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Molecular characterization of nematode fauna associated with the rhizosphere of jamun (*Syzygium cumini* L.) trees from IARI, New Delhi, India

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Abstract
Jamun is an important fruit tree found in many tropical and subtropical regions of the world. It is one of the key indigenous minor fruits of commercial value and also known for its timber and ornamental use. There are very few attempts to characterize the nematode biodiversity associated with Jamun trees. In the present study, molecular approach using DNA markers like ITS (Internal transcribed spacer) rDNA, D2-D3 expansion segments of 28S rDNA are used for identification of nematodes associated with Jamun trees in IARI, New Delhi. Predominant nematodes found are *Paralongidorus* spp, *Discolaimus* spp, *Discolaimoides* spp, *Paractinolaimus* spp, *Ecumenicus* spp, *Tylenchorhynchus* spp, *Rotylenchulus* spp and *Hemicriconemoide* spp.

Keywords: Jamun, *Syzygium cumini*, rhizosphere, nematodes, molecular approach

Introduction
Jamun, *Syzygium cumini* is an evergreen tropical tree and belongs to Myrtaceae family. It is also known as Malabar plum, Java plum, black plum, or jambolan. Jamun is indigenous to the Indian subcontinent from where it has spread overseas. It thrives well under both tropical and subtropical climatic conditions. Jamun is a very hardy fruit tree and grows well even under adverse soil and climatic conditions. Given the fact that Jamun trees are preferred for their fruit, timber and ornamental value, they are still not grown as orchards. These trees are tall and have dense foliage, hence, generally grown as avenue tree or as wind break [1]. Jamun fruits contain good amount of iron and a moderate content of vitamin C. These fruit have tremendous health benefits and used in Ayurvedic medicine. These nutritious wild edible fruits play an important role in maintaining livelihood security for many people in the country [2].
Assessment of nematode fauna in forest trees including Jamun trees has been a neglected area of research. While many of the free-living nematodes and predatory nematodes are beneficial to plants, some nematodes are potentially harmful. Specifically plant-parasitic nematodes (PPNs) are mainly belowground pests and cause insidious damage which in turn may lead to death of the established trees [3]. Therefore, it is very important to identify the nematodes especially PPNs associated with such valuable perennial tree. In this study, an effort is made using molecular approach to highlight the nematode fauna associated with the Jamun trees which are grown as avenue trees in IARI campus, New Delhi.

Materials and Methods
A survey was conducted for the assessment of free-living and plant-parasitic nematode fauna present in the rhizosphere of Jamun trees in IARI, New Delhi. Soil and root samples were collected during October 2019 from the rhizosphere of 20 Jamun trees from a depth of 40-50 cm from the soil surface. An aliquot of 200 cc soil from each sample was processed by Cobb's decanting and sieving method followed by modified Baermann's funnel method for extraction of vermiform stages [4, 5]. Nematodes were picked singly and transferred to 0.5 ml microfuge tubes (PCR tubes) containing 25µl of nuclease free water for further molecular identification.

Molecular characterization
DNA was extracted from individual nematodes using Worm Lysis Buffer (WLB: 0.2M NaCl, 0.2M Tris pH 8.0, 1% β-mercaptoethanol, 800µg/ml of proteinase K) (modified after Castagnone-Sereno *et al*., 1995) [6].

25µl of worm lysis buffer was added to the tube containing nematode/s in 25µl of nuclease free water and incubated at 60 °C for 2-3 h followed by incubation at 100 °C for 5-10 min and stored at-20 °C [7].

PCR analyses were carried out with genomic DNA extracted from nematodes using universal primers to amplify two rRNA gene fragments [ITS (Internal transcribed spacer) rDNA, D2-D3 expansion segments of 28S rDNA], to confirm identity at least up to genus level (Table 1 and 2). PCR amplifications were carried out in a total volume of 25 µl reaction having 2.0 µl of DNA, 1.0 µl of each 10µM primers (forward and reverse), 2.5 µl of 10X buffer,1.5 µl 200mM of each dNTP and 2 units of Taq polymerase enzyme and made upto 25µl using MilliQ water [7].

Table 1: Details of primers used for identification of nematodes associated with Jamun tree rhizosphere

S.No.	Primers
1.	Universal primers (ITS-F/R; D2D3-F/R) [8, 9]
	ITS-F: 5'- TTGATTACGTCCCTGCCCTTT 3'
	ITS-R: 5'- TTTCACTCGCCGTTACTAAGG 3'
	D2D3-F: 5'- ACAAGTACCGTGAGGGAAAGTTG 3'
	D2D3-F: 5'- TCGGAAGGAACCAGCTACTA 3'

Table 2: PCR amplification conditions used for different primer sets

PCR conditions	ITS	D2/D3
Initial denaturation	95 ºC for 5 min	95 ºC for 10 min
Denaturation	95 ºC for 45 sec	94 ºC for 30 sec
Annealing	56 ºC for 45 sec	60 ºC for 45 sec
Extension	72 ºC for 90 sec	72 ºC for 45 sec
Final extension	72 ºC for 10 min	72 ºC for 10 min
Hold	4 ºC	4 ºC
Number of cycle	40	40

5 µl of amplified products were resolved on 1.0% agarose gel prepared in 1X Tris acetate EDTA (TAE) buffer (pH 8.0) containing Ethidium bromide. Electrophoresis was performed at 5V/cm for 45 min. The gels were visualised using Alpha image analyser. The amplified PCR products were sequenced at First base, Malaysia by Sanger dideoxy sequencing method. The sequence information of each marker was analysed by BLASTN analysis at NCBI to confirm nematode identity.

Results and Discussion

Characterization of various nematodes associated with a perennial tree based on morphological characterization is a tedious and time consuming job. It also demands high expertise in accurate genus and species identification. Therefore, molecular approach using specific DNA markers was undertaken to arrive at rapid and accurate identification of both free-living and plant-parasitic nematodes. Individual nematodes were picked and used for molecular identification using DNA based markers i.e. ITS and D2D3.

PCR amplification with ITS primers produced an amplicon of 750bp in all samples. Amplification using D2D3 primer produced amplicons in the range of 1000-1500bp (Figure 1 and 2). PCR products showing specific sharp, single bands were sequenced. Sequence information was analysed using NCBI BLAST tool. Top hit for each sequence with query coverage, E value, percent identity, total score are presented in table 3. Sequence information of each of the markers analysed was submitted to GenBank database; their accession numbers from the GenBank are presented in Table 4.

BLAST analysis of sequence information of ITS rDNA and 28S (D2/D3) rDNA revealed that major nematodes present in the samples are Dorylaimid nematodes such as *Paralongidorus* spp, *Discolaimus* spp, *Discolaimoides* spp, *Microdorylaimus* spp, *Ecumenicus* spp and *Paractinolaimus* spp. Tylenchid nematode such as *Tylenchorhynchus* sp., *Rotylenchulus* sp., and Criconematids such as *Hemicriconemoides rosae* have also been found.

Among these *Discolaimus* spp, *Discolaimoides* spp, *Microdorylaimus* spp, *Paractinolaimus* spp are categorised as predatory nematodes. *Ecumenicus* spp, *Microdorylaimus* spp are under omnivores category [10, 11]. Dorylaimids are known to be present in undisturbed soil such as uncultivated forest soils. Hence presence of predatory and omnivore nematodes in the Jamun rhizosphere indicate an undisturbed soil ecosystem. Omnivores contribute for nutrient recycling in the ecosystem. Predatory nematodes such as *Discolaimus* spp have the potential in bio-control of plant-parasitic nematodes and play a role in maintenance of ecological balance [12].

Paralongidorus spp is migratory ecto-parasites of plant roots. *Tylenchorhynchus* spp, *Rotylenchulus* spp, and *Hemicriconemoides* spp are also parasites of plant roots [10]. While these plant parasites do not cause serious harm, under f avourable conditions they are likely to cause severe harm.

Specifically high organic content, optimum temperature and adequate moisture in the rhizosphere of perennial trees are conducive for nematode survival and infection.

Table 3: BLAST analysis of sequence information of ITS and 28S rDNA taxonomic markers from nematodes fauna of Jamun tree

BLASTn analysis of 28S (D2D3) rDNA marker sequence information						
Sample No.	**Top hits**	**Acession**	**Total score**	**Query value**	**E value**	**% identity**
J1	*Paralongidorus bikanerensis*	JN032584.1	1424	93%	0.0	99.87%
J2	*Discolaimus major* clone 2	AY593026.1	749	92%	0.0	84.72%
J3	*Discolaimoides symmetricus* clone 1214	EF207238.1	887	92%	0.0	88.80%
J4	*Discolaimus* sp. 3 HMM2018 isolate HMM325	KY750793.1	132	24%	3e-26	79.40%
J5	*Microdorylaimus* cf. *modestus* D47	HM235513.1	89.8	24%	2e-13	75.23%
J6	*Rotylenchulus borealis* isolate 193_7	MK558206.1	1112	97%	0.0	94.44%
J7	*Tylenchorhynchus* sp. 2 CCN-2014 isolate SUB582	KJ461561.1	294	60%	4e-75	77.57%
BLASTn analysis of ITS marker sequence information						
Sample No.	**Hits**	**Acession**	**Total score**	**Query value**	**E value**	**% identity**
J8	*Paralongidorus bikanerensis*	JN032585.1	1823	92%	0.0	99.31%
J9	*Rotylenchulus* sp., isolate BMMITS1S0202	KF020186.1	248	29%	4e-61	80.86%
J10	*Hemicriconemoides rosae* isolate Rose_2	MK371816.1	1716	100%	0.0	98.47%
J11	*Ecumenicus* sp. 85G11	MK292127.1	255	24%	2e-63	92.70%
J12	*Paractinolaimus* sp. ESB-2014	KM067902.1	255	15%	2e-63	94.61%

Table 4: Nematodes assemblages associated with Jamun tree rhizosphere and GenBank accession numbers

Sr. No	Species	Marker	Accession number
1	*Paralongidorus bikanerensis*	D2D3	MT776549
		ITS	MT776548
2	*Discolaimus major*	D2D3	MT776558
3	*Discolaimoides symmetricus*	D2D3	MT776559
4	*Discolaimus sp.*	D2D3	-
5	*Microdorylaimus*	D2D3	-
6	*Rotylenchulus borealis*	D2D3	MT775429
		ITS	-
7	*Tylenchorhynchus* sp.	ITS	-
8	*Hemicriconemoides rosae*	ITS	MT776565
9	*Ecumenicus* sp.	ITS	-
10	*Paractinolaimus* sp.	ITS	-

Worldwide very few attempts have been made to highlight the nematode populations associated with Jamun trees. Few investigations showed the association of *Fergusobia* spp, *Filenchus* spp, *Helicotylenchus* spp, *Basiria* spp, *Tylenchus* spp and *Meloidogyne incognita* nematodes with Jamun trees [13, 14].

In the present study, DNA markers such as ITS rDNA and D2D3 expansions of 28S rDNA are used to identify nematode community present in rhizosphere of Jamun tree. Nematodes exist as poly specific community. In such situation, molecular approach using DNA based markers help in identification of multiple species present in the sample. Molecular approach is also a faster and reliable method of nematode identification where there is lack of skilled nematode taxonomist.

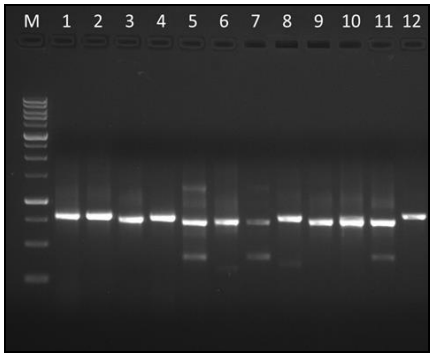

Fig 1: PCR amplification of nematode DNA samples using ITS marker M: 1 kb DNA ladder; Lane 1-12: Amplification of ITS marker

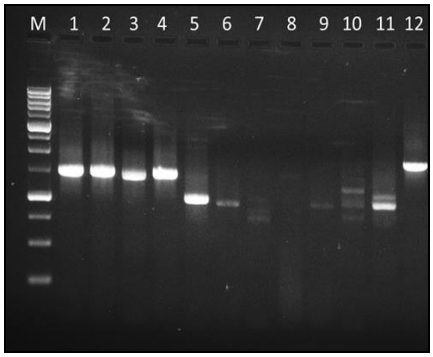

Fig 2: PCR amplification of nematode DNA samples using D2D3 marker M: 1 kb DNA ladder; Lane 1-12: Amplification of 28S (D2D3) marker

Conclusion

To our knowledge, this is the first report of molecular identification of nematodes associated with Jamun tree from Delhi. Present findings indicate that Dorylaimid nematodes are predominant in the undisturbed root zone of Jamun trees in IARI, New Delhi. Such efforts to document nematode assemblages associated with wild, uncultivated trees help to monitor the diversity of nematodes and assist in assessing any unforeseen damage to both the cultivated and uncultivated plants.

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