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Description of a New Anguinid Nematode, *Nothotylenchus phoenixae* n. sp. (Nematoda: Anguinidae) Associated with Palm Date Trees and Its Phylogenetic Relations within the Family Anguinidae

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Abstract: Nothotylenchus phoenixae n. sp. is described and illustrated from soil samples of palm trees in Kermanshah Province, western Iran. The new species is characterized by a body length of 784 (663 to 925) μm in females and 677 to 715 μm in males; a delicate stylet 6 (5 to 7) μm long and six lines in the lateral field; median bulb of pharynx fusiform, nonmuscular, and nonvalvate; isthmus elongate, slender ending to a pyriform basal pharyngeal bulb not overlapping intestine; postvulval uterine sac well developed, 15 (14 to 17) μm long, female tail elongate-conoid with pointed terminus; and male with adanal bursa and spicules 21 to 22 μm long ($n = 2$). The new species comes close in morphology and morphometrics to five known species of the genus, namely *N. affinis*, *N. hexaglyphus*, *N. persicus*, *N. taylori*, and *N. uniformis*. Molecular analyses of the partial 18S, D2/D3 expansion segments of the partial 28S and internal transcribed spacer (ITS) revealed this as a new species. The sequences of the partial 18S and 28S D2/D3 regions confirmed the close phylogenetic relationship between *N. phoenixae* n. sp. and other anguinids, but *Nothotylenchus* is clearly separated from *Ditylenchus* species and should be considered as a valid genus.

Key words: 28S D2/D3, ITS, molecular phylogeny, morphology, new species, partial 18S, plant-parasitic nematode, taxonomy.

The genus *Nothotylenchus* (Thorne, 1941) includes ectoparasitic nematode species of which only a few are parasites of higher plants, whereas the majority of species are mycophagous (Sturhan and Brzeski, 1991). Among more than 45 species presently recognized in the genus (Siddiqi, 2000), some species are poorly characterized and considered as *species inquirendae* (Andrássy, 2007). The number of valid species is uncertain pending a thorough revision of the genus, additional material being necessary for molecular analyses. The two genera *Ditylenchus* (Filipjev, 1936) and *Nothotylenchus* as members of Anguinidae (Nicoll, 1935), are morphologically closely related (Siddiqi, 2000), but they are separated from each other based on the nature of metacorpus (Brzeski, 1981; Fortuner and Maggenti, 1987; Siddiqi, 2000; Andrássy, 2007). *Ditylenchus* has a well-developed, muscular and valvate median bulb, whereas *Nothotylenchus* has a valveless and nondeveloped median bulb (Siddiqi, 2000; Andrássy, 2007). Brzeski (1981) and Fortuner and Maggenti (1987) considered *Nothotylenchus* as a junior synonym of *Ditylenchus*. Here, we followed Siddiqi (2000) and Andrássy's (2007) classification scheme.

The nematode species concept has been widely discussed, suggesting that species delimitation should be based on an amalgamation of principles of polyphasic taxonomy that assembles and assimilates all available data and information (phenotypic, genotypic and phylogenetic) used for delimiting taxa at all levels (Palomares-Rius et al., 2014; Vovlas et al., 2015). Molecular techniques have shown that many presumed monospecific species are in fact sibling or cryptic species, genetically distinct but sharing similar morphology (Subbotin et al., 2005).

By far, 11 *Nothotylenchus* species were reported in various locations in Iran (Ghaderi et al., 2012; Esmaeli et al., 2016). To study the species diversity of this genus in Iran, we conducted several samplings in cultivated and natural areas of Iran during the summer of 2016; as a result, a population of *Nothotylenchus* species was collected from the rhizosphere of palm trees (*Phoenix dactylifera*). This population morphologically resembled a group of *Nothotylenchus* species by having a valveless and nondeveloped median bulb of pharynx, lateral fields with six lines, relatively short postvulval uterine sac, and tail elongate-conoid, with pointed tip. These traits led us to perform much detailed morphological and molecular study to compare with all previously described species. These observations revealed that this species appeared to be morphologically and morphometrically distinct from any existing *Nothotylenchus* species. Thus, it is herein described as *N. phoenixae* n. sp. through morphological observation and molecular characterization by the partial 18S, 28S D2/D3 and ITS rRNA gene sequences.

Materials and Methods

Nematode population sampling, extraction, and morphological identification: Specimens of the *Nothotylenchus* species detected in this study were isolated from the rhizosphere of palm trees (*Phoenix dactylifera*) cultivated in the city of Gilan-e Gharb, Kermanshah Province, western Iran. Nematodes were extracted from soil by the tray method (Whitehead and Hemming, 1965) for 48 hr. The nematodes were handpicked under a stereomicroscope model Olympus SZH and heat killed by adding boiling 4% formalin solution, and then transferred to anhydrous glycerine according to De Grisse (1969) and mounted on permanent slides. The characters of nematodes were observed under a light microscope (Nikon E200). Photographs of nematodes were taken

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by a digital camera attached to the same microscope and the drawings were made using a drawing tube.

Nematode molecular identification: Nematode DNA was extracted from single live individuals. Single nematode specimen was transferred to an Eppendorf tube containing 16 µl ddH_2O, 2 µl 10× PCR buffer, and 2 µl proteinase K (600 µg/ml) (Promega, Benelux, The Netherlands) and crushed for 2 min with a microhomogeniser, Vibro Mixer (Zürich, Switzerland). The tubes were incubated at 65°C for 1 hr, then at 95°C for 10 min. One microliter of extracted DNA was transferred to an Eppendorf tube containing: 2.5 µl 10× NH_4 reaction buffer, 0.75 µl $MgCl_2$ (50 mM), 0.25 µl dNTPs mixture (10 mM each), 0.75 µl of each primer (10 mM), 0.2 µl BIOTAQ DNA Polymerase (BIOLINE, London, UK) and ddH_2O to a final volume of 25 µl. For the first fragment of 18S, the primer 1096F (5′-GGT AAT TCT- GGA GCT AAT AC-3′) was used in combination with the primer 1912R (5′-TTT ACG GTC AGA ACT AGG G-3′) and the second fragment was amplified with forward primer 1813F (5′-CTG CGT GAG AGG TGA AAT-3′) and reverse primer 2646R (5′-GCT ACC TTG TTA CGA CTT TT-3′) (Holterman et al., 2006). The 28S D2/D3 was amplified using forward primer D2A (5′- ACA AGT ACC GTG AGG GAA- AGT TG-3′) and reverse primer D3B (5′-TCG GAA GGA-ACC AGC TAC TA-3′) (Nunn, 1992). The ITS-rRNA-gene was amplified using forward primer TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and reverse primer 5.8MS (5′-GGC GCA ATG TGC ATT CGA-3′) (Tanha Maafi et al., 2003; Vovlas et al., 2008).

PCR cycle conditions were as follows: one cycle of 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing temperature of 55°C for 45 sec, extension at 72°C for 3 min, and finally one cycle of 72°C for 10 min. The PCR products were purified after amplification using ExoSAP-IT (Affmetrix, USB products), quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and used for direct sequencing in both directions using the same PCR primers. The resulting products were purified and run on a DNA multicapillary sequencer (Model 3130XL genetic analyzer; Applied Biosystems, Foster City, CA), using the BigDye Terminator Sequencing Kit v.3.1 (Applied Biosystems), at the Stab Vida sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under accession numbers KX549317-KX549319.

Phylogenetic analyses: DNA sequences were edited with ChromasPro1.5 2003 to 2009 (Technelysium Pty Ltd, Helensvale, Australia) and aligned using ClustalW (http://workbench.sdsc.edu; Bioinformatics and Computational Biology Group, Dept. Bioengineering, UC San Diego, CA). All available species of *Nothotylenchus* and some other anguinid and hexatylenchid species from GenBank were also selected for phylogenetic analysis. The model of base substitution in the sequence data were evaluated using MODELTEST version 3.06 (Posada and Criandall, 1998) based on the Akaike-supported model (Arnold, 2010). Bayesian analysis was performed to confirm the tree topology for each gene

TABLE 1. Morphometrics of *Nothotylenchus phoenixae* n. sp. from Iran. All measurements are in µm and in the form: mean ± SD (range).

Character/Ratio	Holotype female	Paratype females	Paratype males
n	-	15	2
L	772	784 ± 67.9 (663–925)	677–715
a	35.1	36.1 ± 4.1 (30.1–46.3)	44–45
b	6.3	6.3 ± 0.6 (5.4–7.7)	5.5–6.0
c	12.9	14.0 ± 2.0 (11.0–18.0)	11.5–13.2
c′	4.0	4.1 ± 0.6 (3.1–5.0)	5.2–5.4
V or *T* (%)	82	80.6 ± 1.8 (76.6–82.5)	41–43
Lip region height	3.0	2.5 ± 0.6 (2.0–3.5)	3.0–3.5
Lip region width	6.0	5.6 ± 0.5 (5.0–6.0)	5.0–6.0
Stylet length	6.5	6.2 ± 0.7 (5.0–7.0)	5.0–6.0
Stylet conus length	2.8	2.7 ± 0.3 (2.0–3.0)	2.0–2.8
m[a]	46	42.5 ± 2.0 (40–46)	40–46
Body width (BW)	22	21.8 ± 1.2 (20.0–23.0)	15.0–16.0
Nerve ring from anterior end	70	74.2 ± 9.4 (56–92)	79–80
Excretory pore from anterior end	138	111 ± 16.3 (83–138)	93–120
Hemizonid from anterior end	108	109 ± 16.1 (80–135)	90–118
Pharynx length	122	125 ± 13.2 (95–142)	113–130
Vulva–anus distance (VA)	80	94.6 ± 12.3 (76–118)	-
Postvulval uterine sac (PUS)	14	15.3 ± 0.9 (14–17)	-
PUS/VA (%)	17.5	16.4 ± 2.1 (12.0–19.7)	-
PUS/BW (%)	0.6	0.7 ± 0.1 (0.6–0.8)	-
Ovary or testis length	355	382 ± 52.7 (279–480)	290–295
Anal (cloacal) body diameter	13	13.8 ± 1.0 (12.5–15.0)	10–11
Tail length	60	57.2 ± 7.3 (43–65)	54–57
Spicules length (arc line)	-	-	21–22
Gubernaculum	-	-	5–6

[a] Length of conus as percentage of total stylet length.

separately using MrBayes 3.1.0 (Huelsenbeck and Ronquist, 2001) running the chain for 1,000,000 generations and setting the "burnin" at 1,000. Markov Chain Monte Carlo methods were used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees (Larget and Simon, 1999) using the 50% majority rule. The λ^2 test for homogeneity of base frequencies and phylogenetic trees were performed using PAUP* version 4.0 (Sinauer Associates, Inc. Publishers, Sunderland, MA).

Results and Discussion

Systematics

Nothotylenchus phoenixae n. sp.

(Table 1; Figs. 1–5).

Description

Female: Body posture, after fixation and mounting in glycerine, straight to slightly tapering at both ends. Cuticle with fine annulation (annuli 1.2 to 1.4 μm wide). Lip region anteriorly flattened, framework not

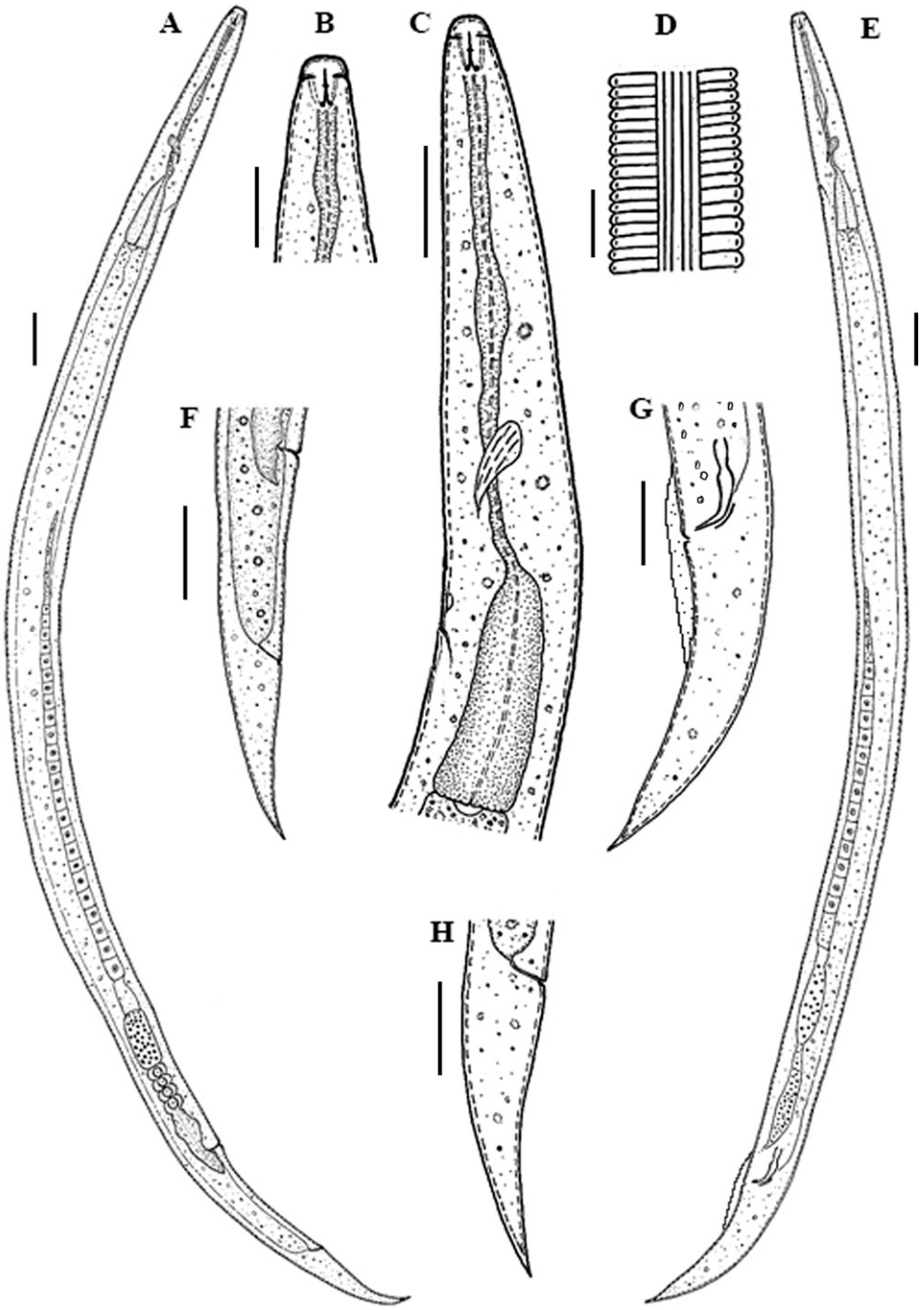

Fig. 1. Line drawing of *Nothotylenchus phoenixae* n. sp. A. Female entire body; B. Lip region; C. Pharyngeal region; D. Lateral field at midbody; E. Male entire body; F. Vulval region to posterior body; G. Male posterior body; H. Female tail region. (A, E, F = 30 μm, B, C, G, H = 15 μm, and D = 10 μm).

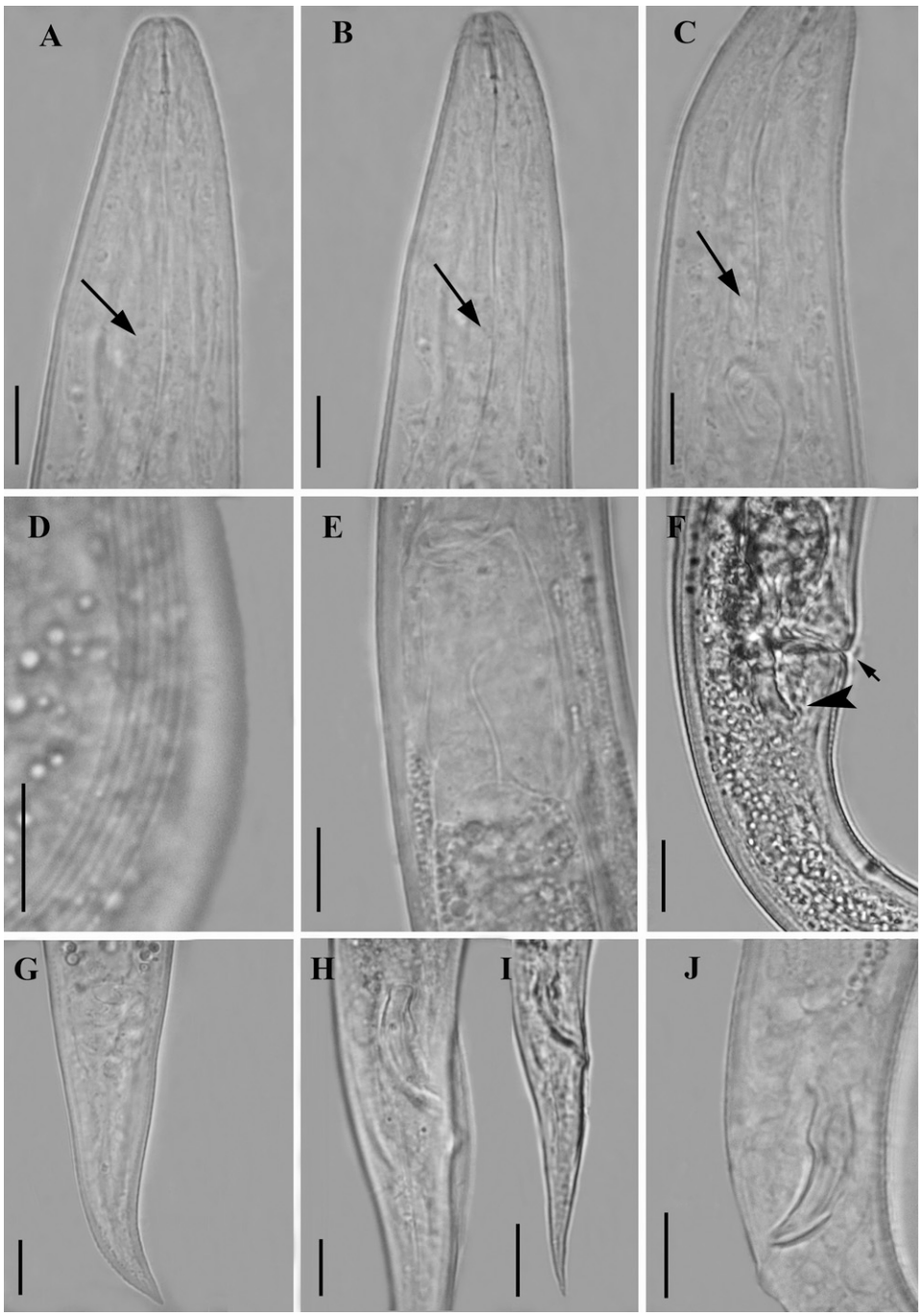

Fig. 2. Light micrographs of *Nothotylenchus phoenixae* n. sp. A to C. Female anterior body region (arrows showing median bulb of pharynx); D. Lateral field at mid-body; E. Pharyngeal basal bulb region; F. Vulval region showing vulva (arrow) and postvulval uterine sac (arrowhead); G. Female tail region; H, I. Male posterior body; J. Spicules. (All scale bars = 10 μm except I = 20 μm.)

sclerotized, lip region continuous with body contour, 5.5 (5 to 6) μm wide and 2.5 (2 to 3) μm high. Lateral field measuring *ca* 1/4 to 1/2 of body diam., marked by six incisures, outer incisures weakly crenate and inner smooth. Stylet short and delicate, knobs rounded and somewhat posteriorly directed, well developed, 1.3 (1.2 to 1.5) μm wide, conical part occupying about 40% to 46% of total stylet length. Dorsal gland orifice very close to stylet knobs. Metacorpus (median bulb) fusiform, nonmuscular, and nonvalvate. Isthmus elongate, slender ending to a pyriform basal pharyngeal bulb not overlapping intestine. Nerve ring around posterior part of isthmus. Hemizonid prominent, about three annuli long, excretory pore (EP) located at the level of anterior third part of glandular lobe, immediately or few annuli behind the hemizonid. Pharyngeal basal bulb pyriform, not overlapping intestine.

Reproductive system characteristic of *Nothotylenchus*, prodelphic, ovary outstretched with oocytes arranged in a single file. Spermatheca elongated *ca* 1.3 (1.2 to 1.5) body diam. in length, filled with large round sperm cells.

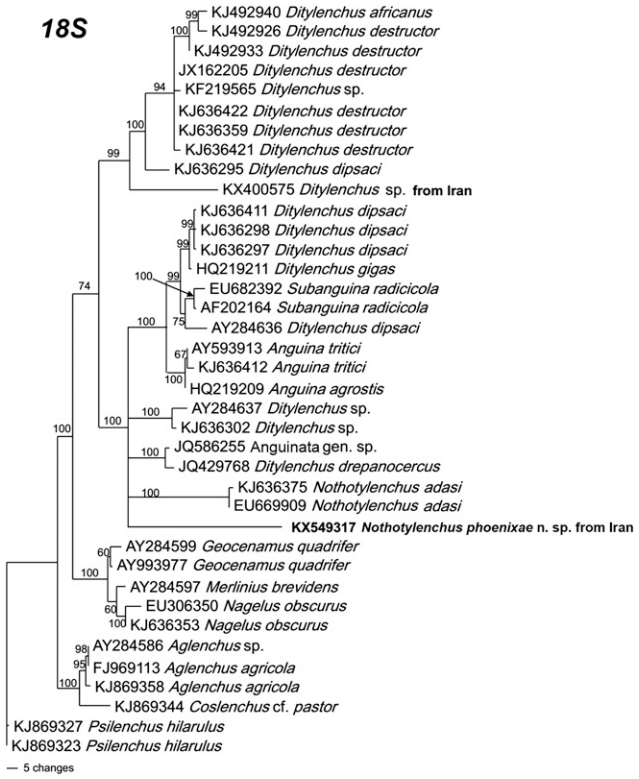

FIG. 3. Bayesian consensus tree inferred from 18S under GTR + I + G model ($-\ln L = 3{,}929.0166$; AIC = 7,878.0332; freqA = 0.2672; freqC = 0.2063; freqG = 0.2606; freqT = 0.2659; R(a) = 1.6289; R(b) = 2.7033; R(c) = 0.8891; R(d) = 0.665; R(e) = 5.3513; R(f) = 1; Pinva = 0.369; Shape = 0.7164). Posterior probability values exceeding 50% are given on appropriate clades.

Uterus with a quadricolumella comprising four rows of four cells. Vulva a transverse slit, vagina somewhat oblique to body axis, reaching more than halfway across body. Postvulval uterine sac well developed, *ca* 0.9 (0.8 to 1.0) vulval body diam. long. Vulva–anus distance *ca* 1.7 (1.3 to 2.3) tail length long. Rectum distinct, somewhat shorter than anal body diam. Tail about 4.1 (3.1 to 5) times the anal body width, elongate-conoid with pointed terminus.

Male: Similar to female in general body characteristics but usually shorter. Lateral field marked by six incisures. Testis single, well developed, outstretched with spermatogonia arranged in a single row. Spicules ditylenchoid, simple, slightly arcuate ventrally. Gubernaculum simple. Bursa leptoderan, extending for 40% to 47% of tail. Tail elongate-conoid, 5.3 (5.2 to 5.4) cloacal body diam. long with pointed terminus.

Type habitat and locality: Rhizosphere of palm trees (*Phoenix dactylifera* L.) in Gilan-e Gharb region, Kermanshah Province, western Iran (GPS coordinates: N 33°59′, E 46°12′, 1,248 m a.s.l.).

Type material: Holotype female (slide ANP201) together with five paratype specimens (three females, two males; slides ANP202-ANP204) were deposited in the Nematode Collection of the Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran. Tow paratype females deposited at the Museum voor Dierkunde, Ghent University, Ghent, Belgium, and three paratype females deposited in the National Nematode Collection of the Department of Nematology, Iranian Research Institute of Plant Protection, Tehran, Iran.

Diagnosis and Relationships

Nothotylenchus phoenixae n. sp. is characterized by the following features: stylet delicate with rounded basal knobs, median bulb of pharynx fusiform, nonmuscular, and nonvalvate, EP located at the level of anterior third of basal pharyngeal bulb, six lines in the lateral field, vulva located at 80.6 (76.6% to 82.5%) of body length, relatively short postvulval uterine sac (*ca* 0.9 [0.8 to 1.0] corresponding vulval body diam. long), and female tail elongate-conoid, with pointed terminus.

Because of the presence of six lines in the lateral fields, stylet length and female tail tip, *Nothotylenchus phoenixae* n. sp. is morphologically and morphometrically similar to five known species in the genus, namely *N. affinis* (Thorne, 1941) Fortuner and Maggenti, 1987, *N. hexaglyphus* (Khan and Siddiqi, 1968) Fortuner and Maggenti, 1987, *N. persicus* Esmaeli et al., 2016, *N. taylori* (Husain and Khan, 1974) Fortuner and Maggenti, 1987 and *N. uniformis* (Truskova and Eroshenko, 1977) Fortuner and Maggenti, 1987. However, the new species differs from *N. hexaglyphus* by its longer body 784 (663 to 925) vs. 660 to 720 μm long, $a = 36$ (30 to 46) vs. 32 to 33, shorter stylet 6.2 (5 to 7) vs. 7.5 to 8.5 μm long, and tail tip pointed vs. finely rounded; from *N. affinis* by its longer body 784 (663 to 925) vs. (420 to 610) μm long, shorter stylet 6.2 (5 to 7) vs. 8 to 9 μm long, $a = 36$ (30 to 46) vs. 30 to 38, $c = 14$ (11 to 18) vs. 9 to 11, longer spicules 21.5 (21 to 22) vs. 15 to 17 μm long, and tail tip pointed vs. finely rounded; from *N. persicus* by its longer postvulval uterine sac 0.9 (0.8 to 1.0) vs. 0.43 (0.4 to 0.6) vulval body diam. long, $a = 36$ (30 to 46) vs. 24 (21 to 27) and EP and hemizonid located anterior to basal pharyngeal bulb vs. posterior to it; from *N. taylori* by its shorter stylet 6.2 (5 to 7) vs. 8 to 9 μm long, shorter postvulval uterine sac (0.9 [0.8 to 1.0] vs. 2 to 3 vulval body diam. long), a more posterior located vulva ($V = 80.6$ [76.6 to 82.5] vs. 75 to 77), bursa extending for <50% of tail vs. >50%, and tail tip pointed vs. rounded; from *N. uniformis* by its shorter stylet 6.2 (5 to 7) vs. 8.4 μm long, shorter isthmus length (*ca* 2 corresponding body diam. long at pharyngeal region vs. 4), basal pharyngeal bulb shape, EP and hemizonid located at the level of anterior third of glandular lobe bulb vs. posterior third of isthmus and male presence vs. absence.

Etymology: The species name is derived from the Latin word of *Phoenix dactylifera*, the plant from which the new species was isolated.

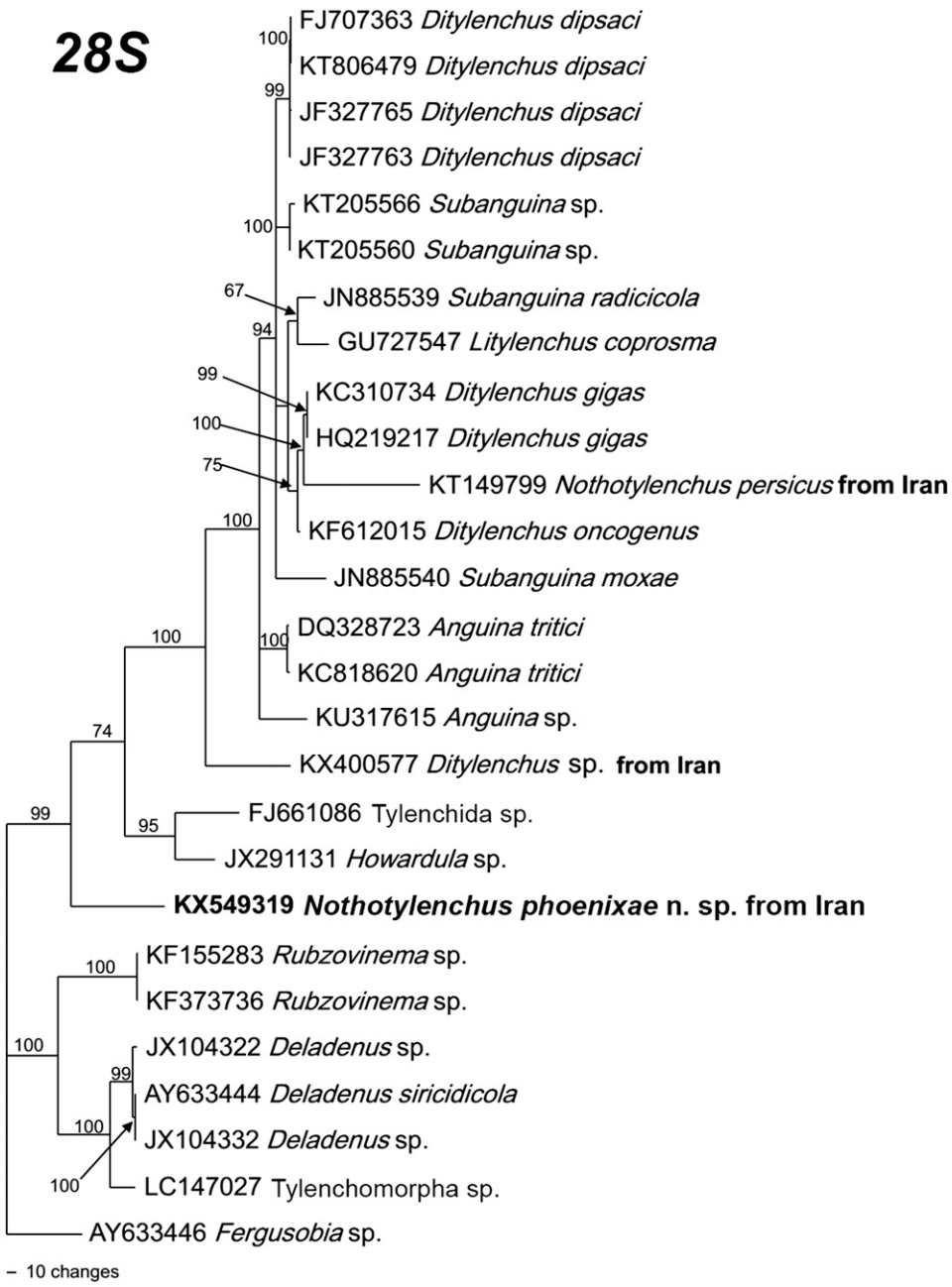

FIG. 4. Bayesian consensus tree inferred from 28SD2/D3 under GTR + I + G model ($-\ln L$ = 6574.5552; AIC = 13169.1104; freqA = 0.1893; freqC = 0.1968; freqG = 0.325; freqT = 0.2889; R(a) = 0.647; R(b) = 4.3615; R(c) = 1.9717; R(d) = 0.7264; R(e) = 6.8176; R(f) = 1; Pinva = 0.2343; Shape = 0.7894). Posterior probability values exceeding 50% are given on appropriate clades.

Molecular phylogeny and discussion: The 925-bp partial 18S rDNA sequence (GenBank accession number KX549317) was used to determine the phylogenetic relationships of *Nothotylenchus phoenixae* n. sp. with other anguinid nematodes. A BlastN search of sequence against the sequence database gave less than 97% similarity with any available DNA sequences from GenBank. It revealed the highest match with *Ditylenchus* sp. 2 and 5 JH-2014 (KJ636299, KJ636302), *Ditylenchus drepanocercus* (Goodey, 1953) (JQ429768), *D. dipsaci* (Kuhn, 1857) Filipjev, 1936 (KJ636295-KJ636298, KJ636411, AY593911, HQ219210, AY593906, AY593908-AY593910, EU669931, AY284636), *D. gigas* Vovlas et al., 2011 (HQ219211) and *Ditylenchus* sp. JH-2003 (AY284637), with 95% identity. The closest sequences per species, along with sequences of genera with morphological similarity, were selected for inclusion in the phylogenetic analyses. Figure 3 presents a Bayesian phylogenetic tree inferred from the multiple alignment of partial 18S sequences of 35 tylenchid taxa, two outgroup taxa and, this study *Nothotylenchus*. In this tree, all *Ditylenchus*, *Anguina* Scopoli, 1777, *Subanguina* Paramonov, 1967, *Anguinata* Siddiqi, 2000 and *Nothotylenchus* in Anguinidae are in a monophyletic clade with 74%

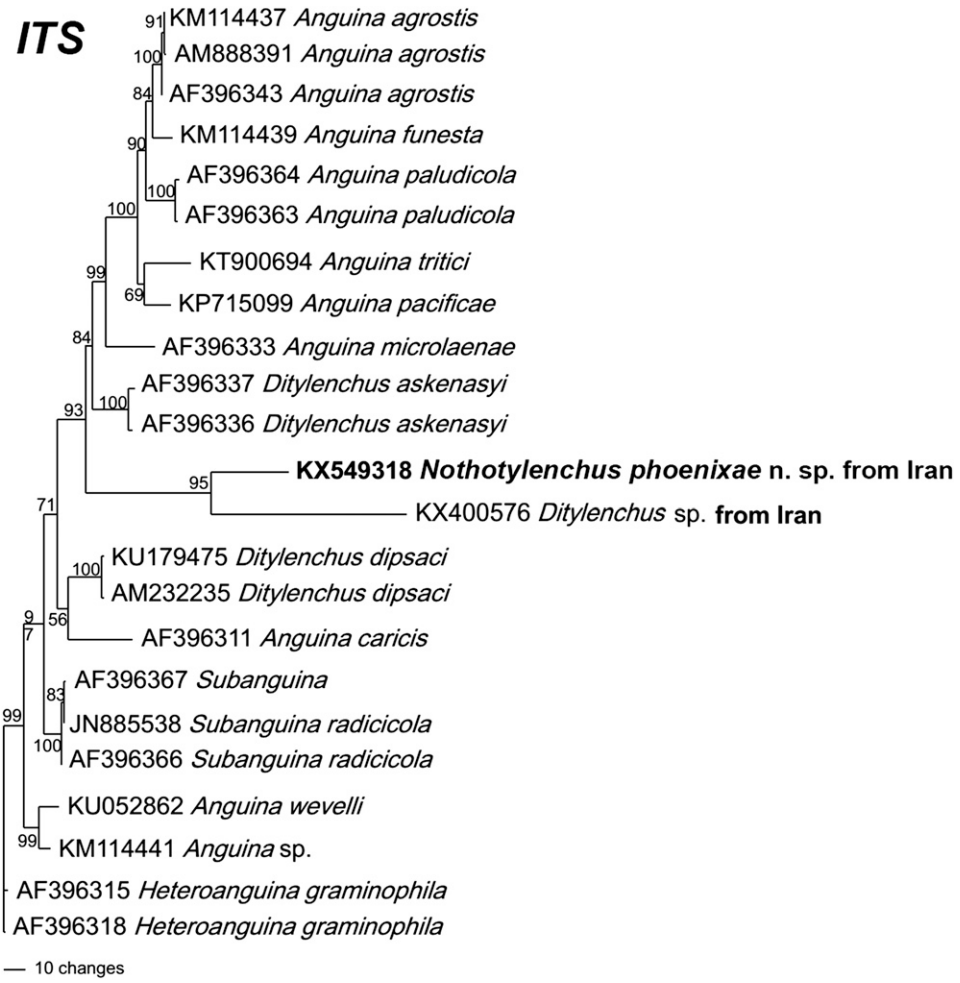

FIG. 5. Bayesian consensus tree inferred from internal transcribed spacer (ITS) under GTR + I + G model (–ln*L* = 2,546.9644; AIC = 5,105.9287; freqA = 0.26851; freqC = 0.21497; freqG = 0.24285; freqT = 0.27366; R(a) = 1.5792; R(b) = 6.3461; R(c) = 4.0583; R(d) = 0.531; R(e) = 9.9289; R(f) = 1; Pinva = 0; Shape = 0.4028). Posterior probability values exceeding 50% are given on appropriate clades.

support. Species in *Ditylenchus* are not monophyletic and were split into two clades. *Nothotylenchus phoenixae* n. sp. is in the same clade with *N. adasi* Sykes, 1980 (KJ636375, EU669909). This clade with 100% support includes *Ditylenchus dipsaci*, *Subanguina radicicola* (Greeff, 1872) Paramonov, 1967, *Anguina tritici* (Steinbuch, 1799) Filipjev, 1936, and *A. agrostis* (Steinbuch, 1799) Filipjev, 1936. The branch length of the new species clade was fairly long compared with other closest clades of species in the Anguinidae. Our 18S region tree was in agreement with the results of other studies (Subbotin et al., 2006; Davies et al., 2009; Chizhov et al., 2010; Vovlas et al., 2015), small differences being attributed to the additional sequences of more species included in this study.

Figure 4 presents the phylogenetic tree of 27 tylenchid taxa based on sequences of 28S D2/D3 region. A BlastN search of the 758 bp D2/D3 of *Nothotylenchus phoenixae* n. sp. (KX549319) was less than 88% homologous from any available DNA sequences from GenBank. It revealed the highest match with *Howardula* sp. CD353 (JX291131), *Deladenus* sp. (JX104331, JX104332, JX104285, JX104298, and JX104302-JX104315), and several isolates of *Ditylenchus dipsaci* with 87% to 88% sequence similarity. *N. phoenixae* n. sp. is at the basal position in a strongly supported monophyletic clade with a 99% support with other genera namely *Ditylenchus*, *Anguina*, *Subanguina*, *Litylenchus* (Zhao et al., 2011), and *Howardula* (Cobb, 1921). Another species of *Nothotylenchus*, *N. persicus* (also from Iran, KT149799) and *N. phoenixae* n. sp. are not monophyletic.

Figure 5 presents the phylogenetic tree of 23 tylenchid taxa based on ITS region. The BlastN search of a 465 bp ITS of *Nothotylenchus phoenixae* n. sp. (KX5493198) was less than 87% homologous from any available DNA sequences from GenBank. The closest matches are *Ditylenchus askenasyi* (Bütschli, 1873) (AF396336, AF396337) and an unidentified species of *Anguina* sp. (KM114441) with 86% to 87% identity. In this tree, *N. phoenixae* n. sp. formed a monophyletic clade with 95% support with *Ditylenchus* sp. (KX400576, unpublished) also from Iran. This clade is grouped with other members of Anguinidae such as *Ditylenchus* spp. and *Anguina* spp. with 93% support. Species in *Ditylenchus* and species in *Anguina* are not monophyletic. These results of ITS sequence generally support the

inclusion of *Nothotylenchus* genus within the Anguinidae as in 18S and 28S D2/D3.

Based on a nonvalvate and nonmuscular median pharyngeal bulb and pharyngeal glands enclosed in a basal bulb, *N. phoenixae* n. sp. belongs to the genus *Nothotylenchus*. This genus is differentiated from *Ditylenchus* by structure and morphology of the median pharyngeal bulb (Brzeski, 1981; Fortuner and Maggenti, 1987; Siddiqi, 2000; Andrássy, 2007). The DNA sequencing data on three gene fragments indicated that *N. phoenixae* n. sp. is unique and is a member of Anguinidae. However, there are very limited data available to examine the relationships in the genus *Nothotylenchus* since *N. adasi* and *N. persicus* are the only other two species with DNA sequence data. Other genera are not monophyletic as observed in previous studies (Vovlas et al., 2015; Esmaeli et al., 2016). Thus, additional molecular data from other genus or species is needed to be conducted to clarify the phylogeny. Siddiqi (2000) stated that most species in the both similar genera, *Ditylenchus* and *Nothotylenchus*, shares fungal feeding habits. So, the new species probably appears to be feeding on fungi, although we could not determine this.

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