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Research Article

Observations on *Hoplolaimus indicus* Sher, 1963 and *Hoplolaimus seinhorsti* Luc, 1958 (Nematoda: Hoplolaimidae) from Southern Iran

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Abstract: Morphological observations are made on several populations of *Hoplolaimus indicus* and *Hoplolaimus seinhorsti*, recovered from rhizosphere of mango, tamarind, sour orange and sugarcane from the southern regions of Iran. Detailed studies on the two species *Hoplolaimus dubius* and *H. indicus* being separated from each other based on some morphological characters, revealed each of them having intra-specific and overlapping variations in morphology and morphometric ranges, enough for not separating two closely related aforementioned species and as a result, *H. dubius* is considered as a junior synonym of *H. indicus*. Observations on *H. seinhorsti* also supported the Siddiqi's decision on the synonymy of *Hoplolaimus sheri* with *H. seinhorsti*. The results of the phylogenetic analyses using D2-D3 expansion segments of 28S rRNA gene were in agreement with the results of previous works, *i.e.* the classic scheme for assigning species of the genus into two "ancestral" and/or "derived" groups was supported. In phylogenetic trees inferred, using different analysis methods, the Iranian populations of *H. indicus* were located in the same clade with *H. seinhorsti* and *H. columbus*, belonging to "derived" group of species of the genus characterized by having six nuclei in pharyngeal glands, less than four incisures at each lateral field and anteriorly situated position of excretory pore to hemizonid.

Keywords: 28S rRNA, *Hoplolaimus dubius*, *H. sheri*, identification, morphology

Introduction

The genus *Hoplolaimus* von Daday, 1905 presently has 29 species according to Sher (1963) and Handoo and Golden (1992), or 32 species in three subgenera, *Basirolaimus*, *Hoplolaimus* and *Ethiolaimus* according to Siddiqi (2000). While revising of the genus and describing four species, Sher (1963) described *H. indicus* based on the specimens associated with sugarcane, banana, pea and guava in India and he distinguished it from *H. columbus* Sher, 1963 by having the shorter female tail, anterior position of the excretory pore, smaller body size, shorter stylet and presence of a functional spermatheca in females and occurring of males. The most closely related species, *H. dubius* Chaturvedi, Singh and Khera, 1979 shares several morphological and morphometric characters with it, but differs from *H. indicus* in some characters such as the number of head annuli, the number of lateral incisures, longitudinal markings on basal annulus of head, nature of epiptygma (single *vs* single or double), extending of the intestine behind the anus,

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position of excretory pore and distance of dorsal gland orifice from knobs base (Chaturvedi and Khera, 1979; Handoo and Golden, 1992). Siddiqi (2000) noted that the large number of species of *Hoplolaimus* (including *H. dubius*) having been described from Southern Asia, need further studies to confirm their identity, since many of them are similar to either *H. seinhorsti* or *H. indicus*. Vovlas (1983) provided SEM data on a population of *H. seinhorsti* collected from Seri Lanka. Anderson (1983) studied a population of *H. indicus* from North America and stated that intra - specific variation of morphology and morphometric data ranges of this species extends the previously known ranges for the species. The sequences of the ITS1 and D2 - D3 segments of 28S rDNA regions have alo been analyzed (Bae *et al*., 2008; 2009b) and used for rapid, easy and reliable identification of several *Hoplolaimus* species (Bae *et al*., 2009a). There is still no molecular data for *H. indicus*.

The review of the Iranian literature revealed that *H. indicus* and *H. seinhorsti* are the common species of the genus occurring in southern Iran. So far, *H. indicus* is reported from Hormozgan, Sistan and Baluchistan and Kerman provinces in association with citrus, banana, other fruit trees, cucurbits, date palm, mango, olive and sapodilla (Tanha Maafi and Kheiri, 1989; 1993; Nowrouzi and Barooti, 1997; Barooti *et al*., 2002; Jahanshahi Afshar *et al*., 2006). The other species, *H. seinhorsti*, occurs in Hormozgan, Khuzestan, Sistan and Baluchistan and Kerman provinces, associated with citrus, banana, sugarcane and field crops (Barooti and Geraert, 1994; Kheiri, 1995; Tanha Maafi *et al*., 2006; Ali Ramaji *et al*., 2006; Jahanshahi Afshar *et al*., 2006). The aims of the present study were morphological and molecular characterisation of Iranian populations of *H. indicus* and morphological observations on *H. seinhorsti* from southern Iran.

Materials and Methods

Specimens of *H. indicus* were collected and identified from the rhizosphere of mango (*Mangifera indica*) in Ghasr - e - Ghand (Sistan and Baluchistan) and Minab (Hormozgan), tamarind (*Tamarindus indica*) in Minab (Hormozgan) and sour orange (*Citrus aurantium*) in Bandar - Abbas (Hormozgan), and those of *H. seinhorsti* from sugarcane (*Saccharum officinarum*) in Ahvaz (Khuzestan). Nematodes were extracted from soil using the tray method (Whitehead and Hemming, 1965), killed and fixed by hot FPG (4:1:1 ratios of formaldehyde, propionic acid and glycerol) and processed to anhydrous glycerol (De Grisse, 1969). The specimens were subsequently mounted on permanent slides using paraffin wax and were studied using a light microscope, equipped with digital camera and corresponding Dino capture 2.0 software. The specimens were identified to the level of species using available identification keys (Anderson, 1983; Krall, 1990; Handoo and Golden, 1992). The voucher slides were deposited at Laboratory of Nematology, Department of Plant Protection, College of Agriculture, University of Shiraz, Shiraz, Iran.

The D2 - D3 expansion regions of 28S rRNA gene of the two *H. indicus* populations (single nematode from each population) were amplified by use of the forward D2A (5'_ACAAGTACCGTGAGGGAAAGTTG - 3') and reverse D3B (5'_TCGGAAGGAACC AGCTACTA - 3') primers according to Tanha Maafi *et al.* (2003) and Subbotin *et al.* (2007). The maximum likelihood (ML), maximum parsimony (MP) and neighbor - joining (NJ) methods were used to reconstruct the phylogenetic relationships of some of *Hoplolaimus* species including newly obtained sequences using MEGA5.05 software (Tamura *et al.*, 2011). The software MrBayes 3.0 (Ronquist and Huelsenbeck, 2003) was used for inferring the Bayesian tree under GTR + I + G model of DNA substitution. Moreover, the MP tree was constructed using Mega5 with Close - Neighbor - Interchange (CNI) on random trees for search method. Consensus tree was bootstrapped 1000 times.

Results and Discussion

Morphometrics of the present population of *H. indicus* fit well with those of original

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description (Sher, 1963), except the value b' slightly smaller in females (5.7 - 8.0 *vs.* 7.0 - 9.1) and males (6.1 - 7.3 *vs.* 6.2 - 9.0). The specimens of *H. seinhorsti* collected during present study have larger body (1480 - 1738 *vs.* 1060 - 1560 µm) and shorter pharynx (b = 10.2 - 12.8 *vs.* 8.8 - 10.1) compared with the data in original description (Sher, 1963). The results are presented in the Table 1 and Figs 1 - 3.

The species *H. dubius* has been separated from *H. indicus* based on some characters that are not so constant and powerful enough for diagnostic purposes. Chaturvedi and Khera (1979) believed that *H. dubius* differs from *H. indicus* in having less number of head annuli (three *vs.* four), variable number of lateral incisures, more longitudinal markings (14 *vs.* 11) on basal annulus of head, variable position of excretory pore and intestine not overlapping the rectum. Handoo and Golden (1992) used the post - anal intestinal sac (absent *vs.* present), O index (9 - 11 *vs.* 13 - 18) and epiptygma (single *vs.* single or double) as diagnostic characters for separating of these two species.

Table 1 Morphometric characters of *Hoplolaimus indicus* and *Hoplolaimus seinhorsti*, collected from southern Iran and their comparisons with the original populations.

Character	*Hoplolaimus indicus*				*Hoplolaimus seinhorsti*	
	Present study		Sher, 1963		Present study	Sher, 1963
	16 ♀♀	7 ♂♂	20 ♀♀	10 ♂♂	8 ♀♀	20 ♀♀
L	1170 ± 63 (1012 - 1271)	1087 ± 64 (1001 - 1202)	950 - 1400	900 - 1300	1631 ± 99 (1480 - 1738)	1060 - 1560
a	31.9 ± 2.1 (28.0 - 36.1)	32.7 ± 2.1 (29.4 - 35.9)	26 - 36	26 - 33	30.6 ± 2.9 (25.5 - 33.8)	25 - 34
b	9.6 ± 0.8 (8.4 - 11.6)	9.0 ± 0.7 (8.3 - 9.9)	9.1 - 12.6	9.4 - 12.0	11.2 ± 1.1 (10.2 - 12.8)	8.8 - 10.1
b'	7.0 ± 0.7 (5.7 - 8.0)	6.6 ± 0.5 (6.1 - 7.3)	7.0 - 9.1	6.2 - 9.0	7.9 ± 0.8 (7.1 - 9.2)	6.0 - 10.1
c	61.3 ± 8.6 (48.7 - 75.4)	39.4 ± 3.4 (35.8 - 44.5)	45 - 74	32 - 38	46.7 ± 8.2 (37.0 - 59.0)	38 - 74
c'	0.7 ± 0.1 (0.6 - 0.9)	1.5 ± 0.1 (1.2 - 1.6)	-	-	1.0 ± 0.2 (0.8 - 1.2)	-
V	56.2 ± 1.1 (54.4 - 58.8)	-	50 - 59	-	56.2 ± 2.9 (53.0 - 60.5)	52 - 60
Stylet	36.4 ± 1.5 (34.3 - 39.0)	33.9 ± 0.9 (33.0 - 35.0)	33 - 40	33 - 37	45.1 ± 1.2 (43.0 - 47.0)	40 - 49
Conus	18.4 ± 0.9 (16.8 - 20.0)	17.6 ± 0.8 (16.5 - 19.0)	-	-	22.6 ± 0.7 (21.0 - 23.0)	-
m	50.7 ± 1.7 (48.6 - 55.6)	51.8 ± 1.7 (49.5 - 54.3)	-	-	50.1 ± 0.9 (48.8 - 51.1)	-
DGO	4.6 ± 0.7 (4.0 - 5.9)	5.2 ± 1.0 (4.0 - 6.2)	-	-	5.3 ± 1.8 (4.0 - 9.0)	-
O	12.8 ± 2.6 (10.3 - 17.1)	15.6 ± 3.2 (11.8 - 18.6)	10 - 18	10 - 16	9.6 ± 1.9 (8.5 - 13.0)	9 - 13
Pharynx	123 ± 8.3 (102 - 133)	122 ± 4.7 (114 - 129)	-	-	147 ± 17 (129 - 170)	-
Phar. glands end	169 ± 15.0 (148 - 200)	166 ± 12.2 (145 - 181)	-	-	207 ± 17.5 (182 - 236)	-
Median bulb	85.1 ± 4.4 (73.1 - 92.0)	82.6 ± 4.1 (78.0 - 88.0)	-	-	110 ± 5.9 (99 - 116)	-
MB	69.5 ± 3.3 (61.9 - 77.6)	68.0 ± 1.6 (65.8 - 70.7)	-	-	75.4 ± 5.8 (68.2 - 81.3)	-
Excretory pore	114 ± 8.9 (99 - 132)	113 ± 8.3 (103 - 128)	-	-	146 ± 5.3 (140 - 153)	-
Hemizonid	127 ± 7.8 (115 - 142)	124 ± 2.2 (121 - 126)	-	-	167 ± 9.5 (157 - 182)	-
Nerve ring	102 ± 5.3 (90 - 112)	101 ± 5.9 (93 - 106)	-	-	130 ± 6.7 (118 - 138)	-
Head - vulva	658 ± 35.0 (572 - 707)	-	-	-	916 ± 56.8 (840 - 1009)	-
Tail length	19.4 ± 2.8 (16.0 - 26.0)	27.8 ± 2.3 (22.8 - 29.3)	-	-	36.0 ± 7.5 (28.0 - 47.0)	-
Body width	36.9 ± 3.3 (30.0 - 42.5)	33.4 ± 2.5 (31.0 - 37.0)	-	-	53.6 ± 4.9 (44.0 - 59.0)	-
Vulval body	35.9 ± 2.9 (30.0 - 40.0)	-		-	53.6 ± 4.9 (44.0 - 59.0)	-
Anal body width	26.8 ± 1.7 (23.5 - 30.0)	19.1 ± 1.1 (17.0 - 20.0)	-	-	35.6 ± 2.1 (33.0 - 38.0)	-
lip region width	13.0 ± 0.6 (12.0 - 13.5)	12.3 ± 0.7 (11.0 - 13.0)	-	-	15.1 ± 0.8 (14.0 - 17.0)	-
lip region height	6.8 ± 0.4 (6.0 - 7.5)	6.8 ± 0.5 (5.6 - 7.3)	-	-	8.0 ± 0.5 (7.0 - 9.0)	-
Annulus width	2.2 ± 0.2 (1.9 - 2.6)	2.2 ± 0.2 (2.0 - 2.4)	-	-	2.3 ± 0.2 (2.1 - 2.5)	-
Tail annuli	11.4 ± 1.9 (9 - 17)	-	13	-	17.4 ± 1.8 (15.0 - 20.0)	10 - 15
Anterior phasmid	30 - 40	-	28 - 44	-	32 - 42	31 - 44
Posterior phasmid	72 - 85	-	76 - 86	-	70 - 78	74 - 83
Spicule	-	39.6 ± 1.6 (37.0 - 41.0)	-	37 - 42	-	-
Gubernaculum	-	17.4 ± 1.5 (15.0 - 19.0)	-	16 - 20	-	-

All measurements are in µm.

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Sher (1963) pointed out that more than half specimens of his studied *H. indicus* population had three annuli on one or both sides of the head. He also noticed that sometimes two or three weakly - developed incomplete incisures could be observed in the lateral field of the specimens. The value of O index, another diagnostic character of *H. indicus*, is 10 - 18 and 10.3 - 17.1 in original description and also present study, respectively, that overlaps with the ranges of that index for *H. dubius*. Khan and Chawla (1975) and Anderson (1983) found that the number of longitudinal markings on basal annulus of head ranged from 6 - 12 and 6 - 20, respectively for populations of *H. indicus* from India and North America. Handoo and Golden (1992) considered the latter range (6-20) in their compendium. The variation in the position of excretory pore is evident in populations of Canada (Anderson, 1983) and Iran (Fig. 1 J - L). Anderson (1983) pointed out that the excretory pore could be observed at 27 µm anterior to 22 µm posterior to the pharyngo - intestinal junction. He also stated that intestine overlaps rectum to varying degrees and finally separated *H. dubius* from *H. indicus* only based on this character. However, he noticed that in the studied population, the intestine overlapping (on rectum) is so short and never extends into tail; moreover, overlapping was not observed in one specimen of Canadian population. Individuals with no or different lengths of overlapping could be found in populations from Iran (Fig. 3 C - H) and it appears that this character is too variable to be used as a character for separating of these two species. Finally, according to the abovementioned arguments, *H. dubius* is regarded as a junior synonym of *H. indicus*.

Suryavanshi (1971) described *H. sheri* from India and distinguished it from the closely related species, *H. seinhorsti* by having more longitudinal striations on the basal annulus of head (20 *vs.* 8 - 12), different number of the pharyngeal glands (five *vs.* six) and the number of lateral incisures (two *vs.* one). Handoo and Golden (1992) added absence of the epiptygma as a further character. On the other hand, variation in number of longitudinal striations of the basal annulus of head has already been considered as intraspecific variation in *H. indicus*, *H. aegypti* Shafiee and Koura, 1969, *H. clarissimus* Fortuner, 1973 and *H. magnistylus* Robbins, 1982 (6 - 20, 13 - 22, 18 - 31 and 22 - 34, respectively). Although usually one incisures could be observed in lateral field of *H. seinhorsti*, but, sometimes two or three incomplete incisures are also visible (Vovlas, 1983; present study). However, specimens with distinct or indistinct epiptygma (Fig. 2 K, L) were observed in the Iranian population (the present study). Regarding the pharyngeal glands' nuclei, Siddiqi (2000) stated that observing the six gland nuclei in the genus *Hoplolaimus* is due to occurrence of four similar - sized nuclei in the dorsal gland instead of one, not because of duplication of the original three nuclei. He further noted that presence of five nuclei, as described for some species, is an error, since one of the two subventral gland's nuclei is overlooked as the two nuclei are not in the same optical level. Based on the given discussion and according to Siddiqi (2000), *H. sheri* is a junior synonym of *H. seinhorsti*.

PCR amplification of the D2 - D3 expansion region of 28S rDNA of the two populations of *H. indicus* yielded a single fragment about 635 nucleotides. The results of the phylogenetic analyses using three maximum likelihood, maximum parsimony and neighbor joining methods revealed the used species of the genus for reconstructing of the phylogenetic trees are clustered in two main clades in all inferred trees using the three abovementioned methods (Figs 4 - 6). Two Iranian populations of *H. indicus* were located in clade II together with *H. seinhorsti* and *H. columbus*. According to Fortuner (1991), there are two ancestral and derived groups within *Hoplolaimus* species. The first group has three nuclei in pharyngeal glands, four incisures at each lateral field and excretory pore posterior to hemizonid (ancestral characters *sensu* Fortuner) *vs.* six nuclei, less than four incisures in lateral fiend and excretory pore anterior to hemizonid (derived characters *sensu* Fortuner) in second group. The three species *H. indicus, H. seinhorsti* and *H. columbus* have derived characters, but *H. stephanus*, *H. magnistylus*, *H. galeatus*, *H. concaudjuvencus* and *Hoplolaimus* sp1, sp2 and

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sp3 studied by Bae *et al*. (2008) have ancestral characters. Molecular results of the present study also support the intraspecies groupings of Bae *et al.*, (2008) and Fortuner (1991) and confirm morphological characters are informative for depicting of phylogenetic relationships inside *Hoplolaimus* species. However, D2 - D3 expansion region of 28S rDNA could not separate closely related species in clade II (*H. colombus*, *H. seinhorsti* and *H. indicus*), a congruent result with previous study using the abovementioned genomic fragment (Bae *et al.*, 2008). Currently, several other molecular markers like multiplex PCR and PCR - RFLP of ITS - rDNA (Bae *et al.*, 2009a) or sequences of ITS1 fragments or the cytochrome c oxidase subunit 1 gene (Holguin *et al.*, 2015) are successfully used for reliable identification of *Hoplolaimus* species.

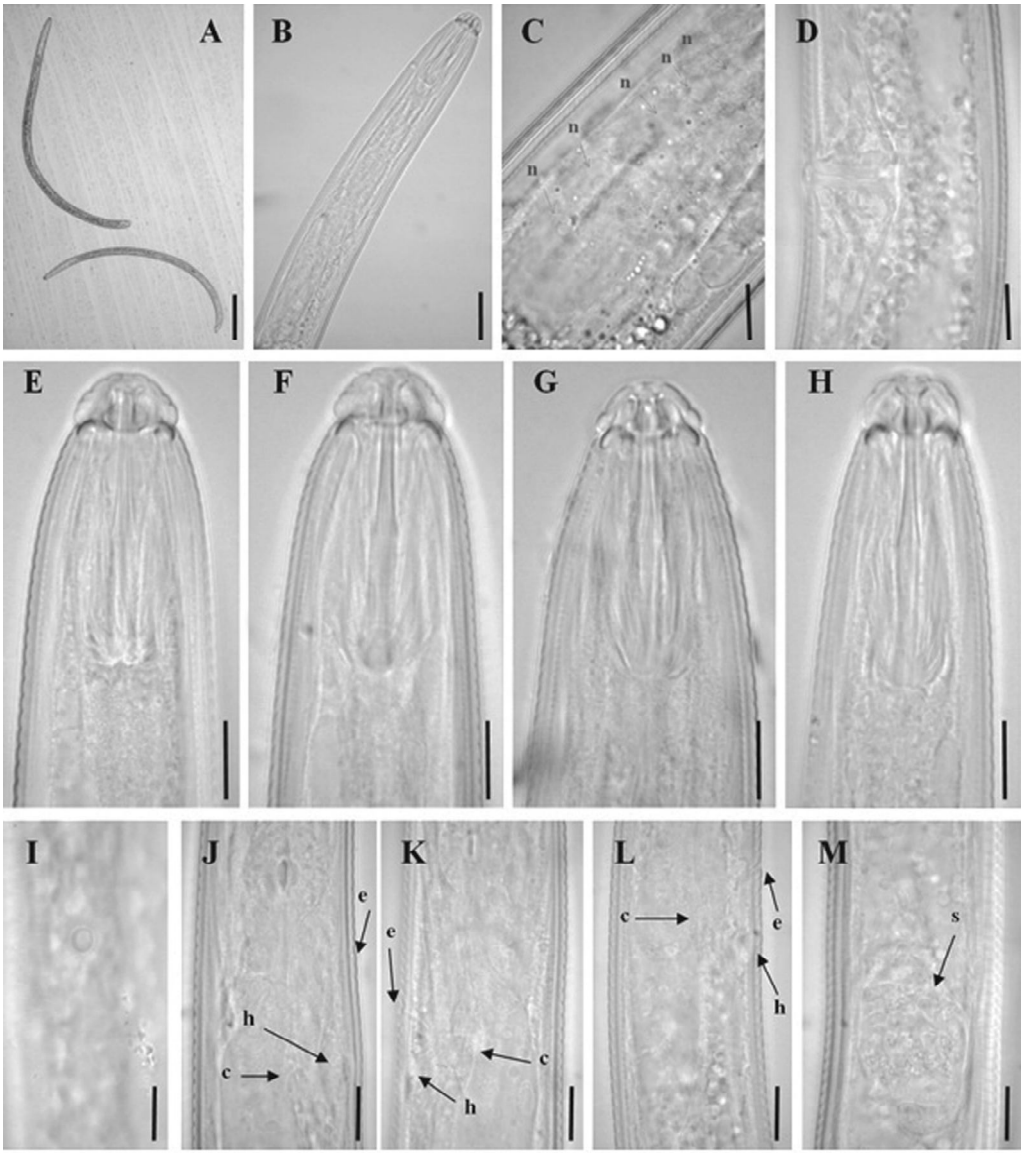

Figure 1 *Hoplolaimus indicus* from southern Iran. A: Female and male entire body; B: Female pharyngeal region; C: Pharyngeal gland nuclei (n); D: Vulva region; E - G: Female anterior end; H: Male anterior end; I: Scutellum; J - L: Position of excretory pore (e), hemizonid (h) and cardia (c); M: Spermatheca (s). Scale bars: A = 200 µm; B = 20 µm; C - M = 10 µm.

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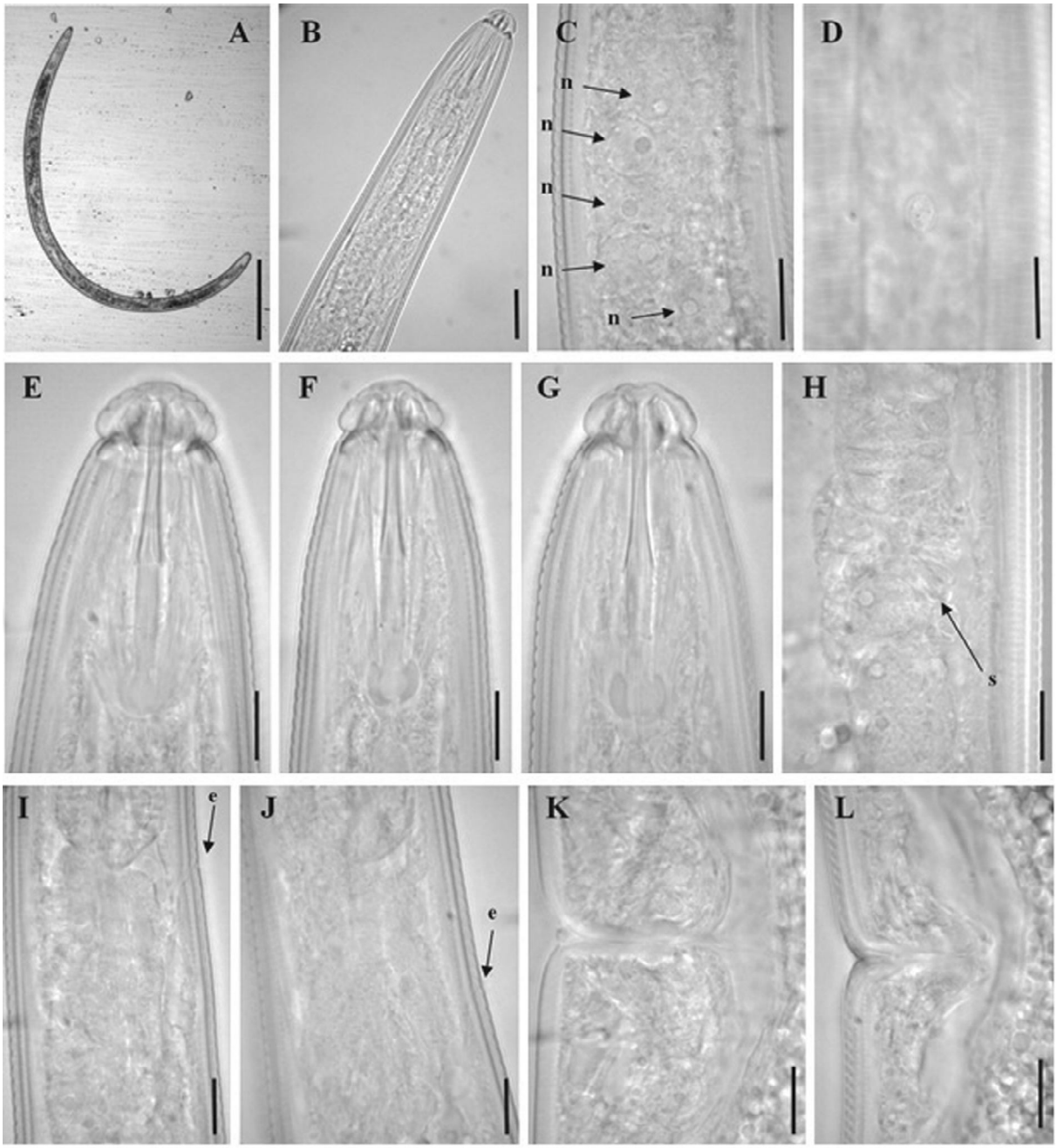

Figure 2 *Hoplolaimus seinhorsti* from southern Iran. A: Female entire body; B: Female pharyngeal region; C: Pharyngeal gland nuclei (n); D: Scutellum; E - G: Female anterior end; H: Spermatheca (s); I - J: Position of excretory pore (e); K - L: Vulva region. Scale bars: A = 200 µm; B = 20 µm; C - M = 10 µm.

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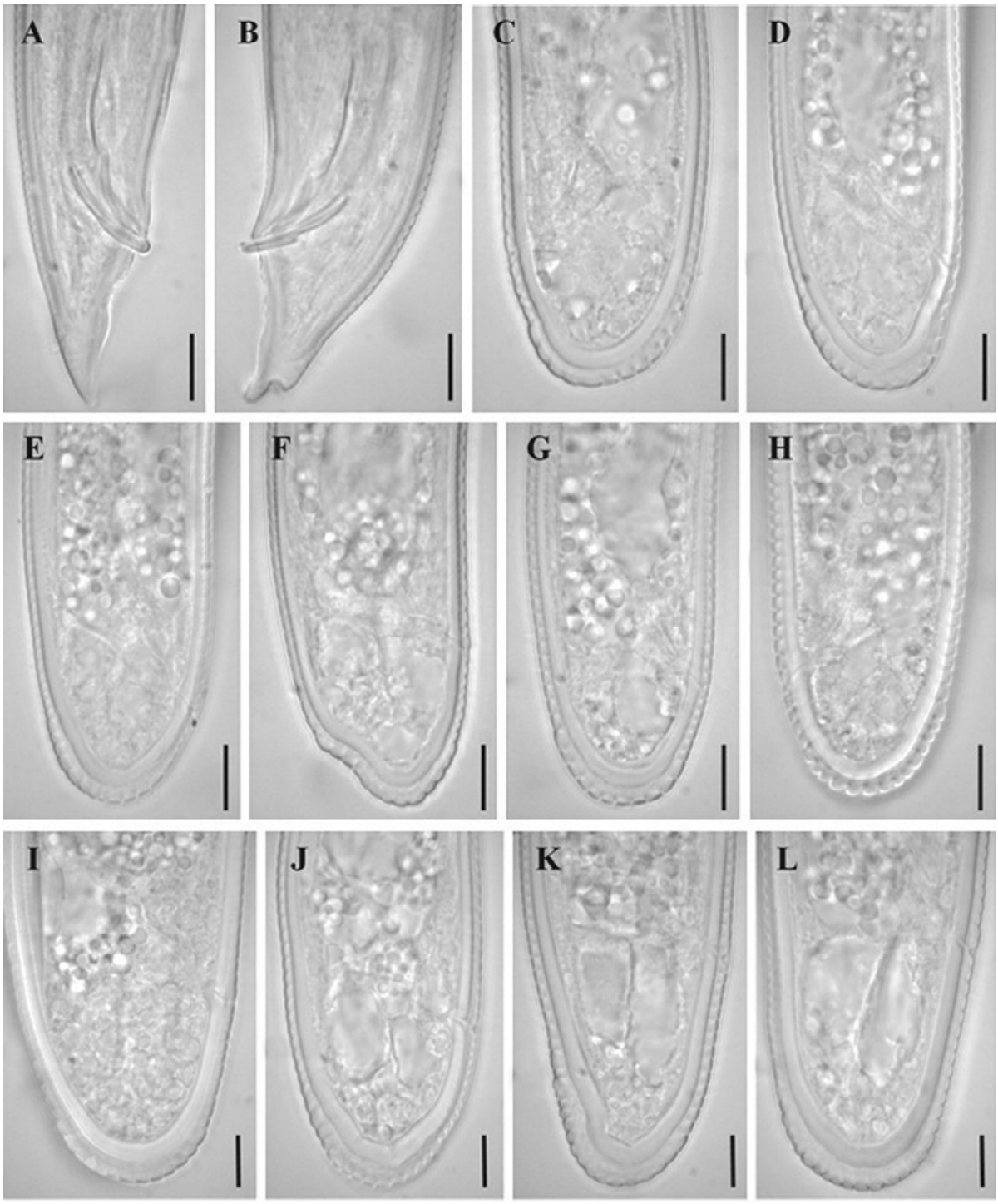

Figure 3 *Hoplolaimus indicus* (A - H; A and G from tamarind, Minab; B, D and E from mango, Ghasr - e - Ghand; C, F and H from sour orange, Bandar-Abbas) and *H. seinhorsti* (I - L from sugarcane, Ahvaz) from southern Iran. A - B: Male posterior end; C - L: Female posterior end and post - anal sac. All scale bars = 10 µm.

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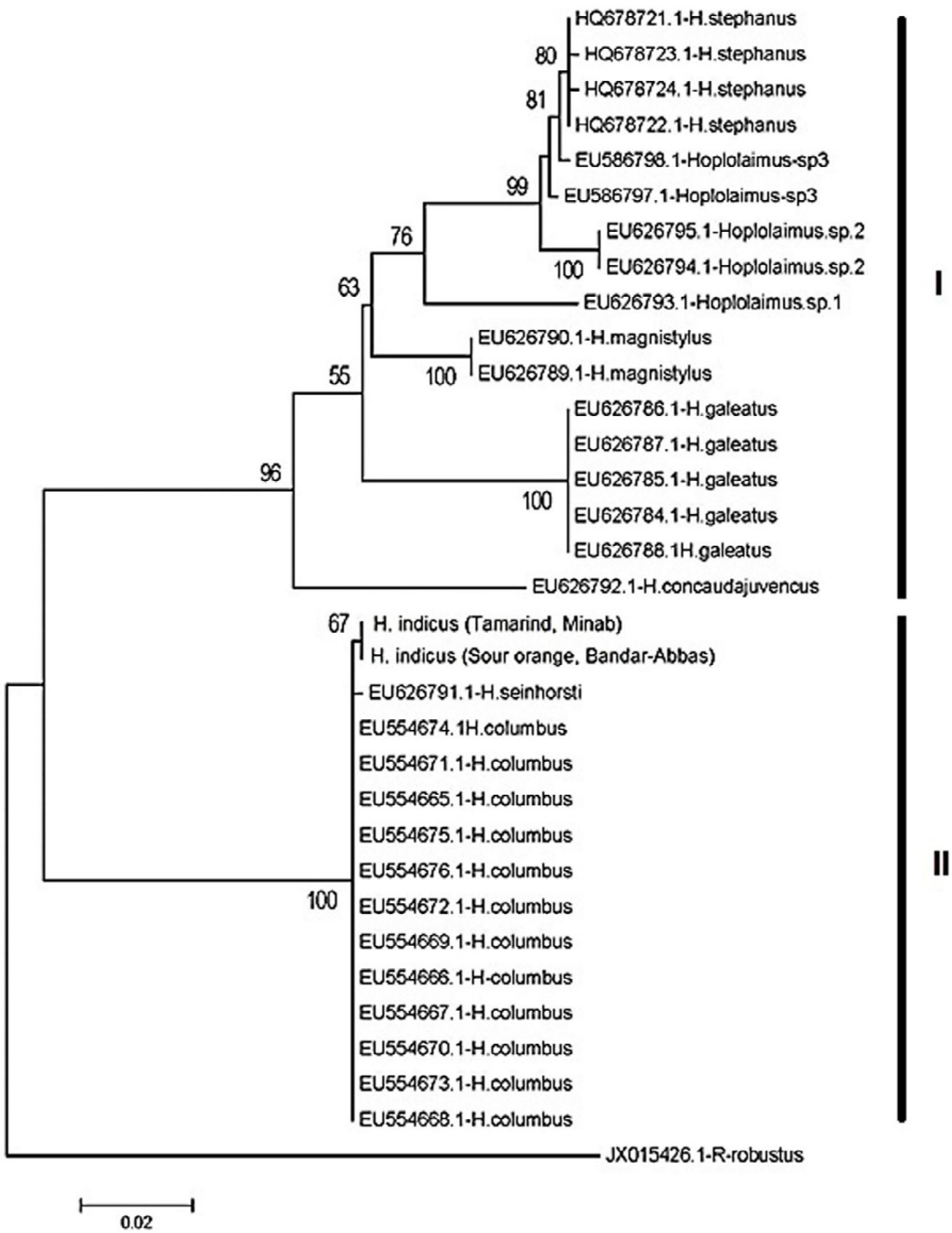

Figure 4 Phylogenetic relationships within *Hoplolaimus* species based on 28S rDNA, reconstructed using maximum likelihood under the GTR + I + G model and 1000 bootstraps. Bootstrap values more than 50% are assigned to the appropriate clades.

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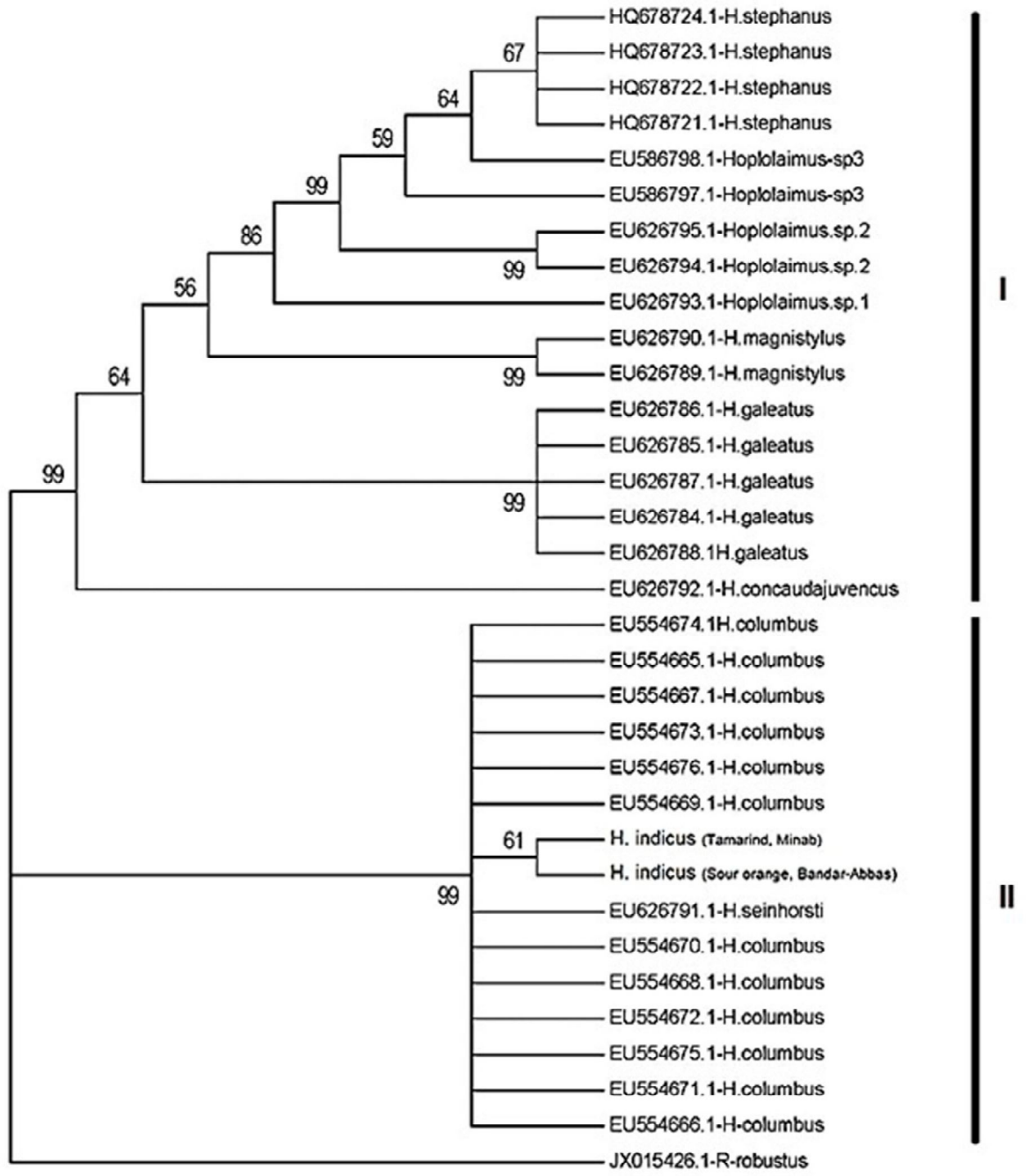

Figure 5 Phylogenetic relationships within *Hoplolaimus* species based on 28S rDNA, reconstructed using maximum parsimony with 1000 bootstraps. Bootstrap values more than 50% are assigned to the appropriate clades.

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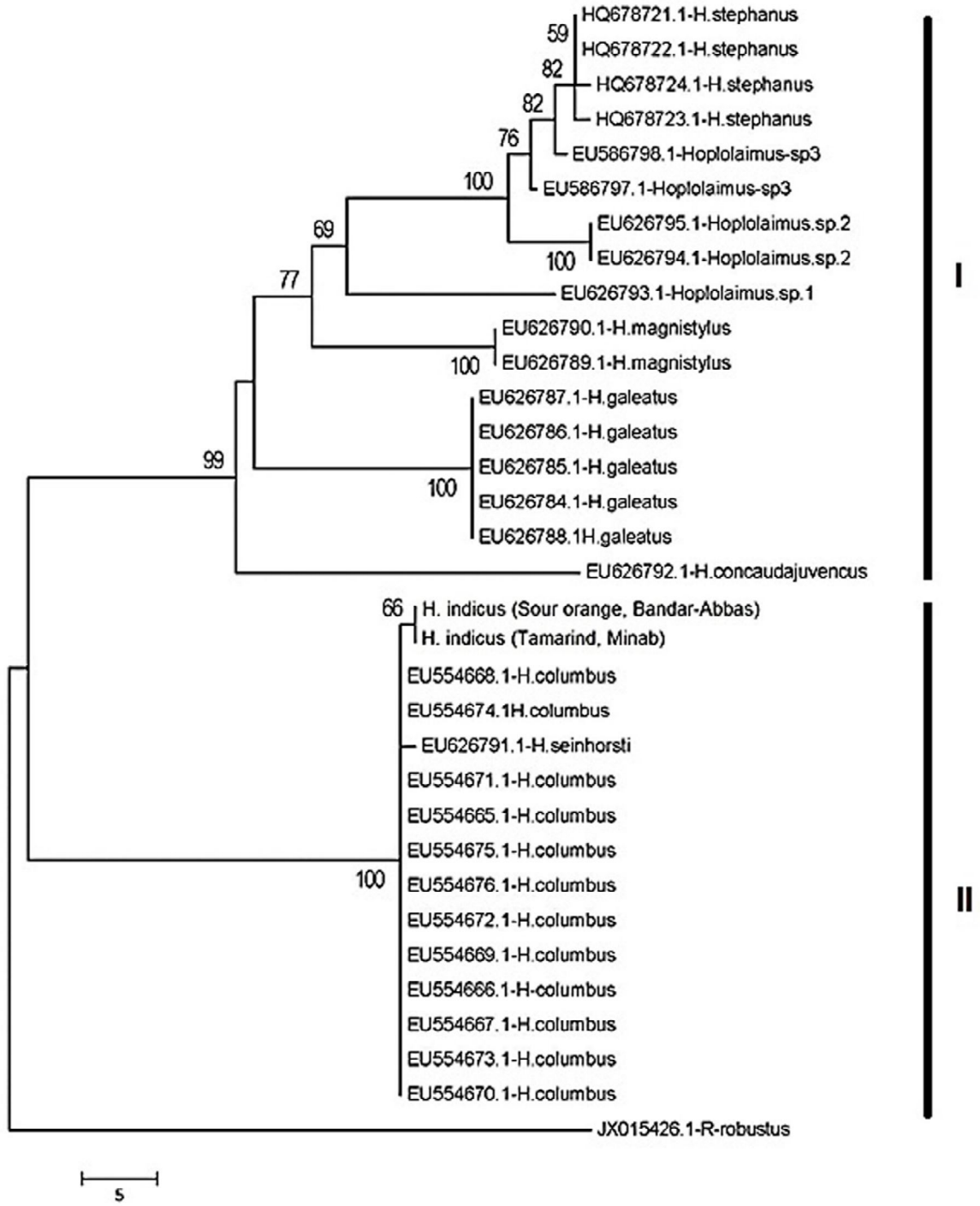

Figure 6 Phylogenetic relationships within *Hoplolaimus* species based on 28S rDNA, reconstructed using neighbor - joining method with 1000 bootstraps. Bootstrap values more than 50% are assigned to the appropriate clades.

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مطالعه *Hoplolaimus indicus* Sher, 1963 و *H. seinhorsti* Luc, 1958 (Nematoda: Hoplolaimidae) جمع‌آوری شده از مناطق جنوبی ایران

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چکیده: چندین جمعیت *Hoplolaimus indicus* و *H. seinhorsti*، جمع‌آوری شده از فراریشه انبه، تمر هندی، لیمو ترش و نیشکر از مناطق جنوبی ایران مورد مطالعه ریخت‌شناختی قرار گرفت. بررسی دقیق *H. dubius* و *H. indicus* نشان داد که این دو گونه براساس صفاتی از یکدیگر متمایز شده‌اند که تغییرات درون گونه‌ای زیادی داشته و برای جداسازی در سطح گونه مناسب نیستند؛ در نتیجه *H. dubius* به‌عنوان مترادف *H. indicus* در نظر گرفته شد. بررسی دقیق جمعیت *H. seinhorsti* نیز نظر صدیقی را درباره مترادف بودن *H. sheri* با *H. seinhorsti* تأیید نمود. هم‌چنین نتایج واکاوی تبارزایی با استفاده از قطعه D2-D3 ژن 28S rRNA، ایده طبقه‌بندی کلاسیک برای قرار دادن گونه‌های جنس *Hoplolaimus* در دو گروه اجدادی (ancestral) و اشتقاقی (derived) را مورد تأیید قرار داد. در درخت‌های تبارزایی ترسیم شده با روش‌های مختلف، جمعیت ایرانی *H. indicus* در کنار *H. seinhorsti* و *H. Columbus* در یک تبار مشترک قرار گرفت. این تبار شامل گونه‌های متعلق به گروه اشتقاقی است که دارای شش هسته در غدد مری بوده، تعداد شیارهای جانبی آنها کمتر از چهار شیار است و روزنه دفعی-ترشحی جلوتر از همی‌زونید قرار گرفته است.

واژگان کلیدی: 28S rRNA، *Hoplolaimus dubius*، *H. sheri*، شناسایی، ریخت‌شناسی

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