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First report of *Xiphinema diffusum* from Pakistan

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Summary

The population of *Xiphinema americanum* species group was detected from the soil surronding the roots of peach (*Prunus persica*) during a survey of plant-parasitic nematodes in Gilgit-Baltistan, Pakistan. From the results of the morphological study, the species was identified as *X. diffusum*. Morphometrics of *X. diffusum* generally agree with those of the type species and the topotype specimens. Molecular analysis of 18S rDNA of *X. diffusum* from Pakistan indicated two nucleotide differences and 99 % similarity with the Chinese (AM086669) and the Australian (AM086685) population of *X. diffusum*. Phylogenetic analysis of 18S rDNA placed the Pakistanis population close to *X. diffusum* from China and Australia. This species is reported for the first time from Pakistan
Keywords: *Prunus persica*; *Xiphinema americanum* group; *X. diffusum*; New record

Introduction

Peach (*Prunus persica*) is the most important among the stone fruits and can be grown in temperate climates. It is considered native to China and probably developed in Persia. It is a traditional crop of the Northern area of Pakistan and occupies the area of 6,330 hectares, with the production of approximately 36,155 tones in 2013 – 2014. Quetta, Kalat, Peshawar, Swat valley and certain parts of Kohistan hills are the main peach-growing areas. Peach is the second most important fruit (after plum) in Pakistan (Habib, 2015).

The genus *Xiphinema* Cobb, 1913, belongs to the family Longidoridae Thorne, 1935 (Meyl, 1961) and subfamily Xiphinematinae Dalmasso, 1961, which is a genus of ectoparasitic nematodes commonly known as the dagger nematodes (Whitehead, 1968). A total of 23 nematode species of the genus *Xiphinema* have so far have been reported from Pakistan. Among them, three species namely, *Xiphinema karachiense* Nasira, Firoza & Maqbool, 1992; *X. cynodonti* Nasira & Maqbool 1994 and *X. pakistanensis* Nasira & Maqbool, 1998 were described from Pakistan, whereas 20 other species of the genus *Xiphinema* have been reported as a new record for Pakistan (Shahina *et al*., 2019). Due to the complexity of the species in the genus, it is imperative that both morphological and molecular characters be used in species identification. The aim of this study was to 1) study *X. diffusum* by morphological and molecular character using 18S rDNA.

Materials and Methods

The soil samples containing the *Xiphinema* species were taken from the root zone of peach trees in Gilgit-Baltistan, Pakistan. Nematodes were extracted with Cobb's wet sieving techniques. Material collected on a 25 μm mesh sieve was placed on a Baermann funnel and nematodes were collected after one day at room temperature. Specimens were transferred to a small amount of water and then killed by heating at 60°C. Nematodes were fixed in

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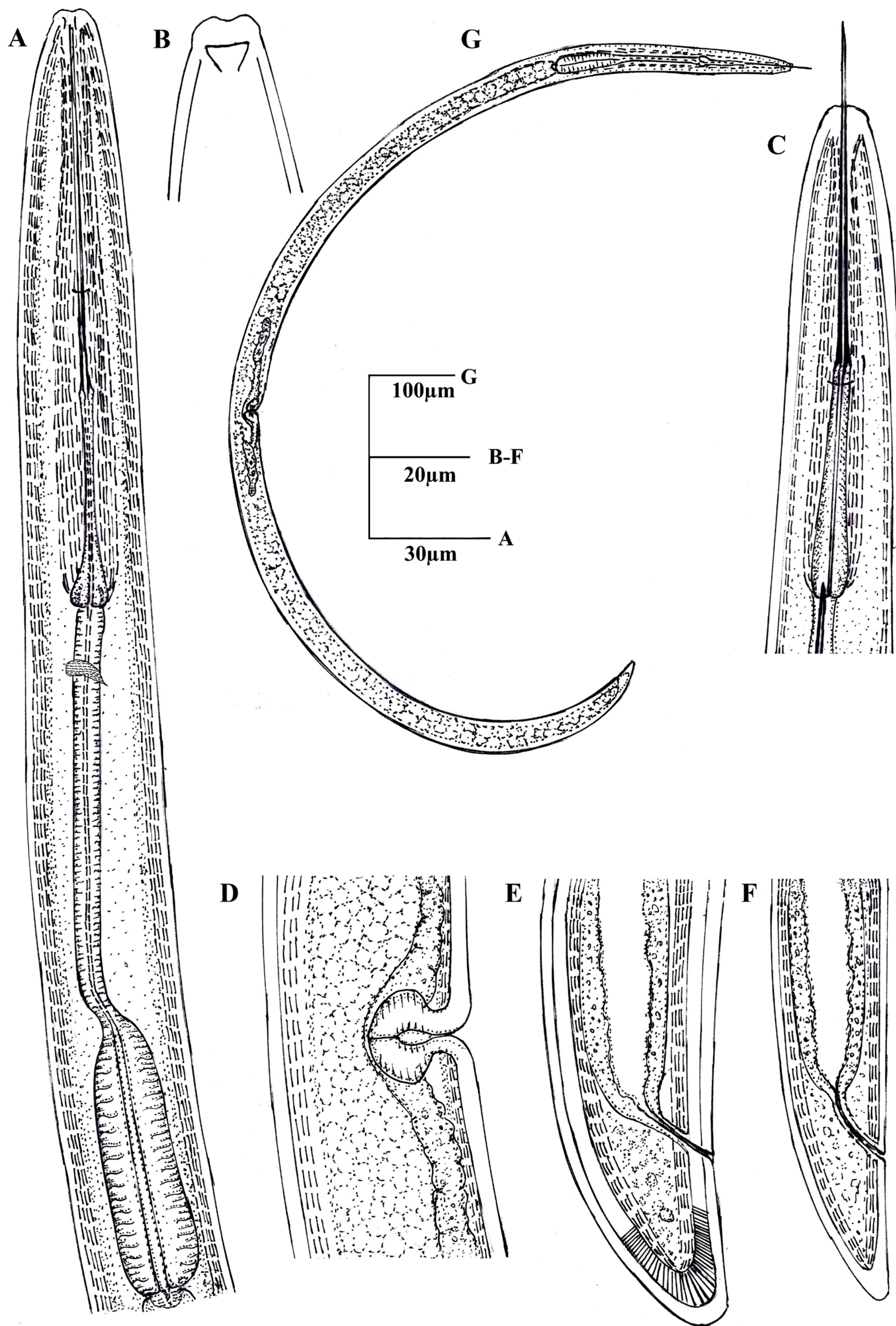

Fig 1. *Xiphinema diffusum* Lamberti & Bleve-Zacheo, 1979. A: Anterior end. B: Amphid. C: Odontostyle and odontophore. D: Vaginal region. E: Female posterior end. F: Juvenile posterior end. G: Entire female.

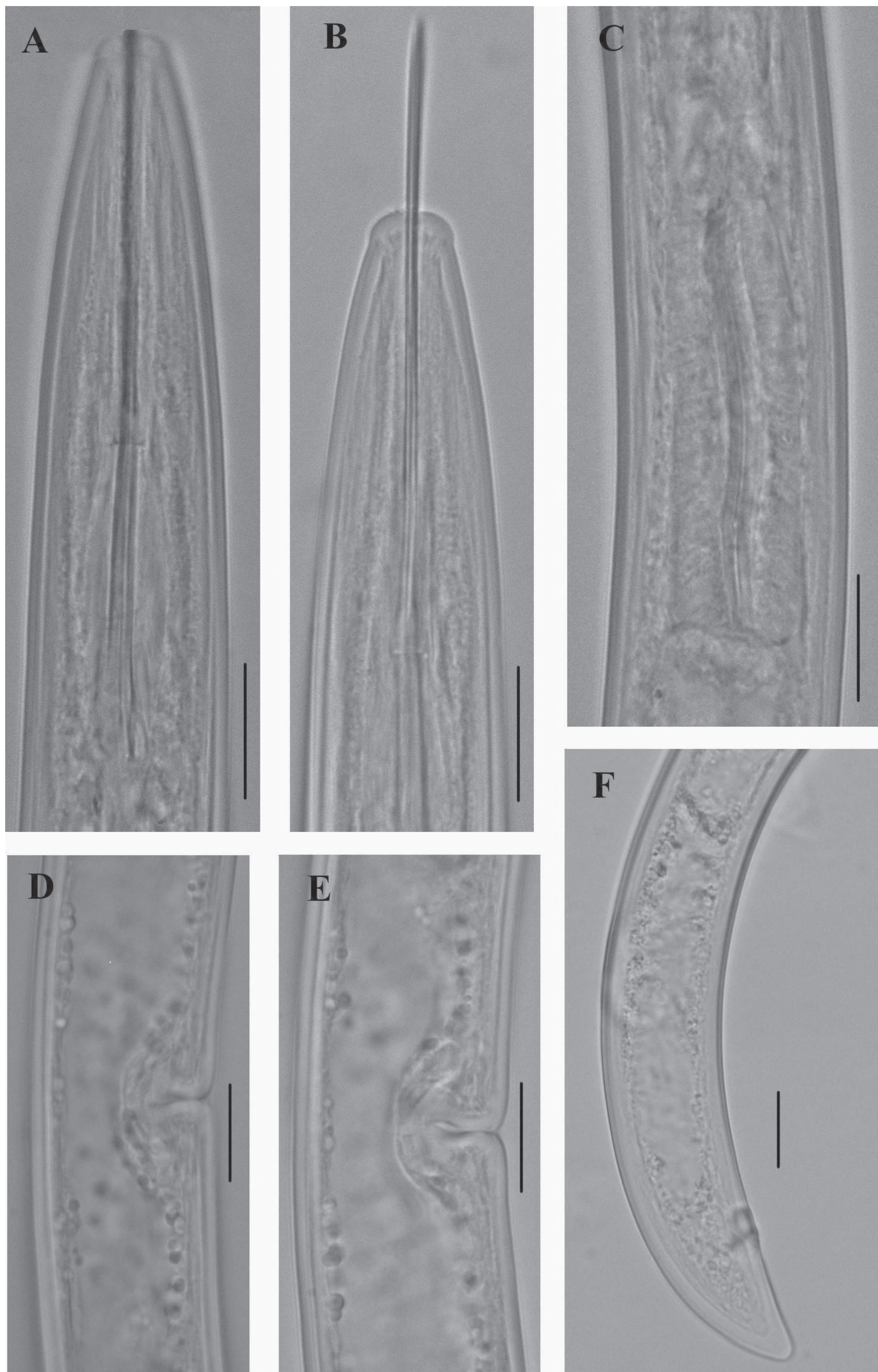

Fig 2. *Xiphinema diffusum* Lamberti & Bleve-Zacheo, 1979. A: Anterior end. B: Odontostyle. C: Pharyngeal-intestinal junction. D, E: Vaginal region. E: Female posterior end. (scale bar = 20 μm)

TAF than processed into glycerine by the ethanol/glycerine method (Seinhorst, 1959; De Grisse, 1969) and mounted in dehydrated glycerine supported with both minute glass beads and paraffin in glass slides. Observations were made using a DIC microscope (B x 51, Olympus Co, Japan). A digital camera was used for measuring, taking LM images and line illustrations of the nematode.

DNA extraction, PCR and phylogenetic analysis

DNA extraction was done using the Chelex method (Straube & Juen, 2013). Five specimens were hand-picked with a fine tip needle and transferred to a 1.5 ml Eppendorf tube containing 20 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 were spun for 2 min at 16000 rpm (Shokoohi *et al*., 2019). The supernatant was collected from each of the tubes and stored at -20 °C. Following this step, the forward and reverse primers, 988F (5-ctcaaagattaagccatgc-3) and 1912R (5-tttacggtcagaactaggg-3) were used for PCR amplification of 18S rDNA (Holterman *et al.,* 2006). Amplification was conducted with eight µl of the DNA template, 12.5 µl of 2X PCR Master Mix Red (Promega, USA), one µl of each primer (10 pmol $µl^{-1}$) and ddH_2O for a final volume of 30 µl. The amplification was processed using an Eppendorf master cycler gradient (Eppendorf, Hamburg, Germany), with the following steps: initial denaturation for 3 min at 94 °C, 37 cycles of denaturation for 45 s at 94 °C; (51 and 55 °C annealing temperatures for ITS rDNA and 28SrDNA respectively); extension for 45 s to 1 min at 72 °C, and finally an extension step of 6 min at 72 °C followed by temperature on hold at 4 °C. After DNA amplification, 4 µl of product from each tube was loaded on a 1.5 % agarose gel in TBE buffer (40 mM Tris, 40 mM boric acid, and one mM EDTA) for evaluation of the DNA bands. The bands were stained with RedGel dye and photographed on a UV transilluminator. The amplicons of the genes were stored at -20 °C. Finally, the PCR products were purified for sequencing by Inqaba Biotech (South Africa). *Longidorus biformis* (AY283162) based on Van Megen *et al*. (2009), was selected as the outgroup for 18S rDNA. The ribosomal DNA sequence was analyzed and edited with BioEdit (Hall, 1999) and aligned using CLUSTAL W (Thompson *et al*., 1994). The length of the alignments were 800 bp. Phylogenetic trees were generated using the Bayesian inference method as implemented in the program Mr Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The GTR+I+G was selected using jModeltest 2.1.10 (Guindon & Gascuel, 2003; Darriba *et al*., 2012). Then, the model was initiated with a random starting tree and run with the Markov chain Monte Carlo (MCMC) for 10^6 generations. The partial 18S rDNA of *X. diffususm* was deposited in GenBank under the accession numbers MT422062.

The authors declare that experiments on animals have been followed according to the international, national and institutional guidelines for the care and use of animals and that a research committee has approved the studies at the institution at which the studies were performed (University of Limpopo, South Africa; University of Karachi, Pakistan).

Results

Xiphinema diffusum Lamberti & Bleve-Zacheo, 1979

(Figs. 1 & 2)

Measurement: Table 1.

Description: Female: The body assumes a ventrally arcuate, open c-shape, or spiral shape when killed by gentle heating. Lip region slightly offset from body contour. Cuticle very finely striated transversely. Amphidial pouches stirrup-shaped with slit-like aperture about half as wide as lip region, occupying 63 – 65 % of the corresponding diameter. Odontostyle needle-like 85 – 90 µm long and furcate at the base. Odontophore distally flanged, flanges 8 – 8.5 µm across. Basal ring of guiding apparatus situated 62 – 76 µm from anterior end. Nerve ring 160 – 170 µm from anterior end. The first of pharynx about three times than pharyngeal bulb diameter. Vulva a transverse slit, about equatorial and vagina occupying almost ½ of the corresponding body diameter. Gonads paired, opposed, reflexed at oviduct; ovaries with a few oogonia with large deformed nuclei, infested with bacteria. Prerectum 104 – 127 µm long, rectum shorter than body diameter at the anus. Tail conoid, with a greater curvature dorsally, rounded terminus, bearing two pairs of caudal pores.

Male: Not found.

Remarks: The specimens of *Xiphinema diffusum* were collected from the soil around the roots of peach (*Prunus persica*) from Gilgit-Baltistan, Pakistan. The Pakistanis *Xiphinema diffusum* resembled the original description of *X. diffusum* described by Lamberti & Bleve-Zacheo, 1979 except for the lower range of the odontostyle (85 – 90 vs 73 – 89 µm) and tail length (30 – 42 vs 19 – 33 µm). Besides, compared with the populations from Malawi, Gambia and Madagascar (Lamberti & Bleve-Zacheo, 1979), they only differ in the tail length (30 – 42 vs 32 µm). Geographical distribution and biometric variability of *X. diffusum* from Paarl (Western Cape, South Africa), Bet Dagan (Israel) and Mataveri Otai (Eastern Island) have been provided by Lamberti *et al*. (1991). In comparison with the South African population, Pakistanis species have smaller odontostyle (85 – 90 *vs* 90 – 95 µm), lower c ratio (57 – 64 *vs* 65.6 – 92.2), smaller body width at the beginning of J = Hyaline portion (12 – 15 *vs* 15.9 – 22 µm), the length of the hyaline portion of the tail (J) (7.5 – 8.0 *vs* 8.8 – 12.9 µm) and longer tail (28 – 30 *vs* 20 – 26 µm). The species differs from Israel population only in a lower c ratio (57 – 64 *vs* 63.8 – 92) and from a population from Eastern Island, it differs only in the smaller tail (30 – 42 vs

Table 1. Morphometric of *Xiphinema diffusum* Lamberti & Bleve-Zacheo, 1979.
Mean ± SD (range) in µm except for L.

	Females (n = 8)	**J1 (n = 1)**	**J2 (n = 4)**	**J3 (n = 1)**
L	1.736 ± 103.02 (1.6 – 1.9)	(1063)	1073 ± 33.5 (1.0 – 1.1)	(1317)
a	44.3 ± 1.8 (42 – 47)	40.8	46.1 ± 0.9 (45.0 – 47.2)	50.6
b	6.2 ± 0.5 (5.7 – 7.1)	5.0	4.7 ± 0.4 (4.3 – 5.3)	4.8
c	60.1 ± 2.5 (57 – 64)	44.2	30.2 ± 3.5 (28.1 – 34.8)	43.9
c'	1.1 ± 0.1 (0.9 – 1.2)	1.7	2.3 ± 0.5 (1.9 – 3.0)	1.6
V	52.4 ± 0.9 (52 – 54)	–	–	–
Odontostyle	87.5 ± 1.7 (85 – 90)	62	62.2 ± 1.8 (60 – 64)	71
Odontophore	52.8 ± 1.1 (51 – 54)	42	37.2 ± 0.8 (36 – 38)	42
Total stylet	140 ± 0 (138 – 144)	104	99.5 ± 1.1 (98 – 101)	113
Replacement odontostyle	–	72	74 ± 3.1 (70 – 78)	86
Oral aperture to guiding ring	68.3 ± 5.41 (62 – 76)	46	47.2 ± 1.9 (45 – 50)	57
Pharyngeal bulb length	66.8 ± 2.6 (64 – 79)	44	51.5 ± 4.0 (46 – 57)	56
Pharyngeal bulb width	19.7 ± 0.6 (19 – 21)	18	15.7 ± 0.4 (15 – 16)	18
Body diameter at lip region	11.8 ± 0.7 (11 – 13)	9	8.5 ± 0.5 (8 – 9)	10
Body diameter at guiding ring	27.7 ± 1.1 (26 – 29)	20	18.5 ± 0.5 (18 – 19)	23
Body diameter at base of pharynx	34.8 ± 1.1 (33 – 36)	26	23.5 ± 0.5 (23 – 24)	29
Body diameter at vulva	37.8 ± 3.4 (34 – 44)	–	–	–
Body diameter at anus	26.8 ± 1.3 (25 – 28)	14	15 ± 0.70 (14 – 16)	19
Body diameter at beginning of J	13.3 ± 1.1 (12 – 15)	6	5.7 ± 0.4 (5 – 6)	7
Genital Primordium (GP %)	–	54.5	53.0 ± 2.2 (49.5 – 55.5)	52.0
Hyaline portion of tail (J)	7.9 ± 0.2 (7.5 – 8.0)	6	7.0 ± 0.70 (6 – 7)	7
Tail	35.5 ± 5.5 (30 – 42)	24	29 ± 0.8 (28 – 30)	30

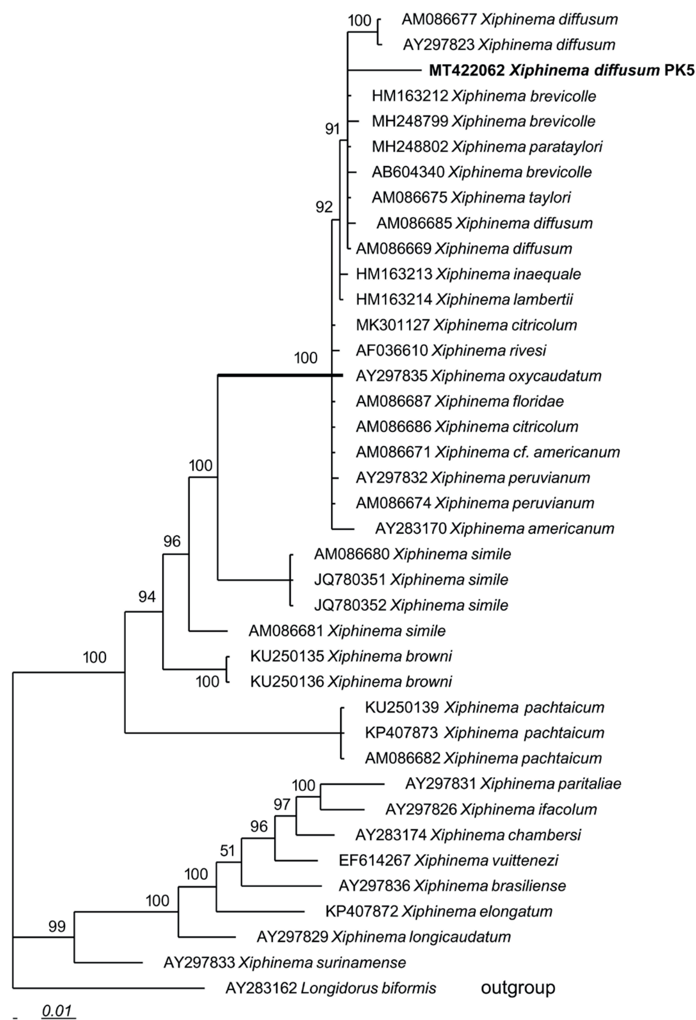

Fig 3. MrBayes tree of the *Xiphinema diffusum* populations from GenBank and newly sequenced from Pakistan based on the 18S rDNA gene under GTR+I+G model ((-*lnL* = 4705.5454; AIC = 9583.090780; freqA = 0.2774; freqC = 0.2134; freqG = 0.2590; freqT = 0.2502; R(a) [AC] = 1.2052; R(b) [AG] = 2.9726; R(c) [AT] = 1.6440; R(d) [CG] = 0.4703; R(e) [CT] = 4.9113; R(f) [GT] = 1.0000; p-inv = 0.5080; gamma shape = 0.2770).

23.5 – 27.1 µm). From a population of *X. diffusum* studied by Luc *et al*., (1998), they differ in the odontostyle length (85 – 90 *vs* 90 – 95 µm). Compared with a Portuguese population of *X. diffusum* (Lamberti *et al*., 1994), they differ in body length (L = 1.6 – 1.9 *vs* 1.7 – 2.2 mm), V value (V = 51.7 – 54 *vs* 47 – 50), body width at beginning of J (12 – 15 *vs* 14 – 20 µm) and in hyaline portion of tail (J = 7.5 – 8.0 *vs* 8 – 12 µm). This species is reported for the first time from Pakistan.

Phylogenetic analysis

The Bayesian inference tree of 18S rDNA of *Xiphinema* (Fig. 3) grouped them into two clades including I) *X. diffusum, X. brevicolle, X. taylori, X. paratylori, X. inaequale, X. lamberti, X. citricolum, X. rivesi, X. oxycaudatum, X. floridae, X. cf. americanum, X. peruvianum, X. simile, X. browni* and *X. pachtaicum* with 100 posterior probability; and II) *X. paritaliae, X. ifacolum, X. chambersi, X. vuittenezi, X. brasiliense, X. elongatum, X. longicaudatum* and *X. surinamense* with 99 posterior probability. Nblast of 18S rDNA of *X. diffusum* revealed two nucleotides differences with the same species of Chinese (AM086669) and Australian (AM086685) population with 99 % similarity. The Pakistanis speciemns of *X. diffusum* placed in Clade I with 100 posterior probability, close to the same species from Brasil (AM086677; AY297823), along with two other sequences of *Xiphinema diffusum* from China (AM086669) and Australia (AM086685) with 91 posterior probability. In addition, our sequence of 18S rDNA of *X. diffusum* grouped with *X. brevicolle* from Czech Republic (HM163212) with 91 % posterior probability.

Discussion

This study is the first report of the occurrence of *X. diffusum* in Pakistan to date. Twenty-three species of *Xiphinema* have been reported from Pakistan. *Xiphinema diffusum*, including our specimens belong to the *X. brevicolle*-subgroup, which includes five species namely [*X. brevicolle*, *X. diffusum*, *X. inaequale*, *X. incognitum*, and *X. taylori*] (Sakai *et al.,* 2011). However, the validity of the respective species is a different matter. Appropriate establishment of subgroups within the *X. americanum*-group will contribute to a more feasible identification process of the member species and requires further research. In the original description for *X. diffusum*, Lamberti & Bleve-Zacheo (1979) indicated that it differs with *X. brevicolle* in smaller size, less expanded lip region, shorter odontostyle and more rounded tail. In addition, the further studies also indicated that the species such as *X. brevicolle* and *X. diffusum* are very similar which distinguished by only minute morphological differences (Brown & Halbrendt, 1997). The previous molecular study revealed that *X. diffusum* and *X. brevicolle* are close to each other (Sakai *et al*., 2011; Zeng *et al*., 2016). The result of the present study indicated that *X. diffusum* grouped with *X. brevicolle*. However, they differ in body length (1.6 – 1.9 *vs* 1.7 – 2.2 mm) and total stylet length (138 – 144 *vs* 144 – 173 μm) (see Sakai *et al*., 2011). Besides, *X. brevicolle* was reported from Pakistan in associated with apple and citrus trees (Lamberti *et al*., 1987) and banana (Pathan *et al*., 2004). Although the morphometrics of *X. brevicolle* was not provided by Lamberti *et al*. (1987), however, our speciemns differ from those studied by Pathan *et al*. (2004) in stylet length (138 – 144 *vs* 98 – 123.6 μm) and c value (57 – 64 *vs* 27 – 41).

Conclusion

In this study, specimens of *X. diffusum*, present in the root zone of peach in Pakistan, were identified using morphological and 18S rDNA. Despite the close relationship of *X. brevicolle* and *X. diffusum* using 18S rDNA marker and phylogenetic analysis, their morphometrics suggesting separate species. Although, our specimens belong to the *X. diffusum* and *X. brevicolle*-subgroup, more populations are needed to be studied using other molecular markers to find out the relationship of the above-mentioned species group.

Conflict of Interest

The authors declare that there is no conflict of interest.

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