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Morphological and Molecular note on the identity of *Mylonchulus sigmaturus* Cobb, 1917 (Nematoda: Mylonchulidae) from Pakistan

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Summary

A species of predatory nematode, *Mylonchulus sigmaturus* Cobb, 1917, was recovered around the soil and roots of banana plants (*Musa paradisiaca*) from four different localities of Pakistan. The male of this species represents a new record from Pakistan. Morphological and morphometric data of the species have been contributed along with the molecular study. The phylogenetic analysis using 18S rDNA placed the Pakistani populations of *M. sigmaturus* with the same species in a clade with 100 posterior probabilities. The first input of 28S rDNA data placed Pakistani *M. sigmaturus* in a separate clade with 100 posterior probability support, however close with *Prionchulus punctatus* **(**Cobb, 1917) Andrássy, 1958 and *Clarkus papillatus* (Bastian, 1865) Jairajpuri, 1970.
Keywords: *Mylonchulus*; morphometric; phylogeny; predatory nematode; rDNA

Introduction

The order Mononchida was proposed by Jairajpuri in 1969. These nematodes are predators in nature and found in different habitats both terrestrial and aquatic (Ahmad & Jairajpuri, 2010).

The predator nematodes are free-living nematodes, they usually use other nematodes and invertebrates as feed (Ahmad and Jairajpuri, 2010) and distributed worldwide (Koohkan *et al*., 2014). The genus *Mylonchulus* Cobb, 1916 comprises of 62 nominal species (Ahmad and Jairajpuri, 2010; Jana *et al*., 2010; Shah and Hussain, 2015) with the type species *M. minor* (Cobb, 1893) Cobb, 1916. From Pakistan, a total of twelve species of the genus *Mylonchulus* Cobb, 1916 have so far been reported from different climatic zones, including *M. amurus* Khan and Jairajpuri, 1979; *M. brachyuris* (Butschli, 1873) Cobb, 1917; *M. contractus* Jairajpuri, 1970; *M. lacustris* (Cobb in Cobb, 1915) Cobb, 1917; *M. minor* (Cobb, 1893) Cobb, 1916; *M. nainitalensis* Jairajpuri, 1970; *M. paitensis* Yeates, 1992; *M. rosensis* Khan, 1975 and *M. sigmaturus* Cobb, 1917 (Shahina *et al*., 2019). A new species, *M. capsicumi* along with two new recorded species *viz*., *M. polinicus* (Stefanski, 1915) Cobb, 1917 and *M. maritimus* Jimenez-Guirado and Murillo-Navarro, 2008 were described and redescribed, respectively (Ishaque *et al*., 2021). *M. sigmaturus* was first reported by Khan and Saeed, 1987 from the soil around the roots of banana plantation, Malir and cultivated soil from Jam Goth Sindh, Pakistan. In the present study, four different populations of *M. sigmaturus* were recovered from the rhizosphere of banana (*Musa paradisiaca* L.) in different localities of Pakistan. The male of this species is reported for the first time from Pakistan. Therefore, the aims of the research were to study *M. sigmaturus* using morphological, multivariate and molecular analysis using 18S and 28S rDNA regions.

Materials and Methods

Nematode extraction and morphological identification

Nematodes were extracted from soil samples by Cobb's wet sieving and decanting methods (Cobb, 1918), followed by the modified Baermann funnel technique (Baermann, 1917). Spec-

* – corresponding author

Table 1. Morphometric data of different populations of *Mylonchulus sigmaturus* along with other populations of *M. sigmaturus* from different ecological zones. All measurements are in µm except body length.

References	**Present specimens**		**Koohkan *et al*., (2014)**	**Ahmad *et al*., (2010)**	**Farahmand *et al*., (2009)**	**De Bruin & Heyns (1992)**	**Chaves (1990)**	**Khan & Saeed (1987)**	**Coetzee (1966)**	**Mulvey (1961)**
Locality	***Pakistan***		***Iran***	***Japan***	***Iran***	***South Africa***	***Argentina***	***Pakistan***	***South Africa***	***Sweden***
n	**40**♀♀	**1**♂	5 ♀♀	10♀♀	11♀♀	1 ♀	18 ♀♀	1♀	4 ♀♀	58♀♀
L	1.22 ±1.056 (1.04– 1.46)	1.21	1.294 ± 4.94 (1.22 – 1.36)	1.30 ± 0.08 (1.16 – 1.46)	1.20 ±1.29 (1.01 – 1.46)	1.25	1.12 ± 0.1 (1.0 – 1.32)	0.95	1.1 (1.1 – 1.2)	1 – 1.7
a	28.04 ±2.02 (25.8 – 33.7)	31.8	31.7 ± 1.3 (30.1 – 33.3)	29.7 ± 1.27 (27.3 – 31.8)	28.0 ± 2.1 (24.1 – 30.7)	34.7	29.7 ± 2.3 (26 – 36.2)	23	23.1 (29 – 33)	24 – 47
b	3.475 ± 0.196 (3.1 – 3.9)	3.4	3.5 ± 0.1 (3.4 – 3.6)	3.37 ± 0.12 (3.16 – 3.55)	3.7 ± 0.2 (3.2 – 3.9)	3.3	3.3 ± 0.2 (3.2 – 3.8)	4	3.2 (3.1 – 3.3)	3 – 4
c	35.17 ± 3.52 (26.0 – 40.93)	28.8	32.8 ± 5.6 (27.8 – 42.1)	35 ± 2.4 (30.5 – 38.6)	27.5 2.6 (26.6 – 30.7)	37.9	30 ± 2.6 (2558 – 32.2)	17.9	26.9 (25 – 29)	24 – 39.2
ć	1.205 ± 0.115 (1.0 – 1.42)	1.2	1.5 ± 0.2 (1.2 – 1.9)	1.21 ± 0.07 (1.1 – 1.3)	1.4 ± 0.2 (1.1 – 1.8)	1.5	1.4 ± 0.2 (1.2 – 1.6)	1.2	1.4	1 – 1.8
V	65.72 ± 2.217 (60.41 – 70.6)	--	64 ± 1.1 (63.1 – 65.9)	65.14 ± 1.4 (62.9 – 67.3)	62.8± 1.9 (59.7 – 66.4)	65	64.6 ± 1.5 (62 – 68)	58.4	66.0 (66 – 67)	50 – 77
G_1	9.92 ± 1.50 (6.1 – 14.0)	--	18.7 ± 1.5 (18 – 20)	14.15 ± 3.69 (7 – 20)	12.1 ± 0.8 (11.1 – 12.9)	-	-	13.7	1.4 (9.2 – 11.8)	-
G_2	9.731 ± 1.77 (5.4 – 14.4)	--	18.0 ± 4.0 (13 – 22)	11.3 ± 2.5 (9 – 18)	10.5± 1.3 (8.5 – 11.7)	-	-	-	9.5 (9.1 – 9.8)	-
Lip region width	24.54 ± 1.44 (22 – 27)	22	24.2 ± 1.5 (23 – 27)	25.1 ± 1.4 (23 – 27)	24.6±1.8 (22.4 – 28)	23	21.5 ± 1.8 (20 – 25)	23.2	19.1	-
Buccal cavity length	25.75 ± 1.198 (25 – 27)	26	24.7 ± 0.8 (24 – 26)	25 ± 1.06 (24 – 27)	22.9± 1.4 (21 – 25.2)	26	22.7 ± 1.4 (20 – 25)	26.5	22.1	20 – 31
Buccal cavity width	16 ± 2.01 (15 – 17)	15	14.1 ± 0.4 (14 – 15)	15.2 ± 0.4 (15 – 16)	15.8±0.7 (15.4 – 16.8)	13	14.3 ± 1.0 (13 – 16)	13.1	9.6	11 – 17
Dorsal tooth apex % of buccal cavity	78.5 ± 1.945% (74 – 80%)	77	81.2 ± 2.6 (79 – 85)		80.2±4.6 (72.2 – 87.6)	15	77.2 ± 2.6 (74 – 81)		20	-
Amphid position from anterior end	9 ± 1.07 (8 – 10)	11	12±0.0 (12)	-	-	-	-	-		-
Amphidial aperture diameter	4.50±0.31 (4 – 5)	5	3.0±0.0 (3)	-	-	-	-	-	-	-
Excretory pore from anterior end	102.5 ± 3.05 (94 – 113)	--	-	-	-	-	-	-	-	-

Nerve ring from anterior end	82.166 ± 3.46 (80– 90)	84	104.9±11.3 (89 – 117)	108 ± 3.6 (102 – 114)	99.6±11.9 (87.5 – 122.5)	-	-		-	-
Neck length	348.8 ± 17.6 (318 – 372)	352	367.5± 10.2 (355 – 379)	386 ± 12 (370 – 410)	330.6±29.3 (297.5 – 392)	-	293.7 ± 12.3 (279 – 309)		-	-
Body diameter at:		--		-	-	-	-	-	-	-
Neck base	37.12 ± 3.91 (30 – 47)	36	37.1±1.8 (35 – 39)	-	-	-	-	-	-	-
Mid body	43.75 ± 3.78 (38 – 50)	38	40.9±2.3 (39 – 44)	-	-	-	-	-	-	-
Anus	29.04 ± 1.92 (26 – 33)	37	27.3±3.0 (24 – 31)	-	30.9±1.4 (28 – 31.5)	-	-	-	-	-
Rectum	21.91 ± 1.49 (20 – 24)	--	21.4±2.1 (18 – 24)	21 ± 2.4 (17 – 25)	24.5±3.1 (21 – 28)	-	-		-	-
Tail length	35 ± 4.20 (30 – 50)	42	40.3±6.2 (31 – 47)	38.6 ± 2.6 (35 – 42)	44.2±5.5 (35 – 52.5)	33	33.2 ± 3.5 (29 – 37)		26.5	-
Tail length as % of total body length	2.76 ± 0.288 (2.26% – 3.38%)	3.46	3.1±0.5 (2 – 4)	-	-	-	-	-	-	-
Spicules	--	46	--	--	--	--	--	--	--	--
Gubernaculum	--	18	--	--	--	--	--	--	--	--

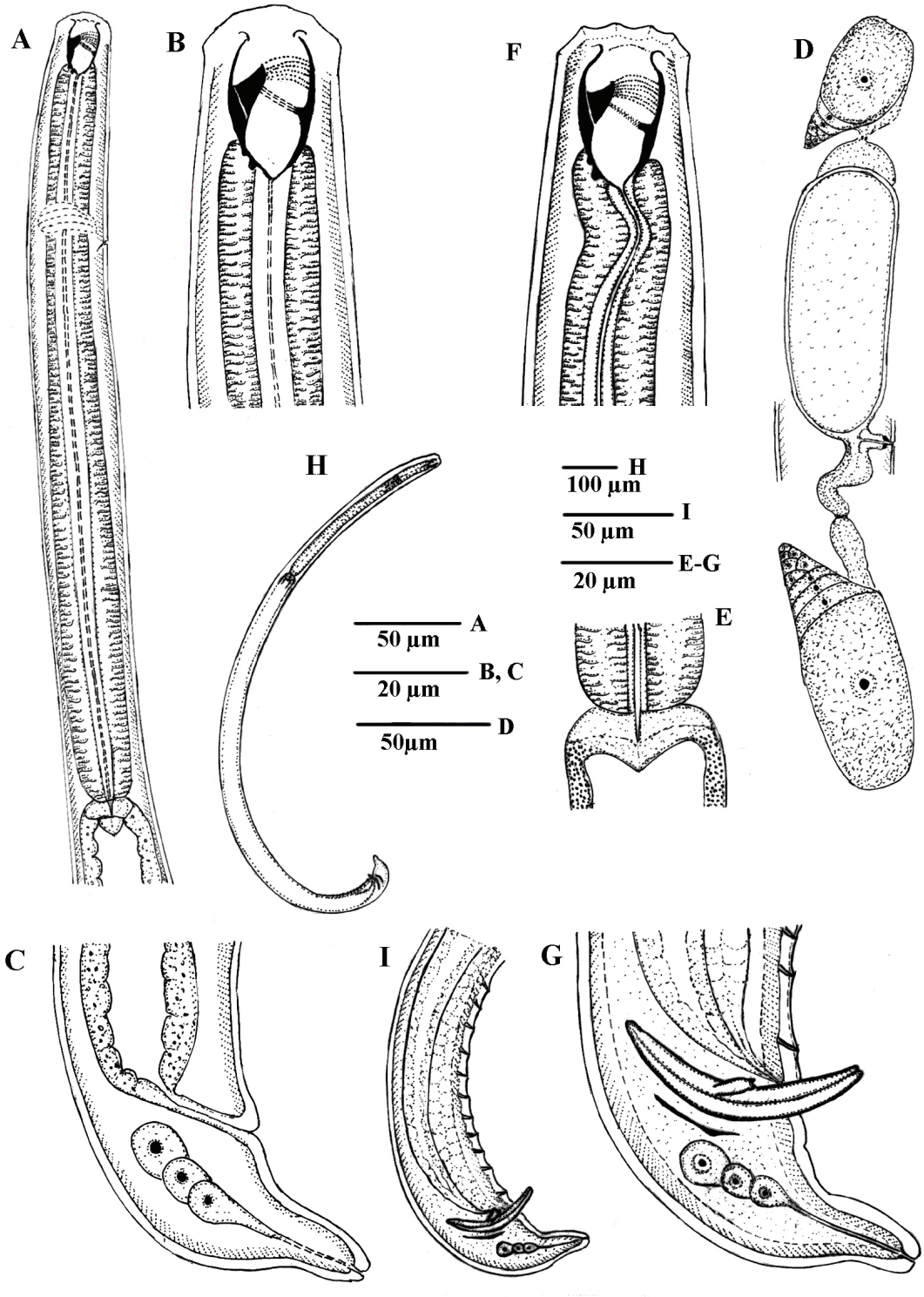

Fig. 1. *Mylonchulus sigmaturus* Cobb, 1917, **Female**: A: Pharyngeal region; B: Anterior region; C. Tail; D: Reproductive system; E. Cardia; **Male**: F: Anterior region, G. Tail region, H. whole body; I. Tail having precloacal genital papillae.

imens were fixed with hot 4 % formaldehyde solution and processed to anhydrous glycerin by the method of Seinhorst's method (Seinhorst, 1959). Preserved specimens were observed under different magnifications of a compound microscope. Measurements were taken directly using an ocular micrometer. De Man's formula was used for denoting the dimensions of the nematodes. Line drawing was made with the aid of a camera lucida attached to the microscope and photomicrographs were taken by Nikon DS L2 camera. Ahmad and Jairajpuri (2010) was followed for taxonomic descriptions.

Statistical analysis

The principal component analysis was used to study the variation among the populations of *M. sigmaturus* and *M. arenicolus*. We have included *M. arenicolus*, because of being placed together with *M. sigmaturus* through molecular analysis of 18S rDNA. A total of fourteen populations were analyzed for this study. Twenty-two morphometric characters, *viz*. body length (L), a, b, c, c', V, G1, G2, lip region width (LW), buccal cavity length (BCL), buccal cavity width (BCW), dorsal tooth apex % (DTA%), amphids opening to anterior end, amphid diameter, nerve ring to anterior end (NR), neck length, neck base diameter (NBD), mid-body diameter (MBD), anal body diameter (ABD), rectum, tail length (TL), and tail length % of body length (TL%BL) were used for analysis. Measurements for the PCA were taken from original descriptions. Data on the morphometric measurements of the species were analyzed using XLSTAT (Addinsoft, 2007). The morphometric measurements for the PCA analysis were taken from the original descriptions. The measures were normalized using XLSTAT software before their analysis (Addinsoft, 2007). The score values were determined for each species based on each of the principal components. The scores for the first two components were used to form a two-dimensional plot (F1 and F2) of each isolate based on the eigenvalues given by the software XLSTAT.

DNA extraction, PCR, and phylogenetic analysis

DNA extraction was done using the Chelex method (Shokoohi, 2021). Two specimens of each species were hand-picked with a fine tip needle and transferred to a 1.5 ml microcentrifuge tube containing 5 µl double distilled water. The nematodes in the tube were crushed with the tip of a fine needle and vortexed. Thirty microliters of 5 % Chelex® 50 and 2 µl of proteinase K were added to each of the microcentrifuge tubes that contained the crushed nematodes and mixed. These separate microcentrifuge tubes with the nematode lysate were incubated at 56 °C for two hours and then incubated at 95 °C for 10 minutes to deactivate the proteinase K and finally spun for 2 min at 16000 rpm (Shokoohi and Abolafia, 2021). The supernatant was then extracted from each of the tubes and stored at –20 °C. Following this step, the forward and reverse primers, SSU F04 (5'-GCTTGTCTCAAAGATTAAGCC–3') and SSU R26 (5'-CATTCTTGGCAAATGCTTTCG–3') (Blaxter *et al*., 1998); D2A (5'-ACAAGTACCGTGAGGGAAAGTTG–3'), D3B (5'-TCGGAAGGAACCAGCTACTA–3') (De Ley *et al*., 1999), were used in the PCR reactions for partial amplification of the 18S rDNA, and 28S rDNA regions. PCR was conducted with 5 µl of the DNA template, 12.5 µl of 2X PCR Master Mix Red (Promega, USA) for the Pakistani specimens, 1 µl of each primer (10 pmol μl^{-1}), and ddH2O for a final volume of 30 µl. The amplification was processed using an Eppendorf master cycler gradient (Eppendorf, Hamburg, Germany), with the following program: initial denaturation for 3 min at 94 °C, 37 cycles of denaturation for 45 s at 94°C; 54 °C, and 56 °C annealing temperatures for 18S, and 28S rDNA, respectively; extension for 45 s to 1 min at 72 °C, and finally an extension step of 6 min at 72 °C followed by a temperature on hold at 4 °C. After DNA amplification, 5 µl of product from each tube was loaded on a 1 % agarose gel in TBE buffer (40 mM Tris, 40 mM boric acid, and 1 mM EDTA) for evaluation of the DNA bands. The bands were stained with GelRed® and visualized and photographed on a UV transilluminator. The amplicons of each gene were stored at –20 °C. Finally, the PCR products were purified for sequencing. Available sequences for other Mononchida were obtained from NCBI GenBank for comparison. Also, *Mermis nigrescens* Dujardin, 1842 (KF583882; AF036641) and (KF886018) were used as outgroups for 18S and 28S rDNA, respectively. Outgroups were selected based on Van Megen *et al*. (2009). The ribosomal DNA

Table 2. Factor loading for different populations of *M. sigmaturus*.

	F1	F2
Body length (BL)	0.486	0.360
a	0.170	0.833
b	0.110	-0.568
c	0.106	0.550
c'	-0.621	0.398
V	0.116	0.430
G1	-0.799	0.256
G2	-0.816	0.231
Lip region width (LW)	0.897	-0.109
Buccal cavity length (BCL)	0.576	0.164
Buccal cavity width (BCW)	0.614	0.350
Dorsal tooth apex (DTA%)	0.141	-0.357
Amphid to anterior end	0.109	0.532
Amphid diameter	0.082	-0.769
Nerve ring to anterior end (NR)	0.412	0.150
Neck length	0.851	0.005
Neck base diameter (NBD)	0.104	0.646
Mid-body diameter (MBD)	0.980	-0.123
Anal body diameter (ABD)	0.867	-0.180
Rectum	0.941	-0.123
Tail length (TL)	0.903	0.083
TL%BL	0.803	0.380

sequences were analyzed and edited with BioEdit (Hall, 1999) and aligned using CLUSTAL W (Thompson *et al*., 1994). The length of the alignments was 1015 and 850 bps for 18S, and 28S rDNA, respectively. Phylogenetic trees were generated using the Bayesian inference method as implemented in the program Mr Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The GTR model was selected using jModeltest 2.1.10 (Guindon & Gascuel, 2003; Darriba *et al*., 2012). Then, the selected model was initiated with a random starting tree and ran with the Markov chain Monte Carlo (MCMC) for 106 generations. The 18S sequence alignments were also used to construct phylogenetic Median Joining Networks using PopART software (http://popart.otago.ac.nz) (Bandelt *et al*., 1999). The partial rDNA sequences of *M. sigmaturus* were deposited in GenBank under the accession numbers: OM278259-OM278262 for 18S rDNA, and OM317560-OM317561 for 28S rDNA.

Taxonomy Section

The genus *Mylonchulus* is characterized by goblet or funnel shaped stoma, dorsal wall thicker than ventral. Dorsal tooth large directed forward with pointed apex in anterior half of stoma. Subventral walls bearing several small rasp like denticles arranged in rather regular transverse rows. Often ventro-sublateral denticles opposite to the base of the dorsal tooth. Pharyngo- intestinal junction non-tuberculate.

Female genital system didelphic, amphidelphic. Spicules slender, arcuate, gubernaculum simple or bidentate with or without lateral accessory pieces. Tails similar in both sexes, short or slightly elongated with usually conspicuous glands and terminal or subterminal spinneret.

Mylonchulus sigmaturus was originally described by Cobb, 1916 from various parts of the United States and Mexico. This species is recognized by the outlandish ventrally bent tail which is similar in both males and females. Tail of the male bears a series of 13 to 15 low mammliform accessary organs which have distinct connections with the inner body tissues. Spicule slightly arcuate, tapering and subacute and about as long as the anal body diameter. The wedge shaped accessory pieces taper at both ends. Besides the accessory pieces, there are furcate lateral guiding pieces. The caudal glands lie tandem in the tail (Thorne, 1924).

Table 3. Factor score for different populations of *M. sigmaturus*.

Observation	F1	F2
P1	-0.629	-1.704
P2	-0.112	-2.305
P3	0.224	0.378
P4	0.436	0.645
Koohkan et al., (2014); Iran	-0.725	1.769
Chaves (1990); Argentina	-2.506	0.567
Coetzee (1966); South Africa	-2.479	-0.303
de Bruin & Heyns (1992); South Africa	-1.260	2.193
Khan & Saeed (1987); Pakistan	-1.636	-4.145
Ahmad et al., (2010); Japan	-0.034	1.920
Farahmand et al., (2009); Iran	0.122	-0.773
Mulvey (1961); Sweden	-0.414	2.491
M. arenicolus	9.168	-0.271

***Mylonchulus sigmaturus* Cobb, 1917**
(Fig. 1. A-I; 2. A-O)

Measurements: Table 1.

Female: Medium sized slender nematodes, with cylindroid, ventrally curved C-shaped body upon fixation. Body truncate anteriorly and arcuate-digitate at posterior end. Cuticle smooth under light microscopy, 1 – 2 µm thick at mid body. Lateral chord about one third of body width at mid body. Lip region is almost continuous with the adjoining body, about 2.5 – 3.0 times as wide as high and about two thirds as wide as body width at neck base. Labial and cephalic papillae distinct and prominent interfering with the labial contour. Amphidial aperture slit like, the openings appear to be higher than the dorsal tooth apex, 16 – 20 % of lip region width. Buccal cavity funnel shaped, thick walled, tapering at base 1.58 – 1.66 times as long as wide. Subventral teeth well developed, 15 – 17 µm from anterior end. Ventro-sublateral denticles arranged in seven rows: a pair of ventro-sublateral foramina present near the oblique basal plate of stoma. Dorsal tooth strong, claw like, obliquely forward directed, sharply pointed terminus, 9 – 11 µm long, 6 – 9 µm wide, situated in the anterior half of buccal cavity and its apex situated at 74 – 80 % from base of stoma. Nerve ring located at 21 – 24 % of the neck length from anterior end. Pharyngo-intestinal junctional non-tuberculate. Excretory pore 94 – 106 µm from anterior end. Genital system didelphic amphidelphic, with both branches almost equally developed. Vulva a transverse slit, vagina strongly muscular, extending inwards to about one third corresponding of body width with *pars refringens* consisting of two small drop shaped sclerotized pieces in lateral view, each measuring 3 – 4 × 1.5 – 2 µm. Ovaries generally small, reflexed with oocytes arranged in a single or two rows. Oviduct with distinctly swollen *pars dialata*; anterior ovary 50 – 80 µm and posterior ovary 60 – 100 µm long. Sphincter at the oviduct-uterus junction, well developed; uterus like structure, with distal part slightly swollen (*pars dialata*). Three females with a uterine egg measuring 100 – 106 × 35 – 38 µm oblong. Vulva a transverse slit, vagina strongly muscular, extending inwards to about one third corresponding of body width with *pars refringens* consisting of two small drop shaped sclerotized pieces in lateral view, each measuring 3 – 4 × 1.5 – 2 µm. Advulval papillae absent. Rectum 0.75 – 0.76 times anal body width long. Tail short conoid, curved ventral, with its ventral side strongly curved and the dorsal one convex, often tapering near the terminus giving a digitate appearance. Caudal glands well developed arranged in tandem spinneret opening terminally.

Male: General morphology similar to that of female except for being more strongly curved ventrally in the posterior region of body.

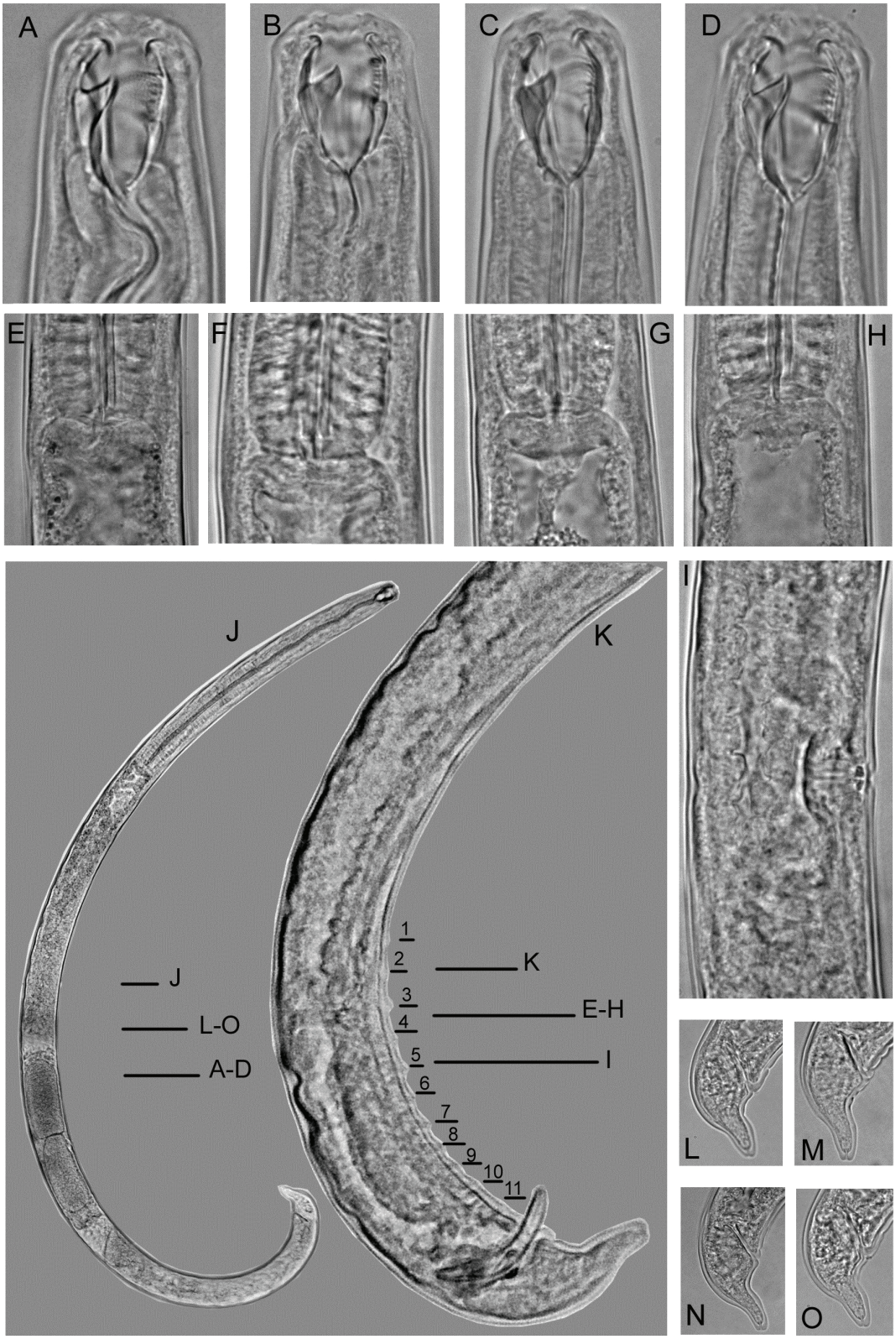

Fig. 2. *Mylonchulus sigmaturus* Cobb, 1917, A-D: Anterior region of P1-P4 populations; E-H: Cardiac Junction (P1-P4); I: Vulval region; J: Whole-body female; K: Male tail; L-O: Female tail (P1-P4).

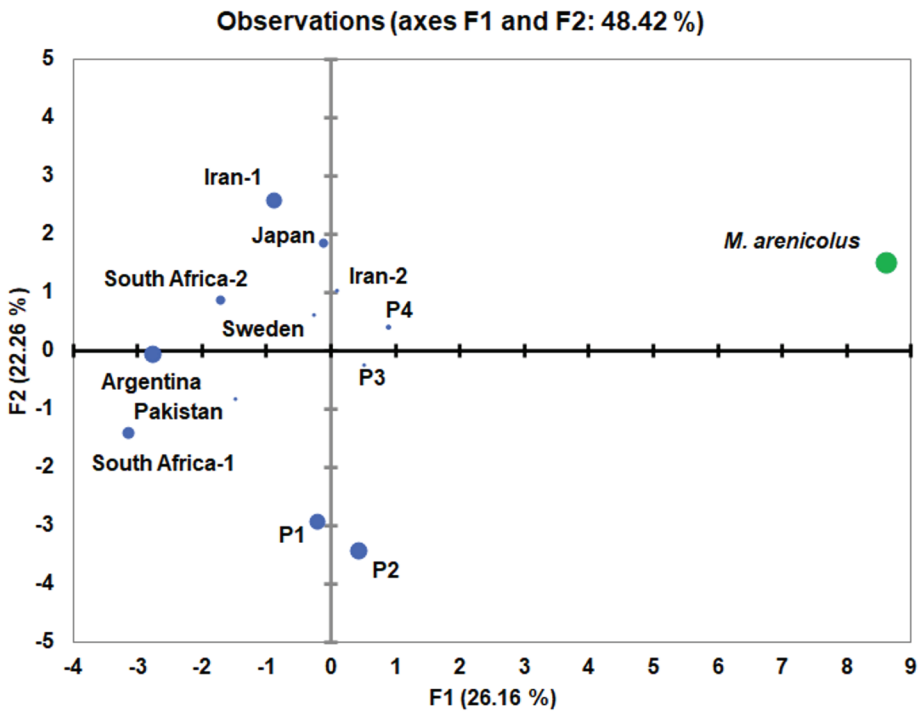

Fig. 3. Principal component analysis biplot for *M Mylonchulus sigmaturus* from Pakistan and *Mylonchulus arenicolus*.

Buccal cavity same as female. Lip region 24 µm wide. Apex of dorsal tooth 77 % from base of stoma. Genital system diorchic, each testis thin walled, contain many immature germ cells and elongated spindle-shaped spermatozoa 6 – 7 long. The two testes join to form a common vas deferens which leads to the ejaculatory duct. Spicules slender, slightly curved ventrally, 1.2 times the body diameter long. Lateral guiding piece about one fifth of spicule length, with bifurcate distal end which are connected to accessory pieces by strong muscular bands. Gubernaculum wedge shaped with more than a third as long as the spicule length, tapering at both ends. Caudal glands lie tandem in the tail leading to a common duct which opens terminally. Eleven ventromedian supplements well developed and regularly spaced. Male tail similar to that of female, provided with two pairs of caudal pores (one dorsal and one ventral) on digitate portion of tail.

Locality and habitat

Four populations of a reported species of predatory nematode, *M. sigmaturus* Cobb, 1917 have been isolated from the different geographical locations of Pakistan *viz*., P1-Thatta (Sindh), P2-Matiari (Sindh), P3-Lasbella (Balochistan), P4-Khanewal (Punjab), from the soil around plantation of banana (*Musa paradisiaca* L.) (Table S1; Table 1; aggregated).

Remarks

M. sigmaturus is a cosmopolitan species described or reported from various parts of the world (Koohkan *et al*., 2014). This species was first reported in Pakistan by Khan & Saeed (1987). In the present study, the specimens were collected from around the roots of banana plants (*Musa paradisiaca* L.) in different localities of Pakistan.

In comparison with the material studied by Koohkan *et al*., (2014) the present specimens have a slightly wider buccal cavity (15 – 17 vs 14 – 15 µm), having slightly anterior position of amphid aperture (8 – 10 vs 12 µm) and more anterior nerve ring (78 – 88 vs 89 – 117 µm). Compared with the specimens studied by Coetzee (1966) present specimens have a wider lip region (25 – 27 vs 19 µm), longer buccal cavity (25 – 27vs 22 µm) and longer tail (30 – 40 vs 26.5 µm). Compared with the material examined by de Bruin & Heyns (1992), the present population differs in lower c' value (1.0 – 1.4 vs 1.5) and smaller buccal cavity width (15 – 17 vs 13 µm). The specimens described by Khan & Saeed (1987) differ from the present population in higher 'a' and 'c' ratios (a=25.8 – 33.7 vs 23; c=26 – 40 vs17.9) and in slightly posteriorly located vulva (V=60 – 70 vs 58). The present specimens examined fits well with description of Ahmad *et al*., (2010), but differs in anteriorly located nerve ring (78 – 88 vs 102 – 114 µm), in shorter neck length (318 – 372 vs 370 – 410 µm) and in shorter G_2 ratio (5 – 14 vs 23 – 27). In comparison with the specimens examined by Farahmand *et al*., (2009), the present specimens have a longer buccal cavity (25 – 27 vs 21 – 25 µm) and nerve ring located more anteriorly (78 – 88 vs 87.5 – 122.5 µm). In the specimens described by Chaves (1990), advulval papillae were observed, but this character was not observed in the Pakistani specimens.

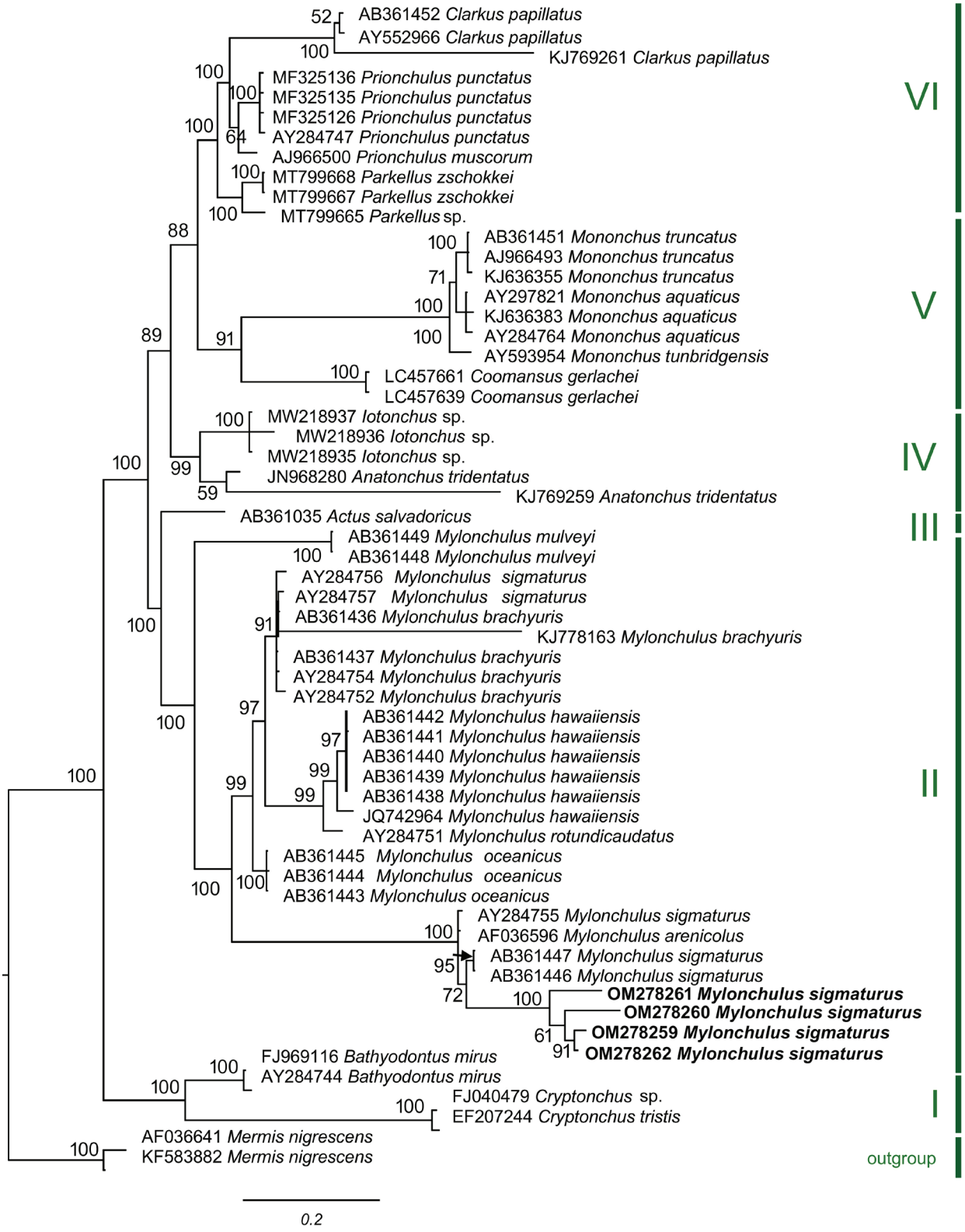

Fig. 4. 18S rDNA Bayesian tree inferred from known and newly sequenced *Mylonchulus sigmaturus* from Pakistan.

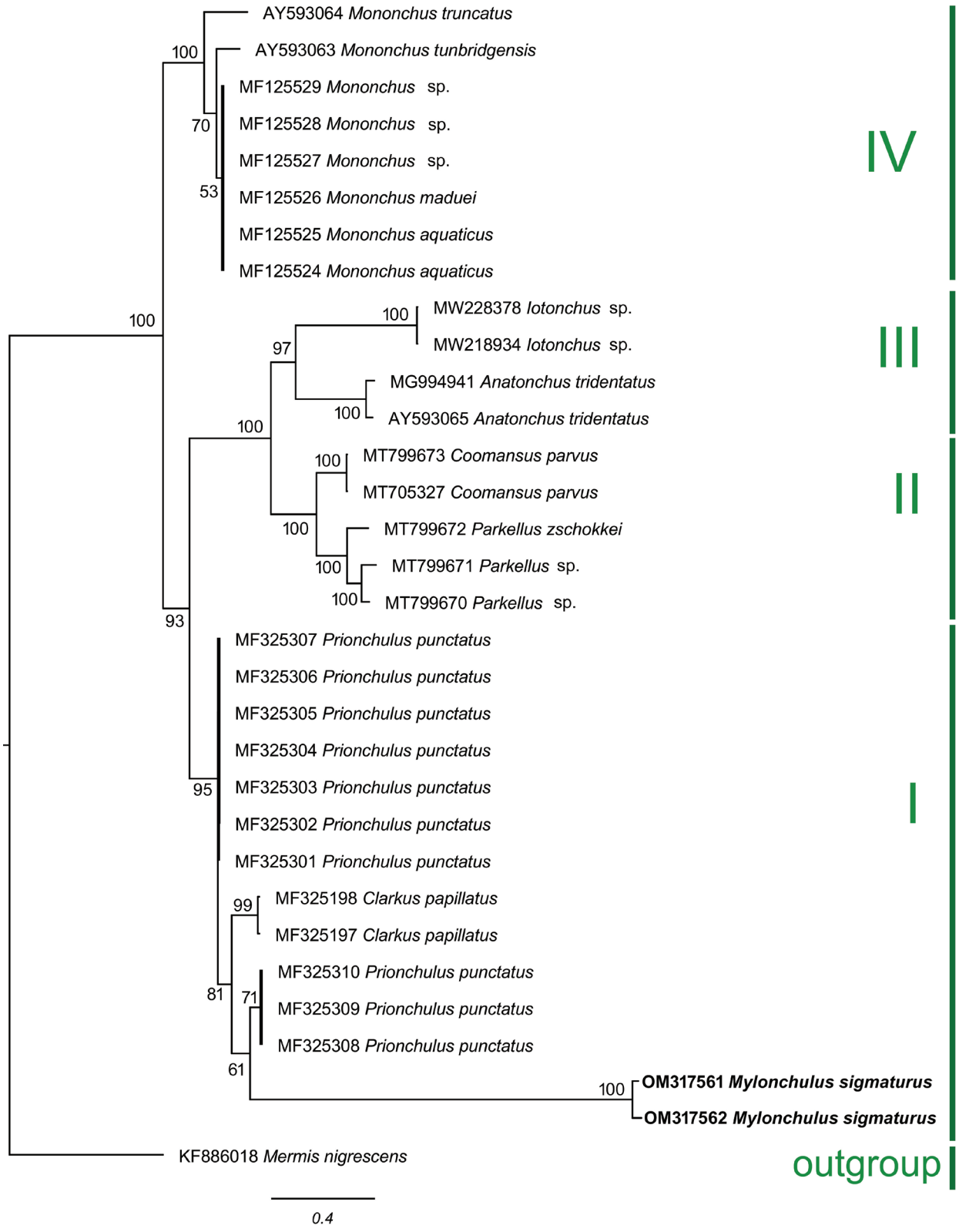

Fig. 5. 28S rDNA Bayesian tree inferred from known and newly sequenced *Mylonchulus sigmaturus* from Pakistan.

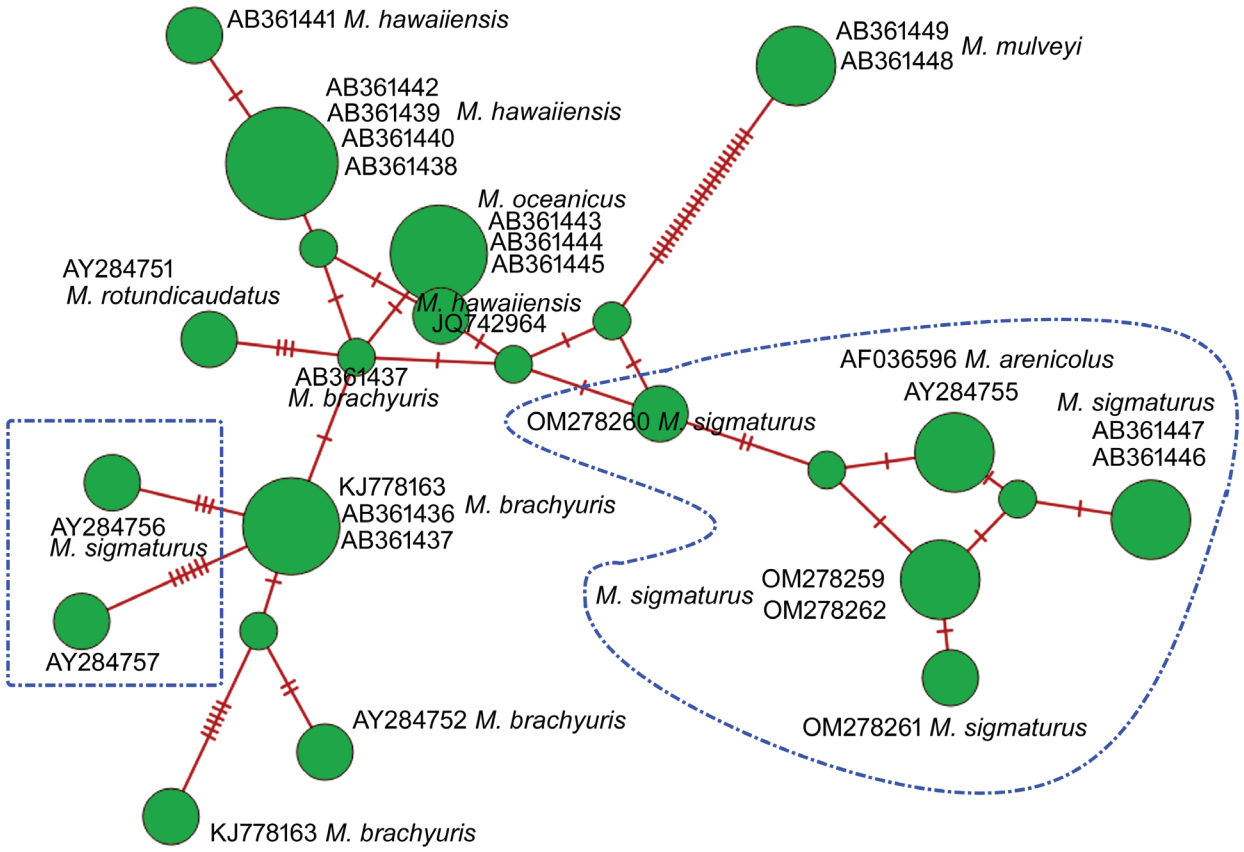

Fig. 6. Phylogenetic median-joining network showing the relationships between the 18S rRNA gene sequences of *Mylonchulus sigmaturus*. The sizes of circles are proportional to the number of identical sequences. The number of changes between haplotypes are given in hatch marks.

The present specimens have a longer neck and long buccal cavity (318 – 372 vs 279 – 309 and 25 – 27 vs 20 – 25 µm), respectively.

Principal Component Analysis of *M. sigmaturus* populations

Principal component analysis using morphometric features of females of *M. sigmaturus* showed a close relationship to these populations.

An accumulated variability of 55.61 % was observed in female-based PCA, 40.02 % in the F1, and 15.69 % in the F2.

The PCA indicated that P1-(Thatta) Sindh and P4 (Khanewal) Punjab are more similar to the South African and Iranian populations. In addition, P2-(Matiari) Sindh and P3-(Lasbella) Balochistan are similar to each other. Overall, the populations of *M. sigmaturus* showed a low morphometric variation based on the female's morphometrics (Fig. 3). The Pakistani populations of *M. sigmaturus* were categorized into two groups on the based on their morphometrics. Furthermore, a population P4 (Khanewal) Punjab from Pakistan stands separately from other *M. sigmaturus* in the present work, possibly belonging to another species than *M. sigmaturus*. The results indicated that *M. arenicolus* stands separate from the *M. sigmaturus* populations, differentiating them into two valid species.

DNA characterization of *M. sigmaturus*

The sequence flanked by the two primers SSU F04 and SSU R26 of the 18S regions of *M. sigmaturus* isolates is 800 bp long. The blastn search revealed that this population has 5 nucleotides differences with *M. sigmaturus* from Japan (acc. no. AB361446; AB361447) with 99 % similarity. Estimates of genetic distance using the maximum composite likelihood method among the 18S rDNA region of *M. sigmaturus* ranges from 0.007 to 0.014. The result showed that Pakistani *M. sigmaturus* (OM278261) has 0.016 genetic distance from other populations in Pakistan, which is the highest variation. In addition, *M. arenicolus* Clark, 1961 (AF036596) showed 0.002 genetic distance from *M. sigmaturus* (AY284755; The Netherlands).

Phylogenetic analysis

The consensus tree inferred using our 18S rDNA marker (Fig. 4) indicated that the mononchids included in the molecular study were grouped into six clades, including I) *Bathyodontus mirus* Andrássy, 1956, and *Cryptonchus tristis* (Ditlevsen, 1911) Filipjev, 1934 and unidentified *Cryptonchus*; II) *Mylonchulus* spp. (e.g., *M. sigmaturus* (Cobb, 1917) Altherr, 1953; *M. oceanicus* Andrássy, 1986; *M. brachyuris* (Bütschli, 1873) Cobb, 1917; and *M. rotuni-*

caudatus Skwarra, 1921; and *M. arenicolus* Clark, 1961, III) *Actus salvadoricus* Baqri & Jairajpuri, 1974, IV) *Iotonchus* species and *Anatonchus tridentatus* (de Man, 1876), De Coninck, 1939, V) *Mononchus aquaticus* Coetzee, 1968; *M. truncatus* Bastian, 1865, and *M. tunbridgensis* Bastian, 1865 and *Coomansus gerlachei* (de Man, 1904) Jairajpuri, 1970, and VI) *Clarkus papillatus* (Bastian, 1865) Jairajpuri, 1970; *Prionchulus punctatus* (Cobb, 1917) Andrássy, 1958; *P. muscorum* (Du Jardin, 1845) Wu and Hoeppli, 1929; *Parkellus zschokkei* (Menzel, 1913) Ahmad & Jairajpuri, 2010 and unidentified *Parkellus*.

The consensus tree inferred using our 28S rDNA marker (Fig. 5) indicated that the mononchids included in the molecular study are grouped into four clades, including I) *Prionchulus punctatus, Clarkus papillatus,* and *Mylonchulus sigmaturus*; II) *Coomansus parvus*, *Parkellus zschokkei* and unidentified *Parkellus*, III) *Anatonchus tridentatus* and *Iotonchus* sp., IV) *Mononchus aquaticus*, *M. truncates, M. tunbridgensis*, *M. maduei* Schneider, 1925 and unidentified *Mononchus*.

A phylogenetic median-joining network showing the relationships between populations of *M. sigmaturus*, is given in Fig. 6. The populations studied could be divided into two groups, including (I) OM278259-OM278262, AY284755, AB361446, and AB361447, and (II) AY284756 and AY284757. The Pakistani *M. sigmaturus* (OM278259 and OM278262) differed from Dutch *M. sigmaturus* (AY284755) and Japanese *M. sigmaturus* (AB361446; AB361447) in 2 nucleotides. Besides, Pakistani *M. sigmaturus* (OM278259 and OM278262) in the first group was represented as an identical sequence (Fig. 6). Nucleotide differences of 18S rRNA sequences of 27 populations of *Mylonchulus* was estimated as Л = 0.338214.

Discussion

Mononchida members are predacious and well-distributed nematodes worldwide (Ahmad and Jairajpuri, 2010). In the present study, we have analyzed the populations of *M. sigmaturus* from Pakistan through morphological, multivariate and phylogenetic analysis. Regarding the PCA, result showed a low variation among *M. sigmaturus*. The previous work showed three species, namely *M. sigmaturus*, *M. brachyuris,* and *M. lacustris* differentiated based on the female morphometrics (Koohkan *et al*., 2014). They have shown a low variation among *M. sigmaturus*. The same result was obtained in the present study. However, the result also indicated that despite the close relationship of *M. sigmaturus* and *M. arenicolus* based on 18S rDNA, they differ in morphometrical features, as shown by PCA plots. This result agrees with Ahmad and Jairajpuri (2010), who indicated both species with different morphological characteristics. Therefore, a further study was conducted to verify the Pakistani populations as *M. sigmaturus,* including molecular approaches.

Regarding the phylogenetic analysis, results showed that *Mylonchulus* group is a monophyletic taxon. However, a close relationship between *M. sigmaturus* and *M. arenicolus* was observed using 18S rDNA. However, despite the similarities of these two species, they differ in female tail length (30 – 44 vs 47 µm), and male (present vs absent). Additionally, the pairwise genetic distance showed a very low variation between *M. arenicolus* (AF036596) and *M. sigmaturus* (AY284755). Therefore, further investigations are needed to determine the relationship between these two species. Furthermore, the population of *M. sigmaturus* from Pakistan forms a strongly supported clade with the other *M. sigmaturus* sequences used in the analysis. This is consistent with the results obtained by van Megen *et al*. (2009), Shokoohi *et al*. (2013), and Koohkan *et al*. (2015). Although, two populations of *M. sigmaturus* (AY284756, AY284757) were placed separately from other *M. sigmaturus*.

The 28S rDNA is reported for the first time for *M. sigmaturus*. Therefore, due to lack of other *Mylonchulus* to compare, the present sequence stands molecularly close with *P. punctatus*. However, morphological, they differ in buccal cavity length (25 – 27 vs 35 – 45 µm), buccal cavity denticle (in a transverse rows vs longitudinal rows), spinneret (present vs absent), and male supplementary organs (11 vs 18 – 21). They also differ with *C. papillatus* in buccal cavity (funnel-shaped vs cup-shaped), denticles (present vs absent), and tail spinneret (present vs absent) (Ahmad and Jairajpuri 2010).

In conclusion, the rDNA helps to separate *Mylonchulus* species.

Table 4. Genetic pairwise distance estimating for different populations of *M. sigmaturus* (numbers 1-7) and *M. arenicolus* (number 8) [upper matrix indicates standard error).

	species	Accession number	Locality	1	2	3	4	5	6	7	8
1	*M. sigmaturus* N18_988	OM278259	Pakistan		0.003	0.004	0.003	0.003	0.004	0.003	0.004
2	*M. sigmaturus* N19_988	OM278260	Pakistan	0.008		0.004	0.003	0.004	0.003	0.004	0.003
3	*M. sigmaturus* N21_988	OM278261	Pakistan	0.012	0.016		0.004	0.007	0.008	0.006	0.007
4	*M. sigmaturus* N22_988	OM278262	Pakistan	0.007	0.010	0.016		0.003	0.004	0.003	0.003
5	*M. sigmaturus*	AB361447	Japan	0.012	0.012	0.045	0.009		0.002	0.000	0.002
6	*M. sigmaturus*	AY284755	The Netherlands	0.014	0.010	0.047	0.012	0.008		0.002	0.001
7	*M. sigmaturus*	AB361446	Japan	0.012	0.012	0.033	0.009	0.000	0.008		0.002
8	*M. arenicolus*	AF036596	UK	0.013	0.010	0.042	0.010	0.007	0.002	0.008	

However, mtDNA can be a good option for distinguishing the close species of *Mylonchulus*. The phylogenetic analysis showed some differences between the Pakistani *M. sigmaturus*, and other related species. However, due to the morphology and morphometrics similarity, we consider all *Mylonchulus* populations from Pakistan, as *M. sigmaturus*, with the male being reported for the first time. In addition, the potential of the pest biocontrol or ecological behavior of *Mylonchlus* needs to be investigated.

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