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Phylogenetic relationships of Telotylenchidae Siddiqi, 1960 and Merliniidae Siddiqi, 1971 (Nematoda: Tylenchida) from Iran, as inferred from the analysis of the D2D3 expansion fragments of 28S rRNA gene sequences

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Summary – Sixteen species, *Amplimerlinius globigerus*, *A. macrurus*, *Bitylenchus parvus*, *Merlinius brevidens*, *M. nanus*, *Neodolichorhynchus phaseoli*, *Paramerlinius neohexagrammus*, *Pratylenchoides alkani*, *P. ritteri*, *P. utahensis*, *Scutylenchus paniculoides*, *S. rugosus*, *S. tartuensis*, *Scutylenchus* sp. A, *Trophurus impar* and *Tylenchorhynchus brassicae*, from the families Telotylenchidae and Merliniidae were collected from different locations in Iran and molecularly characterised using sequencing of the D2D3 expansion fragments of the 28S rRNA gene. Morphometrics and light micrography for studied species are also provided as vouchers. The phylogenetic relationships of Telotylenchidae and Merliniidae with other representatives of the order Tylenchida, as obtained from Bayesian inference and Maximum likelihood analysis of partial 28S rRNA gene sequences, are presented and discussed. The results of phylogenetic analysis were in accordance with classifications in which *Bitylenchus* and *Scutylenchus* are considered as separate genera, but *Tessellus* and *Telotylenchus* were synonyms of *Tylenchorhynchus*. The Shimodaira-Hasegawa test of the 28S rRNA gene sequence alignment and trees rejected a large genus concept of *Tylenchorhynchus* and the constrained monophyly for Belonolaimidae revealed within this family two genera groups: *i*) *Belonolaimus* and *Ibipora*; and *ii*) *Carphodorus* and *Morulaimus*. The present results also support the combination of *Pratylenchoides* and Merliniinae into a single family, the Merliniidae.

Keywords – 28S rRNA gene, *Amplimerlinius*, *Bitylenchus*, *Neodolichorhynchus*, *Paramerlinius*, phylogeny, *Pratylenchoides*, *Scutylenchus*, *Trophurus*, *Tylenchorhynchus*.

The taxonomic positions of the plant-parasitic nematodes known under the common names 'awl' (dolichodorids), 'sting' (belonolaimids) and 'stunt' (tylenchorhynchids) nematodes within the order Tylenchida have been the subject of long discussion and still remain problematic and controversial. Maggenti *et al.* (1987) considered the Dolichodoridae Chitwood *in* Chitwood & Chitwood, 1950 separately from the Belonolaimidae Whitehead, 1960, which had two subfamilies: Belonolaiminae Whitehead, 1960 and Telotylenchinae Siddiqi, 1960. Siddiqi (1986, 2000) treated these nematodes as three different families: Dolichodoridae, Belonolaimidae and Telotylenchidae Siddiqi, 1960 which, along with Psilenchidae Paramonov, 1967, were included in the superfamily Dolichodoroidea Chitwood *in* Chitwood & Chitwood. Andrássy (2007) also recognised these three families, but included them in the Hoplolaimoidea Filipjev, 1934. Decraemer & Hunt (2006), Geraert (2011) and Hunt *et al.* (2013) considered these families (except for Psilenchidae) at subfamily level and put them together with four other subfamilies: Brachydorinae Siddiqi, 2000, Meiodorinae Siddiqi, 1976, Macrotrophurinae Fotedar & Handoo,

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1978 and Merliniinae Siddiqi, 1971 under the Dolichodoridae. In a recent revision, considering congruent morphological and molecular data, Sturhan (2012) removed the subfamily Merliniinae from Telotylenchidae *sensu* Siddiqi, 2000 and the genus *Pratylenchoides* Winslow, 1958 from Pratylenchidae Thorne, 1949 and defined the family Merliniidae as earlier proposed by Ryss (1993). According to Sturhan (2012), the Merliniidae consists of two subfamilies: Merliniinae comprising *Geocenamus* Thorne & Malek, 1968, *Merlinius* Siddiqi, 1970, *Paramerlinius* Sturhan, 2012, *Macrotylenchus* Sturhan, 2012, *Amplimerlinius* Siddiqi, 1976 and *Nagelus* Thorne & Malek, 1968, and Pratylenchoidinae Sturhan, 2012 with *Pratylenchoides*. Subbotin *et al.* (2006) and Sturhan (2012) also concluded that the erection of the Dolichodoroidea *sensu* Siddiqi, 2000 is no longer justified.

In this study we characterised molecularly 16 species of the Telotylenchidae and Merliniidae collected from different locations in Iran. We reconstructed phylogenetic relationships of these two families along with other representatives of the main families of plant-parasitic tylenchids using sequences of the D2D3 expansion fragments of 28S rRNA gene, and tested alternative hypotheses of nematode evolution and classifications previously proposed based on morphology.

Materials and methods

Nematode samples

Nematode isolates were collected from different plants and localities in Iran (Table 1). Nematodes were extracted from soil samples using the tray method (Whitehead & Hemming, 1965). For morphological identification, specimens were killed and fixed by hot FPG (formaldehyde:propionic acid:glycerol = 4:1:1), processed to anhydrous glycerin (De Grisse, 1969), mounted in glycerin on permanent slides and studied by a Zeiss III light microscope equipped with Dino-eye microscope eye-piece camera and its software Dino Capture version 2.0. Nematode species were identified based on morphological and morphometric characters using identification keys (Geraert, 2011).

DNA extraction, PCR and sequencing

For molecular analysis, a single female of each species or population was put in a drop of distilled water on a microscopic slide and was examined under the light microscope. Identification was done based on the morphological and morphometric characters. Each specimen was then transferred into deionised water, washed three times and put into an Eppendorf tube with 25 μl distilled water. Lysis buffer (25 μl) (23.75 μl NaCl 0.2 M and Tris-HCl 0.2 M, 1.0 μl β-mercaptoethanol and 0.25 μl proteinase K) was added to each Eppendorf tubes. The nematode specimen was crushed with a microhomogeniser for 2 min. Tubes were incubated at 65°C (1 h) and then at 95°C (15 min). Detailed protocols for PCR and sequencing are described by Tanha Maafi *et al.* (2003). The D2D3 expansion segments of the 28S rRNA gene were amplified with the forward D2A and the reverse D3B primers (Subbotin *et al.*, 2006). 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 Bio Basic (Ontario, ON, Canada). New sequences were submitted to the GenBank database under the accession numbers KJ585416-KJ585432, as indicated in Table 1 and Figure 4.

Sequence and phylogenetic analyses

The newly obtained sequences of the D2D3 of 28S rRNA were aligned using ClustalX 1.83 (Thompson *et al.*, 1997) with default parameters and corresponding published gene sequences (Subbotin *et al.*, 2006; Majd Taheri *et al.*, 2013; Stirling *et al.*, 2013). Outgroup taxa were chosen according to the result of a previous study (Subbotin *et al.*, 2006). Pairwise divergences between taxa were computed as absolute distance values and as percentage mean distance values based on the whole alignment, with adjustment for missing data, using PAUP* 4.0b 10 (Swofford, 2003). The best fit model of DNA evolution was obtained using the program jModeltest 0.1.1 (Posada, 2008) under the Akaike Information Criterion. The general time reversible substitution model with estimation of invariant sites and assuming a gamma distribution with four categories (GTR + I + G) was selected as the optimal nucleotide substitution model for the analyses. The alignment was analysed with Bayesian Inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) and with Maximum Likelihood (ML) using RAxML version 7.7.1 (available online at http://phylobench.vital-it.ch/raxml-bb/index.php) (Stamatakis, 2006). BI analysis for each gene was initiated with a random starting tree and was run with four chains for 2.0×10^6 generations. Two runs were performed for each analysis. The Markov chains were sampled at intervals of 100 generations. Af-

Table 1. Species used in the present study.

Species	Locality/province	Associated plant	GenBank accession number of the D2D3 of 28S rRNA sequence
Amplimerlinius globigerus	Fars	Walnut	KJ585428
A. macrurus	Mazandaran	Citrus	KJ585424
Bitylenchus parvus	Fars	Wheat	KJ585431
Merlinius brevidens	Kohgiluyeh and Boyer-Ahmad	Wheat	KJ585416
M. nanus	Fars	Potato	KJ585417
Neodolichorhynchus phaseoli	Hormozgan	Turf grass	KJ585429
Paramerlinius neohexagrammus	Kohgiluyeh and Boyer-Ahmad	Eucalyptus	KJ585423
Pratylenchoides alkani	Kurdistan	Almond	KJ585426
P. ritteri	Fars	Wheat	KJ585425
P. utahensis	Kohgiluyeh and Boyer-Ahmad	Meadow	KJ585427
Scutylenchus paniculoides	Fars	Pomegranate	KJ585422
S. rugosus	Fars	Forest trees	KJ585421
Scutylenchus sp. A	Khuzestan	Lebbeck tree (*Albizia lebbek*)	KJ585418
Scutylenchus sp. A	Fars	Eucalyptus	KJ585419
S. tartuensis	Kurdistan	Fruit tree seedlings	KJ585420
Trophurus impar	Fars	Walnut	KJ585430
Tylenchorhynchus brassicae	Fars	Vegetables	KJ585432

ter discarding burn-in samples, other trees were used to generate a 50% majority rule consensus tree. ML analysis was run with 100 bootstrap replicates. For testing of alternative topologies in ML, we applied the Shimodaira-Hasegawa (SH) test as implemented in PAUP* and used an alignment with sequences from Clades I or III (Fig. 4). Trees were visualised using TreeView (Page, 1996).

Results

The following species were identified within the studied samples: *Amplimerlinius globigerus* Siddiqi, 1979, *A. macrurus* (Goodey, 1932) Siddiqi, 1976, *Bitylenchus parvus* (Allen, 1955) Siddiqi, 1986, *Merlinius brevidens* (Allen, 1955) Siddiqi, 1970, *M. nanus* (Allen, 1955) Siddiqi, 1970, *Neodolichorhynchus phaseoli* (Sethi & Swarup, 1968) Talavera & Tobar, 1997, *Paramerlinius neohexagrammus* (Ivanova, 1978) Sturhan, 2012, *Pratylenchoides alkani* Yüksel, 1977, *P. ritteri* Sher, 1970, *P. utahensis* Baldwin, Luc & Bell, 1983, *Scutylenchus paniculoides* (Vovlas & Esser, 1990) Siddiqi, 2000, *S. rugosus* (Siddiqi, 1963) Siddiqi, 1979, *S. tartuensis* (Krall, 1959) Siddiqi, 1979, *Scutylenchus* sp. A, *Trophurus impar* Ganguly & Khan, 1983 and *Tylenchorhynchus brassicae* Siddiqi, 1961. Important diagnostic characters of all 16 species are presented in Figures 1-3. Morphometrics of *M. brevidens*, *N. phaseoli*, *P. utahensis*, *S. paniculoides* and *T. impar* (Ghaderi *et al.*, 2014), and *A. globigerus* and *A. macrurus* (Ghaderi & Karegar, 2014) have already been published. The morphometrics of the other species are given in Tables 2-4.

The alignment of the D2D3 expansion fragments of 28S rRNA gene sequences contained 68 taxa including two outgroup taxa. The length of alignment had 704 positions. A new sequence of *P. ritteri* was identical to that already published for *P. alkani* (Majd Taheri *et al.*, 2013) and sequence variation within *P. ritteri*/*P. alkani* varied from 0.0 to 1.2% (0-8 bp). Sequence differences between *Trophurus impar* and *T. sculptus* were 4.3% (28 bp), between *M. brevidens* and *M. nanus* 2.5% (16 nucleotides), between *A. icarus* and *A. macrurus* 2.6% (15 nucleotides), between *Scutylenchus* sp. A and *S. rugosus* 0.5-0.8% (3-5 bp), and between *S. rugosus* and *S. tartuensis* 0.5% (3 bp). Sequence variation within studied *Scutylenchus* populations and species varied from 0.5 to 3.2% (2-21 bp).

The phylogenetic relationships between the Telotylenchidae and Merliniidae and other tylenchids, as inferred from the BI analysis, are presented in Figure 4. Tree topologies obtained with BI and ML analyses were congruent. Five major clades with high or moderate statistical support were distinguished within the phylogenetic tree. Clade I contained representatives of the suborder Hoplolaimina Chizhov & Berezina, 1988; Clade II included representatives of the suborder Criconematina

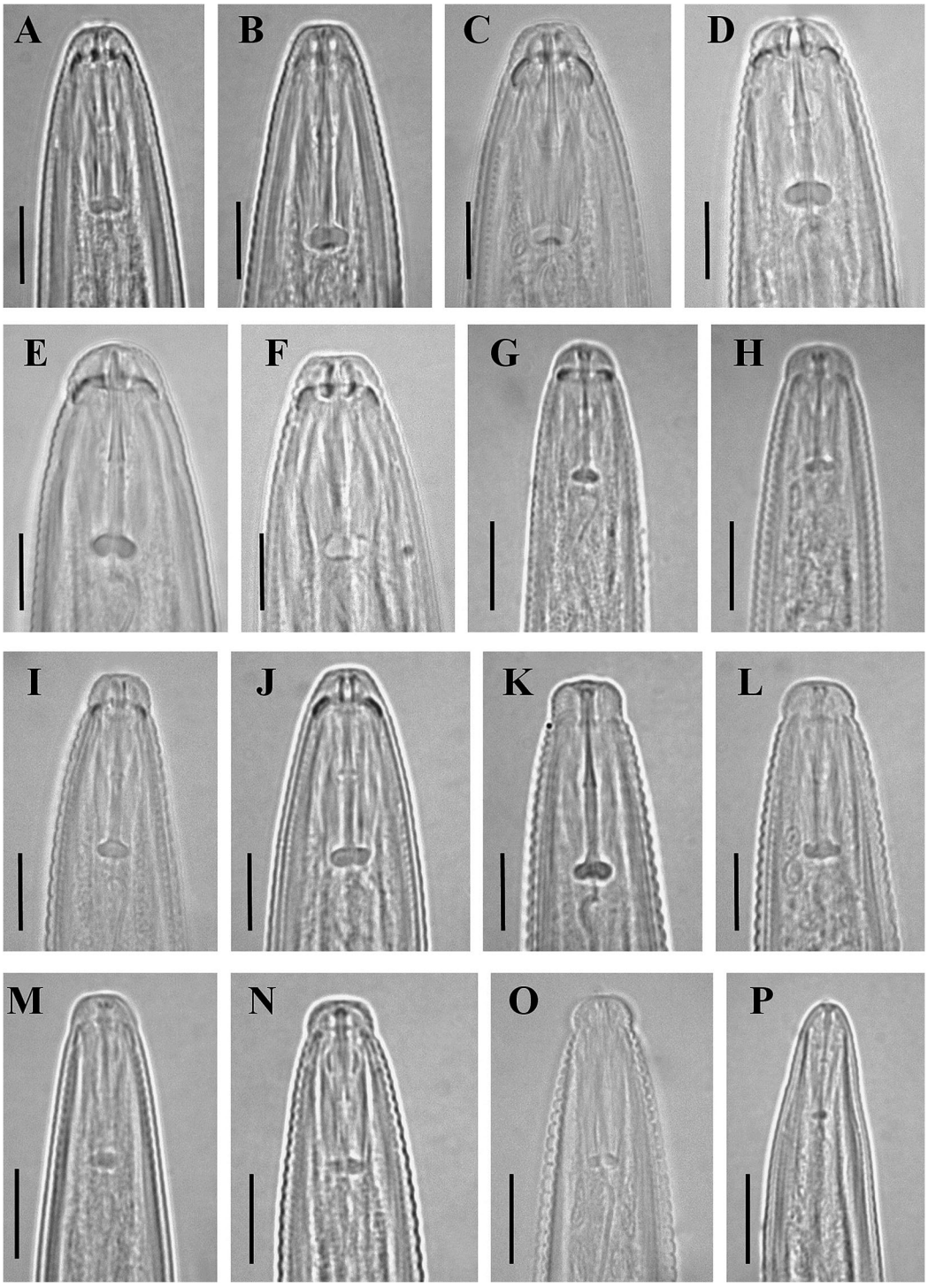

Fig. 1. Female anterior end of several species of Merliniidae and Telotylenchidae from Iran. A: *Amplimerlinius globigerus*; B: *A. macrurus*; C: *Paramerlinius neohexagrammus*; D: *Pratylenchoides utahensis*; E: *P. alkani*; F: *P. ritteri*; G: *Merlinius brevidens*; H: *M. nanus*; I: *Scutylenchus paniculoides*; J: *S. tartuensis*; K: *S. rugosus*; L: *Scutylenchus* sp. A; M: *Bitylenchus parvus*; N: *Tylenchorhynchus brassicae*; O: *Neodolichorhynchus phaseoli*; P: *Trophurus impar*. (Scale bars = 10 μm.)

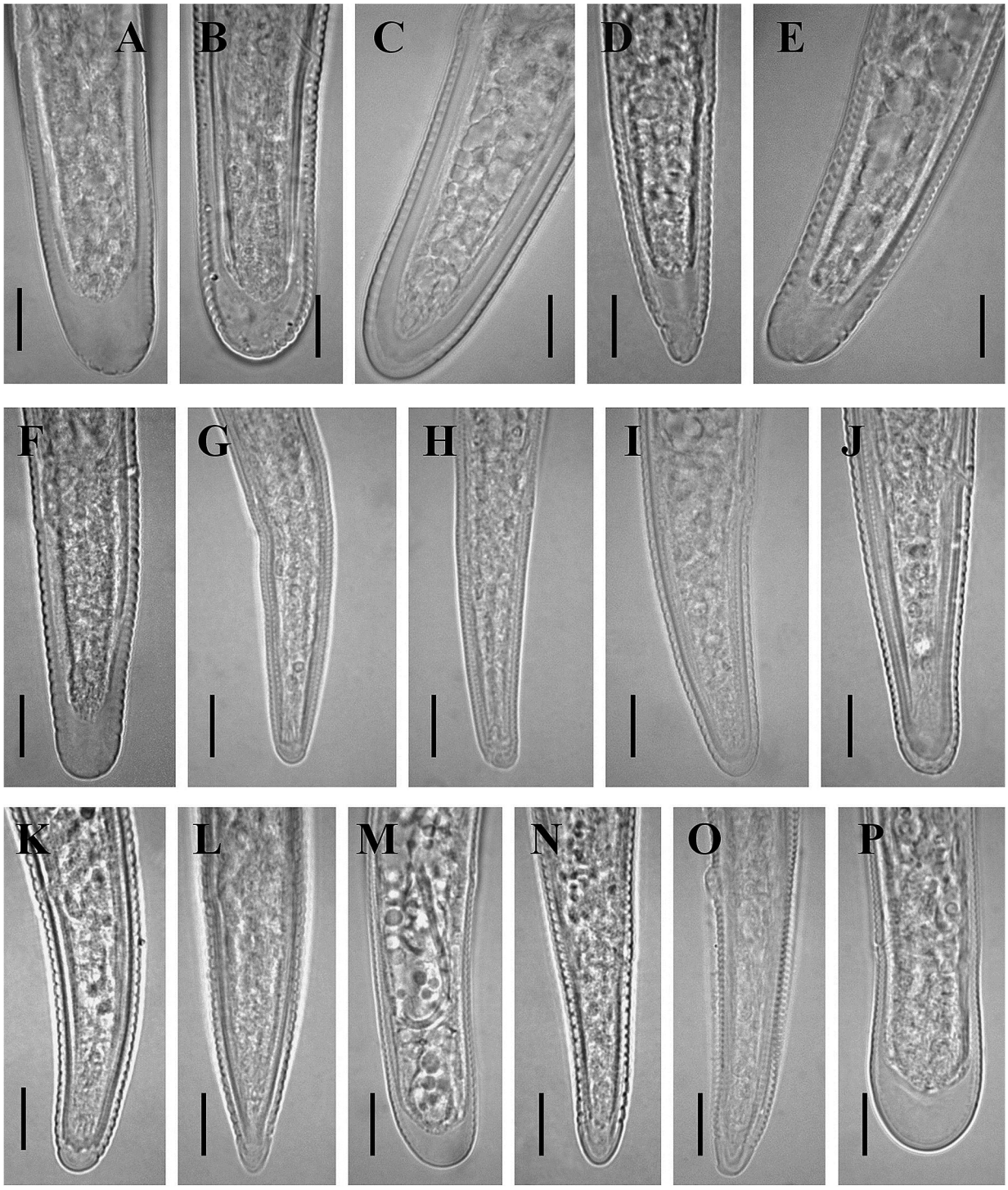

Fig. 2. Female posterior end of several species of Merliniidae and Telotylenchidae from Iran. A: *Amplimerlinius globigerus*; B: *A. macrurus*; C: *Paramerlinius neohexagrammus*; D: *Pratylenchoides utahensis*; E: *P. alkani*; F: *P. ritteri*; G: *Merlinius brevidens*; H: *M. nanus*; I: *Scutylenchus paniculoides*; J: *S. tartuensis*; K: *S. rugosus*; L: *Scutylenchus* sp. A; M: *Bitylenchus parvus*; N: *Tylenchorhynchus brassicae*; O: *Neodolichorhynchus phaseoli*; P: *Trophurus impar*. (Scale bars = 10 μm.)

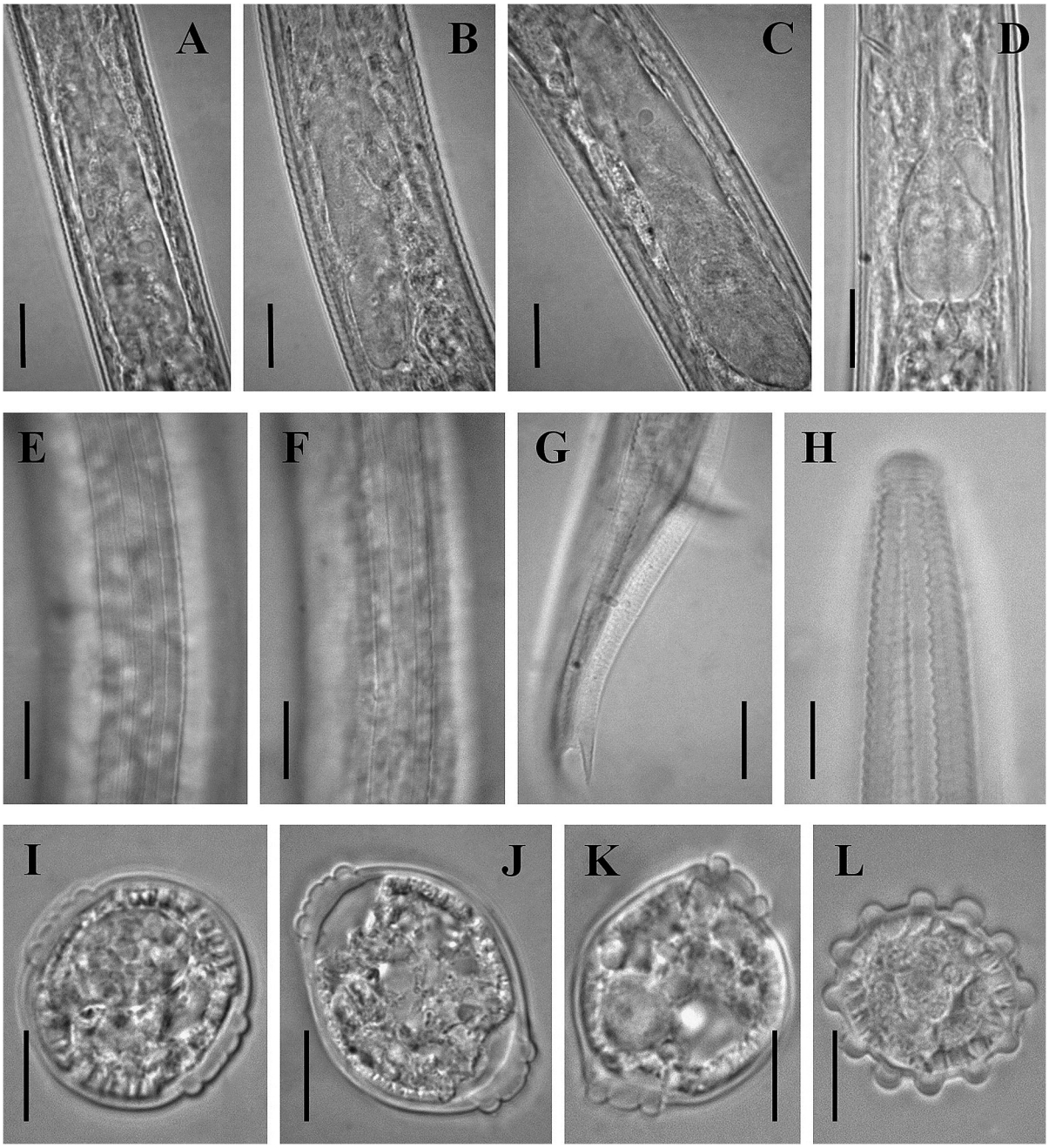

Fig. 3. Species of Merliniidae and Telotylenchidae from Iran. A, I: *Pratylenchoides utahensis*; B, E, J: *P. alkani*; C, F, K: *P. ritteri*; D: *Trophurus impar*; G, H, L: *Neodolichorhynchus phaseoli*. A-D: Pharyngeal region; E, F: Lateral field at mid-body; G: Male posterior end; H: Cuticle longitudinal ridges at anterior end; I-L: Cross-section near mid-body. (Scale bars = 10 μm.)

Table 2. Morphometric characters of *Paramerlinius neohexagrammus*, *Pratylenchoides alkani* and *P. ritteri* collected from Iran. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*Paramerlinius neohexagrammus*		*Pratylenchoides alkani*		*Pratylenchoides ritteri*	
	Female	Male	Female	Male	Female	Male
n	23	9	6	4	8	3
L	1123 ± 61 (991-1247)	1087 ± 71 (984-1216)	1014 ± 100 (828-1090)	855 ± 108 (755-1002)	712 ± 52.8 (621-805)	667 ± 64.6 (596-722)
a	30.6 ± 2.1 (27.4-34.9)	34.6 ± 2.4 (31.7-38.0)	31.6 ± 1.8 (29.8-34.9)	33.0 ± 1.6 (30.6-34.0)	27.6 ± 1.8 (24.8-30.3)	31.1 ± 0.9 (30.4-32.1)
b	6.1 ± 0.3 (5.6-7.0)	5.9 ± 0.3 (5.3-6.3)	7.3 ± 0.9 (6.3-8.5)	7.3 ± 0.6 (6.6-7.9)	5.6 ± 0.6 (4.6-6.1)	6.5 ± 0.8 (5.6-7.1)
b′	–	–	5.3 ± 0.2 (5.0-5.7)	5.8 ± 0.4 (5.5-6.4)	4.0 ± 0.5 (3.4-4.7)	5.5 ± 0.5 (5.0-5.9)
c	18.2 ± 1.4 (16.3-21.8)	14.3 ± 0.9 (12.9-15.5)	16.1 ± 1.5 (14.2-18.4)	14.1 ± 0.3 (13.7-14.5)	14.8 ± 1.2 (13.5-17.3)	13.7 ± 0.9 (13.0-14.7)
c′	2.4 ± 0.2 (1.9-2.9)	3.4 ± 0.4 (2.9-4.0)	2.9 ± 0.3 (2.5-3.3)	3.4 ± 0.2 (3.2-3.7)	2.8 ± 0.2 (2.5-3.2)	3.2 ± 0.1 (3.2-3.3)
V	54.5 ± 1.6 (52.1-57.4)	–	52.3 ± 2.4 (49.0-55.0)	–	55.4 ± 2.1 (51.5-58.9)	–
Stylet	26.3 ± 0.9 (24.8-28.7)	26.2 ± 1.1 (24.0-27.5)	23.7 ± 0.7 (23.0-24.6)	20.7 ± 0.9 (19.6-21.9)	21.3 ± 0.8 (20.0-22.4)	18.1 ± 1.4 (17.0-19.6)
m	–	–	50.1 ± 1.5 (47.9-52.2)	54.1 ± 1.1 (53.1-55.7)	50.8 ± 1.8 (48.2-53.2)	55.3 ± 2.1 (52.9-56.8)
Pharynx	184 ± 8.2 (166-200)	186 ± 8.8 (176-200)	140 ± 15.4 (120-159)	117 ± 16.4 (101-140)	127 ± 13.8 (116-160)	103 ± 6.1 (96-108)
Gland overlap	–	–	50.6 ± 18.4 (32.6-75.1)	30.9 ± 9.9 (21.5-42.4)	51.8 ± 15.2 (24.8-73.8)	16.9 ± 5.3 (13.8-23.0)
Overlap/body diam. at pharynx	–	–	1.7 ± 0.6 (1.1-2.7)	–	2.2 ± 0.7 (1.1-3.4)	–
MB	49.5 ± 2.3 (46.2-56.9)	49.6 ± 1.2 (48.3-52.6)	58.5 ± 4.1 (53.6-63.8)	59.8 ± 5.0 (54.1-66.1)	37.6 ± 3.8 (32.8-43.9)	49.5 ± 1.8 (47.5-51.0)
S.E. pore	151 ± 7.4 (133-163)	147 ± 10.6 (123-162)	129 ± 10.3 (115-141)	119 ± 9.3 (109-132)	107 ± 7.8 (98-119)	104 ± 5.8 (98-110)
Head-vulva	612 ± 31.6 (538-665)	–	529 ± 42 (455-586)	–	394 ± 30.8 (350-446)	–
Tail length	62 ± 5.3 (53-72)	76 ± 7.0 (65-87)	64 ± 11.0 (48-77)	61 ± 7.1 (55-71)	48 ± 3.9 (42-53)	49 ± 2.4 (46-51)
Max. body diam.	36.8 ± 2.4 (31.0-42.0)	31.5 ± 1.6 (28.0-33.7)	32.1 ± 2.6 (27.8-35.7)	25.9 ± 2.6 (23.6-29.5)	25.9 ± 1.9 (23.8-29.0)	21.5 ± 1.8 (19.4-22.5)
Annuli width	2.0 ± 0.2 (1.7-2.3)	1.9 ± 0.2 (1.7-2.2)	1.9 ± 0.2 (1.6-2.0)	1.6 ± 0.2 (1.4-1.8)	1.5 ± 0.1 (1.3-1.7)	1.3 ± 0.1 (1.2-1.4)
Tail annuli	33.9 ± 4.2 (26-47)	–	29.8 ± 4.4 (22-35)	29.0 ± 4.4 (23.5-33.0)	23 ± 2 (20-26)	22.7 ± 0.6 (22.0-23.2)
Phasmid/tail (%)	29.1 ± 4.9 (21.0-42.5)	37.5 ± 4.6 (31.8-45.1)	31.0 ± 6.3 (20.5-38.1)	47.8 ± 4.8 (42.6-54.1)	23.1 ± 2.8 (18.5-25.7)	46.7 ± 1.5 (45.0-47.8)
Spicules	–	30.8 ± 1.5 (28.5-32.5)	–	26.3 ± 0.8 (25.3-27.0)	–	22.8 ± 1.8 (21.0-24.5)
Gubernaculum	–	10.6 ± 0.6 (9.4-11.3)	–	6.9 ± 0.3 (6.5-7.1)	–	6.7 ± 0.2 (6.6-7.0)

Table 3. Morphometric characters of *Scutylenchus paniculoides*, *S. rugosus* and *Scutylenchus* sp. A collected from Iran. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*Scutylenchus paniculoides*		*Scutylenchus rugosus*		*Scutylenchus* sp. A	
	Female	Male	Female	Male	Female	Male
n	24	6	188	2	25	20
L	820 ± 72.4 (680-942)	780 ± 33.0 (734-819)	815 ± 63.8 (622-1022)	680-740	791 ± 62.7 (635-929)	799 ± 70.1 (678-920)
a	30.4 ± 2.3 (27.7-38.8)	33.6 ± 1.5 (31.1-35.2)	30.5 ± 2.6 (24.2-41.3)	30.2-30.2	33.2 ± 2.4 (28.6-38.0)	35.3 ± 2.4 (31.4-41.5)
b	5.5 ± 0.4 (4.7-6.1)	5.4 ± 0.3 (4.9-5.7)	5.3 ± 0.4 (4.3-6.4)	5.8-6.0	5.7 ± 0.4 (5.0-6.7)	5.9 ± 0.5 (4.9-6.7)
c	17.6 ± 1.3 (15.4-20.1)	14.7 ± 0.6 (13.9-15.6)	15.7 ± 1.1 (12.8-19.1)	14.4-14.9	16.5 ± 1.6 (13.3-20.4)	14.5 ± 1.2 (12.7-17.0)
c′	2.5 ± 0.2 (2.0-2.9)	3.4 ± 0.2 (3.1-3.7)	2.9 ± 0.3 (2.2-4.1)	3.0-3.2	2.8 ± 0.3 (2.3-3.4)	3.6 ± 0.4 (3.0-4.2)
V	55.7 ± 1.5 (52.4-58.1)	–	56.2 ± 1.5 (51.5-60.4)	–	55.8 ± 1.7 (53.1-61.2)	–
Stylet	19.8 ± 0.9 (18.0-21.8)	19.1 ± 0.5 (18.7-19.8)	22.0 ± 0.9 (20.0-25.8)	17.5-17.5	18.3 ± 0.8 (16.0-19.8)	18.0 ± 0.8 (16.5-19.5)
m	51.4 ± 1.9 (46.0-55.1)	50.6 ± 1.2 (48.9-51.9)	51.0 ± 1.9 (44.7-55.9)	47.4-48.6	51.1 ± 2.0 (47.6-54.4)	51.7 ± 1.6 (49.4-54.5)
Pharynx	150 ± 11.7 (130-170)	144 ± 6.0 (138-154)	153 ± 10.1 (126-183)	117-123	139 ± 11.3 (109-159)	137 ± 10.4 (114-151)
MB	47.5 ± 1.8 (43.6-50.6)	48.2 ± 1.0 (46.6-49.4)	47.3 ± 1.8 (39.4-54.5)	46.9-48.6	47.8 ± 1.8 (44.8-52.6)	47.1 ± 1.8 (44.9-53.3)
S.E. pore	120 ± 7.9 (100-135)	116 ± 6.5 (110-127)	127 ± 7.9 (107-151)	99-102	115 ± 9.0 (95-138)	116 ± 9.5 (101-135)
Basal bulb length	32.1 ± 3.6 (26.0-38.7)	28.9 ± 2.0 (26.0-32.0)	29.0 ± 2.5 (21.6-38.0)	21.2-21.9	27.5 ± 3.4 (20.4-33.6)	26.4 ± 2.9 (22.0-31.3)
Basal bulb diam.	13.5 ± 1.1 (11.2-16.3)	12.0 ± 0.5 (11.4-12.5)	14.5 ± 1.0 (11.4-16.8)	11.4-11.6	12.1 ± 1.1 (10.0-14.3)	11.6 ± 1.2 (9.7-13.2)
Head-vulva	456 ± 35.2 (378-519)	–	458 ± 35.4 (370-597)	–	441 ± 34.2 (353-503)	–
Tail length	47 ± 4.5 (36-54)	53 ± 4.4 (47-59)	52 ± 4.6 (39-67)	46-51	48 ± 4.4 (39-58)	55 ± 4.3 (48-66)
Max. body diam.	27.0 ± 2.0 (23.5-30.0)	23.3 ± 1.3 (21.0-24.6)	26.9 ± 2.2 (19.7-33.2)	22.5-24.5	23.9 ± 2.4 (19.2-30.0)	22.7 ± 2.5 (16.5-26.5)
Annuli width	2.1 ± 0.2 (1.8-2.5)	1.9 ± 0.1 (1.8-2.2)	2.2 ± 0.2 (1.5-3.0)	1.8-1.8	2.0 ± 0.2 (1.6-2.3)	2.0 ± 0.3 (1.6-2.6)
Tail annuli	25.4 ± 2.8 (20-29)	–	26.2 ± 3.2 (20-35)	–	24.9 ± 3.1 (17-30)	–
Phasmid/tail (%)	34.8 ± 5.0 (22.4-42.9)	41.5 ± 5.7 (34.8-47.8)	30.0 ± 5.0 (17.9-42.4)	32.2-42.8	35.7 ± 5.9 (24.9-48.7)	42.2 ± 6.0 (31.6-55.5)
Spicules	–	25.9 ± 1.5 (24.5-28.5)	–	21.6-22.8	–	24.5 ± 1.4 (21.5-26.8)
Gubernaculum	–	9.3 ± 1.4 (7.0-11.0)	–	7.1-9.4	–	9.0 ± 1.0 (7.7-11.0)

Table 4. Morphometric characters of *Merlinius nanus*, *Bitylenchus parvus* and *Tylenchorhynchus brassicae* collected from Iran. All measurements are in μm and in the form: mean ± s.d. (range).

Character	*Merlinius nanus*		*Bitylenchus parvus*		*Tylenchorhynchus brassicae*	
	Female	Male	Female	Male	Female	Male
n	55	11	26	13	46	19
L	546 ± 45.3 (433-682)	522 ± 70.6 (404-639)	754 ± 49.8 (630-863)	711 ± 46.7 (634-797)	618 ± 40.9 (543-734)	600 ± 48.9 (535-675)
a	28.5 ± 1.7 (25.7-33.0)	31.1 ± 1.5 (28.7-33.8)	31.3 ± 1.9 (28.0-34.9)	32.0 ± 2.6 (28.3-36.7)	30.2 ± 1.9 (25.6-35.3)	31.7 ± 2.4 (27.4-36.3)
b	5.0 ± 0.4 (4.0-5.9)	4.9 ± 0.5 (4.3-5.6)	6.0 ± 0.5 (5.1-7.3)	5.6 ± 0.4 (4.9-6.1)	5.3 ± 0.4 (4.6-6.2)	5.2 ± 0.4 (4.6-6.4)
c	12.6 ± 1.1 (10.5-15.9)	10.8 ± 0.6 (9.7-11.7)	14.6 ± 1.2 (12.0-17.0)	16.1 ± 1.5 (14.4-19.4)	18.3 ± 1.9 (14.8-23.0)	16.8 ± 1.3 (14.2-19.1)
c′	3.4 ± 0.4 (2.7-4.2)	4.2 ± 0.3 (3.7-4.8)	2.9 ± 0.3 (2.4-3.4)	2.9 ± 0.3 (2.3-3.4)	2.6 ± 0.3 (2.1-3.3)	2.9 ± 0.2 (2.6-3.5)
V	56.1 ± 1.6 (52.3-60.2)	–	53.3 ± 1.4 (50.3-55.9)	–	57.1 ± 1.2 (55.0-59.5)	–
Stylet	12.3 ± 1.0 (10.5-14.0)	11.1 ± 0.5 (10.6-12.0)	17.6 ± 0.7 (16.5-18.9)	17.2 ± 0.9 (16.0-19.0)	16.6 ± 0.9 (14.8-18.7)	16.3 ± 0.9 (14.5-18.2)
m	49.9 ± 3.7 (36.9-57.1)	49.1 ± 3.3 (44.3-53.6)	50.5 ± 2.3 (43.6-53.9)	50.2 ± 1.8 (47.5-54.1)	50.5 ± 1.7 (46.7-54.2)	50.4 ± 1.9 (46.9-53.4)
Pharynx	110 ± 8.5 (94-128)	107 ± 8.2 (92-119)	126 ± 7.2 (109-138)	127 ± 7.6 (118-136)	117 ± 7.0 (101-131)	115 ± 6.6 (104-127)
MB	45.6 ± 1.7 (42.1-49.9)	45.7 ± 1.0 (43.6-46.8)	49.8 ± 1.8 (45.9-54.0)	50.1 ± 1.6 (48.2-53.4)	48.2 ± 2.0 (42.7-52.0)	48.1 ± 1.9 (44.7-51.5)
S.E. pore	88 ± 6.4 (75-104)	84.6 ± 9.0 (64.6-96.4)	104 ± 6.4 (90-119)	104 ± 4.7 (96-114)	95 ± 6.1 (79-110)	92 ± 5.2 (84-104)
Head-vulva	306 ± 25.4 (239-376)	–	402 ± 26.2 (344-462)	–	353 ± 22.8 (311-415)	–
Tail length	44 ± 5.2 (34-63)	48 ± 6.0 (40-58)	52 ± 5.6 (41-63)	44 ± 5.5 (36-55)	34 ± 3.8 (28-42)	36 ± 3.4 (31-47)
Max. body diam.	19.2 ± 1.6 (16.2-23.0)	16.8 ± 2.2 (13.6-21.0)	24.2 ± 1.7 (21.0-29.5)	22.3 ± 1.6 (20.5-25.4)	20.5 ± 1.3 (17.0-23.3)	19.0 ± 1.2 (17.1-20.7)
Annuli width	1.0 ± 0.1 (0.8-1.4)	1.0 ± 0.1 (0.8-1.1)	1.1 ± 0.2 (0.9-1.7)	1.2 ± 0.3 (0.9-1.8)	1.7 ± 0.2 (1.4-2.1)	1.7 ± 0.2 (1.3-2.1)
Tail annuli	42.9 ± 5.9 (32.0-59.0)	–	40.3 ± 4.1 (33-48)	–	15.0 ± 2.2 (10-20)	–
Phasmid/tail (%)	45.3 ± 4.7 (36.3-54.2)	47.6 ± 4.0 (40.6-53.0)	38.5 ± 5.4 (29.5-51.2)	37.8 ± 5.6 (28.2-47.5)	33.6 ± 5.5 (19.0-47.5)	40.1 ± 6.0 (32.6-55.5)
Spicules	–	18.7 ± 0.9 (18.0-21.1)	–	23.9 ± 1.2 (21.6-26.1)	–	18.5 ± 1.0 (17.0-20.4)
Gubernaculum	–	6.8 ± 0.4 (6.0-7.5)	–	12.0 ± 0.6 (10.7-13.0)	–	9.8 ± 1.1 (7.2-11.2)

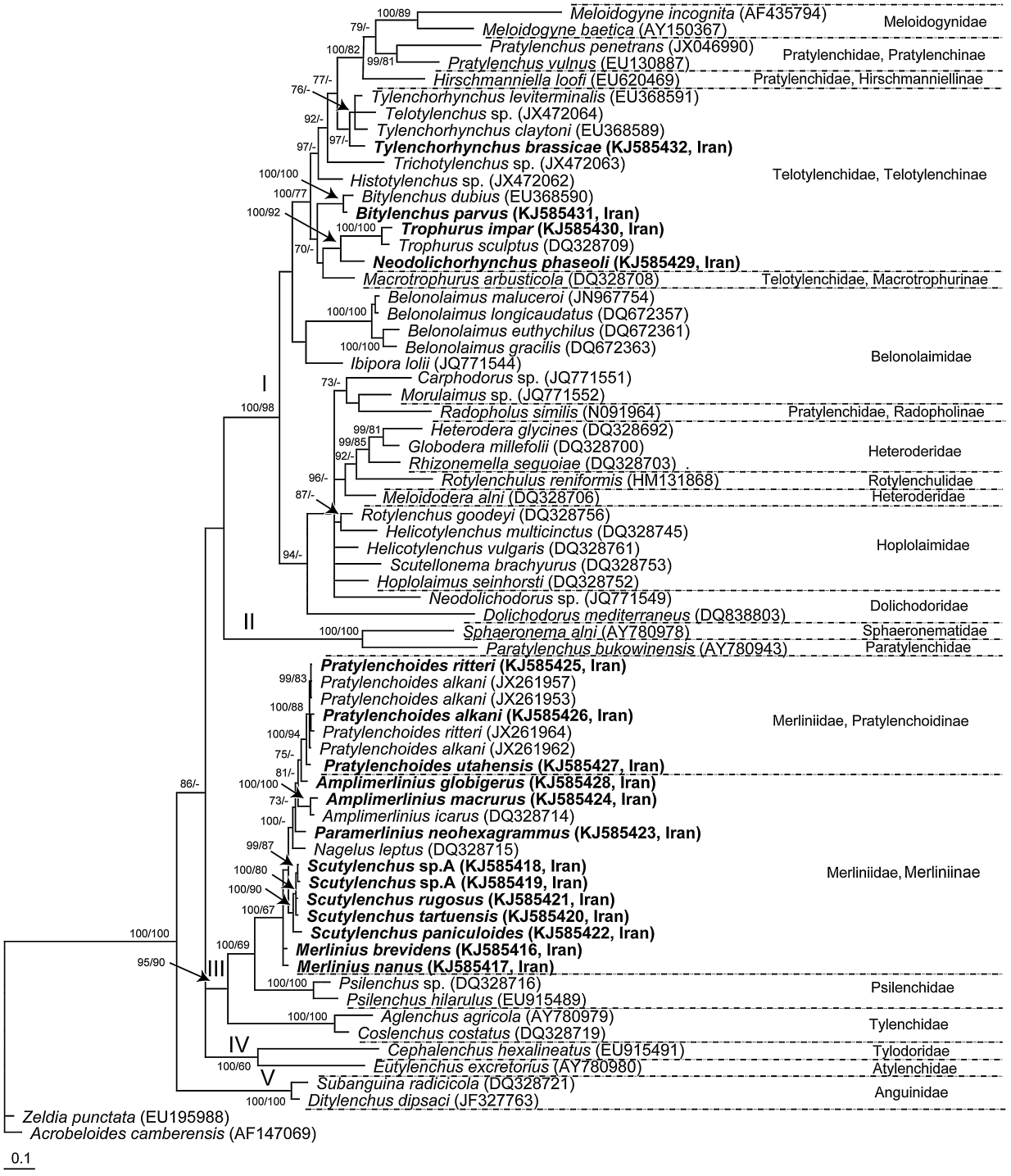

Fig. 4. Phylogenetic relationships within selected species of the order Tylenchida: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of the D2D3 of 28S rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities for BI/bootstraps for ML analyses equal to or more than 70% are given for appropriate clades. New sequences are indicated in bold. Division of the order Tylenchida into families and subfamilies are given according to Siddiqi (2000), except for nematodes of the Merliniidae, which is given according to Sturhan (2012).

Table 5. Results of the Shimodaira-Hasegawa tests for alternative hypotheses using ML trees.

Hypothesis tested	− ln L	Difference of − ln L	P^*
ML tree (Clade I)	10 584.00519	Best	–
Monophyly of Belonolaimidae *sensu* Siddiqi (2000)	10 645.46760	61.46241	0.028*
Monophyly of Dolichodoridae *sensu* Siddiqi (2000)	10 594.56864	10.56345	0.581
Monophyly of Telotylenchinae *sensu* Siddiqi (2000)	10 600.62986	16.62467	0.375
Monophyly of *Tylenchorhynchus sensu* Siddiqi (2000)	10 585.57701	1.57182	0.888
Monophyly of *Tylenchorhynchus sensu* Fortuner & Luc (1987)	10 663.59803	79.59283	0.001*
ML tree (Clade III)	3914.39618	Best	–
Monophyly of Merliniinae *sensu* Sturhan (2012)	3922.38558	7.98941	0.242
Monophyly of *Amplimerlinius sensu* Siddiqi (2000)	3915.70805	1.31188	0.624

ML trees were reconstructed from the partial alignment containing sequences from Clades I and III and the outgroup.
* $P < 0.05$ indicates significant differences between the two inferred tree topologies.

Siddiqi, 1980; Clade III consisted of representatives of the suborder Hoplolaimina (Merliniidae and Psilenchidae) as well as the suborder Tylenchina Chitwood *in* Chitwood & Chitwood, 1950 (Tylenchoidea: Tylenchidae); Clade IV included representatives of the suborder Tylenchina (Tylenchoidea: Tylodoridae and Atylenchidae); and Clade V contained representatives of the suborder Tylenchina (Anguinoidea: Anguinidae). The families Belonolaimidae and Dolichodoridae and the subfamilies Merliniinae and Telotylenchinae were non-monophyletic in the tree. However, the SH test (Table 5) did not exclude monophyly for Dolichodoridae, Telotylenchinae and Merliniinae. The SH test rejected the constrained monophyly for Belonolaimidae, and the monophyly of *Tylenchorhynchus*, Fortuner & Luc (1987), but did not exclude monophylies for *Tylenchorhynchus* and *Amplimerlinius*, Siddiqi (2000).

Discussion

In this study, 11 valid species and two populations of an unidentified species of Merliniidae and four valid species of Telotylenchidae were molecularly characterised. We should note that none of these species was collected from the type localities and, therefore, we are aware that future molecular analyses of type material of the studied species might contradict or confirm the species identification results obtained in this work.

Phylogenetic relationships within tylenchid nematodes reconstructed from the present dataset are mainly congruent with those published by Subbotin *et al.* (2006) and Stirling *et al.* (2013). The present analysis showed that Merliniidae and Telotylenchidae are not closely related, a feature that has also been shown in other molecular phylogenetic studies (Subbotin *et al.*, 2006; Bert *et al.*, 2008; Holterman *et al.*, 2009; Palomares-Rius *et al.*, 2009; van Megen *et al.*, 2009), and thus rejected the opinion of Fortuner & Luc (1987) according to which at least two paths of evolution can be recognised in Telotylenchinae, one towards genera such as *Amplimerlinius* and *Paratrophurus* Arias, 1970 that are closest to Pratylenchidae and Hoplolaimidae, whilst the other followed a divergent path towards typical *Tylenchorhynchus* and *Merlinius* with superficial root-grazing feeding habits. The present molecular analysis does not justify the erection of the superfamily Dolichodoroidea *sensu* Siddiqi (2000) or the Dolichodoridae *sensu* Decraemer & Hunt (2006), or the present genus composition of the Belonolaimidae *sensu* Siddiqi (2000) or Belonolaiminae *sensu* Decraemer & Hunt (2006).

Siddiqi (2000) distinguished four families within the Dolichodoroidea, namely Psilenchidae, Telotylenchidae, Dolichodoridae and Belonolaimidae. The present study indicated that the representatives of these families formed several groups within the tylenchid phylogenetic tree, showing close relationships only between some of them: *viz.*, between Psilenchidae and Merliniidae (former Merliniinae within Telotylenchidae *sensu* Siddiqi, 2000); between Telotylenchidae (except for former Merliniinae *sensu* Siddiqi, 2000) and one group of Belonolaimidae (including *Belonolaimus* Steiner, 1949 and *Ibipora* Monteiro & Lordello, 1977); and between Dolichodoridae and another group of Belonolaimidae (including *Carphodorus* Colbran, 1965 and *Morulaimus* Sauer, 1966). Considering molecular phylogenies, Hunt *et al.* (2013) have already pointed out that the Dolichodoridae evolved separately in at least three different clades within the Tylenchomorpha and is therefore a polyphyletic assemblage.

The family Belonolaimidae *sensu* Siddiqi (2000) was divided into two unrelated groups: *i*) including representatives of *Belonolaimus* and *Ibipora*; and *ii*) containing *Carphodorus* and *Morulaimus*. These groups are differentiated by morphology of the labial region (four-lobed *vs* not four-lobed) and biogeographical distribution (Americas *vs* Oceania). Thus, the results of phylogenetic analysis indicating the paraphyly of the family Belonolaimidae suggest the need for additional study with revision of this group.

The results of our phylogenetic analysis of Telotylenchinae showed grouping of species and genera mainly congruent with the classification of this subfamily proposed by Siddiqi (2000). Our study rejected the concept of the 'large genus' for *Tylenchorhynchus* as proposed by Fortuner & Luc (1987), in which *Bitylenchus* Filipjev, 1934, *Neodolichorhynchus* Jairajpuri & Hunt, 1984, *Telotylenchus* Siddiqi, 1960 and several other genera were considered as synonyms of *Tylenchorhynchus* but, alternatively, supported Siddiqi's view on the genus *Tylenchorhynchus* (Siddiqi, 2000). In the phylogenetic tree the representatives of *Tylenchorhynchus* and *Bitylenchus* formed two separate clades and the position of *Bitylenchus* was phylogenetically distinct from *Tylenchorhynchus*. *Bitylenchus* is distinguished from *Tylenchorhynchus* by having lateral fields with areolated outer bands, a large post-anal intestinal sac containing intestinal granules and fasciculi, relatively more thickened cuticle at the female tail terminus, and gubernaculum lacking a crest (Gomez Barcina *et al.*, 1992; Siddiqi, 2000). Thus, our molecular results support the views of Siddiqi (2000) and Andrássy (2007) on *Bitylenchus* as a separate genus from *Tylenchorhynchus* as opposed to Decraemer & Hunt (2006) and Geraert (2011), who considered *Bitylenchus* a junior synonym of *Tylenchorhynchus*.

In the taxonomic review of stunt nematodes having longitudinal lines or ridges on the cuticle, Jairajpuri & Hunt (1984), together with two known genera, *Dolichorhynchus* Mulk & Jairajpuri, 1974 and *Trilineellus* Lewis & Golden, 1981, described two new genera *Neodolichorhynchus* Jairajpuri & Hunt, 1984 and *Tessellus* Jairajpuri & Hunt, 1984. *Neodolichorhynchus* was erected for those species previously in *Dolichorhynchus* that have no lateral vulval flaps and a normal bursa. *Tessellus* was proposed for *T. claytoni* Steiner, 1937 and *T. pachys* Thorne & Malek, 1968 as the only remaining *Tylenchorhynchus* species with longitudinal cuticular lines. Siddiqi (2000) considered *Tessellus* as a synonym of *Tylenchorhynchus* and, within the genus *Neodolichorhynchus*, he distinguished three subgenera *Neodolichorhynchus*, *Mulkorhynchus* Jairajpuri, 1988 (= *Dolichorhynchus*) and *Prodolichorhynchus* Jairajpuri, 1985 differentiated by the number of incisures in the lateral field, presence or absence of vulval lateral cuticular membranes, and having a normal or notched bursa.

The results of our study indicate that *Neodolichorhynchus* (*Mulkorhynchus*) *phaseoli* is in a different phylogenetic position from *Tylenchorhynchus* species. Similar relationships between the two genera were observed by Carta *et al.* (2010) in the 18S rRNA gene tree. Moreover, two studied species of *N.* (*N.*) *microphasmis* and *N.* (*M.*) *lamelliferus* failed to group together. In light of the divergent position, Carta *et al.* (2010) concluded that the subgenus *Mulkorhynchus* might change rank in the future. The subgenus *Mulkorhynchus* can be distinguished from the subgenus *Neodolichorhynchus* by having lateral vulval flaps and a notched bursa (Jairajpuri & Hunt, 1984).

Our phylogenetic analysis does not support the validity of the genus *Tessellus* as *T. claytoni* clustered with other *Tylenchorhynchus* species. Longitudinal lines have been observed in other species of *Tylenchorhynchus* (*T. tobari* and *T. brevilineatus*), although in the latter two species they are limited to the neck region and are not so prominently visible along the whole body as in *Neodolichorhynchus*.

The genera *Telotylenchus*, *Histotylenchus* Siddiqi, 1971 and *Trichotylenchus* Whitehead, 1960 differ from *Tylenchorhynchus* in having pharyngeal glands extending over the intestine (Fortuner & Luc, 1987; Geraert, 2011). This difference remains the only diagnostic character differentiating *Telotylenchus* from *Tylenchorhynchus*, whereas *Histotylenchus* and *Trichotylenchus* are supported by additional characters. *Histotylenchus* has an asymmetrical stylet conus and intestine extending over the rectum into the tail, and *Trichotylenchus* has three incisures in the lateral field, an attenuated stylet, a different form to the gubernaculum, distinct *en face* view and elongate-subclavate to cylindroid female tail. In the tree, *Telotylenchus* clustered within *Tylenchorhynchus* species, whereas *Histotylenchus* and *Trichotylenchus* were in separate lineages. It may be suggested that overlapping pharyngeal glands are not a suitable character at generic level. Seinhorst (1971) noted that intermediate forms exist between the two glandular morphologies described as typical in the two genera *Telotylenchus* and *Tylenchorhynchus*, and Fortuner & Luc (1987) pointed out that there is no structural difference between forms with abutting glands and others with glands overlapping the anterior part of the intestine. They

also claimed that the two kinds of arrangements may coexist in the same family, in the same genus or even in the same species. For this reason, they proposed *Telotylenchus* as a synonym of *Tylenchorhynchus*, an action that was accepted by Brzeski (1998), but not by others (Siddiqi, 2000; Andrássy, 2007; Geraert, 2011; Hunt *et al.*, 2013).

Trophurus is characterised by its reduced posterior genital branch presented by a post-uterine sac, which make it unique among Telotylenchinae. As noted by Loof (1956), the V ratio is equal to about 50 in some monodelphic Tylenchidae, but in those cases the long filiform tail is responsible for this unusual situation. *Trophurus* has a short tail and yet the vulva is in the mid-region of the body (Fortuner & Luc, 1987). Bert *et al.* (2008) indicated that monodelphy is an ancestral character for tylenchid nematodes. The position of the *Trophurus* clade within the Telotylenchinae and a sister relationship between *Trophurus* and *Neodolichorhynchus* might indicate that monodelphy in *Trophurus* is the result of a secondary loss of the posterior genital branch during evolution.

In the present tree, representatives of the family Merliniidae clustered with a high statistical support with Psilenchidae, as has also been shown in the D2D3 of 28S rRNA gene tree (Subbotin *et al.*, 2006). Our phylogenetic analysis revealed the subdivision of Merliniidae into two subfamilies: Merliniinae and Pratylenchoidinae as proposed by Sturhan (2012). *Pratylenchoides* and *Amplimerlinius* are related taxa in the tree. This is in support of some findings concerning the morphological affinities of *Pratylenchoides* species with *Amplimerlinius* (Baldwin *et al.*, 1983; Ryss & Sturhan, 1994; Fortuner & Luc, 1987) and the transference of *Pratylenchoides* to the Merliniidae (Sturhan, 2012). In spite of distinct morphological differences between these two genera in structure of the lip region and labial framework, form of the pharyngeal glands and number of incisures in the lateral field, the differences in the D2D3 of 28S rRNA gene sequences are rather small for separating the two subfamilies Merliniinae and Pratylenchoidinae. Additional molecular studies using more taxa are required to clarify relationship between representatives of the Merliniidae.

Scutylenchus was originally proposed for *Tylenchorhynchus mamillatus* by Tobar-Jiménez (1966). However, Anderson (1977) and Sturhan (2012) considered this genus as a junior synonym of *Merlinius* or *Geocenamus*, respectively. Siddiqi (1979, 2000) revalidated *Scutylenchus* and listed the diagnostic characters as presence of longitudinal striae or grooves in the body cuticle and the absence of deirids. In the present study, three valid and one undescribed species of *Scutylenchus* formed a distinct clade within Merliniinae, thus supporting the view of Siddiqi (1979, 2000) on *Scutylenchus* as a distinct genus. However, the relationships of *Scutylenchus* with other genera should be further studied and tested by inclusion of additional sequences of species of *Merlinius* and *Geocenamus*. Three *Scutylenchus* species, namely *S. rugosus*, *S. tartuensis* and *Scutylenchus* sp. A, were very closely related in the tree, despite having distinct differences in the morphology of their labial region and tail shape (Figs 1, 2).

In this study, three *Pratylenchoides* species (*P. alkani*, *P. ritteri* and *P. utahensis*) were included. Brzeski (1998) and Karegar (2006) synonymised *P. alkani* with *P. ritteri*, although these species were considered as valid by Siddiqi (2000), Ryss (2007), Majd Taheri *et al.* (2013) and Geraert (2013). After studying Iranian isolates of *P. alkani* and *P. ritteri*, Majd Taheri *et al.* (2013) concluded there were no precise diagnostic characters to differentiate these two species, although they could be distinguished molecularly. However, comparison of the sequences provided by Majd Taheri *et al.* (2013) with those obtained in this study from new Iranian isolates of *P. alkani* and *P. ritteri* indicated that these species are molecularly indistinguishable and previous noticed differences might be treated as intraspecific variation of *P. ritteri*. Additionally, the results of morphological and biological studies have also shown that *P. alkani* should be considered as a synonym of *P. ritteri* (Ghaderi, unpubl.). The other species, *P. utahensis*, was placed in a separate clade from the six isolates of *P. ritteri* and *P. alkani*. This molecular difference is linked to obvious morphological diagnostic characters such as position of the subventral gland nuclei (at same level *vs* one anterior to the other) and intestinal-pharyngeal junction (lateral *vs* ventral), absence of fasciculi and male labial region shape (similar to female labial region *vs* conical and higher than that of female).

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