Colletotrichum fusiforme JAYAWARDENA, BHAT, N.TANGTHIR, K.D.HYDE, A NEW FUNGAL RECORD FROM THE INDIAN SUBCONTINENT

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ABSTRACT

This communication deals with the description and illustration of a new fungal record Colletotrichum fusiforme Jayawardena, Bhat, N.Tangthir, K.D.Hyde. The fungus was isolated from the leaves of a medicinally and aesthetically important Srilankan Chest Wood plant (Mesua ferrea L., Family – Calophyllaceae), during the survey of the Botanic garden of Dr Harisingh Gour University, Sagar, Madhya Pradesh, India. The study was carried out by the conventional technique (morpho-mycotaxonomy) and it was confirmed by ITS sequence analysis, the phylogenetic relationships are made with MEGA-X.

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KEYWORDS

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1 Introduction

*Mesua ferrea* L., Family – Calophyllaceae, is a very important medicinal plant. It is a medium-sized evergreen plant distributed in almost all Asian countries like Burma, Cambodia, Indonesia, Malaysia, Sri Lanka, Myanmar, Nepal, Philippines, Sumatra and Thailand (Asif et al., 2017). *M. ferrea* is widely used as an antipyretic, antimicrobial, anticancer, carminative, cardiotonic, diuretic and expectorant (Rahman et al., 2008; Chahar et al., 2012). In India also, *M. ferrea* is widely used in the number of ayurvedic medicines for the treatment of many diseases like bleeding piles, cough, cardiovascular disorder, dysentery, excessive thirst, scabies (Joseph et al., 2010; Lim, 2012). Presence of phytochemicals like xanthones, terpenoids and sterol justify its medicinal properties (Keawsa-ard et al., 2015).

*M. ferrea* tree is threatened by many fungal pathogens among these foliar pathogens are also well reported. Presence of the fungal genus *Colletotrichum* sp. was also reported from the tree (Sunkar et al., 2017). Genus *Colletotrichum* was first reported by Tode (1790) as the genus *Vermicularia*, while its current name was established by Corda (1831) for *C. lineola* (Cannon et al., 2012). This genus is considered as the plant pathogenic genus which causes diseases in various economically important plants (Jayawardena et al., 2016). According to Dean et al. (2012), its host range varies from trees to the small grasses. The *Colletotrichum* sp. exhibits various natures such as endophytic, parasitic, saprophytic lifestyles (Kumar, 2014). The inoculums of *Colletotrichum* are disseminated through wind or rain from one to another host and for the extensive growth and higher degree of infection; it requires warm and humid conditions (Purkayastha & Sen Gupta, 1973; Farr et al., 2006; Kumar, 2014). During the intensive survey of Dr Harisingh Gour University, Sagar botanical garden vegetation, conspicuous pinhead dots was reported on the leaves of *M. ferrea*. Aim of this investigation was to identify fungal pathogens associated with these pinheads dots.

2 Materials and Methods

2.1 Sample collection:

The weather of Sagar M.P. is highly humid and rainy from July to November which favours the growth and development of various fungal pathogens (Figure 1). The infected leaves of *M. ferrea* plants were collected in new unused polythene bags from the botanical garden of Dr Harisingh Gour University, Sagar. Each polythene bag was marked by paper tag on which descriptions of the host plant, local name, place, collection date, symptoms and serial number were noted (Mall & Kumar, 2014; Sabeena et al., 2018). After returning from the field visit, sample specimens were kept between blotters for proper pressing, this absorbs moisture which helps in avoiding saprophytic cross contaminators (Sabeena et al., 2018).

2.2 Isolation of fungi & Morphological characterization

The collected samples were processed according to the standardized procedure (Savile, 1962; Hawksworth 1974). The detailed and critical morphological identification was carried out with the help of Scanning Electron Microscope (NOVA NANOSEM 450). For this, specimens were dehydrated and coated.
with conducting material (Gold) to prevent charge built from electron beam (Hall & Hawes, 1991). The SEM microphotograph revealed the unseen structures of the fungus and their measurements. With the help of standard monographs of Coelomycetes (Sutton, 1980) and some other important available literature, the comparison list was prepared (Table 2). The Holotype of the specimen has been deposited for accession number in Ajrekar Mycological Herbarium (AMH) of Agharkar Research Institute (ARI), Pune, Maharastra India.

2.3 Molecular characterization

For molecular studies, the fungal organism was isolated from the infected leaf of *M. ferrea* and aseptically transferred to the PDA media. The pathogen grows prolifically on PDA media under a controlled environment at 25-28°C in the incubator. Vigorous growth was obtained after the fifth day of inoculation, by subsequent subculture process, pure culture of the desired isolate obtains. The pure culture has also been deposited to the National fungal culture collection of India (NFCCI), Agharkar Research Institute (A.R.I.) Pune, Maharastra (India) for the accession. DNA from the pure culture was isolated by using the CTAB extraction buffer (Carlier et al., 1996). This isolated DNA was used for the sequencing and distinguishes differences between various *Colletotrichum* species by Mills et al. (1992) method. The variation in the sequence of the ITS1 region of rDNA used for the detection of polymorphisms in the same region between various strains of *Colletotrichum* (Sreenivasaprasad et al., 1992). ITS4 & ITS5 universal primers (White et al., 1990) were used to amplify the ITS gene in sequencing PCR ABI3100 automated DNA sequencer with ABI-BigDye® Terminator 3.1 Cycle sequencing kit. The sequence of the Universal ITS primers used in the current study are:

ITS 4 (5’TCTTCCGCTTTATGATGC3’) (Forward primer)

ITS 5 (5’GGAAAGTAAAGTCGTAACAGG3’) (Reverse primer)

To render the similarities between different species the DNA sequence BLAST (Basic Local Alignment Search Tool) is done on the NCBI website (https://blast.ncbi.nlm.nih.gov). The DNA sequence was also deposited in the NCBI database and obtain genebank accession no. MH352480. The DNA sequences of the other similar species were downloaded and utilized for making a phylogenetic tree by using MEGA-X. Host records were checked for the novelty of the taxa by using the Index Fungorum website (http://www.indexfungorum.org) and the USDA website (https://nt.arsgrin.gov/fungal databases).

2.4 Phylogenetic analysis

For the conversion of the raw DNA sequence into the fasta format EMBOSS programs-EMBL-EBI online software is used, DNA Sequence (Fasta format) is used for the NCBI BLAST (Basic Local Alignment Search Tool). NCBI BLAST hits provide the sequence similarity percentage, query coverage, E-value (Table 3) in between the sequences already submitted by the different workers, with these data, it is easier to identify the isolate at the species level, it also provides the facility to download the most related sequences for making the phylogenetic tree by aligning them using Muscle (Edgar, 2004) in MAGA X (Hall, 2013) software, which gives the clear cut estimation of the relationship between the taxa (Sequences) (Nei & Kumar, 2000; Felsenstein, 2004; Hall, 2011). Now the test of phylogeny was done by the bootstrap method using the maximum likelihood statistical method, with 1000 replications.

2.5 Pathogenicity test

The conidial suspension is spread over the artificially damaged *M. ferrea* leaves and keeps in incubation at room temperature (25°C – 28°C) for the proper spreading of the fungal infection. After 72 hours of inoculation, fungal infections start appearing over the leaf surface same as obtained during the investigation and at the end of the seventh-day ambient infection observed. This test was applied in triplet sets in which the optimum temperature varied (Table 1)

Table 1 showing growth variations at the different temperature range

| Set     | Temperature variance | Growth visualization               |
|---------|----------------------|------------------------------------|
| Set 1   | 22°C – 25°C          | Little to moderate growth observed  |
| Set 2   | 25°C – 28°C          | Ambient growth observed            |
| Set 3   | 28°C – 31°C          | Initially growth increases but later on it starts declining |

3 Results

3.1 Morphotaxonomic Identification:

Leaf spots are amphigenous, irregular, starts from the acute apex of the leaf and further extending back (Figure 2). The colony is epiphyllous with fine pinhead dots, dark brown to dark black with internal mycelium. Conidiomata are acervular, cuticular, dehiscence irregular, 37.50 – 75 x 22.5 – 50 µm. Sclerotia are indistinct. Setae are present and arise from the base of conidiomata, abundant, small to long, straight to flexuous, base
swollen, tapering apex, dark brown to black, aseptate, 25 – 175 x 2.5 – 7.5 µm. Conidiophores are mostly indistinct, 8.7 – 16.43 µm in size in SEM imaging (Figure 4). Conidiogenous cells are enteroblastic, phialidic, and the average size is 1.411 µm in SEM images, hyaline, determinate. Conidia are hyaline, single-celled (aseptate), smooth, and straight to falcate acute at both ends with 12.5 – 25 x 2.5 – 5.0 µm size (Figure 3). The comparison with the allied taxa is shown in table 2. Based on the above-said characteristics, morpho-taxonomic identification suggested that isolated fungal species is *Colletotrichum fusiforme* Jayawardena, Bhat, N. Tangthir, K.D.Hyde, *Holotype, AMH – 9939.*

### 3.3 Molecular Identification:

NCBI BLAST analysis of top five BLAST hits indicates that the isolated DNA sequence show 100 per cent query coverage and identity with the *Colletotrichum fusiforme*, Fungal endophyte isolate LIES529, *Colletotrichum sp.* WF134, *Colletotrichum sp.* WF115, *Colletotrichum sp.* VNCF2 (Table 3). The evolutionary history was inferred by using the maximum likelihood method and the Tamura-Nei model (Tamura & Nei 1993). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the analyzed taxa (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50%
bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. This analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1182 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018). Culture accession and deposition number is 4272, while Genebank accession no. MH352480.

Tajima's neutrality test is a population genetic evolution test (Table 4) that shows whether the genes are evolving randomly (Naturally) or non-randomly. The randomly evolving DNA sequences have mutations with no effect on the health and survival of the organism.

Figure 2 Symptoms of *Colletrotrichum fusiforme* Jayawardena, Bhat, N.Tangthir, K.D.Hyde on *M. ferrea* L. (Holotype, AMH – 9939), a - Infected host plant, b-e - infection spots along with colonies on different leaves.

Figure 3 showing the morphology of acervulus, conidia and setae, a-b - acervulus with abundant setae, c - numerous conidia, d - seta (Scale Bars a-d – 20 µm)

Figure 4 SEM visualization of *Colletrotrichum fusiforme* Jayawardena, Bhat, N.Tangthir, K.D.Hyde on *M. ferrea* (Holotype, AMH – 9939), a - colony over leaf surface (Scale = 50 µm), b – setae, c – conidiophores, d – acervulus with numerous setae, e – conidiogenous cell.
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while the DNA sequences evolving by non-random process having mutations that have an impact on the health and survival of the organisms then, in this case, natural selection process occurs. When Tajima's D = 0, it indicates that observed variation in the organism is similar to the expected variation and population of the organism evolving as per mutation-drift equilibrium. No natural selection observed here. When Tajima's D<0 then negative Tajima's D signifies an excess of a low frequency of polymorphic organisms after a bottleneck or a selective sweep, indicating homozygous population size expansion. Tajima's D>0 the positive value of Tajima's D signifies a high frequency of polymorphic organisms, indicating a decrease in population size and/or balancing selection. In the current study, the value of Tajima's D is negative which indicates the increase in the homozygous population size (Figure 6).

3.4 Classification: Based on morphological, molecular and genetic characteristics, the fungal parasite isolated from the infected M. ferrea leaves was identified as Colletotrichum fusiforme and detail classification of this is as follows:

Kingdom: Fungi
Division: Ascomycota
Class: Sordariomycetes
Order: Phyllachorales
Family: Phyllachoraceae
Genus: Colletotrichum
Species: fusiforme

| Gene Bank Accession No. | Description                  | Max score | Query cover | Query coverage | E value | Identity (%) |
|-------------------------|------------------------------|-----------|-------------|----------------|---------|--------------|
| NR_138010.1             | Colletotrichum fusiforme     | 868       | 868         | 100%           | 0.0     | 100%         |
| KX816018.1              | Fungal endophyte isolate LIERS29 | 868       | 868         | 100%           | 0.0     | 100%         |
| HQ130691.1              | Colletotrichum sp. WF134     | 868       | 868         | 100%           | 0.0     | 100%         |
| HQ130674.1              | Colletotrichum sp. WF115     | 868       | 868         | 100%           | 0.0     | 100%         |
| DQ463363.1              | Colletotrichum sp. VNCF2     | 868       | 868         | 100%           | 0.0     | 100%         |

Table 4 Tajima's Neutrality Test

| m  | $S$  | $p_s$ | $\Theta$ | $\pi$ | $D$   |
|----|------|-------|----------|-------|-------|
| 6  | 186  | 0.157360 | 0.068917 | 0.050987 | -1.688390 |

$S$ = Number of segregating sites; $p_s$ = $S/n$; $\Theta = p_s/\alpha$; $\pi$ = nucleotide diversity; and $D$ is the Tajima test statistic (Tajima, 1989).
4 Discussion

The isolated ITS sequence show 100 per cent sequence similarity with C. fusiforme (Altschul et al., 1990) and clusters with the C. fusiforme with 77 per cent bootstrap value in the phylogram generated with Maximum Likelihood Method using MEGA-X (Tamura & Nei 1993). Tajima’s D test gives the negative value which indicates the increase in the homozygous population size. Morphologically the isolate showing great similarity with C. fusiforme in its dimensions of setae, conidia but at the same time the isolate show some differences in some of the characters such as setae (aseptate) and conidia (only smooth-walled and egutulate). The detailed phylogenetic analysis on morphological as well as a molecular basis, the isolate was identified as Colletotrichum fusiforme Jayawardena, Bhat, N. Tangthir, K.D.Hyde. The available mycological literature revealed that this is the first record from India and the first report on the host M. ferrea (Index fungorum website on June 2020).

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Conflict of interest

The authors declare that they have no conflict of interest

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