First report on molecular identification of *Fusarium* species causing fruit rot of mandarin (*Citrus reticulata*) in Bangladesh

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Abstract

**Background:** Fusarium rot is a newly introduced, devastating disease of citrus fruits. The current investigation was undertaken to characterize the microbes responsible for fruit rot in *Citrus reticulata*.

**Methods:** Pathogens were isolated from infected citrus fruits using morphological and molecular approaches. For confirmation of the isolated fungi, polymerase chain reaction (PCR) amplification and internal transcribed spacer gene sequencing techniques were used.

**Results:** The isolated fungus was grown on potato dextrose agar for three days and it produced clamp hyphae, hyphae and macroconidia. PCR amplification of isolated fungal DNA gave a 650 bp product. The sequence obtained from isolated fungi had 99.42% similarity with the reference *Fusarium concentricum* sequence in NCBI GenBank. The obtained sequence was deposited in GenBank (Accession No. MT856371). Two isolates showed virulence capability on fresh guava, sweet orange and tomato fruits, which confirmed species identification and Koch's postulates. Artificially inoculated fungal species grown on tested fruits showed typical *Fusarium* species symptoms.

**Conclusions:** Outcomes of the present study are beneficial for the detection of this detrimental disease in postharvest *Citrus reticulata* fruits. Further research is needed for the control of this economically important disease. This is the first study of fruit rot in *Citrus reticulata* caused by *Fusarium* in Bangladesh.

**Keywords**

Citrus reticulata, Fruit Rot, Fusarium sp., PCR, ITS rRNA gene
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Introduction

*Citrus reticulata* Blanco, commonly known as mandarin, is an oblate fruit resembling other oranges, belonging to the family of Rutaceae (Ahmed et al., 2014) and originating from hybridization with *Citrus maxima* (Wang et al., 2018). Citrus fruits contain different vitamins, minerals and trace elements. *Citrus aurantifolia* fruits are usually eaten fresh or used in salads and also used as flavoring in some liqueurs (Morton, 1987). In traditional medicine, they are also used for the treatment of rheumatoid arthritis and obesity (Srinivasan et al., 2008).

*Fusarium* species can cause superficial infections in plants and animals with high mortality in persistently and severely neutropenic patients (Dignani & Anaissie, 2004). *Fusarium* species are highly competent at contamination, possessing several mycotoxins (O’Donnell et al., 2009) and different fruits decay in different storage and postharvest conditions (Whiteside et al., 1988). Citrus fruits lose their market value due to damage incurred by different pathogens, including fungi and bacteria. *Fusarium* fruit rot is a very common and destructive problem for mandarin after harvesting and marketing in Bangladesh (Ahmed et al., 2014).

The novel *Fusarium* fungi were isolated and identified through applying advanced methods on different crops from different countries (Akaruzzaman et al., 2018; Al-Najada & Gherbawy, 2015; Geiser et al., 2004; Sun et al., 2018). To develop biosafety and biosecurity management strategies, isolation, identification and characterization of fruit rot causing microbes are needed. The objectives of the study were to identify the microbes responsible for fruit rot of mandarins in Bangladesh using morphological and molecular approaches.

Methods

**Fungi isolation from the infected fruits**

In 2018, 10 rotten *Citrus reticulata* fruits (Figure 1A) were collected from fruit market stores in Rajshahi, Bangladesh. Out of 10 fruits, three showed symptoms of rot. Collected fruits were cleaned under running tap water to remove foreign agents and kept in a Biosafety Cabinet (Esco, Singapore). Moreover, the fruits were disinfested with 1% sodium hypochlorite (NaOCl) for 30 seconds, followed by five rinses in autoclave distilled water. Disinfested tissue was excised and plated on potato dextrose agar (PDA) (Hi-Media, India) at 35°C in the dark for three days. The colonies showing typical morphological characteristics including, colony color, pigmentation, growth rate and size of macroconidia of *Fusarium* species were selected (Hafizi et al., 2013) and isolated using the single spore technique (Chowdhury et al., 2019). Isolated colonies were transferred onto a Petri plate with PDA and incubated for seven days at 35°C in dark conditions. Isolates were grouped into two on the basis of morphological color (blackish color in the first group and white in the second group). Finally, one isolate from each of the two groups was selected for morphological analysis.

**Characterization of isolates**

The selected fungal colony was characterized by macromorphological and micromorphological investigation (Al-Najada & Gherbawy, 2015). The isolate was sub-cultured in fresh PDA medium and three-day-old cultures were mounted using the lacto-phenol cotton blue (LPCB) staining method (Sathy et al., 2017). The mounted microscope slide was covered with a cover slip and conidia were observed under a light microscope (LABOMED LX400, USA) at 40X magnification.

Genomic DNA was extracted from 15 gm of mycelia, collected from day three-day-old PDA cultures. DNA was extracted using a MaxiMaxwell® 16 LEV Plant DNA Kit (Cat No. AS1420, Promega, USA) and DNA quantity and quality were checked using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, USA).

To amplify the internal transcribed spacer (ITS) gene, primer pairs ITS4 (5′-TCCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) were used (Gardes & Bruns, 1993; White et al., 1990). The PCR reaction was performed using the method described by Hassan et al. (2018) using hot start green master mix (dNTPs, Buffer, MgCl2, Taq Pol) (Cat # M7432, Promega, USA). A total of 25 µl reaction volume containing 2 µl genomic DNA, 2.5 µl 1X PCR buffer, 1.0 µl MgCl2, 1.5 µl dNTPs, 0.5 µl of each primer, 0.5 µl of *Taq* polymerase and 16.5 µl of deionized water was used. The PCR was programmed with an initialization step at 95°C for 2 min, followed by 32 cycles of denaturation at 95°C for 30 seconds, primer annealing at 48°C for 30 seconds, and extension at 72°C for 45 seconds and a final extension at

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**Figure 1.** Naturally infected postharvest mandarin fruit showing symptoms of *Fusarium* rot and morphological phenotypes (F1). (A) Infected mandarin fruit; (B) fungus growth; (C) clamydospores, hyphae, appressoria; and (D) conidia under the microscope from seven-day-old culture at 35°C on potato dextrose agar.
Results

Morphological characterization

Collected samples were incubated on PDA medium following the single spore technique, and after seven days, white colored fungal colonies appeared (Figure 1B).

Isolated fungus was whitish in color and produced clamydospores, hyphae, appressoria and macroconidia (Figure 1C–D) at day three on PDA medium. Isolates produced microconidia that were 0-septate, oval, obovoid with a truncate base, elliptical or reniform. Macroconidia were sporodochia and fusiform. Chlamydospores were monophialides.

Molecular characterization

DNA amplification through PCR produced a bright band at approximately 650 bp where a 1kb DNA ladder was used as marker. No band was found in the negative control where water was used instead of template DNA (Figure 2).

The dendrogram tree showed a close relationship with *Fusarium concentricum* and dissimilarity with *Fusarium begoniae* (Figure 3). Therefore, molecular identification confirmed the isolates as *Fusarium* sp. The sequence of the total isolate was compared to *Fusarium* sequences in GenBank using BLASTN, which revealed closely related sequences and 99.42% homology with the reference sequence for *F. concentricum* (Accession No. NR_111886.1).

Virulence test

The virulence test was conducted to characterize the fungus as pathogenic or saprophytic on mature, fresh and healthy guava, lemon and tomato. All fruits showed similar morphological characteristics of *Fusarium* symptoms (Figure 4A–C). Isolated ribosomal DNA (rDNA) of fungus from artificially inoculated guava, lemon and tomato showed clear bands of approximately 650bp in length (Figure 5).

Discussion

Traditionally identification based on colony morphology, conidial morphology and other phenotypic characteristics has been used previously for different fungi of citrus fruits (Leslie & Summerell, 2006; Tafinta et al., 2013). Further confirmation of the isolated fungi using advanced morphological and molecular approaches is required for characterization and differentiation of closely related *Fusarium* species (Geiser et al., 2004). rDNA sequences of *Fusarium*, isolated from eggplant, lemon and onion (frequencies of occurrence ranging from 40% to 100%) were reported by Al-Najada & Gherbawy (2015).

The present *Fusarium* sp. responsible for mandarin fruit rot was identified using morpho– molecular approaches. *Fusarium* appeared as white or blackish-white and showed chlamydospores and macroconidia on PDA after seven days of culture. Huda-Shakirah et al. (2020) found 3-5 long, thin walled, septate macroconidia on a *F. concentricum* fugal stain under microscopic observation, which supports our present findings. Zhu et al. (2014) also found similar morphological characteristics for *Fusarium* isolated from *Eleocharis dulcis*. Phylogenetic analysis was done using comparative analysis with different
ITS-rDNA regions of sequences published in NCBI databases. The sequence of the fungal ITS-rDNA region was 546bp in size and matched the sequence of *Fusarium concentricum* in the database with 99.42% similarity. Huda-Shakirah et al. (2020) reported 99.53% similarity with *Fusarium concentricum* isolated from *Hibiscus sabdariffa*. The results of PCR and ITS sequencing confirm the isolated fungus as *F. concentricum*, which is supported by some other researcher’s findings (Chowdhury et al., 2019; Hasan et al., 2020; Hyun et al., 2000).

**Conclusions**

Fusarium fruit rot is a big problem for the citrus fruit industry in Bangladesh. In this study, *Fusarium* species was found to cause citrus fruit rot in Bangladesh. Moreover, pathogenicity was confirmed according to Koch’s postulates using three different types of fresh fruits. *Fusarium* species fruit rot leads to declines in the Bangladeshi fruit industry as well as fruit markets. Therefore, the current study may help the development of control measures for postharvest mandarin rot.
PCR amplification of internal transcribed spacer region yielded ~650 bp product for virulence test. 1kb DNA marker is used for size determination of inoculated fungus in different fruit samples. M - marker, G - guava, C - lemon, T - tomato, N - negative control.

**Figure 5.** PCR amplification of internal transcribed spacer region yielded ~650 bp product for virulence test. 1kb DNA marker is used for size determination of inoculated fungus in different fruit samples. M - marker, G - guava, C - lemon, T - tomato, N - negative control.

**Data availability**

**Underlying data**


**References**


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Leslie JF, Summerell BA: *The Fusarium laboratory manual*. Blackwell

This project contains the following underlying data:

- Figure 2.jpg (original, unedited gel image from Figure 2)

This project contains the following underlying data:

- Figure 3.jpg (original, unedited gel image from Figure 3)

This project contains the following underlying data:

- Micro.imag.1.jpg (original, unedited microscopy image showing conidia from Figure 1D)

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).


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General comment:
I found the article well-written and cohesive. I was happy to read about the whole article and I am glad to recommend for indexing with minor corrections.

Specific comments:

Title and abstract: Title is very informative and abstract gives good insight into the study.

Introduction: Author describe the introduction very well. Importance of the study, background and objectives are very clear. It provided important information.
Need to add some recent references.

Methods: I think this section was clearly describe with relevant references.

Results: This study describes the isolation and identification of a proposed new species of Fusarium. The isolate has been characterized using molecular approaches. The obtained sequence was deposited in GenBank (Accession No. MT856371). PCR and sequencing confirm the isolated stain. At the genomic level, the proposed new species was 99.42% similarity with the reference Fusarium concentricum sequence. Virulence test showed the fungus as pathogenic on healthy guava, lemon and tomato. Therefore, the proposed species meets the molecular and morphological requirements of being designated as a new species.
Need to add outgroup in the phylogenetic tree.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes
Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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