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Morphological and Molecular Identification of Fungi for their Association with Postharvest Fruit Rots in Some Selected Citrus Species

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ABSTRACT

Purpose: *The availability of fresh Citrus fruits is limited by their susceptibility to invasion by microbial pathogens which leads to cause serious postharvest losses. The present study was carried out to isolate and morphologically identify postharvest fungal associations from selected Citrus fruit species (C. sinensis, C. limon, C. crenatifolia and C. medica) and to confirm their identity by molecular characterization.*

Research Method: *Postharvest fungal associations of selected Citrus fruit species were isolated, and identification was done based on morphological characteristics. Confirmation of fungal associations was done through phylogenetic analysis of newly generated ITS sequencing data. Further, frequency of occurrence of each fungal isolate was calculated in three different districts in Sri Lanka.*

Findings: *From the morphological and molecular identification, Collectrichum fructicola, Collectrichum gloeosporioides, Lasiodiplodia theobromae, Aspergillus niger and Pestalotiopsis sp. were recorded from C. sinensis. Neofusicoccum parvum, Collectrichum gigasporium and Aspergillus clavatus were isolated from C. crenatifolia. Further, Lasiodiplodia theobromae and L. pseudotheobromae were the only fungal association isolated from C. limon and C. medica fruit species, respectively. It is worthwhile noting that this is the first report of association of the C. gigasporium and Pestalotiopsis sp. from Citrus fruits in Sri Lanka.*

Research Limitations: *Since this research was mainly focused on the isolation and identification of the potential fungal associations, pathogenicity evaluation could not be carried out.*

Originality/value: *Findings of potential disease causative agents in citrus will be valuable for agriculture sector, to adopt and practice effective strategies to minimize postharvest losses of citrus fruits.*

Keywords: *Citrus fruits, Fungal associations, Molecular identification, Postharvest fungi*

INTRODUCTION

Citrus is the largest genus in the family Rutaceae and it is one of the most traded horticultural products in the world (Turner and Burri, 2013). Citrus fruits are native to the tropical and subtropical areas of Asia and later they spread to other parts of the world (Liu *et al.*, 2012). Most of the citrus fruits are highly preferred as fresh juice and as a flavour enhancer in food (Herath *et al.*, 2016). Due to the delicious taste and nutritional value it possesses, citrus has become one of the most desirable fruits in the local as well as international markets. Citrus fruits have higher contents of nutrients such as

vitamin A and C, sugars, minerals, and amino acids with beneficial properties for health such as antioxidant, anticancer and antimutagenic (Paul and Shaha, 2004; Herath *et al.*, 2016).

Citrus sinensis (Green cultivar is locally known as *"Pani dodam"*) is considered one of the most important fruit crops due to its sweet taste and other benefits such as nutritional and medicinal

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values. It is more popular among consumers as a fresh juice due to its succulent nature (Shravan *et al.*, 2018). *C. limon* (*"Lemon"*) is commonly used in both home and commercial recipes as a food ingredient. Further, lemon acts as an excellent preventative medicine and it has a wide range of uses in the indigenous medicine as well (Chaturvedi and Shrivastava, 2016). *C. crenatifolia* (locally known as *"Heen naran"*) is supposed to be native to Sri Lanka, which is well known for a wide range of medicinal properties, and has been used to treat vomiting, nausea and liver diseases.

The availability of fresh citrus fruits in the local and international markets have been limited by their susceptibility to physical, chemical, and pathological damages, causing serious postharvest losses. Any postharvest deterioration or decay may result in citrus fruits with an undesirable quality and a shortened storage life, thereby subjected to rejection of fruits in local and international trade (Al-Mouei, 2014). Citrus fruit loss has mainly been attributed to postharvest diseases caused by various pathogens which accounts for approximately 25 % - 30 % of the total wastage in the world (Saroj and Ambadas, 2008). Citrus fruits are more susceptible to infections by microbial pathogens due to the high nutrient composition and readily available water content, during the period between harvest and consumption (Tripathi and Dubey, 2004). They are more prone to fungal deterioration since citrus fruits are acidic having a pH range of 2 – 4. Anthracnose, stem end rot, sour rot, green and blue mold, brown rot, and Alternaria rot can be considered as common fungal diseases of citrus (Palou *et al.*, 2001; Ladaniya, 2008).

Anthracnose is mainly caused by *Colletotrichum gloeosporioides*. However, *Colletotrichum karstii,* has also been reported to be involved in the infection and disease progress (Baroncelli *et al.*, 2017). Anthracnose is a latent infection and symptoms appear as lesions that are irregular in shape and of various sizes (Timmer *et al.*, 1998). Stem-end rot (SER) is known to be caused by a complex of fungal pathogens including *Lasiodiplodia theobromae, Diaporthe citri, C. gloeosporioides* and *Phomopsis* sp. These pathogens endophytically colonise during fruit development in the orchard or field and remain quiescent until the onset of fruit ripening. In advanced stages of ripening, SER progresses to decay resulting in heavy losses of citrus fruits (Galsurker *et al.*, 2018). Citrus brown rot is caused by several *Phytophthora* species (*P. palmivora, P. citrophthora, P. nicotianae,* and *P. hibernalis*) while *Geotrichum citri* and *Alternaria citri* are supposed to be the main causal agents of sour rot and Alternaria rot respectively. Green and blue mold cause significant economic losses during fruit storage and marketing due to both *Penicillium digitatum* and *P. italicum* respectively (Papoutsis *et al.*, 2019). Rind injuries and impact bruising are the major contributors to fruit deterioration, since they accelerate water loss, stimulate higher respiration and ethylene production rates, and help pathogen development. Postharvest diseases represent the major causes of postharvest losses in local and international markets. It contributes to the fruit waste in the marketplace and cause quality and safety losses of fruits throughout the supply chain, resulting in high economic loss (Negi and Anand, 2014). Consumers are reluctant to accept fruits of low quality resulting in decreases in export.

The availability of significant detailed research studies on the characterization of postharvest fungal pathogens from selected citrus fruit species in Sri Lanka, under both molecular and morphological aspects are comparatively low. Therefore, the main objective of this study was to isolate and identify the postharvest fungal pathogens commonly inhabiting selected citrus fruit species through both morphological and molecular parameters. An attempt was also made to assess the frequency of occurrence of the isolated fungi from the three districts viz. Colombo, Gampaha and Kandy in Sri Lanka.

MATERIALS AND METHODS

Sample collection

Citrus fruits (*C. sinensis, C. crenatifolia, C. limon, C. medica*) with visible symptoms of post-harvest diseases were collected from different local markets in Kelaniya, Kiribathgoda, Kadawatha, Gampaha, Kegalle, Mawanella,

Kadugannawa and Kandy areas in Sri Lanka. The collected samples were brought to the laboratory in sterile polythene bags with proper packing and labelling.

Isolation of fungi

Diseased citrus fruit tissues (4 mm^2) were cut from margins of symptomatic areas under aseptic conditions. The tissues were surface sterilized by soaking the sections in freshly prepared NaOCl (3% w/v) for 3 minutes. After 3 serial washings in sterile distilled water, citrus tissues were placed (2 pieces per plate) on sterile petri plates containing Potato Dextrose Agar (PDA) medium. Inoculated plates were incubated at room temperature 28±2 ºC for 5 to 7 days. After incubation, pure cultures of the isolates were prepared by transferring 5 mm^2 mycelial discs on to freshly prepared, sterile PDA plates. Colony characters of pure cultures grown on PDA plates were observed and recorded. The associated fungi were identified by observing their morphological characteristics such as conidial shape, hyphal septations, and other structures under Phase Contrast Microscope under×400 magnification (Olympus CX41 model, Tokyo, Japan) by comparison with Atlas of pathogens and relevant literature (Ploetz, 2003; Abeywickrama, 2006; Ekanayake *et al.*, 2019).

DNA extraction

DNA was extracted from isolated fungal cultures grown on PDA plates using modified CTAB protocol (Daranagama *et al.*, 2015). The total genomic DNA was extracted from 0.05 – 0.10 g of growing mycelium by scraping from the edge of the growing culture. Mycelium was ground with half volume of polyvinylpyrrolidone (PVP), sterile quartz sand and 200 μL of pre-heated (65 ºC) 2% CTAB buffer in sterilized micro centrifuge tubes. 400 μL of pre-heated (65 ºC) CTAB buffer was added and incubated in 65 ºC for 60 minutes. The tubes were centrifuged at 12000 rpm for 10 minutes. The supernatant was treated with an equal volume of Phenol: Chloroform: Iso amyl alcohol (25:24:1) and the solution was mixed by inversion. The tubes were

centrifuged at 10000 rpm for 10 minutes and the supernatant was transferred to fresh micro centrifuge tubes. An equal volume of Chloroform: Isoamyl alcohol (24:1) was added, mixed by inversion and tubes were centrifuged at 10000 rpm for 10 minutes. The upper aqueous phase containing DNA was transferred to fresh micro centrifuge tubes and an equal volume of ice-cold iso-propanol was added and incubated for 1 hour at -20 ºC. After the samples cooled down to room temperature, precipitated DNA was recovered by centrifugation at 12000 rpm for 10 minutes followed by three steps of purification with 70% ethanol. The air dried, precipitate was dissolved in 25 μL of nuclease free water and stored at -20 ºC until use for amplification reactions.

PCR amplification

Extracted DNA was used for the amplification of ITS region using ITS 1 and ITS 4 primers (F- 5' CTTGGTCATTTAGAGGAAGTAA 3' and R- 5' CAGACTTGTACATGGTCCAG 3'). A total volume of 25 μL reaction mixture [5X PCR buffer, 1.5 mM MgCl$_2$, 0.25 mM dNTP, 0.4 μM of each primer, Taq polymerase, and 1.0 μL template DNA] was used for PCR with adjustments of components' volumes and concentration when needed. PCR was carried out in a thermal cycler (Veriti 96-Well Thermal Cycler, ABI Biosystems, USA) following initial denaturation at 95 ºC for 5 minutes, 37 cycles of denaturation at 95 ºC for 30 seconds, annealing at 54 ºC for 30 seconds, extension at 72 ºC for 1 minute 30 seconds and final extension at 72 ºC for 10 minutes (Daranagama *et al.*, 2015).

Agarose gel electrophoresis

The agarose gel electrophoresis was carried out at 90 V, 300 mA and 60 W for 60 minutes in a horizontal gel electrophoresis system. Amplified PCR products were visualized in agarose gel (1%) in 1X TBE containing ethidium bromide using gel documentation system (QUANTUM ST5, Germany). Hundred base pair ladder (Promega, USA) was used for quantification of the PCR products.

DNA sequencing of the isolated fungal strains

Sequencing was performed at Genetech Pvt. Ltd., Colombo, Sri Lanka and Macrogen Inc. Seoul, Republic of Korea using the same primers for successful PCR products. Sequences obtained were manually edited using BioEdit sequence Alignment Editor (Version7.2.5). Sequences generated from this study were analyzed comparatively with other homologous sequences retrieved from GenBank by NCBI Blast search (https://www.ncbi.nlm.nih.gov/) (Table 01).

Sequence alignment and phylogenetic analysis

Sequence homologies for the assembled consensus sequences were analyzed using the BLAST search engine available at the NCBI for the initial identification of fresh isolates, used in the analyses. Newly generated sequences were aligned using MUSCLE (Multiple Sequence Alignments) Alignment tool of MEGA sequence Alignment Editor (Version 7.0) along with reference taxa of *Lasiodiplodia, Aspergillus, Pestalotiopsis* and *Neofusicoccum* species, obtained from the GenBank. The alignments were visually checked and manually improved where necessary. Phylogenetic analysis using Maximum Likelihood (ML) trees, was constructed using the MEGA software (Version 7.0). Kimura 2-parameter model with bootstrap values obtained for 1000 bootstrap replicates were applied following Kimura (1980). The best scoring tree was selected. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and Bio NJ 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. Trees were constructed in MEGA (Version 7.0) software (Ekanayake *et al.*, 2019).

Frequency of occurrence of fungal isolates

Research was conducted to investigate disease causative agents associated with four citrus fruit species from different markets in Gampaha, Kegalle, and Kandy Districts. Fungal associations isolated from each species and the number of isolates were recorded separately. Frequency of occurrence of each fungal isolate was calculated using the following equation (Tafinta *et al.*, 2014; Muhammad *et al.*, 2018).

Frequency of occurrence=

$$\frac{\text{(Number of isolations of a particular pathogen in a corresponding disctrict)}}{\text{(Number of total isolations of pathogens in the corresponding district)}} \times 100$$

RESULTS AND DISCUSSION

Isolation and purification of the isolated fungi

Five fungal strains (CS 01, CS 02, CS 03, CS 04, and CS 05) were isolated and purified from spoilt *C. sinensis* while CC 01, CC 02 and CC 03 were recorded from *C. crenatifolia* fruits. Further, CL 01 was the only fungus isolated from *C. limon* whereas isolate CM 01 was recorded from *C. medica*. Identification of the isolated fungal strains was performed observing morphological characters and performing internal transcribed spacer (ITS) sequencing.

Morphological identification of fungal isolates

CS 01 and CS 02 were similar in appearance and grew rapidly on PDA medium. CS 01 isolate was in filamentous form at the beginning with white mycelium which turned yellowish to orange on maturity. Colony produced dark-orange pigments with production of conidia, which were immersed throughout the plate exhibiting circular rings (Figure 01.A). In CS 02, colonies contained cottony, creamy-white mycelia which formed concentric rings gradually on the PDA medium on maturity. The center rings attained a yellow colour with time. Colony produced numerous dark black acervuli on the culture plate with dark-orange pigmented mycelia in the center of the plate (Figure 01.C). Vegetative hyphae were hyaline, septate, smooth walled and branched. Conidia were hyaline, straight to cylindrical, and rounded at both ends in both CS 01 and CS 02 (Figure 01.B and D).
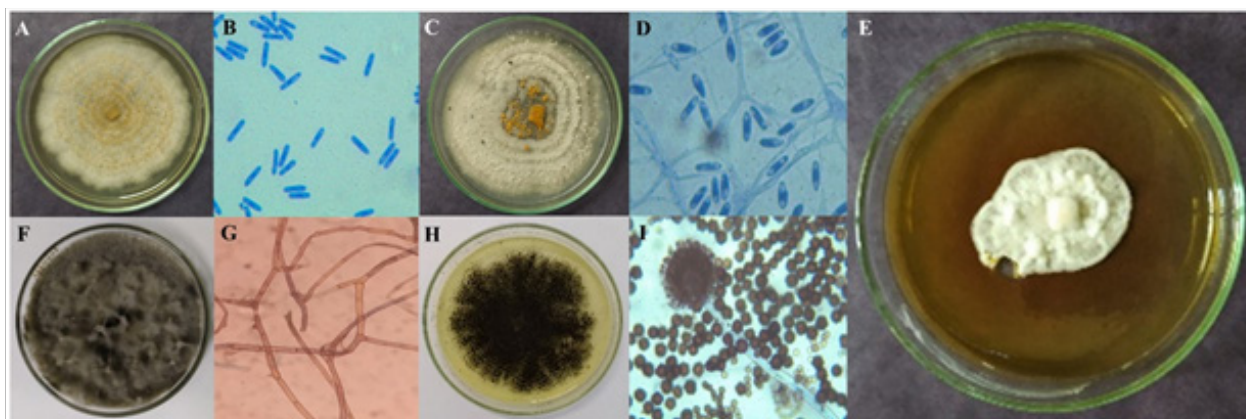
Figure 01: Morphological variation of fungal isolates (A, C, E, F and H) from *C. sinensis* fruits and phase contrast microscopic view of *C. fructicola* with conidia (A and B), *C. gloeosporiodes* with conidia (C and D), *Pestalotiopsis* sp. grown on PDA (E), *L. theobromae* with mycelia (F and G), *A. niger* with conidiophores and conidia (H and I).

In CS 03, colony grew very slowly on PDA and attained a diameter of 15 – 22 mm after 21 days of incubation. Colonies were white, wrinkled and the mycelia were thick and rough in texture (Figure 01.E). The reverse of the colony was orange in colour on PDA. CS 04 grew rapidly on PDA producing light grey, cottony, mycelia, which turned into dark grey within 2 – 3 days (Figure 01.F). The hyphae were branched, septate and dark brown (Figure 01.G). During the period of study under laboratory conditions, no reproductive structures were visible.

The isolated colonies of CS 05 were wooly, initially being pale green and turned to black with the formation of conidia. The reverse of the colony was green to pale yellow. The edges of the colonies were pale yellow producing radial fissures (Figure 01.H). Hyaline and septate hyphae produced long erect conidiophores that became dark at the apex terminating in a globose vesicle. Carbon black conidia were arranged in chains (several rows) at maturity (Figure 01.I).

At the first appearance, CC 01 colony was white cottony to fluffy and with maturity (5 – 6 days) turned to olivaceous grey. Culture grew rapidly on PDA producing moderately dense, light grey mycelia. The center of the colony attained a greyish yellow colour with time (Figure 02.A). The reverse of the colony was greyish yellow to black in colour. The hyphae were branched, septate and irregular in shape (Figure 02.B).

CC 02 colonies contained greyish green mycelia which formed concentric rings gradually on the PDA medium on maturity (Figure 02.C). The center rings attained dark brown colour with time. The reverse of the colony was yellowish brown in colour. Colony attained a filamentous form and powdery nature. Hyphae were branched, hyaline and aseptate (Figure 02.D). During the period of study under laboratory conditions, no reproductive structures were visible.

The isolated colony of CC 03 grew rapidly throughout the PDA plate and mycelia were initially grey and turned powdery black with sporulation (Figure 02.E). The reverse of the colony was greenish black. Hyphae were septate and hyaline with a long erect conidiophore that turned dark at the apex terminating in a globose vesicle. Black to dark brown conidia were arranged in columns (several rows) on the sterigmata at maturity (Figure 02.F and G).

The isolated CL 01 and CM 01 cultures were similar in appearance and they grew rapidly on PDA. Light grey, cottony, mycelia, which turned dark grey was observed on the PDA in both CL 01 and CM 01 isolates (Figure 03.A and B). The reverse of the colony was dark grey/black in colour. The hyphae were branched, septate and dark brown in colour (Figure 03.C and D). During the period of study under laboratory conditions, no reproductive structures were visible.
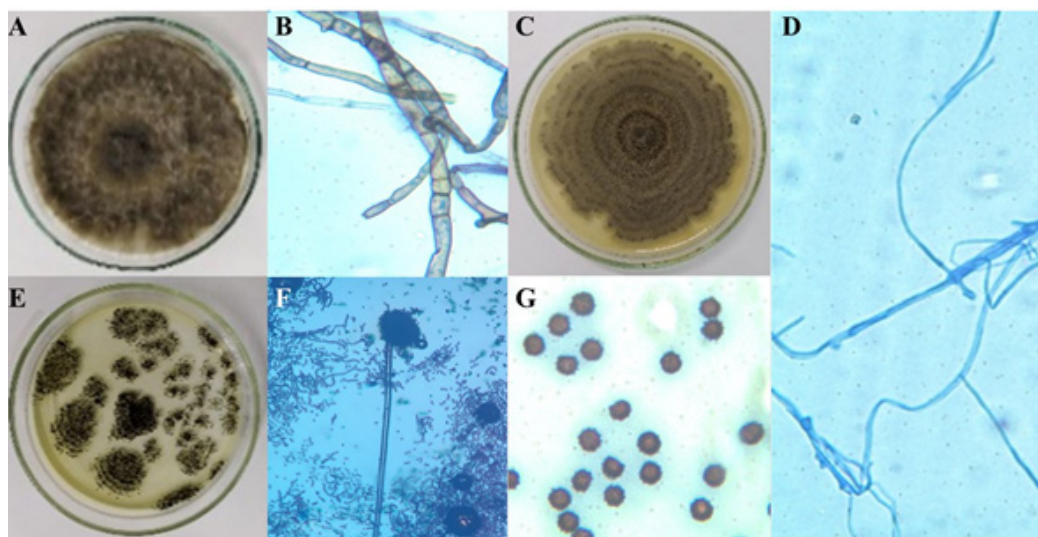
Figure 02: Morphological variation of fungal isolates (A, C and E) from *C. crenatifolia* fruits and phase contrast microscopic view of *N. parvum* with mycelia (A and B), *C. gigasporium* with mycelia (C and D), *A. clavatus* with conidiophore and conidia (E, F and G).
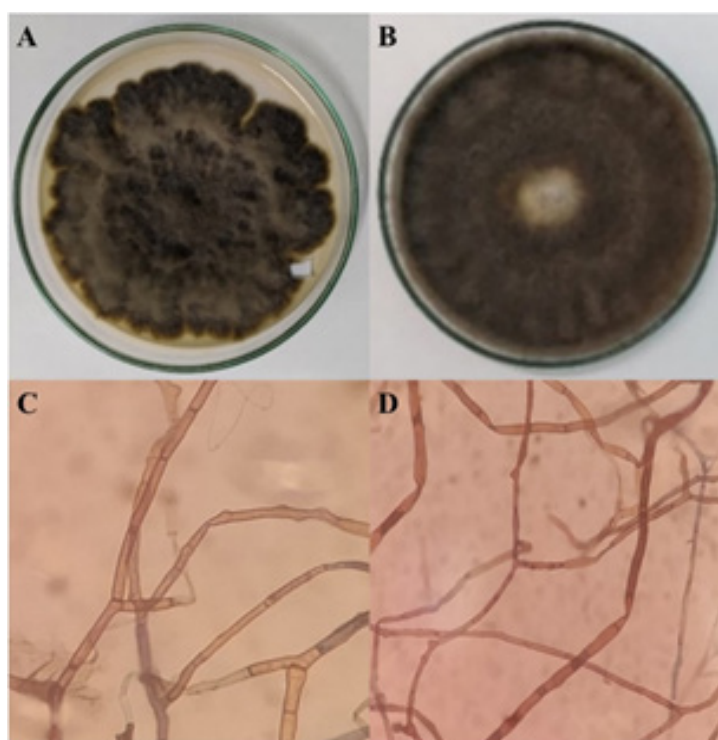
Figure 03: Phase contrast microscopic view of *L. theobromae* with mycelia (A and C) from *C. limon* and *L. pseudotheobromae* with mycelia (B and D) from *C. medica*

Based on the comparison of colony morphology, phase contrast microscopic micrographs and sporulation structures, both CS 01 and 02, were identified as *Colletotrichum* sp. whereas CS 04 and CS 05 isolated from *C. sinensis* were identified as., *Lasiodiplodia* sp. and Aspergillus sp. respectively. Further, CC 01 and CC 03 isolated from *C. crenatifolia* were identified as *Neofusicoccum* sp. and *Aspergillus* sp. CL 01 isolated from *C. limon* and CM 01 isolated from *C. medica* were identified as *Lasiodiplodia* sp. However, CS 03 and CC 02 could not be identified morphologically as there were no reproductive structures visible during the time of study.

Preliminary identification of the fungal isolates based on sequence data

According to the BLAST search performed, sequence analysis of the ITS regions exhibited significant sequence similarities for CS 01 (100 %), CS 02 (99 %), CS 03 (99 %), CS 04 (98 %) and CS 05 (98 %) with previously reported *Colletotrichum fructicola* (MK611680.1), *Colletotrichum gloeosporiodes* (MT254840.1), *Pestalotiopsis* sp. (KU377578.1), *Lasiodiplodia theobromae* (LC074359.1) and *Aspergillus niger* (MK886749.1), respectively. ITS sequences of CC 01 (99 %), CC 02 (98 %) and CC 03 (100 %) showed sequence similarities with previously reported isolates of *Neofusicoccum parvum* (MH712271.1), *Colletotrichum gigasporium* (KF687724.1) and *Aspergilus clavatus*

(MT620752.1). Further, CL 01 and CM 01 exhibited a 98 % and 100 % sequence similarities with previously reported isolates of *Lasiodiplodia theobromae* (KY655203.1) and *Lasiodiplodia pseudotheobromae* (MT043794.1), respectively.

Phylogenetic analysis

Phylogenetic analyses were performed for the genera *Lasiodiplodia, Aspergillus, Neofusicocum* and *Pestalotiopsis* using the MEGA software (Version 7.0). However, phylogenetic analysis was not performed for the genus *Colletotrichum* as only the ITS sequence data were not sufficient for reliable resolution of the terminal taxa (Crouch *et al.*, 2009). The bipartition trees generated are shown in Figures 04 – 07.

Table 01: **Details of the locally collected fungal isolates and comparison with previously reported isolates**

Sample code	Accession number (GENBANK)	Highest match with NCBI database	Location of previous isolate	Percentage similarity	Reference
CS 01	MZ081366	*Colletotrichum fructicola* (MK611680.1)	China	100%	Luo, 2020
CS 02	MZ081368	*Colletotrichum gloeosporiodes* (MT254840.1)	China	99%	Wang, 2020
CS 03	MZ081371	*Pestalotiopsis* sp. (KU377578.1)	China	99%	Deng *et al.*, 2018
CS 04	MZ081369	*Lasiodiplodia theobromae* (LC074359.1)	India	98%	Prathibha *et al.*, 2015
CS 05	MZ081370	*Aspergillus niger* (MK886749.1)	Egypt	98%	Bendary *et al.*, 2019
CC 01	MZ081372	*Neofusicoccum parvum* (MH712271.1)	China	99%	Deng *et al.*, 2018
CC 02	MZ150510	*Colletotrichum gigasporium* (MZ150510)	China	98%	Liu *et al.,* 2014
CC 03	MZ081373	*Aspergilus clavatus* (MT620752.1)	China	100%	Li, 2020
CL 01	MZ081374	*Lasiodiplodia theobromae* (KY655203.1)	Brazil	98%	Santos, 2017
CM 01	MZ081375	*Lasiodiplodia pseudotheobromae* (MT043794.1)	Malaysia	100%	Nisam *et al.,* 2020
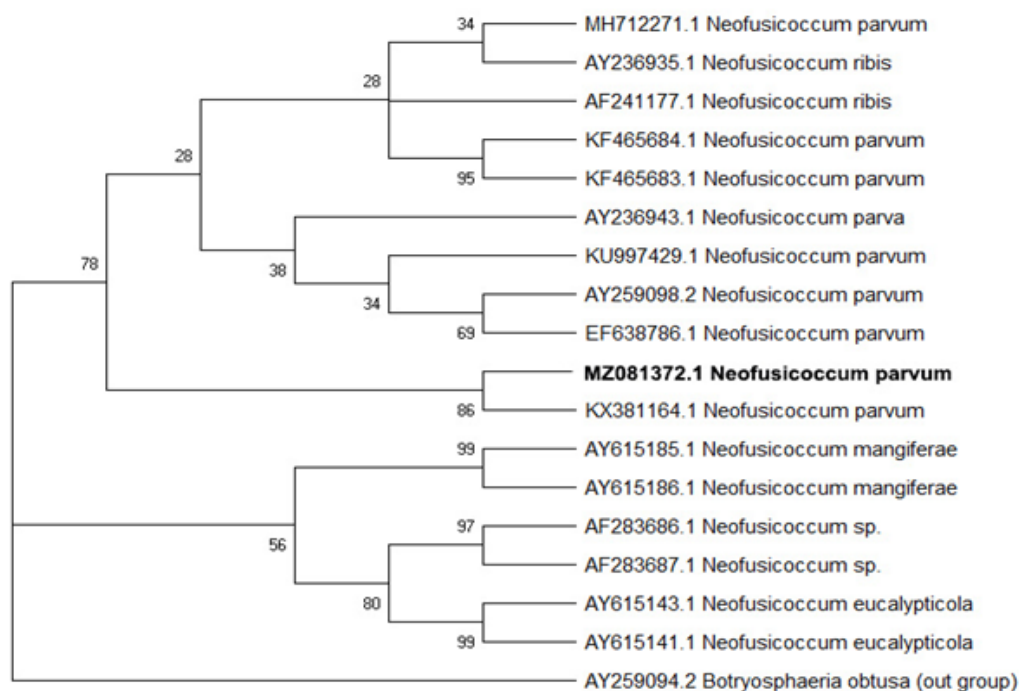
Figure 04:   **The phylogenetic tree of *N. parvum* (MZ081372.1) inferred from likelihood analysis using ITS sequences. Bootstrap values are given on the branches. The tree is rooted to *Botryosphaeria obtusa* (AY259094.2) (Lazzizera *et al.*, 2008).**

According to the phylogenetic analysis, the isolate CC 01 resulted from this study clustered with *Neofusicoccum parvum* (KX381164.1) with a high bootstrap support of 86%, which identified as *N. parvum* (MZ081372.1). The subclade containing *N. parvum* (MZ081372.1) and *N. parvum* (KX381164.1) falls well within the main clade comprising other *N. parvum* strains with bootstrap support as high as 78 % (Figure 04). Further, the isolate CS 03 appeared as a singleton within the main clade comprising many other *Pestalotiopsis* sp. Therefore, this isolate was identified as *Pestalotiopsis* sp. (MZ081371.1) (Figure 05).

The isolates CS 05 and CC 03 were identified to be *Aspergillus* sp. in the initial identification. During the phylogenetic analysis, CS 05 isolate clustered into a clade of *A. niger* with a high (83 %) bootstrap support, hence confirmed its phylogenetic position as *A. niger* (MZ081370.1). The other isolate CC 03 was identified as *A. clavatus* (MZ081373.1) (66 % bootstrap support) which was clustered with several other *A. clavatus* species from different regions of the world (Figure 06).

The isolate CL 01 clustered with many strains of *Lasiodiplodia theobromae* with 66 % bootstrap support, and was identified to be *L. theobromae* (MZ081374.1). Two isolates (CS 04 and CM 01) were identified as *L. pseudotheobromae* (MZ081369.1 and MZ081375.1) which formed a monophyletic clade with reference strains of *L. pseudotheobromae*. Isolate CS 04, clustered with *L. pseudotheobromae* with 72 % bootstrap support while the isolate CM 01 clustered in this same clade with low bootstrap support of 18 % (Figure 07).

From the phylogenetic trees generated, isolates clustered with reference sequences with bootstrap support of more than 70 % were confirmed since clades with bootstrap support of more than 70 % are true for over 95 % of the time with high accuracy and those with bootstrap values >50 % are considered probably significant (Hillis and Bull, 1993). The use of concatenated alignment of the ITS region with one or more of the protein-coding genes might have been effective for finer scale species-level identification of the specific fungi and might have accounted for higher resolution among species (Tekpinar and Kalmer, 2019).
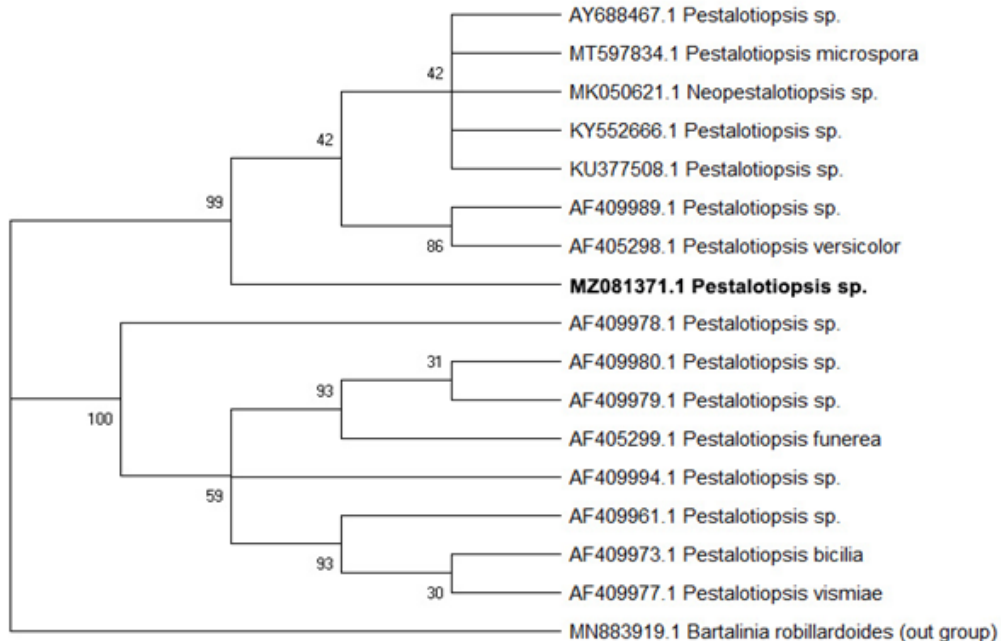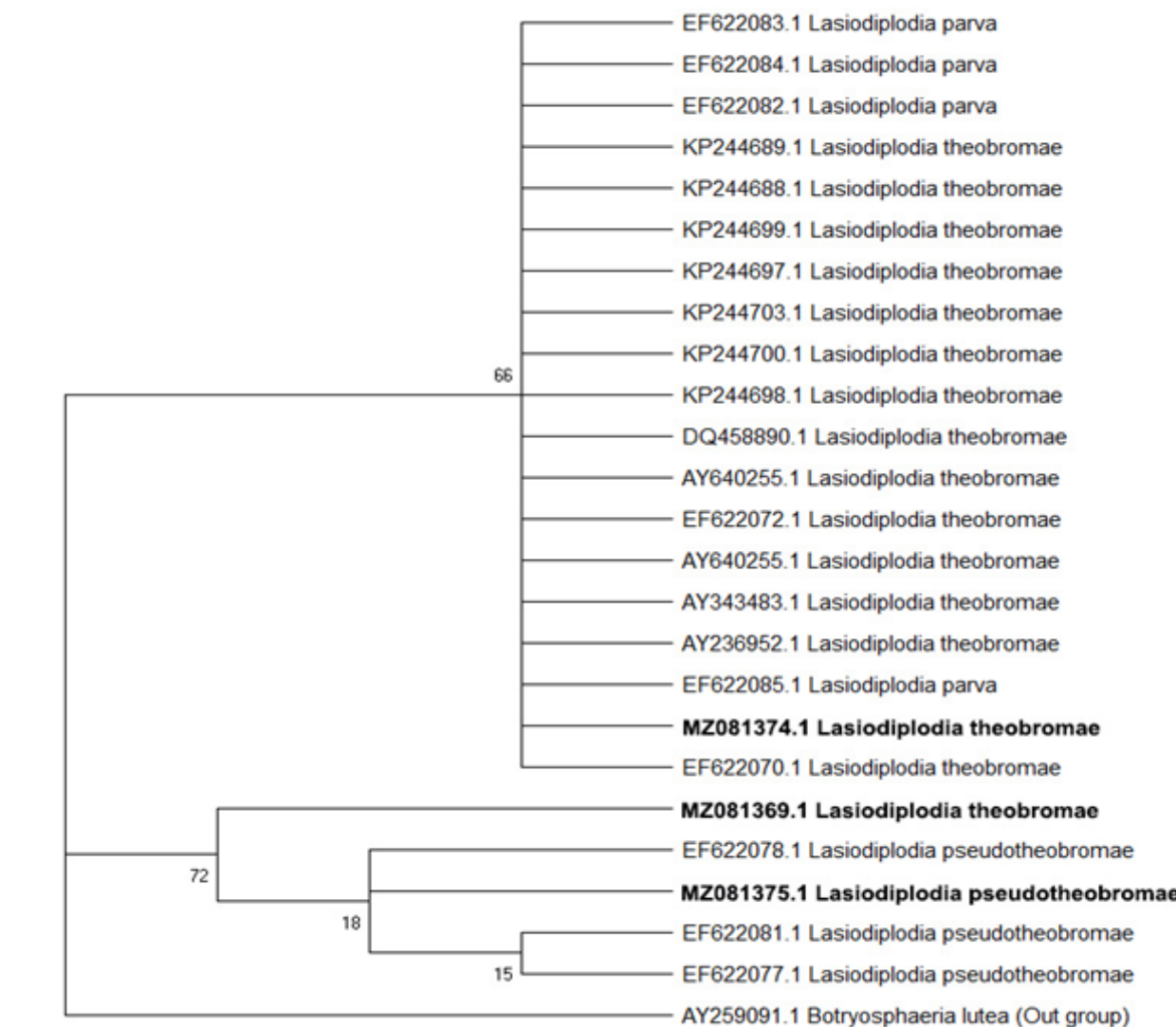
Figure 05: The phylogenetic tree of *Pestalotipsis* sp. (MZ081371.1) inferred from likelihood analysis using ITS sequences. Bootstrap values are given on the branches. The tree is rooted to *Bartalinia robilardoides* (MN883919.1) (Kamhawy *et al.*, 2011).
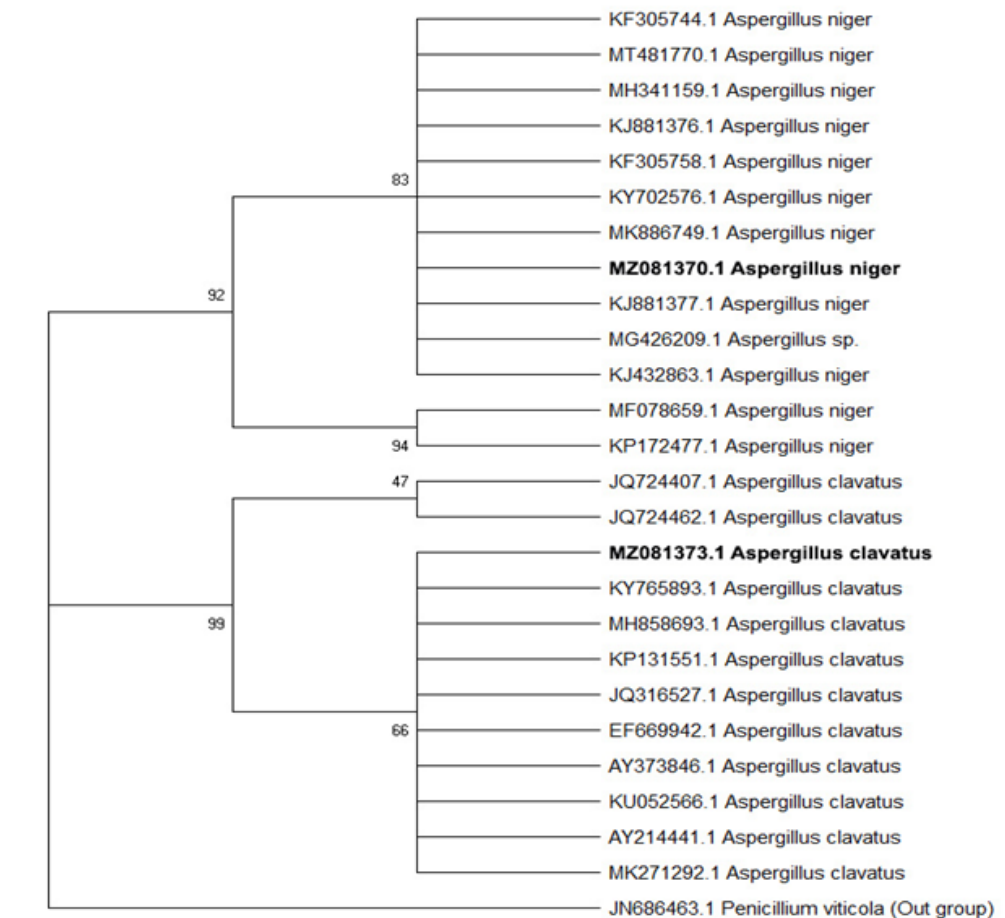
Figure 06: The phylogenetic tree of *A. niger* (MZ081370.1) and *A. clavatus* (MZ081373.1) inferred from likelihood analysis using ITS sequences. Bootstrap values are given on the branches. The tree is rooted to *Penicillium viticola* (JN686463.1) (Khan *et al*., 2019).

Figure 07: The phylogenetic tree of *L. theobromae* (MZ081374.1/MZ081369.1) and *L. pseudotheobromae* (MZ081375.1) inferred from likelihood analysis using ITS sequences. Bootstrap values are given on the branches. The tree is rooted to *Botryosphaeria lutea* (AY 259091.1) (Qian *et al.*, 2014).

Frequency of occurrence and distribution of the fungal isolates

Based on the results, the isolated fungi from the spoilt citrus fruit species and their frequencies of occurrence are shown in Tables 02 – 04. *Lasiodiplodia theobromae, A. niger, N. parvum, A. clavatus* and *L. pseudotheobromae* were the fungal strains isolated from the spoilt citrus fruits in the Kegalle District. Of the isolated fungi, *L. theobromae* isolated from the *C. sinensis* had the highest frequency of occurrence (66.7 %). *A. niger* isolated from *C. sinensis* and *A. clavatus* isolated from *C. crenatifolia* had the lowest frequencies of occurrence (16.7 %) indicating that they are less troublesome spoilage fungi on

citrus. *Lasiodiplodia pseudotheobromae* which was isolated from *C. medica* recorded only from Kegalle District.

The results showed that the presence of a very interesting fungal biodiversity as there were eight fungal strains recorded from the three citrus species at different markets in the Gampaha District. Based on the fungal distribution, four strains (*C. fructicola, C. gloeosporiodes,*

L. theobromae and *Pestalotiopsis* sp.) were recorded from *C. sinensis* while three fungal strains (*N. parvum, A. clavatus* and *C. gigasporium*) were recorded from *C. crenatifolia. L. theobromae* was the only fungus isolated

from *C. limon*. Of the isolated fungi, *N. parvum*, isolated from *C. crenatifolia* had the highest frequency of occurrence (75 %) while three fungal strains (*C. fructicola, Pestalotiopsis sp.,* and *C. gigasporium*) showed the lowest frequencies of occurrence (13 %).

Based on the data, a limited number of fungal strains could be isolated from three citrus species at different markets in the Kandy District. Of the isolated fungi, *N. parvum* isolated from *C. crenatifolia* had the highest frequency of occurrence (75 %) while *L. theobromae* isolated from *C. limon* had the lowest frequency of occurrence (25 %). *Lasiodiplodia. theobromae* and *C. fructicola* strains showed similar frequencies of occurrence (50 %). Based on the results of identified fungal strains, it can be concluded that *L. theobromae, C. fructicola* and *N. parvum* were recorded in all three districts. Lasiodiplodia theobromae was the only fungus isolated from *C. limon* in all three districts and the presence of *L. pseudotheobromae* was low in frequency. This could be attributed to less availability of *C. medica* variety in local markets which was found only from the Kegalle District.

According to the findings of previous research, many fungal strains have been identified from spoilt *C. sinensis* fruits. And similar fungal strains have been reported such as *Colletotrichum* sp., *L. theobromae* and *A. niger*. Further, *A. flavus, A. fumigatus, P. chrysogenum, P. digitatum, Fusarium* sp., and *Alternaria* sp. have been reported in spoilt *C. sinensis* fruits (Timmer *et al.*, 1998; Tafinta *et al.*, 2014; Muhammad *et al.,* 2018). There have been no reports of pathogenic activities in *Pestalotiopsis* sp. related to postharvest diseases in citrus fruits. However, several previous studies have reported that *Pestalotiopsis* sp. has been associated with crown rot disease of strawberry, stem-end rot disease of mango and fruit rot of grape (Deng *et al.*, 2013; Ara *et al.*, 2017; Kodituwakku *et al.*, 2020). According to the best of the knowledge of the authors, this is the first report on the identification of Pestalotiopsis from selected citrus fruit species in Sri Lanka. In the present study, *L. theobromae* was the only fungus that was identified from the *C. limon.* However, many fungal strains have been reported as pathogens in previous studies

such as *C. gloeosporioides, A. alternata, A. citri, P. italicum, P. digitatum* and *Botrytis cinerea* in *C. limon* (Kaur *et al.,* 2020; Taha *et al.*, 2019; Wang *et al.*, 2021).

Three fungal strains were identified in *C. crenatifolia* such as *N. parvum, C. gigasporium* and *A. clavatus* in this research. Although the isolate of *C. gigasporium* has not been recorded related to postharvest diseases of citrus fruits, there is some evidence of *C. gigasporium* strain being associated with anthracnose disease in Avacado (Zakaria, 2021). Lasiodiplodia pseudotheobromae was the only fungi isolated from *C. medica* species in the present study. Furthermore, Rhizopus oryzae has been reported as the first isolated fungus in *C. medica* species (Manghwar *et al.*, 2015). This may be the first report on the association of *L. pseudotheobromae* in *C. medica* fruits in Sri Lanka as no previous reports are available.

Isolated and characterized *Colletotrichum* sp. were recognized as causative agents of anthracnose disease and stem-end rot disease based on previous literature. Furthermore, *L. theobromae* has been known to be associated with stem-end rot disease in citrus fruits. The above two pathogens are considered as endophytes on a wide range of plant hosts and they have the ability to develop latent infections to become active during fruit ripening (Wang *et al.*, 2021; Zhang, 2014;). *Lasiodiplodia pseudotheobromae, N. parvum,* and *Aspergillus* sp. have previously been identified as causative agents of fruit rot in citrus fruits (Chen *et al.,* 2021; Zhai and Zhang, 2019). *Aspergillus* sp. are not considered as postharvest pathogens, however, as saprophytes they are responsible for significant postharvest losses of fruits.

CONCLUSIONS

Based on the morphological and molecular identification, the associated fungal strains, MZ081366, MZ081368, MZ081369, MZ081370, and MZ081371 which were isolated from spoilt *C. sinensis* were identified as *C. fructicola, C. gloeosporiodes, Pestalotiopsis* sp., *L. theobromae,* and *A. niger* respectively. Further, MZ081372,

MZ150510 and MZ081373 were identified as *N. parvum, C. gigasporium* and *A. clavatus* in spoilt *C. crenatifolia* fruits. MZ081374 isolated from *C. limon* and MZ081375 isolated from *C. medica* were identified as *L. theobromae* and *L. pseudotheobromae* respectively. It is worthwhile noting that this is the first report on isolation and identification of *L. pseudotheobromae* from *C. medica* fruits as well as *Pestalotiopsis* sp. and *C. gigasporium* from citrus fruits. The findings need to be confirmed through pathogenicity studies to authenticate their pathogenic association with postharvest fruit rot of citrus fruits.

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Conflicts of Interest

The authors declare no conflict of interest.

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