RESEARCH ARTICLE

First report on molecular identification of *Fusarium* species causing fruit rot of mandarin (*Citrus reticulata*) in Bangladesh [version 1; peer review: 1 approved, 1 approved with reservations]

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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 chlamydospores, 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

Open Peer Review

Approval Status

	1	2
version 2 (revision) 15 Feb 2023		 view
version 1 08 Oct 2020	 view	 view

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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 (Aktaruzzaman *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 whitish 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 (Sathya *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 MaxMaxwell® 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'-TCCTCCGCTTATTGATATGC-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

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.

72°C for 10 minutes using a G02 GeneAtlas PCR machine (Astec, Japan). PCR products were run by horizontal electrophoresis (Mini-Gel, CBS Scientific, USA) on 1% agarose (Cat # V3125, Promega, USA) gel with 0.5% ethidium bromide solution (Cat # H5041, Promega, USA) in 1x TAE buffer (Cat # V4251, Promega, USA) using a 1kb DNA ladder (Cat # G5711, Promega, USA) as marker and visualized under alpha imager UV trans-illumination (Mini, Protein Simple, USA). PCR products were purified from the agarose gel, using the Wizard SV Gel and PCR Clean-Up System (Cat # A9281, Promega, USA).

Purified PCR products were sequenced commercially by Sanger sequencing (Apical Scientific, Malaysia). Sequences were used in a search using the [NCBI BLAST tool](#). Sequences were submitted to GenBank and compared with the GenBank database. A phylogenetic tree was constructed using MEGA 10 software using the neighbor joining method ([Kumar et al., 2016](#); [Tamura et al., 2004](#)).

Virulence test

Virulence competency of the isolate was carried out using the method described by [Tafinta et al. \(2013\)](#). The surfaces of mature, fresh guava, lemon and tomato were sterilized using water and 70% ethanol. The fruits were holed using a 2 mm sized cork borer and selected fungal inoculums were aseptically placed in the holes. The inoculated samples and the control were placed in sterile polythene bags and incubated at 35°C for seven days in dark. Isolates from the fruits and colonies from the diseased lesions were sub-cultured in PDA. The isolated fungal stains were identified based on colony features, growth rates and pigmentation. For confirmation, genomic DNA was isolated from subcultured colonies, which were isolated from artificially infected fruits. PCR amplification was performed using same procedure described in our previous article ([Hasan et al., 2020](#)) for virulence potency test through ITS rDNA gene amplification.

Figure 2. PCR amplification of internal transcribed spacer region yielded ~650 bp product (F1). 1kb DNA marker is used for size determination, M - marker, I - isolate, N - negative control.

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 chlamydospores, 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* fungal 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

Figure 3. Phylogenetic tree based on the internal transcribed spacer region of rDNA showing closest relatives of fungal species isolated from citrus fruit samples (F1). The tree was constructed by neighbor joining method. The scale bar on the rooted tree indicates a 0.20 substitution per nucleotide position.

Figure 4. *Fusarium* rot symptoms in artificially inoculated fruits (F1). (A) Guava, (B) lemon and (C) tomato, the image was taken 10 days after inoculation.

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.

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

Fusarium sp. pure cultured isolate containing small subunit ribosomal RNA, internal transcribed spacer 1 and 5.8S ribosomal RNA on GenBank. Accession number, MT856371: <https://www.ncbi.nlm.nih.gov/nucleotide/MT856371.1?report=genbank>.

Figshare: PCR amplification of ITS region yielded ~650 bp product. <https://doi.org/10.6084/m9.figshare.13014209.v1> (Hasan, 2020a).

This project contains the following underlying data:

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

Figshare: PCR amplification of ITS region for virulence test. <https://doi.org/10.6084/m9.figshare.13008458.v1> (Hasan, 2020b).

This project contains the following underlying data:

- Gel doc.2.jpg (original, unedited gel image from Figure 5)

Figshare: Molecular identification of *Fusarium* species causing fruit rot. <https://doi.org/10.6084/m9.figshare.12990746.v1> (Hasan, 2020c).

This project contains the following underlying data:

- Micro.imag.1.jpg (original, unedited microscopy image showing chytrid spores, hyphae and appressoria from Figure 1C)
- Micro.imag.2.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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Barbara Sawicka

Department of Plant Production Technology and Commodities Science, University of Life Science in Lublin, Lublin, Poland

The work entitled: "The first report on the molecular identification of *Fusarium* species causing mandarin fruit rot (*Citrus reticulata*) in Bangladesh" is an important contribution to the research on identifying the causes of mandarin fruit rot. However, the authors made several mistakes that need to be corrected before the work is published.

Detailed notes:

- 1) The abstract is properly structured.
- 2) The introduction should introduce the reader to the subject of the work and end with a clear purpose of the work.
- 3) The purpose of the research should be clearly explained. The authors should also put forward an alternative research hypothesis to the null hypothesis in order to verify it later in the work.
- 4) The methodology of work should be extended to the methodology of sampling for testing (the number of samples and isolates should be sufficient to make statistical calculations) and the methodology of statistical calculations.
- 5) Research results should be thoroughly discussed with statistical justification.
- 6) The "Discussion" section needs to be expanded. It should include references to the latest literature in the field.
- 7) The application should be general and summative and should include a clear indication for the manufacturing practice. In addition, it should contain an indication (proposal) for the future.
- 8) The list of literature should be enriched with the latest items in the researched area.

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

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

No

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

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: agriculture, agronomy, commodity sciences, food safety, plant protection

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, however I have significant reservations, as outlined above.

Author Response 02 Jan 2023

Md. Faruk Hasan

Dear Reviewer,

Thank you for your valuable comments and suggestions about our manuscript. We make some correction according to your comments in different parts of our manuscript which are marked as red color. We are submitting the new version of our MS.

Regards

Authors

Competing Interests: Authors have no competing interest.

Reviewer Report 07 January 2021

<https://doi.org/10.5256/f1000research.29219.r73841>

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Shuvra Kanti Dey

Department of Microbiology, Jahangirnagar University, Dhaka, Bangladesh

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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