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# Morphological and molecular characterisation of stubby root nematode (*Paratrichodorus* Siddiqi, 1974) parasitizing citrus (*Citrus* sp.) from IARI, New Delhi, India

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**ABSTRACT: Populations of *Paratrichodorus* were collected from the root zones of citrus plants (*Citrus* sp.) cultivated at the Indian Agricultural Research Institute (IARI) farm in New Delhi. The study aimed to characterize these populations both morphologically and molecularly for accurate species identification. Morphology and morphometrics (21 females and 20 males) were studied and compared with the species described under the genus. The *Paratrichodorus* female population body lengths of 500.0-690.4 µm, with a = 16.5-22.6 and b = 4.5-6.2. Vulval position averaged at 47% of body length, while the Onchiostyle measured 28-39 µm. In contrast, *Paratrichodorus* males measured 498.2-645.2 µm, with a = 16.0-22.1 and b = 3.9-6.0. Their spicule length ranged from 30.1 to 36.9 µm, with an average of 32.9 µm. We considered the *Paratrichodorus* population is quite different from the known and described species. At the molecular level, amplified sequence fragments encompassing the 18S region for the *Paratrichodorus* isolate iari123. Subsequent phylogenetic analysis of the obtained 18S rRNA sequence (MZ567177) suggests a potential correlation with *Paratrichodorus teres* (Hooper, 1962) Siddiqi, 1974. This particular sequence has been formally submitted and documented in GenBank under the accession number MZ567177.**

**Keywords:** 18S rRNA, *Paratrichodorus*, morphometrics.

## INTRODUCTION

*Paratrichodorus* is type of stubby-root nematodes belonging to the family Trichodoridae. They are also considered as economically important plant parasites nematodes as well as vector of plant viruses. Females are didelphic, and distributed worldwide. More than 100 species under the family Trichodoridae have been divided into five genera; they are *Trichodorus* Cobb, 1913; *Monotrichodorus* Andrassy, 1976; *Allotrichodorus* Rodriguez, Sher and Siddiqi, 1978; *Paratrichodorus* Siddiqi, 1974, and *Ecuadorus* Siddiqi, 2002 (Decraemer and Robbins 2007). The genus *Trichodorus* is the type genus of the family Trichodoridae. Siddiqi (1974) *Trichodorus* split into two genera - *Trichodorus* and *Paratrichodorus*. *Paratrichodorus* is consisting 26 species so far. Identification of stubby root nematodes based on morphological features requires expertise for the group, and it is relatively difficult and more complex due to minor differences among closely related species (Decraemer and Baujard 1998). Therefore, molecular methods for species identification have been used based on the variability in the ribosomal RNA (rRNA) sequences such 18S, ITS-I &ITS-II of I8S, and D2-D3 region of 28S rRNA (Boutsika *et al.,* 2004; Duarte *et al.,* 2011; Holeva *et al.,* 2006; Kumari and Subbotin 2012; Riga *et al.,* 2007). Therefore integration of morphological and morphometric and molecular data helps in diagnosis, identification and confirmation of species. Of the 26 species known under genus *Paratrichodorus*, only four species have been described from India.

**Systematics of *Paratrichodorus* Siddiqi, 1974**

| | | |
|---|---|---|
| Phylum | : | Nematoda |
| Class | : | Enoplea |
| Subclass | : | Dorylaimia |
| Order | : | Triplonchida |
| Sub order | : | Diphtherophorina |
| Superfamily: | | Diphtherophoroidea |
| Family | : | Trichodoridae |
| Genus | : | *Paratrichodorus* |
| Species | : | *Paratrichodorus teres* |

## MATERIALS AND METHODS

*A. Sampling, nematode extraction*

The measured quantity of sub-sample (200 cc) from the composite sample was washed by Cobb's decanting and sieving technique (Cobb, 1918); the sieves of 20, 60, 200 and 325 mesh size were used to catch the target nematode populations. The catches of 60, 200 and 325 were further cleaned through modified Baermann's technique (Schindler, 1961). Here the residues passed through a double layer tissue paper supported on an aluminium wire-gauge, and the entire assembly was

kept over a glass Petriplate of 10 cm diameter filled with tap water. It was left undisturbed for overnight at room temperature. Nematode suspension thus collected was labeled with details of host, date of collection, locality and collector's name and kept properly for further studies.

*B. Killing and fixing*

Several fixatives have been recommended for killing and fixing the nematodes. However, TAF (7.6 ml formalin, which contains 37% formaldehyde, 2 ml Tri-ethylamine, 90.4 ml Distilled water for 100 ml solution) was used for killing and fixing of nematode specimens. Mass fixation of nematode in the suspension was done with the help of hot TAF at 60°C (for simultaneously killing and fixing). The fixed nematodes were then observed under stereobinocular compound microscope and identified the target nematode genera based on morphological characters from the mixed populations (Seinhorst *et al*., 1959).

*C. Statistical Analysis*

Morphometric data recorded for the populations of *Paratrichodorus* were further analyzed on Microsoft Excel for determining values of De Man's, maximum-minimum (range), mean, standard deviation (SD) etc.

*D. Molecular characterization*

**DNA Extraction and PCR Amplification.** Five to six nematodes of genus *Paratrichodorus* were lysed with worm lysis buffer (0.2M NaCl, 0.2M Tris pH 8.0, 1% β-mercaptoethanol, 800µg / ml of proteinase K) following the protocol described by Castagnone-Sereno *et al*. (1995). Then the lysates containing DNA were stored at –20°C for downstream molecular analysis. The 25µl PCR reaction composition is given in Table 1.

**Table 1: The 25µl reaction composition for PCR amplification.**

| | |
|---|---|
| GoTaq Green Master mix | 12.5 µl |
| Forward primer | 1 µl |
| Reverse primer | 1 µl |
| Crude extracted genomic DNA | 2 µl |
| **Nucleus free water** | **8.5 µl** |

PCR amplification of the molecular markers, the small subunit 18S rRNA gene was carried out using the primers listed in Table 2. The reaction conditions for PCR amplification are given in Table 3. Amplified products were separated by electrophoresis on 1.2% agarose gel, stained with ethidium bromide at a potential of 1.5 V $cm^{-1}$. The gel was visualized under UV light (UV transilluminator).

**DNA purification by centrifugation.** Wizard® SV Gel and PCR Clean-Up System (Catalogue No. A9281) was used for DNA purification from amplified PCR products following manufacture protocols. The purified PCR products were directly sent for sequencing using the same forward and reverse primer set.

**Table 2: Details of the primers used for this study.**

| Primer | Gene | Sequence (5'-3') | References |
|---|---|---|---|
| 18s 965 | 18s rRNA | GGCGATCAGATACCGCCCTAGTT | Powers *et al.* (2005) |
| 18s 1573R | 18s rRNA | TACAAAGGGCAGGGACGTAAT | Powers *et al.* (2005) |

**Table 3: PCR amplification conditions.**

| ***c. Paratrichodorus*** | **Marker** |
|---|---|
| | **18S** (Decraemer *et al.*, 2019) |
| Initial denaturation | 94 ºC for 2 min |
| Denaturation | 94 ºC for 30 sec |
| Annealing | **54 ºC for 30 sec** |
| Extension | 72 ºC for 3 min |
| Final extension | 72 ºC for 5 min |
| Total cycles | 40 |

**Sequencing and analysis of sequences.** The sequence information obtained for each marker was compared with the available sequences in the NCBI GenBank database using BLASTN search. Phylogenetic analyses were carried out in the MEGAX platform (Kumar *et al.*, 2018) using those retrieved sequences along with sequences used in recent publications. 18S SSU consensus tree for *Paratrichodorus* was constructed with the sequences retrieved from Ilieva-Makulec *et al*. (2017) and Decraemer *et al*. (2019). The sequences were aligned using ClustalW (Thompson *et al.*, 1994), and the evolutionary relationship was deduced by the Maximum-Likelihood (ML) method with the selection of a suitable model using Modeltest (Posada and Crandall 1998). The phylograms were bootstrapped 1,000 times (Felsenstein, 1985) to assess the degree of support for the phylogenetic branching as indicated in the consensus tree.

## RESULTS

**Genus *Paratrichodorus* Siddiqi, 1974**

A population of *Paratrichodorus* recovered from the rhizosphere of citrus plants was attempted to

characterize based on morphology, morphometrics and molecular studies.

**Description** (Fig. 1-2, for measurement sees Tables 4).

**Female:** *Paratrichodorus* sp. population recovered from IARI, New Delhi is characterized by a relatively short 586 (500.-690µm) and slender body (a= 16.5-22.6µm). Cuticle usually swollen and granular appearance upon fixed; cuticle thick at mid body region 4.5(4-5µm) and narrow in the region of tail tip 3.3 (3.1-3.8µm). Lip region rounded 9.0(7.9-10.1) µm wide, onchiostyle ventrally curved 33.3(28.1-39.1) µm long. Female reproductive system didelphic–amphidelphic, anterior genital branches smaller, 102(85-112µm) than posterior genital branch 118(97-161µm). Lateral body pore are not present. Very short tail, broadly rounded tip with terminal anal opening.

**Male:** Body straight, with posterior end ventrally curved and assumed J shaped. Cuticle granular appearance when fixed in hot TAF. Lip region rounded, 9.1(7.9-10.2) µm wide, onchiostyle curved ventrally, 31.7 (29.1-37.5) µm long. Cervical papillae (CP) as well as supplement papillae (SP) were not seen. Spicule medium sized 32.9 (30.1-36.9) µm long (Both ventral as well as lateral view of spicule sees (Fig. 2).

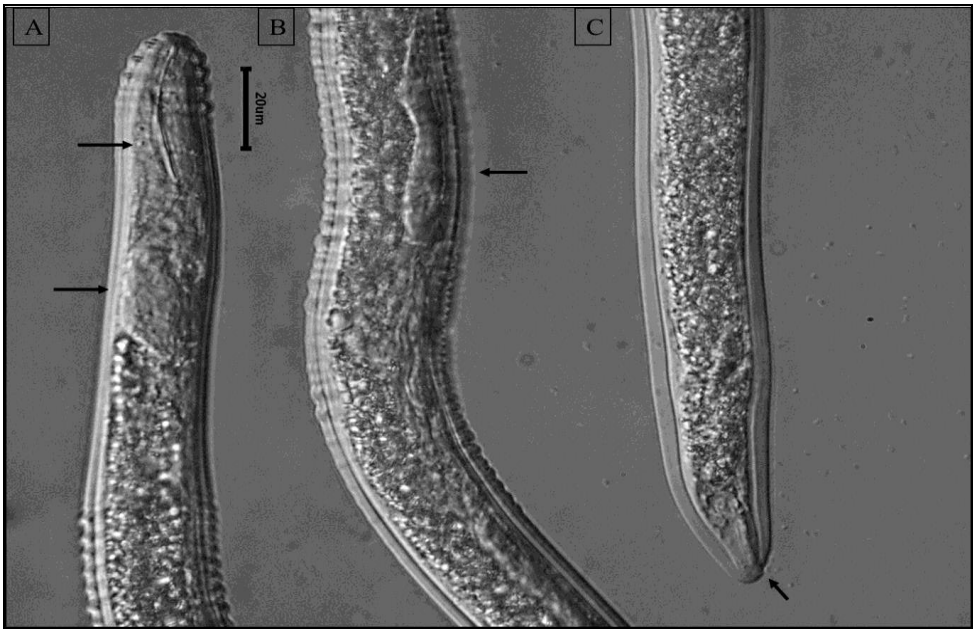


**Fig. 1.** Light microphotographs of *Paratrichodorus* sp. (adult female) **A-C** Cuticle showing fixation impact **A** Anterior body region with arrows pointing position onchiostyle and pharyngeal bulb **B** Anterior genital branch with sperm **C** Female tail with arrow pointing position of terminal anal opening (Scale bars: A, B& C= 20 µm).

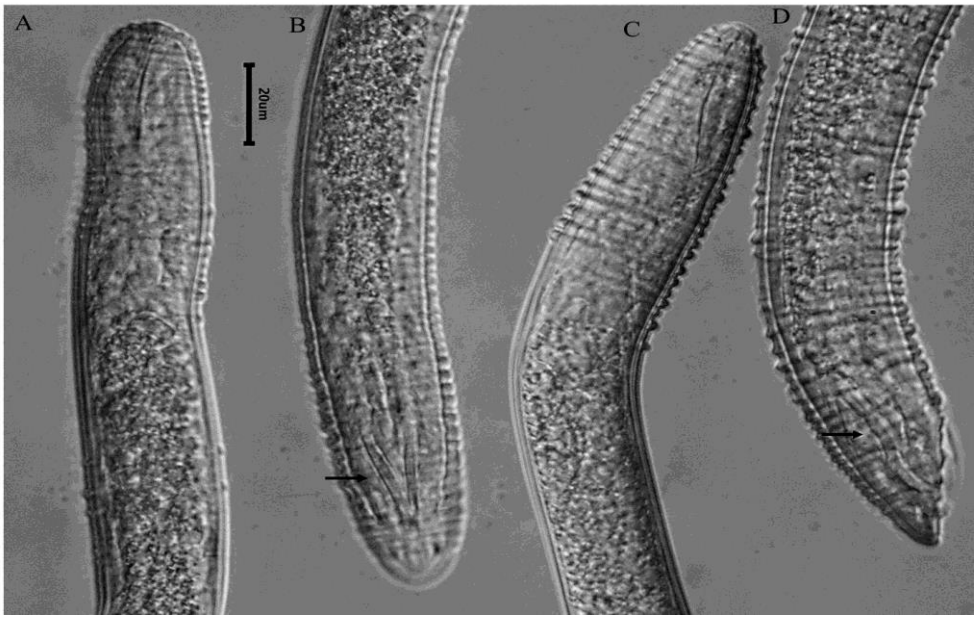


**Fig. 2.** Light microphotographs of *Paratrichodorus* sp. male **A-D** Cuticle showing impact of TAF fixation **A** Male anterior body region **B** Male posterior body region (in ventral view) with arrow pointing to paired spicule **C** Male anterior body region **D** Posterior body region (in lateral view) with arrow pointing position of spicule. (Scale bars: A, B, C & D = 20 µm).

**Table 4: Measurements of *Paratrichodorus* sp. (mean ± standard deviation, with range) All measurements are in µm.**

| Characters | Female (n=21) | Male (n=20) |
|---|---|---|
| L | 586.3± 62.4 (500.0-690.4) | 584.3±44.1 (498.2-645.2) |
| a | 19.3±1.6 (16.5-22.6) | 19.2±1.7 (16.0-22.1) |
| b | 5.4±0.4 (4.5-6.2) | 5.2±0.5 (3.9-6.0) |
| c | - | 30.3±2.3 (25.1-34.5) |
| V | 47.5±5.4 (38.6-53.3) | - |
| Lip width | 9.0±0.7 (7.9-10.1) | 9.1±0.7 (7.9-10.2) |
| Onchiostyle | 33.3±3.2 (28.1-39.1) | 31.7±5.8 (29.1-37.5) |
| Pharynx length | 109.0±5.8 (101.1-120.1) | 112.7±6.0 (103.2-126.2) |
| Pharyngeal bulb length | 29.9±3.0 (25.2-34.9) | 33.5±2.2 (30.2-37.1) |
| Pharyngeal bulb diam. | 13.2±1.3 (11.1-15.1) | 14.9±0.7 (13.5-16.2) |
| Body dia. at cardia | 27.7±1.6 (24.4-30.3) | 27.8±1.3 (26.1-29.8) |
| Body dia. at mid-body | 30.4±1.5 (27.1-32.8) | 30.5±1.0 (28.2-32.2) |
| Length of Anterior genital branch | 102.2±10.3 (85.4-112.1) | - |
| Length of posterior genital branch | 118.2±22.6 (97.9-161.5) | - |
| Anterior end to vulva | 282.6±23.8 (238.4-308.1) | - |
| Anal body dia. | - | 19.3±0.9 (17.8-20.9) |
| Spicule length | - | 32.9±2.1 (30.1-36.9) |
| Tail length | - | 12.5±0.8 (11.5-13.9) |
| Cuticle thickness at tail tip | 3.3±0.3 (3.1-3.8) | - |
| Cuticle thickness at mid-body | 4.5±0.4 (4.0-5.1) | - |

**Molecular Characterization and Phylogenetic Relationship**

The partially amplified sequence fragment of 18S for *Paratrichodorus* isolate iar123 was ca 800 bp. The sequence was submitted to GenBank under the accession number MZ567177 (18s rRNA). A BLAST search against the NCBI database showed that the18s rRNA sequence of P. isolate iari123 was 98.08% identical with *Paratrichodorus teres* (KJ636338.1; query coverage =99%, e-value = 0). Our 18s rRNA phylogenetic analysis (Fig. 3) has not resolved the relationship between *P. teres*, *P. allius* and *P. porosus* (Cluster II-A as in Duarte *et al.,* 2011).

## DISCUSSION

*Paratrichodorus* Siddiqi, 1974, commonly known as stubby-root nematode is an ectoparasite of many crops. More than 100 species have been described across the world. Identification of *Paratrichodorus* is relatively difficult based on morphological features alone due to little differences among closely related species. Of the 26 species known around the world, only four species namely *Paratrichodorus acaudatus*, *P. mirzai*, *P. paramirzai*, and *P. porosus* have been described from India. Morphological and morphometrical data were generated based on 20 females and 21 males in this study but these observations are not matching with morphometrics and descriptions of known and described species so far. Further, we could not make out the ventromedian cervical papilla or lateral cervical pores in the male specimens. We attempted to generate molecular sequence data for 18S rRNA, ITS-I & ITS-II of rRNA, and D2-D2) for the population, but only 18Sof rRNA could be sequenced. The phylogenetic analysis indicated that the population could be *Paratrichodorus teres* (Hooper, 1962; Siddiqi, 1974). However, the morphology and morphometric data did not support the molecular observations.

Therefore, additional marker gene sequence information is needed for comparison and designation of a new species.

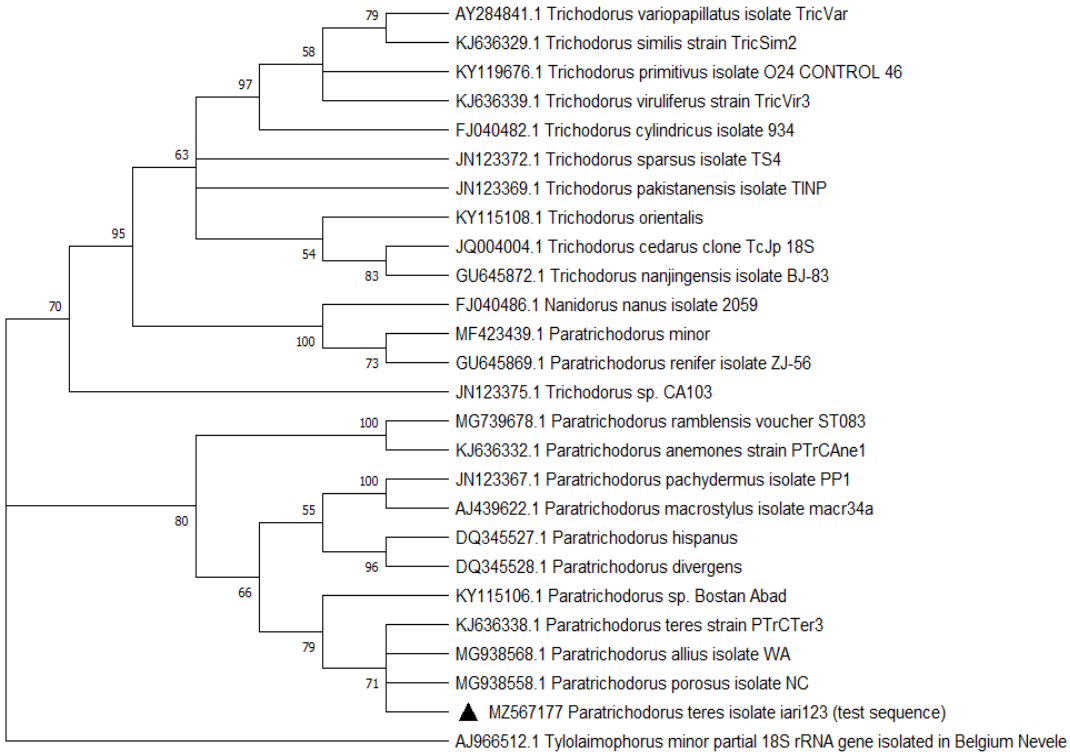


**Fig. 3.** Evolutionary relationship of *Paratrichodorus* isolate iari123 using the 18S rRNA sequence. The evolutionary history was determined by following the Maximum Likelihood (ML) method and Kimura 2-parameter model. The tree with the highest log likelihood (-6422.28) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join (NJ) and BioNJ algorithms to a matrix of pairwise distances estimated following the MCL approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was adopted to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.5898)). The rate variation model allowed for some sites to be evolutionarily not variable ([+*I*], 38.09% sites). This analysis adopted 26 nucleotide sequences. Evolutionary analyses were undertaken in MEGA X.

## CONCLUSION

A population of *Paratrichodorus* recovered from the rhizosphere of citrus plants was attempted to characterize based on morphology, morphometrics, and molecular studies. Morphology and morphometrics (21 females and 20 males) were studied and compared with the species described under the genus. We considered the *Paratrichodorus* population is quite different from the known and described species. Further, we attempted to generate the molecular information for further characterization. The amplified sequence fragments of 18S for *Paratrichodorus* isolate iari123 were *ca* 800 bp. The sequence was submitted to GenBank under the accession number MZ567177 (18s rRNA). A BLAST search against the NCBI database showed that the 18s rRNA sequence of *Paratrichodorus* isolate iari123 was 98.08% identical with *Paratrichodorus teres* (KJ636338.1; query coverage = 99%, e-value = 0). However, 18s rRNA phylogenetic analysis has not resolved the identity of the species. Therefore, the population needs additional sequence information from the ITS-I & ITS-II of 18S, D2-D3 of 28S regions, and *COI* of mt DNA for confirmation of the species.

## FUTURE SCOPE

**Enhanced Molecular Characterization**. Expand the molecular analysis by sequencing ITS-I & ITS-II of 18S, D2-D3 of 28S, and COI of mt DNA to confirm species identity and resolve potential ambiguities.

**Population Diversity**. Investigate samples from various locations and hosts to unveil potential subspecies or genetic variations within the Paratrichodorus population.

**Ecological Interactions**. Explore the nematode's ecological role in citrus plant health, soil dynamics, and disease transmission for practical implications in citrus cultivation.

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