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Molecular and phylogenetic studies on Pratylenchidae from Iran with additional data on *Pratylenchus delattrei*, *Pratylenchoides alkani* and two unknown species of *Hirschmanniella* and *Pratylenchus*

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Summary – Thirteen species of Pratylenchidae: *Pratylenchus coffeae*, *P. delattrei*, *P. loosi*, *P. neglectus*, *P. penetrans*, *P. pseudopratensis*, *P. thornei*, *P. vulnus*, *Pratylenchus* sp., *Pratylenchoides alkani*, *P. ritteri*, *Hirschmanniella* sp. and *Zygotylenchus guevarai* were collected from different crops and plants throughout Iran. The specimens were identified using morphological and molecular methods. Morphometrics and morphology are given for *Pratylenchus* sp., *P. delattrei*, *Pratylenchoides alkani* and *Hirschmanniella* sp. The D2-D3 expansion segments of the 28S rRNA gene were amplified and sequenced for all 13 species studied. Diagnostic PCR-ITS-RFLP profiles are given for *Pratylenchus delattrei*, *P. penetrans*, *P. pseudopratensis*, *Pratylenchus* sp., *Pratylenchoides alkani* and *P. ritteri*. *Pratylenchus neglectus* and *P. thornei*, collected from cereal fields, *P. loosi* from tea plantations, *P. coffeae* from banana, *P. penetrans* from ornamental plants, *P. vulnus* from pines and *Z. guevarai* from almonds showed a high level of similarity in the D2-D3 sequences with corresponding GenBank sequences. Nucleotide differences between Iranian populations and reference species were in the intraspecific range. *Pratylenchus delattrei*, found in vegetable fields, and *Pratylenchus* sp. from palm rhizosphere, formed a highly supported clade with *P. zeae*, the two former species being morphologically very close to the latter except in tail shape. *Pratylenchus pseudopratensis*, from cereal fields, clustered with *P. vulnus* with low support. Phylogenetic relationships within *Pratylenchus* species were mainly congruent with those obtained in previous studies. Despite the morphological similarities between *P. ritteri* and *P. alkani*, the D2-D3 of 28S rRNA gene sequences differed by 5 bp. *Hirschmanniella* sp., from a rice field, formed a clade with *H. loofi* and *H. kwazuna*.

Keywords – D2-D3, description, molecular, morphology, morphometrics, PCR-RFLP, phylogeny, *Zygotylenchus*.

Plant-parasitic nematodes of the Pratylenchidae have been reported from various wild plants and crops in Iran, with 25 species of this family being found so far. This list includes one species of *Zygotylenchus* Siddiqi, 1963, three species of *Pratylenchoides* Winslow, 1958, three species of *Hirschmanniella* Luc & Goodey, 1964, 17 species of *Pratylenchus* and an unidentified *Radopholus* sp. (Ghaderi *et al.*, 2012). The cosmopolitan species *Pratylenchus thornei* Sher & Allen, 1953 and *P. neglectus* (Rensch, 1924) Filipjev & Schuurmans Stekhoven, 1941 have frequently been reported from various crops throughout the country (Kheiri, 1972; Niknam & Kheiri, 1997; Pourjam *et al.*, 1999a, Tanha Maafi *et al.*, 2009). *Praty-*

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lenchus loosi Loof, 1960 is widespread in tea plantations in the northern part of Iran and is considered to be one of the most important nematode pests of tea in this region (Hajieghrari *et al.*, 2005). *Pratylenchus vulnus* Allen & Jensen, 1951 is another pathogenic species frequently reported from fruit trees, *Acer platanoides* L., *A. cappadocicum* Geld, *Robinia pseudoacacia* L. and pine trees (Barooti, 1998; Kheiri *et al.*, 2003; Bakouei *et al.*, 2008). All reported *Pratylenchus* species in Iran, except for *P. loosi* and *P. pseudocoffeae* Mizukubo, 1992, were identified based only on morphology and morphometrics. Populations of *P. loosi* isolated from tea plantations were studied and compared with other pratylenchids using the D2-D3 expansion segments of 28S of rRNA gene sequence (Pourjam *et al.*, 1999b; Hajieghrari *et al.*, 2007). *Pratylenchus pseudocoffeae*, infecting chrysanthemum, was morphologically and molecularly studied with PCR-ITS-RFLP and sequencing of the D3 region of the 28S rRNA gene (Mohammad Deimi *et al.*, 2009).

The morphological similarities and overlapping of morphometric characters among species of Pratylenchidae result in difficulties in species determination. As the number of nominal species increases, so do the challenges for precise identification and the establishment of parameters to discriminate morphologically closely related species. Recently, different molecular markers were applied to assist in the discrimination of morphologically closely related species (see Waeyenberge *et al.*, 2000; Subbotin *et al.*, 2008; Palomares-Rius *et al.*, 2010).

Several nematode species belonging to Pratylenchidae were collected during nematological surveys in different regions of Iran. The results of morphological, morphometric and molecular studies of some of these species are given here. The main objectives of this study were to: *i*) confirm identification of species of the Pratylenchidae by analysis of the D2-D3 expansion segments of 28S of rRNA gene sequence *ii*) provide morphological, morphometric and molecular characterisation of *Pratylenchus delattrei* Luc, 1958, *Pratylenchoides alkani* Yüksel, 1977 and other species; and *iii*) study phylogenetic relationships within the species of Pratylenchidae using the D2-D3 of 28S rRNA gene sequences.

Materials and methods

Nematode samples

Soil and root samples were collected from different crops in the north, west, south and central parts of Iran. The samples were processed by the Whitehead tray method (Whitehead & Hemming, 1965) and after 48 h the nematodes at the bottom of tray were washed with tap water and kept for both morphological and molecular analysis. *Hirschmanniella oryzae* originated from a rice field in Myanmar and was kindly provided by Dr T. Kyndt, Ghent University, Ghent, Belgium.

Light microscopy

In morphological studies the nematodes were fixed in heated TAF (triethanolamine 2 ml, formaldehyde 7 ml, distilled water 91 ml), then transferred to glycerin (De Grisse, 1969). Mature females and males were mounted in a small drop of dehydrated glycerin on a microscopic slide. Morphometric features were measured using a *camera lucida* installed on an Olympus BH-2.

DNA extraction, PCR, RFLP and sequencing

For DNA extraction, 4-5 females from each species and population were put into 8 μl dd H_2O on a microscopic slide, cut in 2-3 pieces, then transferred to a 0.2 μl micro tube including 12 μl WLB and homogenised with a micro-homogeniser. DNA extraction, PCR and sequencing protocols were described by Tanha Maafi *et al.* (2003). The D2-D3 expansion segments of the 28S rRNA gene were amplified with the forward D2A and the reverse D3B primers (Subbotin *et al.*, 2006) and the ITS rRNA gene was amplified with the forward TW81 and reverse AB28 primers (Tanha Maafi *et al.*, 2003) for some species. The PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instruction and used for direct sequencing. The PCR products were sequenced at MWG Biotech (Ebersberg, Germany). The newly obtained sequences were submitted to the GenBank database under accession numbers JX261946-JX261965 as indicated in Table 1.

Restriction Fragment Length Polymorphism (RFLP) of amplified ITS product was carried out for *Pratylenchus delattrei*, *P. penetrans*, *P. pseudopratensis* and *Pratylenchus* sp. Three to 6 μl of PCR product was digested by one of the following restriction enzymes: *Hha*I, *Hin*dIII, *Hin*fI or *Pst*I, in the buffer stipulated by the manufacturer. For further discrimination of two morphologically similar species, *P. alkani* and *P. ritteri*, the purified PCR product of D2-D3 expansion segments of 28S rRNA-ITS gene was restricted by the restriction enzymes *Bsh*NI and *Hph*I. The digested DNA was run on a 1.5% TAE-buffered agarose gel, stained with ethidium bromide, visualised on gel documentation and photographed.

Table 1. Species of Pratylenchidae found in this study.

Species	Host plant rhizosphere	Locality/province	GenBank accession number for D2-D3 of 28S rRNA gene sequences
Hirschmanniella sp.	Rice	Khuzestan	JX261958
Pratylenchus coffeae	Banana	Sistan and Baluchestan	JX261950
P. delattrei	Vegetables	Hormozgan	JX261948
P. delattrei	Vegetables	Hormozgan	JX261949
P. loosi	Tea	Gilan	JX261952
P. neglectus	Rapeseed	Khorasan	JX261951
P. neglectus	Wheat	Lorestan	JX261946
P. neglectus	Wheat	Mazandaran	JX261947
P. penetrans	Chrysanthemum	Markazi	JX261961
P. pseudopratensis	Wheat	Lorestan	JX261965
P. thornei	Wheat	Golestan	JX261954
P. thornei	Wheat	Golestan	JX261955
P. thornei	Wheat	Lorestan	JX261960
P. thornei	Pomegranate	Lorestan	JX261963
P. vulnus	Pine	Gilan	JX261945
Pratylenchus sp.	Palm	Khuzestan	JX261959
Pratylenchoides ritteri	Wheat	Golestan	JX261964
P. alkani	Wheat	Kermanshah	JX261957
P. alkani	Wheat	Kermanshah	JX261962
P. alkani	Wheat	Lorestan	JX261953
Zygotylenchus guevarai	Walnut	Markazi	JX261956

SEQUENCE AND PHYLOGENETIC ANALYSIS

The newly obtained D2-D3 of 28S rRNA sequences for each nematode genus were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with default parameters with published 28S rRNA sequences for corresponding nematode groups (Subbotin *et al.*, 2006, 2008; Van den Berg *et al.*, 2009; De Luca *et al.*, 2010). Only a single sequence for each reference species, except for *P. zeae*, was taken for this analysis. Outgroup taxa for each dataset were chosen according to the results of previously published data (Subbotin *et al.*, 2006). Three sequence datasets were generated. Each sequence dataset was analysed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) under the GTR + I + G model. BI analysis for each gene was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 10^3 generations. After discarding burn-in samples and evaluating convergence, the remaining samples were retained for further analysis. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades.

Results

NEMATODE SPECIES

Based on morphological and molecular analyses, 13 species of Pratylenchidae were identified: *Pratylenchus coffeae*, *P. delattrei*, *P. loosi*, *P. neglectus*, *P. penetrans*, *P. pseudopratensis*, *P. thornei*, *P. vulnus*, *Pratylenchus* sp., *Pratylenchoides alkani*, *P. ritteri*, *Hirschmanniella* sp. and *Z. guevarai* (Table 1). *Pratylenchus delattrei* and *Pratylenchoides alkani* were reported in Iran for the first time. Morphological descriptions of these two species, as well as for the *Pratylenchus* sp. and *Hirschmanniella* sp., are provided.

***Pratylenchus delattrei* Luc, 1958**
(Fig. 1A-G)

MEASUREMENTS

See Table 2.

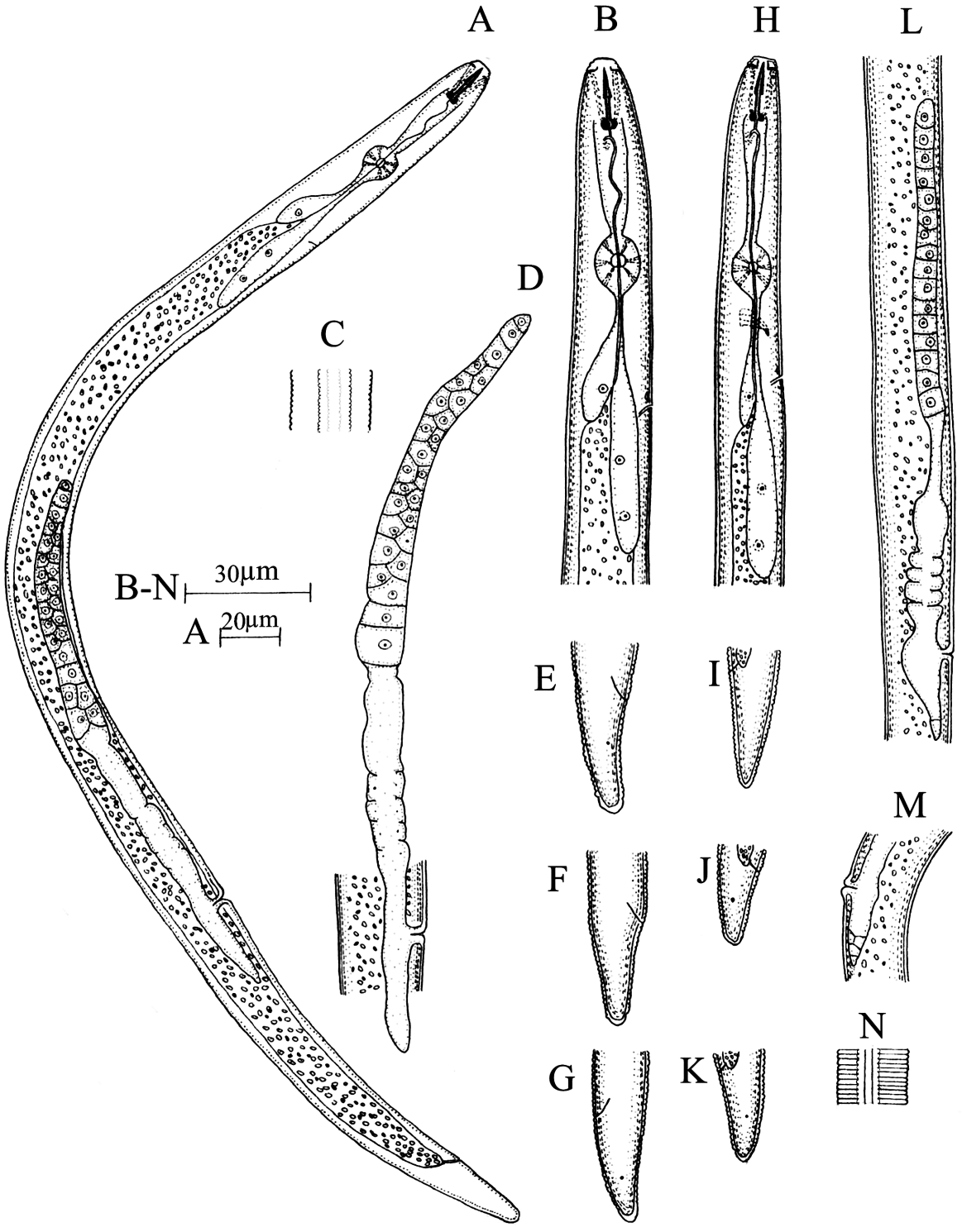

Fig. 1. *Pratylenchus delattrei* (A-G) and *Pratylenchus* sp. (H-N). A: Entire female; B: Anterior region; C: Lateral field at mid-body; D: Genital tract and vulva region; E-G: Variation in tail shape; H: Anterior region; I-K: Variation in tail shape; L, M: Genital tract showing vulval position; N: Lateral field at mid-body.

Table 2. Morphometrics of *Pratylenchus delattrei*, *Pratylenchus* sp. and *Pratylenchoides ritteri*. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*Pratylenchus delattrei*		*Pratylenchus* sp.	*Pratylenchoides ritteri*	
	Hormozgan 1	Hormozgan 2		Golestan population	
	Female	Female	Female	Female	Male
n	7	12	6	5	3
L	543 ± 55 (467-616)	508 ± 49.2 (434-576)	416 ± 40.8 (371-473)	832 ± 75.5 (714-921)	677 ± 75.6 (590-722)
a	23.8 ± 2.1 (21.2-26.9)	22.6 ± 1.0 (21.1-25)	24.9 ± 2.9 (21.7-29.2)	30.4 ± 1.9 (27.7-32.9)	30.4 ± 2.2 (28.8-32.8)
b	6.1 ± 0.7 (5.0-7.2)	5.9 ± 0.6 (5.2-6.9)	5.0 ± 0.7 (4.1-6.0)	6.6 ± 0.6 (5.6-7.0)	6.2 ± 0.5 (5.6-6.6)
b′	4.3 ± 0.2 (4.0-4.6)	4.1 ± 0.4 (3.6-4.9)	3.7 ± 0.6 (3.0-4.6)	4.6 ± 0.4 (4.2-5.1)	5.1 ± 0.4 (4.7-5.5)
c	20 ± 2 (18.1-23.1)	19.7 ± 2.6 (16.7-24.1)	18.4 ± 2.1 (15.2-20.6)	16.5 ± 1.4 (14.6-18.4)	12.3 ± 0.5 (12.0-12.8)
c′	2.2 ± 0.3 (1.9-2.6)	1.9 ± 0.2 (1.6-2.2)	2.1 ± 0.3 (1.8-2.4)	2.6 ± 0.4 (2.1-3.0)	–
Lip region height	2.1 ± 0.4 (2-3)	2	2	3.6 ± 0.6 (3-4)	4
Lip region diam.	8.3 ± 0.5 (8-9)	7.9 ± 0.5 (7-9)	7.4 ± 0.4 (7-8)	10.8 ± 0.5 (10-11)	7.3 ± 0.6 (7-8)
Stylet	16.3 ± 0.8 (15-17)	16.0 ± 0.6 (15-17)	14.7 ± 0.5 (14-15)	23.2 ± 0.5 (23-24)	20 ± 1 (19-21)
Anterior end to pharynx	125 ± 7.3 (116-135)	123 ± 8.3 (113-138)	114 ± 14.5 (96-131)	180 ± 8.6 (172-194)	134 ± 19.3 (117-155)
Anterior end to pharyngeal junction	89 ± 10.4 (76-105)	87 ± 4 (82-94)	83 ± 6.3 (74-91)	126 ± 3.7 (123-132)	108.3 ± 3.1 (105-111)
Anterior genital branch	173 ± 34.7 (135-229)	169 ± 46.8 (107-223)	133 ± 25.1 (102-168)	147 ± 31.1 (125-169)	–
V	75.1 ± 1.9 (71.4-77.1)	75.9 ± 1.3 (74-78.7)	75.1 ± 1.6 (72.6-76.9)	56.1 ± 1.5 (53.9-57.7)	–
Vulva-anus distance	102 ± 13.6 (85-116)	90 ± 11 (82-110)	81 ± 9 (70-92)	–	–
Max. body diam.	23 ± 3.3 (20-29)	22.5 ± 2.1 (19-26)	16.8 ± 1.4 (15-18)	27.4 ± 2.6 (23-30)	–
Body diam. at anus	12.4 ± 1.5 (11-14)	13 ± 0.9 (12-14)	11.2 ± 1.3 (10-13)	20 ± 2.9 (15-22)	–
Median bulb	51 ± 6.2 (41-56)	51 ± 3.6 (46-59)	46 ± 3.9 (41-51)	72 ± 4.9 (67-79)	–
Excretory pore from anterior end	81 ± 8.2 (71-95)	80 ± 2.6 (76-84)	72 ± 7.7 (62-82)	118 ± 10.5 (102-130)	–
Post-vulval uterine sac	27 ± 6.2 (20-33)	32 ± 6.1 (25-37)	17	–	–
Tail	26.6 ± 2.3 (23-29)	24.8 ± 2.3 (23-29)	23 ± 4.3 (18-29)	50.8 ± 7.1 (45-63)	55.3 ± 8.1 (46-60)
Tail annuli	20 ± 2.1 (18-23)	19 ± 1.5 (17-21)	21 ± 3.6 (17-25)	24 ± 1.8 (22-27)	–
Hyaline tail region	–	–	–	10.6 ± 1.1 (9-12)	–

Table 2. (Continued.)

Character	*Pratylenchus delattrei*		*Pratylenchus* sp.	*Pratylenchoides ritteri*	
	Hormozgan 1	Hormozgan 2		Golestan population	
	Female	Female	Female	Female	Male
PUS/Body diam. at anus	1.2 ± 0.3 (0.8-1.6)	1.4 ± 0.4 (1.0-1.7)	–	–	–
Spicules	–	–	–	–	26.3 ± 3.2 (24-30)
Gubernaculum	–	–	–	–	5.3 ± 0.6 (5-6)

DESCRIPTION

Female

Vermiform, curved ventrally, in some cases with dorsal curvature after fixation. Lip region with three annuli, continuous with body contour. Stylet well developed with distinct rounded knobs slightly directed anteriorly. Lateral field with four incisures, outer two fully crenate, inner lines finely striated, an extra line often observed before vulval region and ending around vulval region, areolation visible only at tail level. Metacorpus oval to rounded, isthmus rather short, surrounded by nerve ring, pharyngeal glands well-developed, with rather long ventral overlap. Excretory pore either opposite pharyngo-intestinal junction or slightly anterior. Genital branch with two rows of oocytes, spermatheca inconspicuous, vagina a straight tube. Vulva transverse slit. Post-vulval uterine sac rather long, not differentiated. Tail subcylindrical, terminus rounded to conical, smooth. Phasmids pore-like in first half of tail, in one specimen at one-third of tail length.

Male

Not found.

REMARKS

Pratylenchus delattrei is morphologically very similar to *P. zeae*, from which it differs by the subcylindrical tail shape with rounded to conical terminus *vs* narrowly rounded to subacute and bluntly pointed terminus. The ranges of some key diagnostic morphometric characters of *P. delattrei* and *P. zeae* reported by several researchers overlap (Sher & Allen, 1953; Luc, 1958; Fortuner, 1976; Das & Sultana, 1979; Loof, 1991; Zarina & Maqbool, 1998; Van den Berg & Quénéhervé, 2000). We conclude that the discrimination of *P. delattrei* and *P. zeae* based only on morphological and morphometric characters is difficult.

Pratylenchus delattrei was reported from southern Madagascar on cotton and from several Asian countries, i.e., South Korea, Pakistan, Oman, and regions of India from the rhizosphere of various plants: vegetables, corn, date palm, medicinal and spicy plants (Castillo & Vovlas, 2007). In the present study it was found from tomato and eggplant fields in Hormozgan province in southern Iran, an area with almost the same environmental conditions as Oman.

***Pratylenchus* sp.**

(Fig. 1H-N)

MEASUREMENTS

See Table 2.

DESCRIPTION

Female

Vermiform, curved slightly ventrally after fixation, in some individuals irregularly shaped. Lip region with three annuli, first annulus from base rather large, separated from two other annuli by a slight constriction. Stylet robust, stylet knobs round, sloping on anterior face. Median bulb rather large, oval, pharyngeal glands overlapping intestine ventrally. Excretory-secretory pore at end of isthmus. Lateral field with four incisures, no extra or oblique broken lines in between, outer lines finely crenate. Reproductive system composed of one genital branch outstretched anteriorly, spermatheca empty, rather inconspicuous, vagina straight, vulva a transverse slit with almost two slightly prominent lips, post-vulval uterine sac small, less than vulval body diam., mostly undifferentiated. Phasmids in

middle of tail. Tail conoid with bluntly rounded, smooth terminus.

Male

Not found.

REMARKS

Pratylenchus sp. generally resembles *P. zeae*, sharing most morphological and morphometric characters with this monosexual species, although having some differences in tail shape, which is conoid and terminates in a bluntly rounded end *vs* narrowly rounded to subacute and bluntly pointed terminus; lower value of c′ (2 *vs* 2.1-3) (Hashim, 1983), (2 *vs* 3.0-3.5) (Ryss, 1988), (2 *vs* 2-4) (Van den Berg & Quénéhervé, 2000). The anal body diam. in *Pratylenchus* sp. is wider than *P. zeae* and the tail is not tapered abruptly at anus level.

Pratylenchoides alkani **Yüksel, 1977**
(Figs 2, 3)

MEASUREMENTS

See Table 3.

DESCRIPTION

Female

Vermiform, body straight or curved ventrally sometimes dorsally after fixation, rarely coiled in posterior portion. Lip region highly sclerotised, rather high, flattened, with 4-5 annuli, 10-12 μm wide and 2-4 μm high. Stylet well developed, strong with robust knobs, rounded sloping posteriad. Dorsal pharyngeal gland orifice 2-3 μm posterior to stylet knobs. Lateral field with six incisures, outer lines crenate and areolated in middle of body, inner lines visible as double bands close to one another, as confirmed by cross section in middle of body. Median bulb rounded to oval, muscular with conspicuous valve, isthmus long, slender surrounded by nerve ring at mid-point. Hemizonid 2-3 annuli anterior to excretory pore. Deirids conspicuous, 2-4 annuli anterior to excretory pore. Pharyngeal glands well developed, overlapping intestine dorsally, all three pharyngeal glands nuclei located posterior to pharyngo-intestinal junction. Two genital tracts opposed, equally developed, spermatheca rounded, filled with round sperm. Phasmids pore-like at middle of tail. Tail cylindrical with rounded annulated terminus, hyaline portion 10-14 μm long.

Male

Vermiform, sexually dimorphic in shape of head and lesser development of stylet and pharynx. Lip region high, well sclerotised, with 4-5 annuli, overall head shape conical with rounded surface, unlike female head, which is flattened. Tail conical with rounded to acute terminus, spicules typically tylenchoid, gubernaculum moderately curved, bursa with fine striation, enveloping entire tail.

REMARKS

Pratylenchoides alkani was first reported from soil around snap bean (*Phaseolus vulgaris* L.) in Turkey by Yüksel (1977). Castillo & Barcina (1988) also reported *P. alkani* from the rhizosphere of *Pinus halepensis* in Spain. In this study, the species was found in large numbers from wheat fields in Kermanshah and Lorestan provinces in western Iran.

In morphology, the Iranian specimens agree with the descriptions of *P. alkani* by Yüksel (1977) and Castillo & Barcina (1988). *Pratylenchoides alkani* is a bisexual species characterised by a long dorsal overlap of the pharyngeal glands. It is very close to *P. ritteri* Sher, 1970 and shares many morphological and morphometric characters, most characters overlapping between the two species. Yüksel (1977) separated *P. alkani* from *P. ritteri* by the presence of punctations on the lateral field, posteriad directed stylet knobs and the presence of six lines in the lateral field. In this study, specimens of both species collected from west and north Iran were closely examined. The punctations which Yüksel (1977) mentioned in the description of *P. alkani* were also observed, but they are not present exactly at lateral field level, being located below the lateral field and close to the surface of the intestine (Fig. 3F). They are not always as wide as the lateral field and appear as a continuous band. It appears that these spots are not specific to *P. alkani* as they can also be seen in *P. ritteri* specimens. The number of lines in the lateral field is also variable in both species, ranging from four to six, although in *P. alkani* it is more consistent and usually has six lines. In the original description of *P. ritteri* the stylet knobs were described as flattened anteriorly *vs* posteriorly in *P. alkani*, whereas in our study most specimens of *P. ritteri* showed posteriad directed stylet knobs. Several populations of *P. ritteri* from Iran were closely examined and described in detail by Pourjam *et al.* (2000) and Karegar (2006). In the description of the Iranian population of *P. ritteri* given by Pourjam *et al.* (2000), the stylet knob shape was similar to the original description by Sher

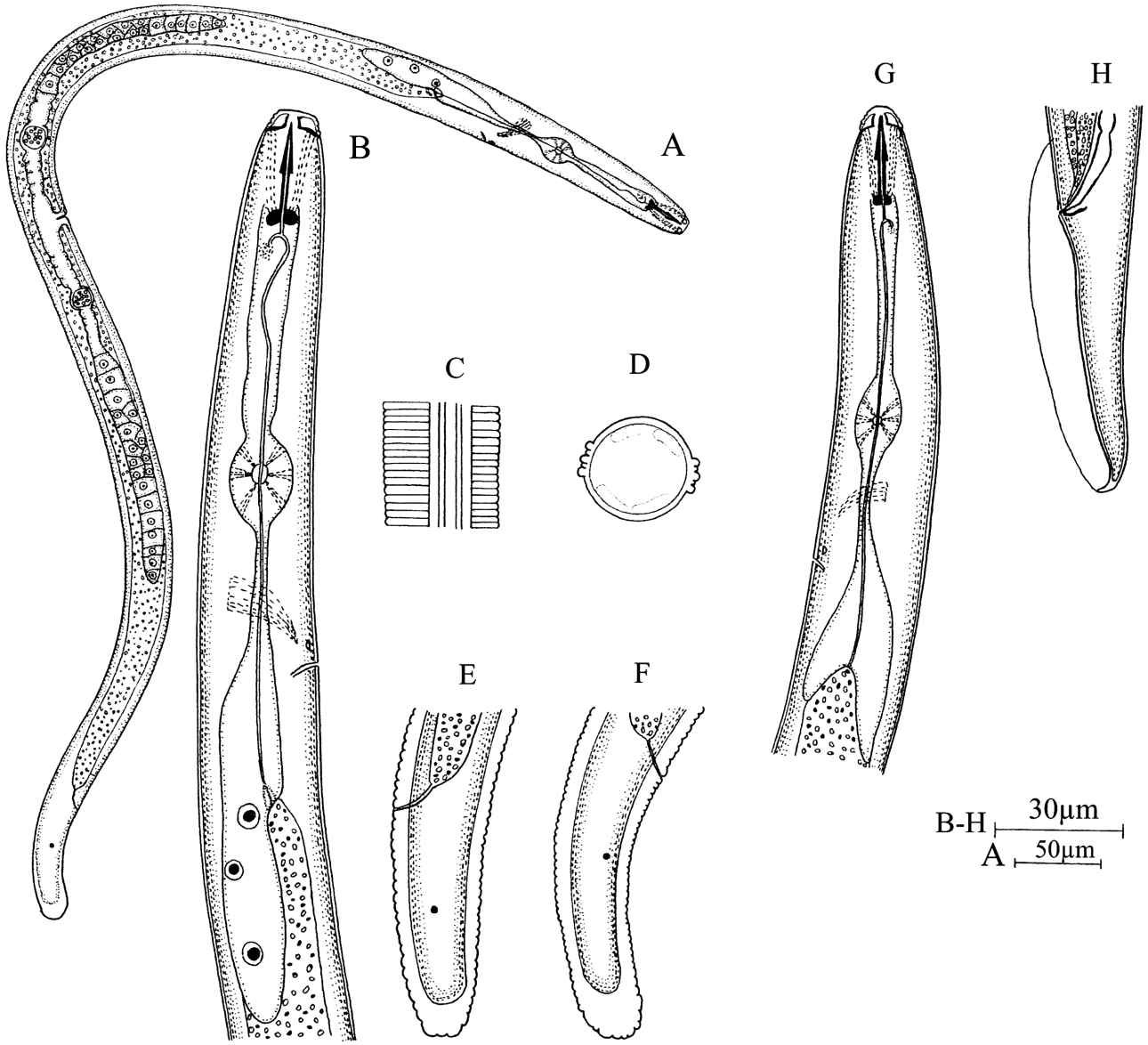

Fig. 2. *Pratylenchoides alkani*. A-F: Female. A: Entire body; B: Anterior end of body; C: Lateral field at mid-body; D: Cross section at mid-body showing six incisures in lateral field; E, F: Tail showing phasmids and hyaline tail region; G, H: Male. G: Anterior end; H: Tail.

(1970). High variability in morphological characters was reported in the Iranian populations of *P. ritteri* (Pourjam *et al.*, 2000), the number of incisures in the lateral field, regarded as one of the two diagnostic characters, showing the most variability. Stylet length has been considered as a diagnostic character for differentiating *P. alkani* and *P. ritteri* (Pourjam *et al.*, 2000), but the ranges overlap between the two species 22-25 μm in Yüksel (1977) and present study; 20-22 μm in Castillo & Barcina (1988) *vs* 21-24 μm in Sher (1970), 20-23 μm in Pourjam *et al.* (2000) and 23-24 μm in the present study. Brzeski (1998) and Karegar (2006) proposed the synonymy of *P. ritteri* and *P. alkani* as they shared most morphological and morphometric characters. We were not able to define exact diagnostic characters to differentiate these two species, yet they showed clear molecular differences and were distin-

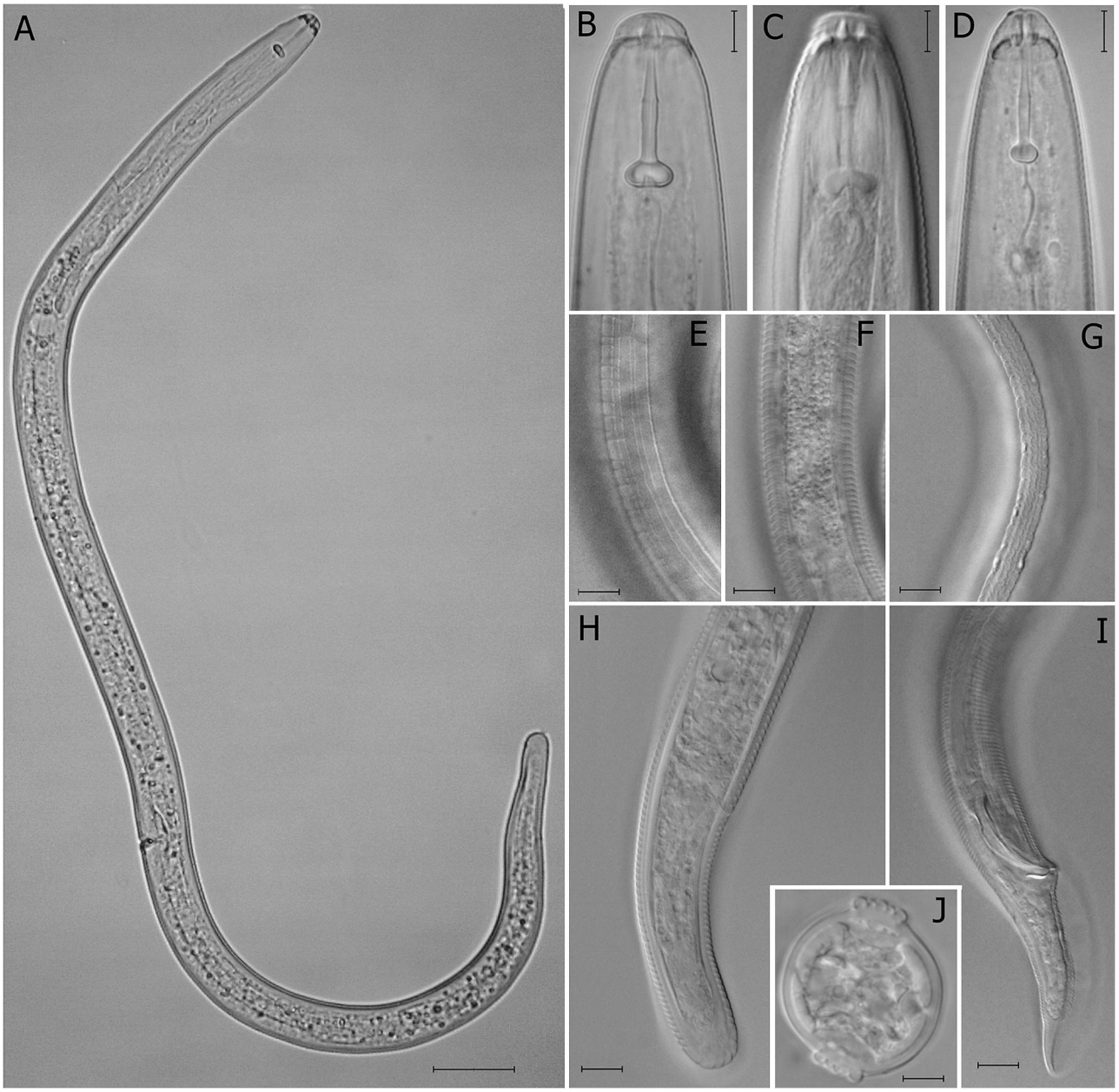

Fig. 3. *Pratylenchoides alkani*. A-F, H, J: Female. A: Entire body; B: Head showing stylet knob shape in live specimen placed in temporary water mount; C: Head showing stylet knob shape in fixed specimen; E, F: Lateral field and punctation at mid-body; H: Tail; J: Cross section at mid-body showing six incisures in lateral field; D, G, I: male. D: Head; G: Lateral field at mid-body; I: Tail. (Scale bars: A: 40 μm; B, D, J: 6 μm; C, E-I: 10 μm.)

Table 3. Morphometrics of *Hirschmanniella* sp. and *Pratylenchoides alkani*. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*Hirschmanniella* sp.		*Pratylenchoides alkani*		
			Kermanshah population		Lorestan population
	Female	Male	Female	Male	Female
n	9	5	8	5	6
L	1448 ± 158.3 (1206-1644)	1450 ± 215.4 (1294-1820)	810 ± 92.7 (719-951)	784 ± 137.9 (624-937)	713 ± 54 (646-781)
a	58.1 ± 4.2 (53.4-65.8)	60.6 ± 13 (48.2-82.7)	29.8 ± 1.6 (27.5-31.9)	30.7 ± 2.6 (27.1-32)	26.6 ± 2.3 (23.1-30)
b	12.5 ± 0.9 (10.9-13.7)	12.8 ± 1.6 (11.1-15)	6.0 ± 0.6 (5.3-6.8)	6.8 ± 0.4 (6.2-7.3)	6.0 ± 0.4 (5.6-6.7)
b′	5.3 ± 1.3 (3.5-8.2)	4.9 ± 0.9 (4.0-6.4)	4.0 ± 0.2 (3.7-4.3)	5.3 ± 0.4 (4.9-5.9)	4.9 ± 0.4 (4.4-5.4)
c	16.6 ± 1.4 (15.4-19.8)	19.9 ± 3.3 (15.8-24.9)	14.3 ± 1.5 (12.5-16.7)	13.5 ± 2.0 (11.1-16)	15 ± 1.5 (13.3-17.8)
c′	5.2 ± 0.4 (4.4-5.5)	–	3.0 ± 0.3 (2.6-3.6)	–	–
Lip region height	3	3	3.4 ± 0.5 (3-4)	4.4 ± 0.6 (4-5)	3.3 ± 0.5 (3-4)
Lip region diam.	9.2 ± 0.4 (9-10)	8.6 ± 0.6 (8-9)	10.5 ± 0.8 (10-12)	9.2 ± 0.8 (8-10)	9.0 ± 0.6 (8-10)
Stylet	18.8 ± 0.7 (18-20)	18.4 ± 0.9 (17-19)	23.3 ± 1.2 (22-25)	21.4 ± 1.3 (20-23)	20.2 ± 0.4 (20-21)
DGO	–	–	2	2.2 ± 0.5 (2-3)	2.3 ± 0.5 (2-3)
Anterior end to pharynx	281 ± 54.1 (181-361)	297 ± 23 (272-328)	204 ± 14.4 (188-223)	149 ± 35.3 (119-190)	145 ± 9.9 (136-162)
Anterior end to pharyngeal junction	116 ± 8.4 (105-128)	113 ± 6.2 (105-121)	135 ± 9.6 (118-149)	115 ± 18.3 (100-140)	118 ± 5.8 (109-125)
Anterior genital branch	372 ± 33.3 (308-401)	–	171 ± 28.5 (134-206)	–	148 ± 12.7 (139-157)
Posterior genital branch	327 ± 6.2 (210-394)	–	160 ± 18.7 (132-181)	–	132
V	52 ± 2.3 (47.5-55.5)	–	56.8 ± 1.4 (54.1-58.6)	–	54.2 ± 1.4 (52.8-56.2)
Max. body diam.	25 ± 2 (22-27)	24 ± 1.9 (22-27)	27.5 ± 3.9 (23-33)	26 ± 2.9 (23-29)	27 ± 1.5 (25-29)
Diam. at anus	17 ± 1.6 (14-19)	–	19 ± 3.1 (15-23)	–	18.7 ± 0.5 (18-19)
Median bulb	68 ± 5.9 (59-76)	69 ± 2.3 (65-71)	81 ± 5.3 (75-89)	70 ± 8.3 (61-79)	69 ± 4.6 (62-75)
Excretory pore from anterior end	97 ± 9.2 (85-111)	91 ± 8.2 (77-98)	121 ± 12.4 (108-139)	111 ± 18.4 (94-133)	104 ± 4.3 (100-110)
Tail	87 ± 8.5 (77-103)	74 ± 9.9 (63-90)	57 ± 8.3 (46-69)	58 ± 7.9 (46-65)	48 ± 4.2 (41-51)
Tail annuli	59 ± 5 (56-72)	–	28 ± 6.3 (22-42)	–	–
Hyaline tail part	–	–	11.6 ± 1.2 (10-14)	–	12.7 ± 1.6 (10-15)

Table 3. (Continued.)

Character	*Hirschmanniella* sp.		*Pratylenchoides alkani*		
			Kermanshah population		Lorestan population
	Female	Male	Female	Male	Female
Spicules	–	28.4 ± 1.1 (27-30)	–	27 ± 2.5 (24-30)	–
Gubernaculum	–	10	–	6.6 ± 0.6 (6-7)	–

guished from each other by sequences of the D2-D3 expansion regions of rRNA and RFLP.

***Hirschmanniella* sp.**
(Figs 4, 5)

MEASUREMENTS

See Table 3.

DESCRIPTION

Female

Various shapes after fixation, open C to open coiled and irregular, transverse striations deeply annulated posterior to anus. Lip region strongly developed, robust, composed of 4-5 annuli, flattened with rounded edges. Stylet strong with rounded knobs sloping slightly posteriad. Median bulb large, round with prominent valve, pharyngeal glands long, overlapping intestine ventrally, three glands arranged an equal distance from each other. Isthmus surrounded by nerve ring, excretory pore at beginning of basal bulb, hemizonid 4-6 annuli anterior to excretory pore. Lateral field with four lines, fully crenate and areolated throughout entire length of body, areolation more distinct at body extremes. Genital tract with two branches, outstretched, oocytes in one row, spermatheca round to oval, filled with round sperm, vagina straight, vulva a transverse slit. Thorneian cells observed all over body, *ca* 18 cells in first half of body. Tail elongated conoid, with 56-72 annuli extending to rounded terminus which ends in a fine ventral to central mucro. Phasmids at almost two-thirds of tail length from posterior end.

Male

Lip region and pharynx similar to female, lip region with 4-5 annuli, lateral field with four lines fully crenate and areolated along body length. Single testis situated at two-thirds of body length from anterior end, spermatocytes in double rows except for short region near posterior end. Edge of crenate bursa starting from beginning of spicular pouch and extending to second half of tail, rarely close to tail terminus. Spicules strongly sclerotised, typically tylenchoid, shaft arcuate with ornamentation, *i.e.*, wart-like projections or depressions at proximal end, more prominent in ventral side. Gubernaculum simple with curved distal end. Tail terminus rounded, often curved dorsally, with a fine ventral mucro or rarely a distinct finger like projection.

REMARKS

Hirschmanniella sp. is morphologically close to *H. oryzae* Sher, 1968, *H. loofi* Sher, 1968 and *H. kwazuna* Van Den Berg, Subbotin, Handoo & Tiedt, 2009. The female of *Hirschmanniella* sp. can be distinguished from *H. oryzae* by: lip region with 4-5 *vs* 3-4 annuli, stylet knobs rounded, sloping slightly posteriad *vs* rounded, slightly sloping anteriorly, and completely areolated lateral field *vs* occasional incomplete areolation in tail region. *Hirschmanniella* sp. males differ from *H. oryzae* by spicule length, which are longer than those of *H. oryzae* at 28.4 (27-30) *vs* 23 (18-26) μm, 23 (20-25) μm (Sher, 1968) and 22-23.4 μm (Chen *et al.*, 2006).

From two populations of *H. loofi*, as described by Sher (1968), and a population from Germany (Sturhan & Hallmann, 2010), the female of *Hirschmanniella* sp. can be separated by: shorter body length of 1448 (1206-1644) *vs* 2360 (2120-2580), 2240 (1810-2550) and 2620 (2130-3240) μm respectively, 4-5 *vs* 6 or 6-9 lip region annuli, shorter stylet length of 18.8 (18-20) *vs* 36 (35-37), 35 (34-36), or 37.7 (35-41.6) μm respectively. It further differs from *H. loofi* by the following male characters: shorter stylet length (18.4 (17-19) *vs* 32 (31-33), 33 (31-34),

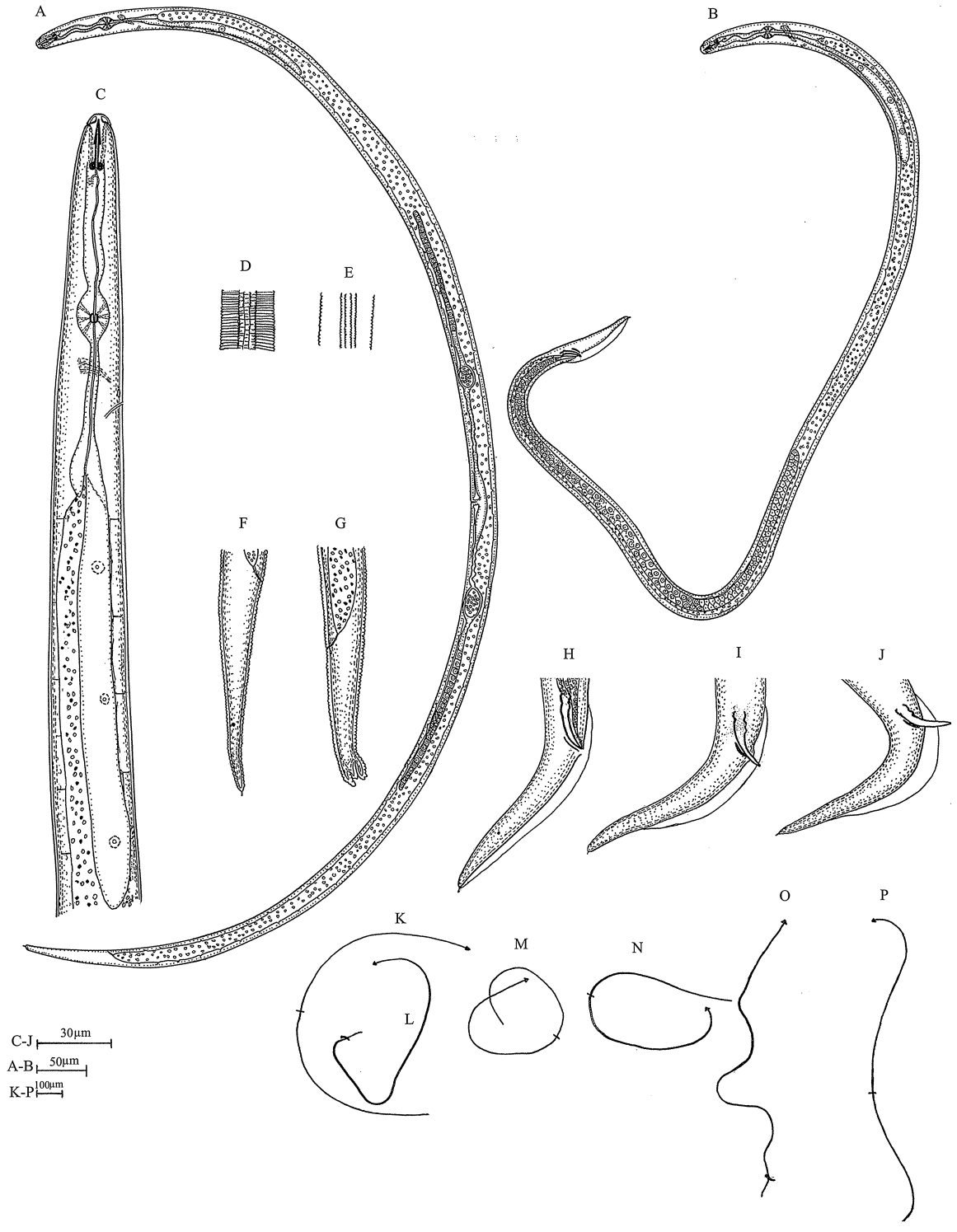

Fig. 4. *Hirschmanniella* sp. A: Entire female; C: Anterior region of female; D, E: Female lateral field at mid-body; F, G: Variation in female tail; K, M, N, P: Various shapes of heat-relaxed female; B: Entire male; G: Male lateral field at mid-body; H-J: Male tail showing variation in tail shape and extension of caudal alae; L, O: Heat-relaxed male.

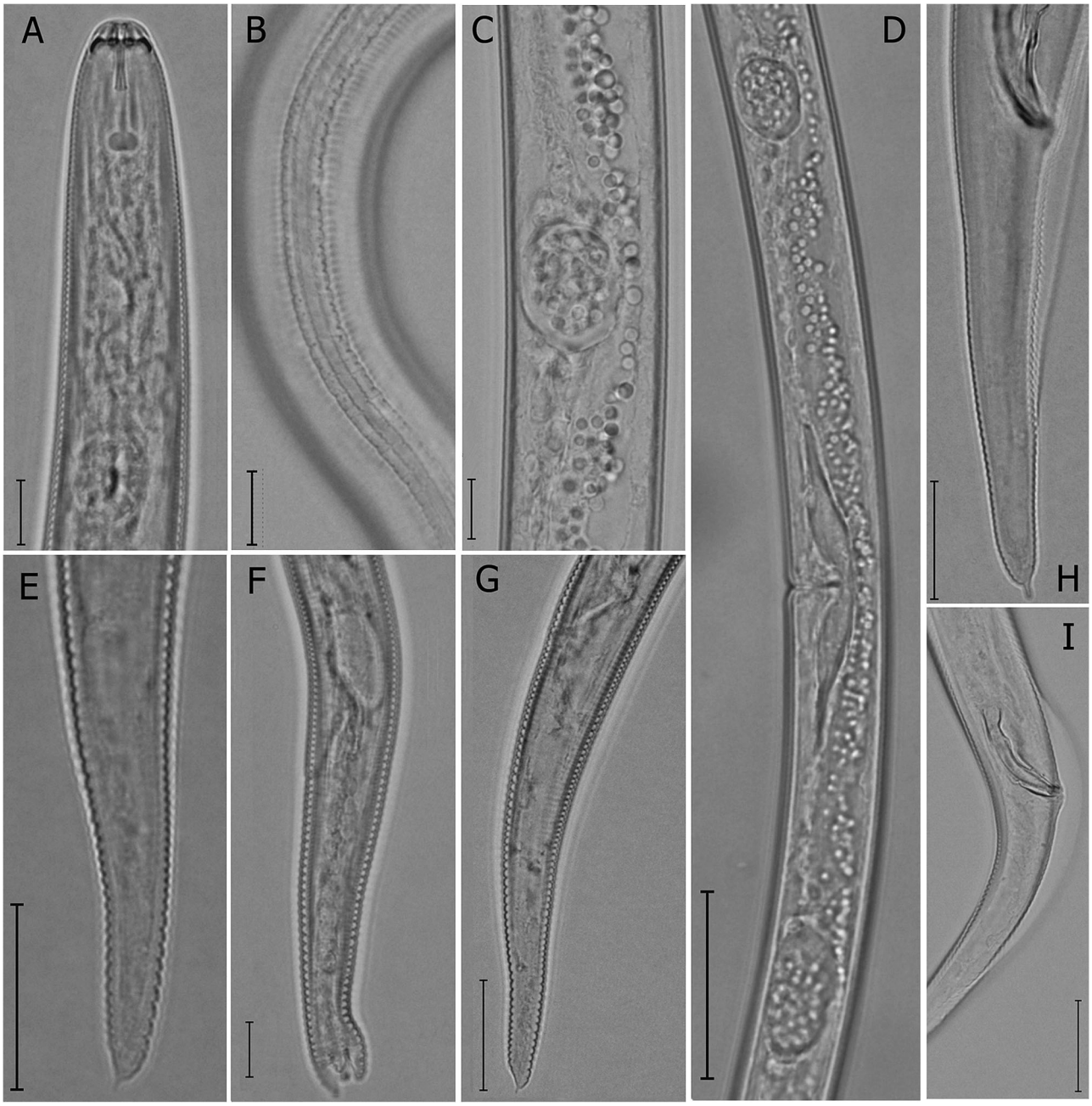

Fig. 5. *Hirschmanniella* sp. A-G: Female. A: anterior end; B: Lateral field at mid-body showing crenation and areolation; C: Anterior spermatheca; D: Vulval region showing rounded anterior and rather oval posterior, spermatheca; E-G: Variation in tail shape showing mucro and abnormality in tail terminus; H, I: Male tail. (Scale bars: A-C, F = 10 μm; D = 50 μm; E, G-I = 20 μm.)

35.5 (31.3-38) μm, respectively), shorter spicules (28.4 (27-30) *vs* 43 (42-44), 40 (38-43), 42.5 (37.5-50) μm, respectively), and shorter gubernaculum (10 *vs* 14 (12-16), 13 (12-14), 13.5 (11-16) μm, respectively).

Hirschmanniella sp. differs from *H. kwazuna* by the following female characters: L = 1448 (1206-1644) *vs* 1805 (1522-2049) μm, stylet length = 18.8 (18-20) *vs* 20.5 (18-22.5) μm, more anterior excretory pore at 97

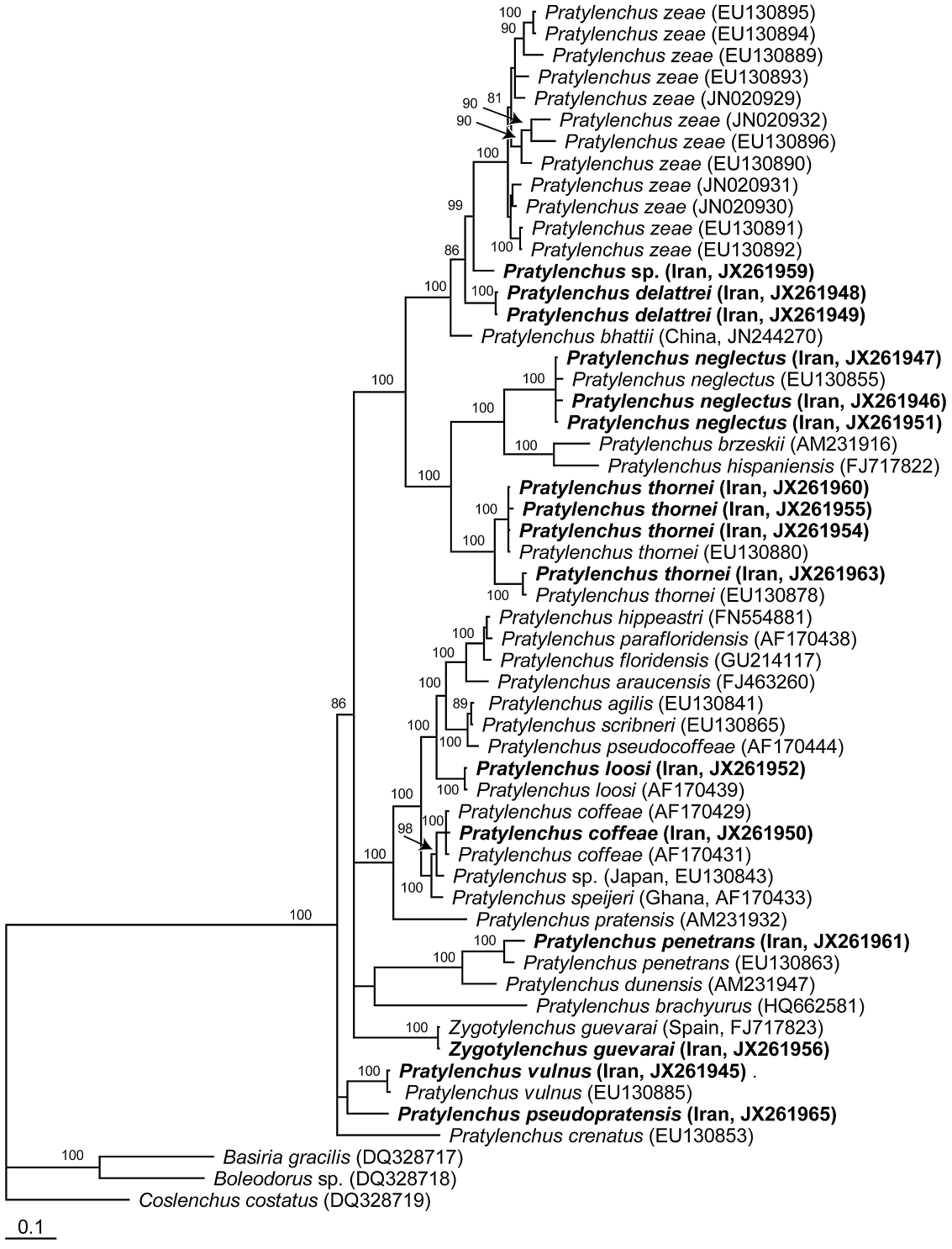

Fig. 6. Phylogenetic relationships between *Pratylenchus* species: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to/or more than, 70% are given for appropriate clades. New sequences are indicated in bold.

(85-111) *vs* 116 (59-151) μm, and lower value of c at 16.6 $\pm$ 1.42 (15.4-19.8) *vs* 20.6 (17.6-26.4). Males of *Hirschmanniella* sp. differ from those of *H. kwazuna* by a lower c value of 16.6 $\pm$ 1.42 (15.4-19.8) *vs* 22.5 (16.7-25.2), c$'$ = 5.2 $\pm$ 0.35 (4.4-5.5) *vs* 4.1 (3-4.9), and spicule length = 28.4 $\pm$ 1.14 (27-30) *vs* 31 (28-34.5) μm.

Hirschmanniella sp. was collected from a rice field in Khuzestan province in southern Iran. *Hirschmanniella oryzae* was previously reported from this region (Minassian & Barooti, 1997).

PHYLOGENETIC RELATIONSHIPS

Pratylenchus

The D2-D3 of 28S rRNA alignment included 51 sequences of *Pratylenchus*, two sequences of *Zygotylenchus* and three sequences of outgroup taxa and was 787 bp in length. *Pratylenchus neglectus*, *P. thornei*, *P. loosi*, *P. coffeae*, *P. penetrans*, *P. vulnus* and *Z. guevarai* clustered with corresponding species, the sequences of which were obtained from GenBank. The nucleotide differences between sequences of the Iranian populations and the corresponding reference species were in the intraspecific range. *Pratylenchus delattrei* and *Pratylenchus* sp. formed a highly supported clade with *P. zeae*. *Pratylenchus pseudopratensis* clustered with *P. vulnus* with low PP. Phylogenetic relationships within *Pratylenchus* species (Fig. 6) were congruent with those obtained in previous studies (Subbotin *et al.*, 2008; Múnera *et al.*, 2009).

Pratylenchoides

The D2-D3 of 28S rRNA alignment included four sequences of *Pratylenchoides*, one sequence of *Amplimerlinius*, one sequence of *Nagelus* and three sequences of outgroup taxa and was 774 bp in length. Sequences of *P. ritteri* and *P. alkani* differed by 5 bp. Phylogenetic relationships within *Pratylenchoides* and related genera are presented in Figure 7.

Hirschmanniella

The D2-D3 of 28S rRNA alignment included 14 sequences of *Hirschmanniella* and three sequences of outgroup taxa and was 721 bp in length. *Hirschmanniella* sp. from Iran formed a clade with *H. loofi* and *H. kwazuna* (Fig. 8). Phylogenetic relationships within *Hirschmanniella* species were congruent with those obtained in a previous study (Van den Berg *et al.*, 2009).

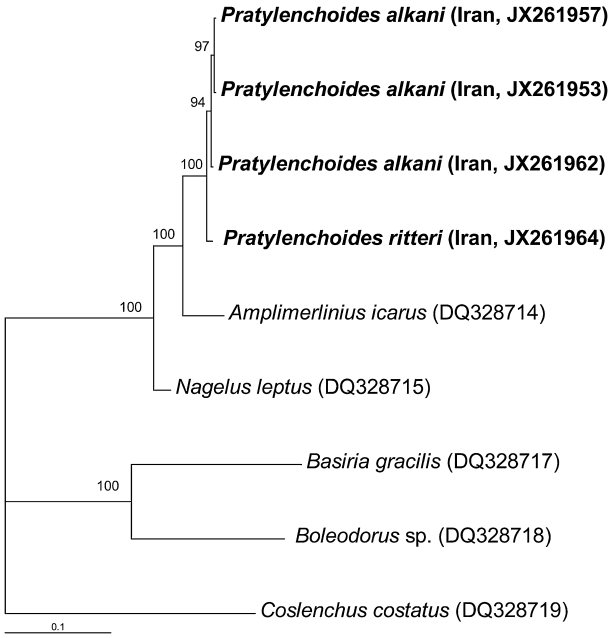

Fig. 7. Phylogenetic relationships between *Pratylenchoides*, *Amplimerlinius* and *Nagelus*: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. New sequences are indicated in bold.

PCR-ITS-RFLP

The PCR-RFLP-ITS results from four species of *Pratylenchus*, *viz*., *P. delattrei*, *P. penetrans*, *P. pseudopratensis* and *Pratylenchus* sp., are shown in Figure 9. Two restriction enzymes, *Hin*dIII and *Hin*fI, discriminated *P. penetrans* from *P. pseudopratensis* and *P. delattrei* from *Pratylenchus* sp., respectively. *Hin*dIII did not restrict *P. penetrans* but could generate two fragments of *ca* 500 and 200 bp in *P. pseudopratensis*. *Pratylenchus delattrei* and *Pratylenchus* sp. could be differentiated from each other by restriction enzyme *Hin*fI which generated two different RFLP patterns.

The enzymes *Bsh*NI and *Hph*I generated different RFLP patterns for *Pratylenchoides alkani* and *P. ritteri* and clearly distinguished these two similar species from each other (Fig. 10). *Bsh*NI produced two and three fragments for *P. ritteri* and *P. alkani*, respectively, which differed from patterns generated by *Hph*I.

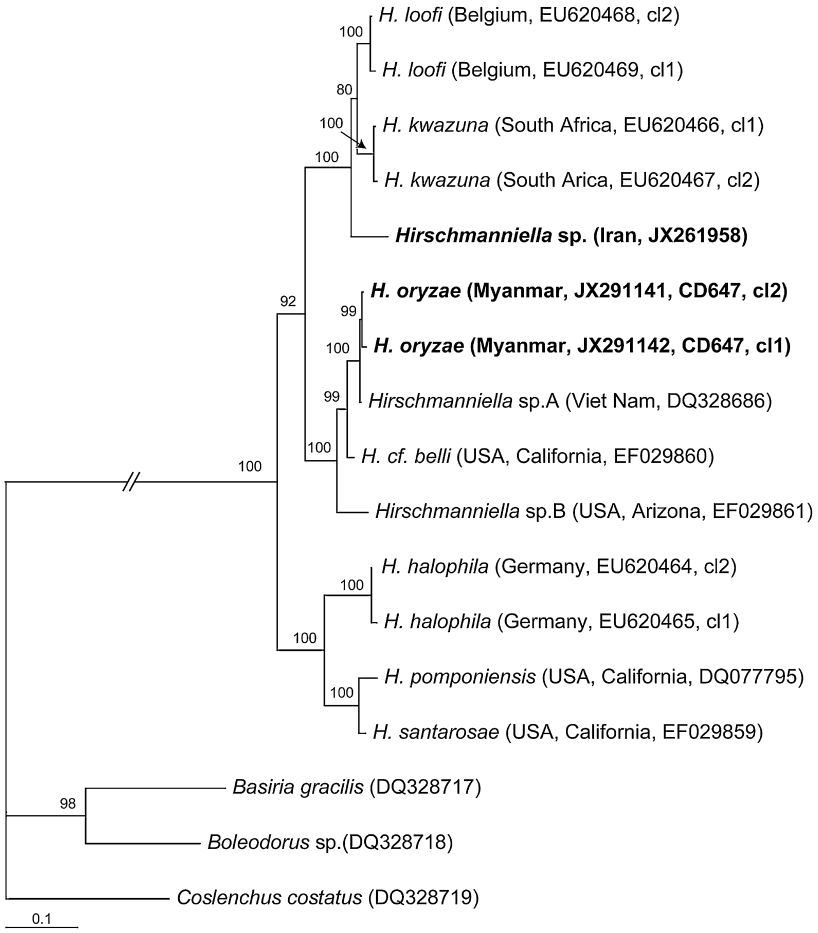

Fig. 8. Phylogenetic relationships between *Hirschmanniella* species: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2-D3 of 28S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal to, or more than, 70% are given for appropriate clades. New sequences are indicated in bold.

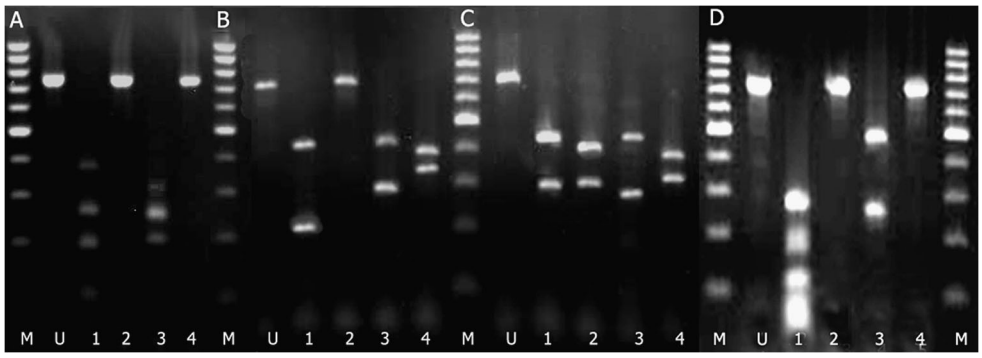

Fig. 9. PCR-ITS-RFLP for *Pratylenchus* species. A: *P. delattrei*, B: *P. penetrans*; C: *P. pseudopratensis*; D: *Pratylenchus* sp. M = 100 bp DNA ladder (Fermentas); U = unrestricted PCR products; 1 = *Hha*I; 2 = *Hin*dIII; 3 = *Hin*fI; 4 = *Pst*I.

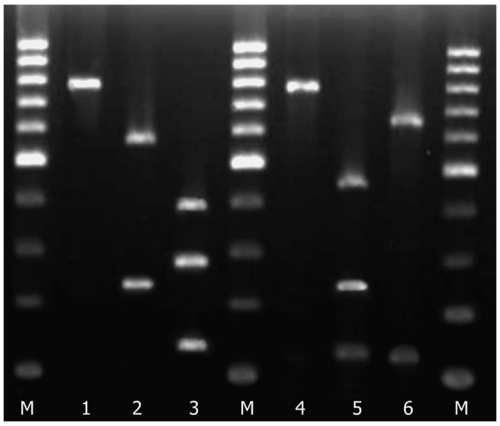

Fig. 10. PCR-LSU (D2-D3)-RFLP for *Pratylenchoides ritteri* and *P. alkani*. 1-3: *P. ritteri*, 4-6: *P. alkani*. M = 100 bp DNA ladder (Fermentas); 1, 4 = unrestricted PCR products; 2, 5 = *Bsh*NI; 3, 6 = *Hph*I.

Discussion

Root-lesion nematodes are considered to be amongst the most important and destructive migratory endoparasites. Accurate identification of this group is important in order to apply appropriate control strategies. Our studies clearly show that morphological differences among some species of *Pratylenchus*, *Pratylenchoides* and *Hirschmanniella* are often uncertain and relying only on such characters runs the risk of mis-identification. Several authors have demonstrated the diagnostic difficulties for discrimination of *Pratylenchus* species (Castillo & Vovlas, 2007; Inserra *et al.*, 2007; De Luca *et al.*, 2010). In this study, we provide molecular data on 13 species of Pratylenchidae, including *Pratylenchus delattrei* and *Pratylenchoides alkani* which were recorded from Iran for the first time. Our study proved the validity of *P. alkani* as a valid species, despite morphological similarities with *P. ritteri*. Our research also showed the existence of an unidentified species of *Hirschmanniella* in rice fields, a species that might be present in a mixture with *H. oryzae* in rice fields. Additional studies are needed for further characterisation of the unidentified *Pratylenchus* and *Hirschmanniella* species found in this research.

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