Additional data on an Afghani population of *Merlinius brevidens* (Allen, 1955) Brzeski, 1991 (Rhabditia: Merliniidae), with molecular study

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Summary. An Afghani population of *Merlinius* was collected from Bala orchard in Kabul. Based on morphological characters, it corresponds well with *M. pyri*, which was deemed similar to *M. brevidens* by Geraert (2011) in page 114. The characters were as follows: straight to arcuate body, lip region truncate or rounded bearing 5 annuli, lateral field consisting of 6 incisures, the outer two areolate, lip region continuous, stylet length 14-17 µm, DGO 1-2 µm, deirids situated above excretory pore, vulva with epiptygma and lateral membrane, spermatheca rounded to saccate, tail bearing 48-73 annuli, phasmid at middle of tail. The present of crenate outer incisures, greater number of tail annuli (70) in females, vulva membrane and developed epiptygma are new characters for *M. brevidens*. According to molecular phylogenetic analyses based on partial LSU rDNA and near full length SSU rDNA data the Afghani population of *Merlinius* and *Merlinius brevidens* formed a clade with high support. The partial LSU sequences of Afghani population were similar to available sequences of other populations of *M. brevidens* in gene bank and only had zero to 15 indels in comparison to them. Two newly LSU rDNA generated (MN947623, MN947624) and available sequences of *M. brevidens* fell in a clade including *M. brevidens*, *M. microdorus* and *M. nanus*. On the other hand, two newly SSU generated sequences (MN944559, MN944560) formed a clade with some species of *Amplimerlinius*, *Geocenamus*, *Merlinius*, *Nagelus* and *Pratylenchoides*. We propose that *M. pyri* be regarded as a junior synonym of *M. brevidens* based on morphological characters and on the comments of Geraert (2011). We propose new morphological and morphometric characters for *M. brevidens*.

Key words: molecular, morphology, morphometric, new synonym, phylogenetic tree, taxonomy.

Allen (1955) provided the first revision of the genus *Tylenchorhynchus* Cobb, 1913. Thorne and Malek (1968) drawn extract from it two genera, *Geocenamus* Thorne & Malek, 1968 and *Nagelus* Thorne & Malek, 1968. Afterwards the genus *Merlinius* Siddiqi, 1970 was proposed by morphologic characters for 32 species of *Tylenchorhynchus sensu lato*. The number of genera was increased tandemly.

The subfamily Merliniinae was erected by Siddiqi (1971) for the genus *Merlinius* Siddiqi, 1970. This subfamily is differentiated from Tylenchorhynchinae Eliava, 1964 based on six incisures in the lateral fields and usually presence of deirids. Siddiqi (1976) introduced five genera in Merliniinae: *Merlinius*, *Nagelus*, *Geocenamus*, *Scutylenchus* Jairajpuri, 1971 and *Amplimerlinius* Siddiqi, 1976.

The molecular studies of Subbotin *et al.* (2006), Holterman *et al.* (2009) and van Megen *et al.* (2009) demonstrated not only that Merliniinae and genera maintained in Telotylenchidae are arranged in different clades, but also that *Pratylenchoides* species are close to genera belonging to Merliniinae. Genera identification in Merliniidae is problematic and Sturhan (2012) provided the species included in the genera in agreement with the respective genus diagnosis and thus an allocation of individual nematode species to particular genera has been available. Sturhan (2012) confirmed *Merlinius* as a separate genus, while synonymising *Scutylenchus* with *Geocenamus*. The inability of attributing an interesting new Merliniinae species recovered in Germany to one of the accepted genera in this subfamily initiated the necessity of clarifying some more general taxonomic difficulties (Sturhan, 2011). The first results of these studies, which mainly concentrated on deirids and the incisures in lateral fields, were presented by Sturhan (2011, 2012).

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The type species of *Merlinius* is *Merlinius brevidens* (Allen, 1955) Siddiqi, 1970. This species has an extensive range of morphometric characters (Geraert, 2011), with body linear to arcuate, six incisures (up to ten in some specimens) in lateral field, head low and broadly rounded, vulval cavity and epiptygma small, spermatheca irregularly saccate, typically bi- or tri-lobed, often filled with sperm. The tail is sub-cylindrical, rounded or with flattened tip, and the ventral surface of tail is marked by 39-66 fine annuli. Males have a narrow bursa with finely crenate margins; and the gubernaculum is of uniform thickness, not protrusible. *Merlinius pyri* Fatema & Farooq, 1992 was shown to be very similar to *M. brevidens* by Geraert (2011) and should be regarded as a junior synonym of *M. brevidens*.

The study of plant-parasitic nematodes in Afghanistan has been conducted since 1971 (Faizyar, 1971). Khan (1982) and Khan and Khan (1977) reported several tylenchid genera in the Hirat region. Khan and Khan (1985) described *Hoplolaimus jalalabadiensis* (Khan & Khan, 1985) Ebsary, 1991 from the roots of fig trees in Jalalabad. Shahina and Maqbool (1993) described *Criconemoides afghanicus* from poplar host plant. Roivainen *et al.* (1988) gave a list including 88 genera and species of plant-parasitic and free-living nematodes from Afghanistan. Asghari *et al*. (2012) reported several tylenchid species from Bamyan and Mazari sharif provinces of Afghanistan. The present study is based on the analysis of several samples of soil and root collected from Kabul province. This is the first study of plant-parasitic nematodes in this province.

MATERIAL AND METHODS

Sampling, extraction, mounting, and drawing. During May and June 2018, ten soil samples were collected from Kabul province, Afghanistan. The nematodes were extracted by centrifugal flotation technique, killed and fixed by FGA (formalin, glycerin and acetic acid) and transferred to glycerin according to the Seinhorst (1959) and De Grisse method (1969). Permanent slides were made and studied using a light microscope (Nikon E600) and measured by means of a drawing tube attached to the microscope. The microphotographs were prepared using an Olympus BX51 light microscope having differential interference contrast (DIC) optics, equipped with an Olympus DP72 digital camera. Drawings were made by Corel DRAW® software version 12.

DNA extraction, PCR, and sequencing. Genomic DNA was extracted from DESS-preserved specimens with worm lysis buffer (Yoder *et al.*, 2006). The extraction was done with a fast method: a single nematode specimen was picked out from DESS and for 5 min placed in Petri dish containing distilled water, then transferred to a temporary slide with a small drop of AE buffer (10 mMTris-Cl, 0.5 mM EDTA; pH 9.0) on a clean slide. DNA samples were used as polymerase chain reaction (PCR) templates. To amplify D2-D3 domains of LSU rDNA and the near full-length fragment of SSU rDNA, three sets of primer pairs were used in the PCR reactions. The D2-D3 domains of the LSU rDNA were amplified using forward primer D2A (5'-ACA AGT ACC GTG AGG GAA AGT-3') and reverse primer 1006R (5'-AGG GGC GAA AGA CTA ATC GAA C-3') (Holterman *et al.*, 2008). For amplification of SSU rDNA forward 1813F (5'-CTG CGT GAG AGG TGA AAT-3') and reverse primer 2646R (5'-CTA CCT TGT TAC GAC TTT T-3') (Holterman *et al.*, 2006) and forward primer F22 (5'-TCC AAG GAA GGC AGC AGG C-3') (Dorris *et al.*, 2002) and reverse primer 1573R (5'-TAC AAA GGG CAG GGA CGT AAT-3') (Mullin *et al.*, 2005) were used. The PCR reaction and sequencings were performed as described by Panahandeh *et al.* (2016). The PCR products and amplicon sizes were verified in a 1.2% agarose gel and visualised by staining with Green Viewer™ (0.05 µl ml^{-1}). Sequencings were performed with Bioneer Corporation. The generated sequences were deposited into the GenBank database under the accession numbers: MN944559 and MN944560 for the near full-length SSU rDNA, and MN947624 and MN947624 for the partial LSU rDNA, respectively.

Phylogenetic analyses. The near full length SSU and partial LSU rDNA D2-D3 sequences of our specimens were compared with those of other tylenchid sequences available in GenBank using the basic local alignment search tool (BLAST) program. The alignment of selected sequences for both datasets was performed by Q-INS-i algorithm of MAFFT v.7.205 (Katoh & Standley, 2013). The poorly aligned and divergent regions were eliminated using the online version of Gblocks 0.91b (Castresana, 2000) using all three less stringent parameters (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). The best-fitting substitution model was selected using PAUP∗/MrModeltest.2 (Nylander, 2004). Bayesian analysis was performed using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) under the GTR + I + G model for both phylogenies with five independent runs and 4 × 106 generations. The Markov chains were sampled every 100 generations. The burn-in phase was set at 25% of the converged

runs. A 50% majority rule consensus tree was generated and posterior probabilities (PP) were calculated for each clade using the Markov chain Monte Carlo (MCMC) method within a Bayesian framework. The Tracer v1.5 software (Rambaut & Drummond, 2009) was used to visualise the results of each run in order to check the effective sample size of each parameter. The maximum likelihood (ML) analyses were performed using raxmlGUI 1.1 software (Silvestro & Michalak, 2012) with the same nucleotide substitution model as in the BI with 1 × 103 bootstraps (BS) for both datasets. The output files of the trees were visualised using Dendroscope V.3.2.8 (Huson & Scornavacca, 2012). The Bayesian posterior probability (BPP) and ML BS values exceeding 0.60 and 50%, respectively, were plotted on Bayesian 50% majority rule consensus trees.

Table 1. Morphometrics of *Merlinius brevidens* Allen, 1955 (all measurements are in µm and in the form: mean ± s.d. (range), except for the ratios a, b, c, c', V).

Characters	Afghani population of *Merlinius brevidens*			*M. pyri* (Geraert, 2011)		*M. brevidens* (Allen, 1955)		*M. brevidens* (Ghaderi *et al.*, 2014b)	
	Females		Males	Females	Males	Females	Males	Females	Males
n	12	CV	5	–	–	11	1	106	10
Body length	613.3 ± 47.9 (533-689)	7.8	599 ± 18.7 (578-626)	650-660	–	540-690	660	485-728	506-653
a	29.5 ± 2.4 (24.2-31.8)	8.1	33.0 ± 2.4 (30.8-36.8)	26.5-30	–	23-27	33	25-36	29-40
b	4.8 ± 0.3 (4.6-5.4)	5.4	4.7 ± 0.2 (4.4-5.0)	5.2-5.5	–	4.2-5.2	5.4	4.2-5.9	4.2-5.1
c	14.4 ± 2.2 (11.1-17.8)	15.4	12.8 ± 1.3 (11.0-15.5)	14.8-20.6	–	11-13	12	11-16	10-14
c'	3.3 ± 0.5 (2.7-4.2)	15.8	3.5 ± 0.4 (2.9-3.7)	2.3-2.7	–	–	–	2.5-4.6	
V/T	56.0 ± 1.5 (52.2-57.9)	2.7	42.6 ± 3.9 (39.4-49.5)	54-54.3	–	52-58	–	51-60	
Stylet	15.3 ± 1.1 (13.0-16.5)	7.1	14.9 ± 0.5 (14.0-15.5)	14-15	–	14-16	13	12-16	13-15
DGO	1.9 ± 0.3 (1.5-2.5)	16.6	1.8 ± 0.3 (1.5-2.0)	1.5	–	–	–		
Pharynx length	126.8 ± 7.7 (111-139)	6.1	127.2 ± 10.1 (116-142)	120-125	–	–	–	–	
MB	45.6 ± 1.8 (42.6-49.2)	4.0	47.0 ± 2.1 (44.4-50.0)	45	–	–	–	–	
Secretory-excretory pore	97.0 ± 6.4 (88-106)	6.6	93.8 ± 0.8 (93-95)	–	–	–	–	–	
Body width	20.9 ± 2.1 (17-25)	10.1	18.2 ± 0.8 (17-19)	23-25	–	–	–	–	
Head-vulva	343.2 ± 27.9 (293-392)	8.1	–	–	–	–	–	–	
Head-anus/cloaca	569.3 ± 48.8 (485-648)	8.6	549.4 ± 14.9 (537-574)	–	–	–	–	–	
Tail length	43.1 ± 4.6 (35-49)	10.7	49.6 ± 5.9 (40-55)	40	–	–	60	–	
Tail annuli	58.7 ± 11.6 (38-73)	19.8	58.8 ± 6.1 (50-65)	70	–	–	–	32-55	
Anal or cloacal body diam.	13.3 ± 1.4 (10-15)	10.3	14.2 ± 0.8 (13-15)	–	–	–	–	–	
Phasmid/tail%	48.9 ± 2.6 (45.5-52.5)	5.2	47.5 ± 2.8 (44.8-54.3)	–	–	–	–		
Spicule length	–	–	20.6 ± 0.5 (20-21)	–	20	–	15		17-22
Gubernaculum length	–	–	7.1 ± 0.5 (6-7.5)	–	6	–	10		

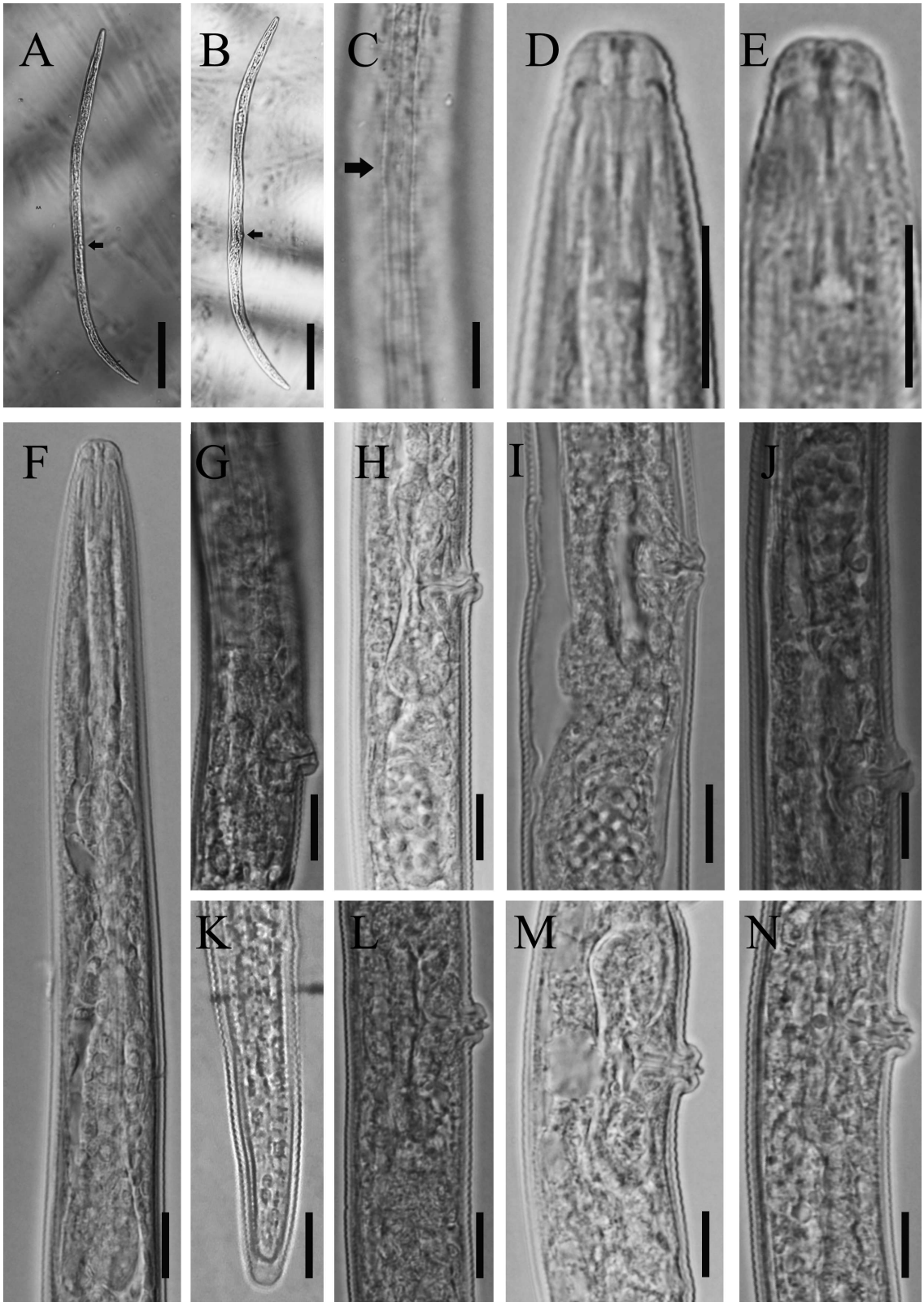

Fig. 1. Light microphotographs of *Merlinius brevidens*, females. A & B: entire female body, arrow showing protruding walls; C: crenate or areolated outer lateral lines, arrow showing deirids; D & E: rounded and truncate cephalic region, respectively; F: anterior region of body; G-J & L-N: vulva with epiptygma and lateral membrane, see variation of vulva cavity; K: tail. Scale bars: A & B = 100 µm; C-N = 10 µm.

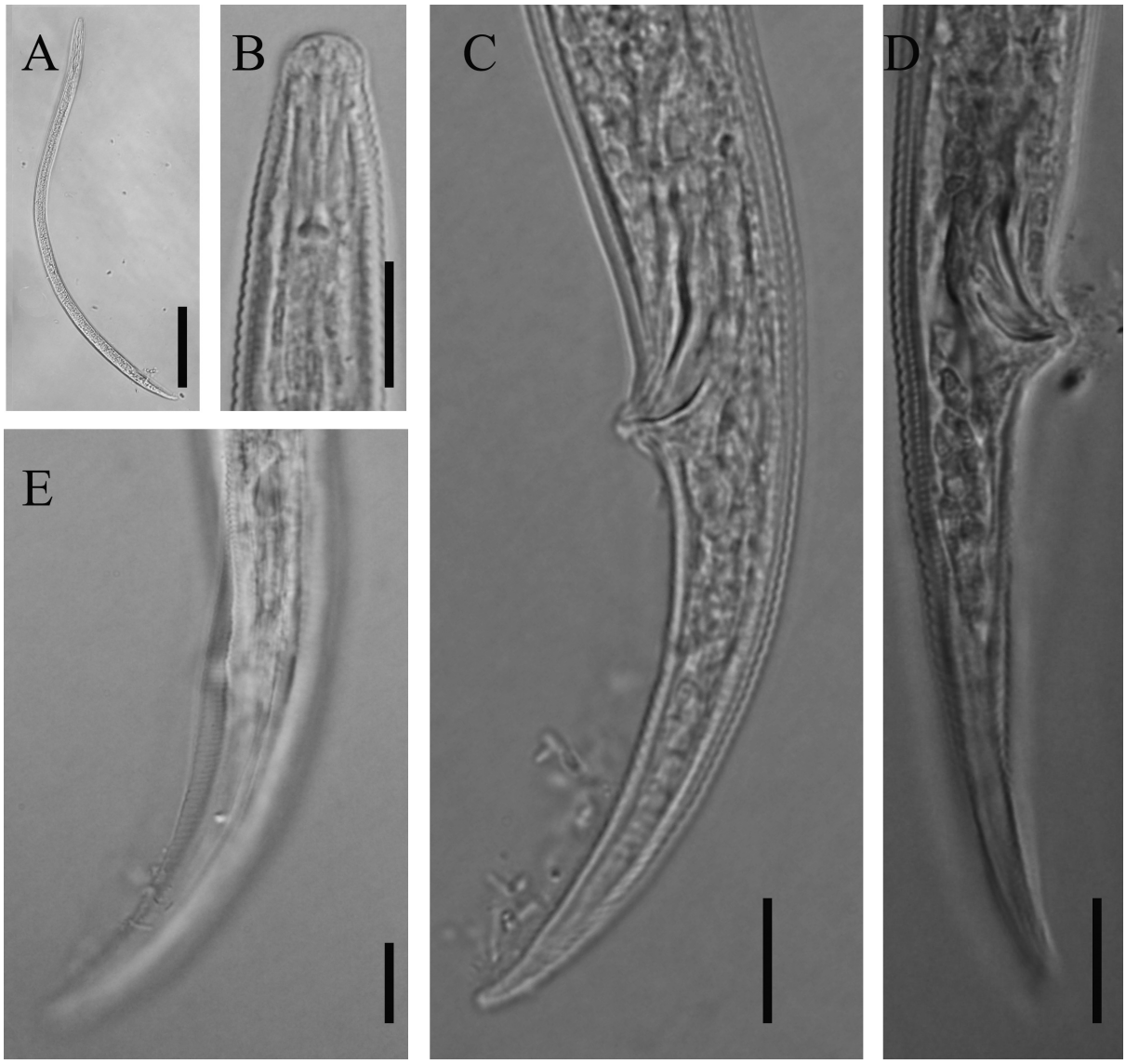

Fig. 2. Light microphotographs of *Merlinius brevidens*, males. A: entire body; B: cephalic region; C-E: male posterior end, C & D, showing hooked gubernaculum and protruding cloacal lips, E, showing bursa with crenate margin and phasmid. Scale bars: A = 100 µm; B-E = 10 µm.

DESCRIPTION

Merlinius brevidens (Afghani population) (Figs 1 & 2; Table 1)

Female. Body straight to C-shaped. Cuticle finely annulated, annuli 0.9-1.1 µm apart near mid-body. Lateral fields 4-6 µm wide or 25-32% of body width, consisting of six incisures, the outer two crenate. Lip region truncate or rounded, continuous, bearing 5-6 annuli, 6-8 µm wide and 3.5-4.5 µm high, usually with apex flattened or somewhat rounded, cephalic framework slightly sclerotised. Stylet slender, conus 7-9 µm, as long as shaft (m = 50-54.5), knobs 2-3.5 µm across, rounded, laterally directed, rarely anterior surfaces slightly sloping backward. Orifice of dorsal pharyngeal gland 1.5-2.5 µm from the base of stylet. Deirids at level of secretory excretory pore or one to three annuli anterior to the level of it, lateral fields with four lines at this level. Hemizonid one annuli long, two to three annuli anterior to the secretory-excretory pore is seen in two females. Median pharyngeal bulb oval, with plates, 12-17 × 9-12 µm, distance from the anterior end to centre of median bulb 57.8 (50-65) µm. Isthmus slender, 0.8-1.1 times as the length of the basal bulb. Nerve ring 70-77 µm from the anterior end is seen in five females. Basal pharyngeal bulb cylindrical, length/width ratio 2.4-2.9, offset from intestine, cardia prominent, discoid.

Vulva transverse slit, vulval lips strongly elevated with epiptygma or with both lateral membrane and epiptygma. Vagina 8-12 µm long and less than half body-width long, not sclerotised, usually dilated inwards as its walls curve out to uterus. Spermatheca round to saccate, axial and bilobed with sperm cells; unfilled in some individuals. Ovaries outstretched, with a single row of oocytes, except in multiplication region. Rectum about half of the anal body width, no post-anal intestinal sac. Tail sub-cylindrical, with 59.9 (38-78) annuli along the ventral side and tail terminus smooth, phasmids at middle of tail.

Male. Similar to females in general morphology, except for the reproductive system and posterior body end being slightly curved ventrally. Stylet length and development comparable with that of females. Spicules slightly arcuate with notched tips, 20-21 µm long. Gubernaculum slightly flattened at distal end, 6-7.5 µm long. Bursa enveloping entire tail, hypoptygma distinct. Phasmids at middle of tail length.

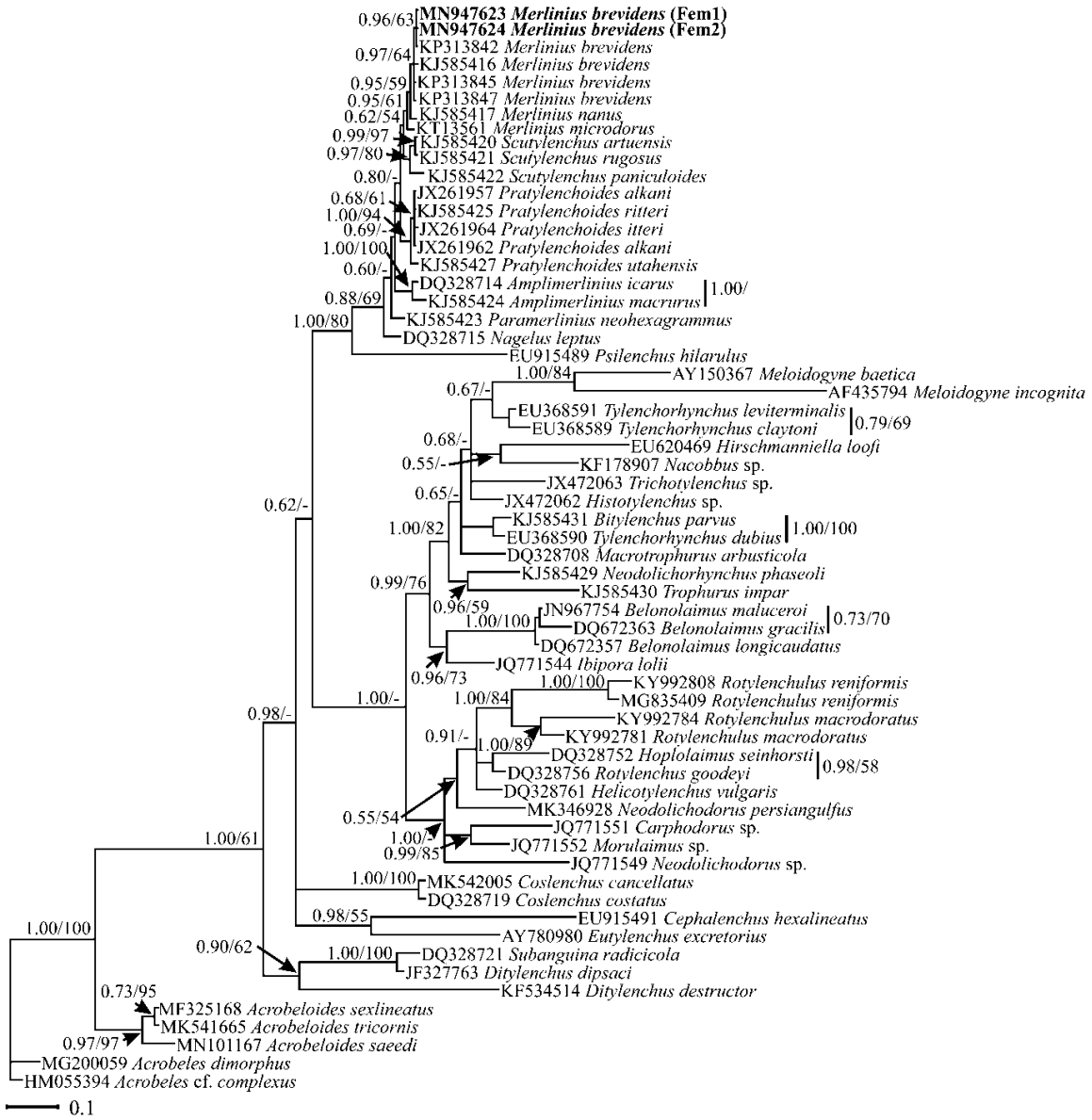

Fig. 3. Bayesian 50% majority rule consensus tree inferred from 60 rhabditid LSU D2-D3 sequences under GTR + G + I model. The Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap (ML BS) values exceeding > 0.5 and > 50 are given for appropriate clades in the shape BPP/ML BS. The Afghani population is in bold font.

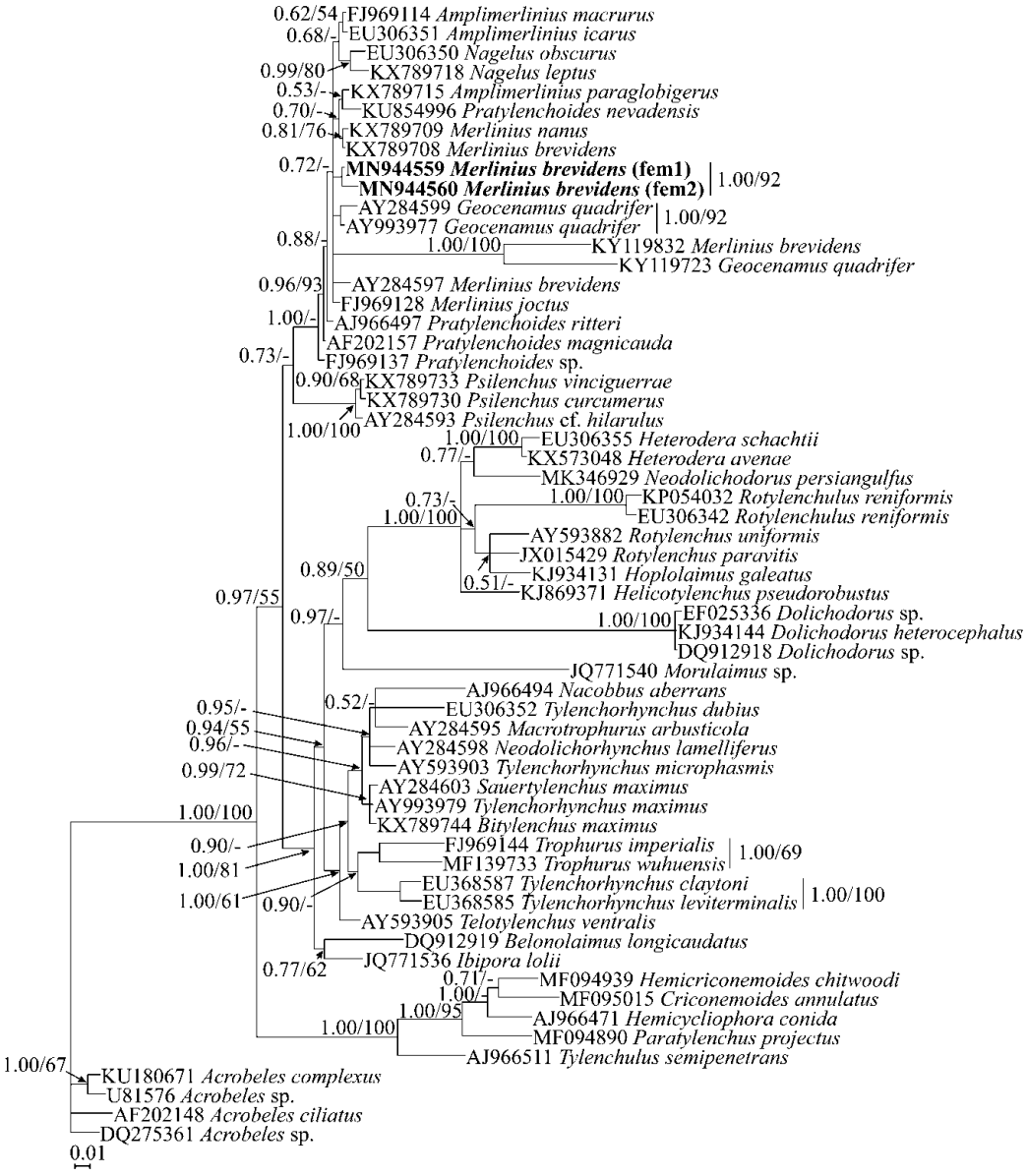

Fig. 4. Bayesian 50% majority rule consensus tree inferred from 60 rhabditid SSU sequences under GTR + G + I model. The Bayesian posterior probabilities (BPP) and maximum likelihood bootstrap (ML BS) values exceeding > 0.5 and > 50 are given for appropriate clades in the shape BPP/ML BS. The Afghani population is in bold font.

Juvenile. Head, pharynx, stylet and tail similar to females; tail sub-cylindrical and always with smooth terminus. Lateral field with four incisures in all of stages.

Type habitat and locality. Twelve females and five males collected from grapevine orchard (Bala orchard) in the western of Kabul city, Kabul province in southern east of Afghanistan. GPS coordinates: N 34°53'15531", E 69°12'66542".

Molecular characterisation and phylogeny. Sequencing of LSU rDNA D2-D3 expansion domains of two females of *M. brevidens* yielded two fragments, 958 (fem1, MN947623) and 788 bp long (fem2, MN947624). Both sequences were identical in their overlapping region after alignment. A BLAST search using one of the newly obtained D2-D3 sequences (fem2, MN947624) revealed it had 98.4-100% identity with unpublished isolates of *M. brevidens* (KP313841-KP313847). The other highly similar sequences belonged to a *M. brevidens* isolate (KJ585416) (Ghaderi *et al.*, 2014a) with 98.49% identity, a *M. brevidens* isolate

(MG770485) (Tzortzakakis *et al*., 2018) with 97.54% identity, and an unpublished *M. brevidens* isolate (MN262457) with 97.92% identity. A total of 60 sequences of *Merlinius*, other Merliniidae genera, other Tylenchomorpha taxa and five rhabditid taxa as outgroup were used for resolving the LSU phylogeny (Fig. 3). The two newly generated (MN947623, MN947624) and available sequences of *M. brevidens* fell in a clade, including *M. brevidens* and *M. nanus* (Allen, 1955) Siddiqi, 1970. The Afghani population of *M. brevidens* was the closest relative to other *M. brevidens* sequences in the LSU rDNA tree and had zero to 15 indels in comparison with their sequences in gene bank.

Sequencing of the SSU rDNA fragment of two females yielded two fragments, 1281 (fem1, MN944559) and 1028 bp long (fem2, MN944560). A total of 60 sequences of Tylenchomorpha taxa and four species of *Acrobeles* von Linstow, 1877 as outgroup were used for resolving the SSU phylogeny. The corresponding dataset was composed of 1600 characters. Figure 4 represents the phylogenetic SSU tree. The two newly generated sequences (MN944559, MN944560) fell in a clade including other Merliniidae genera specially *M. brevidens*.

DISCUSSION

Currently, around 32 nominal species have been described under the genus *Merlinius* (Sturhan, 2012). The elevated vulva lips with vulva membrane and conspicuous epiptygma has been reported for a few of these species; according to Geraert (2011) this character is seen in *M. indicus* (Zarina & Maqbool, 1995) Brzeski, 1998, *M. niazae* (Maqbool, Fatima & Hashmi, 1983) Brzeski, 1991, *M. pistaciei* Fatema & Farooq, 1992 and *M. pyri*, a species close to *M. brevidens*. The Afghani population of *Merlinius* is most similar to *M. pyri* that was similar to *M. brevidens* based on Geraert, 2011. The Afghani population of *M. brevidens* exhibits characters not seen before in *M. brevidens*, namely the developed vulval membrane, the epiptygma, with crenate outer incisures and greater number of tail annuli (70) in females. We therefore propose to amend the diagnosis *M. brevidens* to include a wider range of characters.

According to the phylogenetic tree (Fig. 3) *M. brevidens* and the Afghani population of *Merlinius* formed a clade with strong support and further studies revealed that they have the same molecular sequences in LSU rDNA. The partial LSU rDNA sequences of our population are almost identical to *M. brevidens* isolates available in GenBank corroborating the identity of the present population. According to the phylogenetic tree (Fig. 4) Afghani population sequences fell in a clade including other Merliniidae genera and *M. brevidens*.

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Z. Lotfi and A. Gharahkhani. Дополнительные данные по афганской популяции нематод *Merlinius brevidens* (Allen, 1955) Brzeski, 1991 (Rhabditia: Merliniidae) и их молекулярное изучение.

Резюме. Популяция нематод рода *Merlinius* была собрана в садах Бала, в Кабуле (Афганистан). По морфологическим особенностям эти нематоды близки к *M. pyri*, который считается сходным с *M. brevidens* (Geraert, 2011, стр. 114). Признаки сходства: тело прямое или изогнутое, губной отдел усеченный или закругленный с 5 кольцами, не отделенный от тела, латеральное поле с шестью инцизурами, причем две наружных линии – с насечками (продолжение кольчатости), длина стилета 14-17 мкм, отверстие дорсальной железы 1-2 мкм, дейриды перед экскреторной порой, вульва с эпиптигмой или боковыми мембранами, сперматека округлая или мешковидная, хвостовой отдел с 48-73 кольцами, фазмиды в средней части хвостового конца. Наружные инцизуры с насечками, большое число хвостовых колец кутикулы (70) у самок, мембраны на вульве и развитая эпиптигма – новые признаки для *M. brevidens*. В соответствии с анализом частичных нуклеотидных последовательностей LSU rDNA и почти полной последовательности SSU rDNA афганская популяция *Merlinius* и *Merlinius brevidens* образуют единую группу с высокой поддержкой. Частичная последовательности LSU афганской популяции сходна с таковыми у других популяций *M. brevidens*, будучи идентичной или имея до 15 нуклеотидных замен. Вновь полученные последовательности LSU rDNA (MN947623, MN947624) и имеющиеся в ГенБанке последовательности *M. brevidens* образуют группу, состоящую из *M. brevidens*, *M. microdorus* и *M. nanus*. Две полученные последовательности SSU (MN944559, MN944560) формировали кладу с видами родов *Amplimerlinius*, *Geocenamus*, *Merlinius*, *Nagelus* и *Pratylenchoides*. Предложено рассматривать *M. pyri* как младший синоним *M. brevidens*, в соответствии с морфологическим сходством и замечаниями Geraert (2011). Приводятся морфологические и морфометрические данные для *M. brevidens*.