First report on molecular identification of *Fusarium* species causing fruit rot of mandarin (*Citrus reticulata*) in Bangladesh [version 2; peer review: 2 approved]

Mohammed Faruk Hasan¹, Mohammed Asadul Islam², Biswanath Sikdar²

¹Department of Genetic Engineering and Biotechnology, Faculty of Biological Sciences, University of Rajshahi, Rajshahi, 6205, Bangladesh
²Professor Joarder DNA & Chromosome Research Lab., Dept. of Genetic Engineering and Biotechnology, University of Rajshahi, Rajshahi, 6205, Bangladesh

**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 clamydospores, 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
Corresponding author: Biswanath Sikdar (sikdar2014@gmail.com)

Author roles: Hasan MF: Conceptualization, Investigation, Methodology, Writing – Original Draft Preparation; Islam MA: Conceptualization, Data Curation, Supervision; Sikdar B: Funding Acquisition, Methodology, Supervision, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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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).

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).

Fusarium species are one of the most imperative pathogenic fungi responsible for fruits rots of citrus causing lose their market value (Ezrari et al., 2022). Numerous Fusarium species have been reported with citrus and other fruits decay in different countries (Fogliata et al., 2013; Kurt et al., 2020; Mahmud et al., 2021; Paul et al., 2022). Fusarium fruit rot is a very common and destructive problem for mandarin due to harvesting and marketing in Bangladesh (Ahmed et al., 2014).

To the best of our knowledge, there is no report of Fusarium based mandarin fruit rot in Rajshahi, Bangladesh. Therefore, this study aims to give a clear understanding of the Fusarium species association with mandarin rot in Bangladesh using molecular approaches.

Methods

Fungi isolation from the infected fruits

Research location and samples collection. The research was conducted at Professor Joarder DNA and Chromosome Research Lab., Department of Genetic Engineering and Biotechnology, University of Rajshahi, Bangladesh during 2018 to 2019. The ten different rotten Citrus reticulata fruits (Figure 1A) were collected from fruit market in Rajshahi, Bangladesh. Out of 10 fruits, three showed symptoms of rot which were used for pathogen isolation.

Fungi isolation from the infected fruits. 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 and molecular analysis.

Morphological 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...
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.

The study performed using a completely randomized design (CRD). Data were analyzed using Analysis of Variance (ANOVA) to see the differences between *Fusarium* species pathogenicity (Kasiamdari & Sangadah, 2015). The analysis was performed using SAS software version 9.4.

**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, obvoid with a truncate base, elliptical or reniform. Macroconidia were sporodochia and fusiform. Chlamydospores were monopodial.

**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 dendogram 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.
Figure 3. Phylogenetic tree based on the internal transcribed spacer region of rRNA 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.

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 µm 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 dry root rot of citrus, lemon rot, dragon fruit rot, sugarcane wilt, eggplant rot and onion rot (frequencies of occurrence ranging from 40% to 100%) were reported by several researcher (Al-Najada & Gherbawy, 2015; Ezrari et al., 2022; Huda-Shakirah et al., 2022; Hasan et al., 2020; Hyun et al., 2000; Paul et al., 2022).

The present *Fusarium* spp. responsible for mandarin fruit rot was identified using morpho-molecular approaches. To confirm the morphological characterization, mandarin isolate of *Fusarium* were culture on PDA medium for seven days. *Fusarium* appeared as white or blackish-white and showed chlamydospores and macroconidia on PDA after seven days of culture. Microconidia were septate, oval and elliptical. Macroconidia were sporodochia and fusiform. Huda-Shakirah et al. (2020) found 3–5 µm 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*. To confirm the individuality of the isolated fungi, the ITS4 and ITS5 were amplified using primers ITS4F/ITS5R. PCR amplification of ITS regions of the isolated fungal strains gave ~650 bp products in size and the sequences showed 99.42% similarity with the *Fusarium concentricum* sequence in the database. Kurt et al. (2020) obtained approximately 538 bp PCR amplicon on *Fusarium*

isolated from mandarin using ITS regions. Huda-Shakirah et al. (2020) reported 99.53% similarity with *Fusarium concentricum* isolated from *Hibiscus sabdariffa*. These results are very similar to the present findings. Phylogenetic analysis was done using comparative analysis with different ITS regions of sequences published in NCBI database. Present findings of phylogenetic analysis showed that isolates of *Fusarium* species were in same clade with robust bootstrap support. Results of PCR products and ITS sequencing confirm the isolated fungus as *F. concentricum*, which is supported by some other researcher’s findings (Chowdhury et al., 2019; Ezrari et al., 2022; Hasan et al., 2020; Hyun et al., 2000; Paul et al., 2022). *Fusarium* species are considered one of the most varied fungal species. It was related with numerous plant hosts and is a thoughtful risk to *Citrus reticulata* production due to rot. It is also responsible for twig rot, decline dieback, blight and wilt of citrus. *Fusarium* rot of citrus is fetching a significant worldwide problem. This report for the first time confirmed that *Fusarium* species are the causative microbe of citrus fruit rot in Bangladesh. This report has significance to develop suitable management practices to control the *Fusarium* rot diseases of citrus fruits.

**Conclusions**

Fusarium fruit rot is a big problem for the citrus fruit industry in Bangladesh. In this study, *Fusarium* species were found to cause mandarin fruit rot. 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.

**Data availability**

**Underlying data**


Figshare: PCR amplification of ITS region yielded ~650 bp product. [https://doi.org/10.6084/m9.figshare.13014209.v1](https://doi.org/10.6084/m9.figshare.13014209.v1)

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](https://doi.org/10.6084/m9.figshare.13008458.v1)

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](https://doi.org/10.6084/m9.figshare.12990746.v1)
This project contains the following underlying data:

- Micro.imag.1.jpg (original, unedited microscopy image showing clamydospores, hyphae and appressoria from Figure 1C)

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

References


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Current Peer Review Status: ✔ ✔

Version 2

Reviewer Report 27 February 2023

https://doi.org/10.5256/f1000research.142811.r163583

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

The work has been corrected and in its current version it is suitable for printing.

Competing Interests: No competing interests were disclosed.

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.

Version 1

Reviewer Report 19 December 2022

https://doi.org/10.5256/f1000research.29219.r158099

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Barbara Sawicka
1 Department of Plant Production Technology and Commodities Science, University of Life Science in Lublin, Lublin, Poland
2 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

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Shuvra Kanti Dey

1 Department of Microbiology, Jahangirnagar University, Dhaka, Bangladesh
2 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.

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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