iranian population of *E. dolichurus* (accession numbers KR184124 and 25). A total number

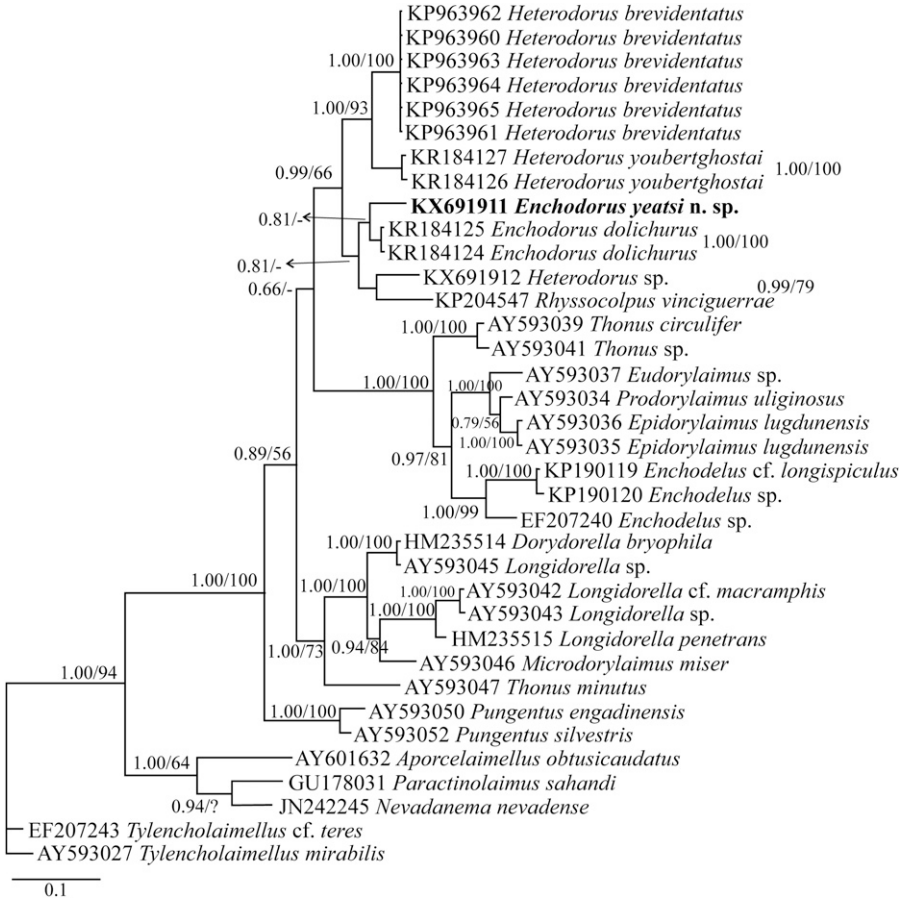

Fig. 3. Bayesian 50% majority rule consensus tree inferred from LSU rDNA D2/D3 under the GTR+G+I model. Bayesian posterior probability and maximum likelihood bootstrap values more than 50% are given for appropriate clades.

of 36 sequences of species/isolates of nordiids and several other dorylaim species/genera (including one sequence of the new and two tylencholaim outgroup species) were included in phylogenetic analyses. The alignment of the abovementioned sequences had 744 total characters which 330 characters were variable. In Fig. 3, the Bayesian phylogenetic tree inferred using the aforementioned dataset, the new species has formed a clade with two Iranian isolates of the type species with moderate (0.81) BPP, and no ML BS. The clade of the genus *Enchodorus* is a sister clade to the clade of *Rhyssocolpus vinciguerrae* Pedram, Pourjam, Robbins, Ye, and Peña-Santiago, 2011 (KP204547), and an unidentified isolate of the genus *Heterodorus* Altherr, 1952 (KX691912). Currently, there are no molecular data for most of nordiid species and genera, but according to presently available data, the family Nordiidae is clearly not a natural group.

The partial ITS1 sequencing of two isolates of the new species yielded two fragments of 673 and 667 nt. The size of this fragment for two isolates of *E. dolichurus* was 653 and 701 nt. With paucity of ITS1 sequences for nordiid taxa, four other available sequences already deposited in GenBank database were used for reconstructing of the ITS1 Bayesian tree. The ITS1 sequences of the new species and *E. dolichurus* had basic differences (several indels and gaps) and both species formed a highly (0.89 BPP) supported clade in this tree.

Remarks: Beside the type species, *E. yeatsi* n. sp. was morphologically compared with the species *E. neodolichurus* too. The latter species has morphological similarities with the genus *Heterodorus* Altherr, 1952, and there is no discussion in its original description by Ahmad and Wu (1999) on assigning of the species to either of the

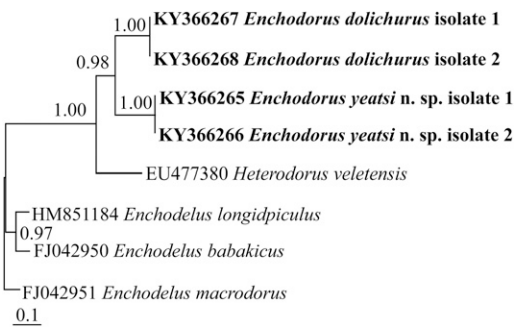

Fig. 4. Bayesian 50% majority rule consensus tree inferred from ITS1 rDNA under the GTR+G model. Bayesian posterior probabilities more than 50% are given for appropriate clades.

genera. Present study, underscores usefulness of fast-evolving genomic regions for separating morphologically-indistinguishable/cryptic species. Compared to sequences of 28S rDNA D2/D3, even with enough differences for separating two species, the sequences of ITS1 rDNA confidently separated the new species from its closely related species, *E. dolichurus*.

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