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FIRST TESTIMONY OF NEW HOST PLANT OF *COLLETOTRICHUM MAGNUM* (S.F. Jenkins & Winstead) Rossman & W.C. Allen CAUSING ANTHRACNOSE IN *CARICA PAPAYA* L. FRUITS IN BALI, INDONESIA

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SUMMARY

Anthracnose forms a group of fungal diseases that affect a variety of crop plants in warm and humid areas. Anthracnose causes the wilting, withering, and dying of tissues of plants, commonly infecting the developing shoots, leaves, and fruits. It critically affects the quality and production of *Carica papaya* fruits in Bali, Indonesia. The pathogenic fungi belonging to the genus *Colletotrichum* cause anthracnose fungal disease. Research on morphology and molecular identification of pathogenic fungi is very important to determine the type of pathogenic fungus that causes anthracnose disease in the papaya fruit. The latest study aims to identify the pathogen that caused anthracnose on papaya fruits in Bali, Indonesia, carried out during July – December 2021 at the Belok Village, District of Petang, Badung Regency, and in Laboratory of Biochemistry, Udayana University, Badung Regency, Bali, Indonesia. The study used Koch's postulates to confirm the pathogenicity and continued with macro- and micro-morphological characterization. Based on macro- and micro-morphological characterization, the pathogen has shown similar traits to the genus *Colletotrichum*. Molecular identification took place using the ITS sequences to increase data robustness. Based on the molecular analysis, the DNA sequences of the genus *Colletotrichum* isolated from papaya fruits showed the symptoms of anthracnose disease. Based on the latest findings, the pathogen causing anthracnose disease in *Carica papaya* fruits showed as the species *Colletotrichum magnum* in Bali, Indonesia.

Keywords: *Carica papaya*, *Colletotrichum magnum*, anthracnose, molecular identification

Key findings: The latest study identified the pathogenic fungi (species *Colletotrichum magnum*) causing anthracnose disease on *Carica papaya* fruits through morphological and molecular approaches.

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INTRODUCTION

The papaya (*Carica papaya* L.) fruit plant is commonly known to the public in Bali, Indonesia. The papaya fruit is consumed fresh or processed, such as, vegetables. During the last three years—2018, 2019, and 2020—the papaya fruit production has decreased from 16,789 t to 10,282 t and 6,888 t, respectively, in Bali, Indonesia (BPS, 2021). However, the papaya fruit production in Indonesia ranks low compared with other producing countries, with a share at only 6.87% (0.9 million t) of the world's papaya production (13.16 million t).

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Meanwhile, in India, Brazil, and Mexico, papaya fruit production amounts to 43.31%, 10.87%, and 7.23%, of the world's production, respectively (Atlasbig, 2022). Several factors severely affected papaya fruit production in Indonesia, of which the most crucial consists of insect pests and diseases.

Anthracnose forms a group of fungal diseases that affect a variety of plants in warm and humid regions. Papaya anthracnose is a critical fungal disease caused by the species complexes *C. gloeosporioides* L*.*, *C. acutatum* L., and *C. magnum* L. In Indonesia, one of the reasons for papaya's decreased production emanates from the presence of diseases that infect papaya fruit, particularly the papaya anthracnose caused by *Colletotrichum magnum* L. (Sudha *et al*., 2015; Rangkuti *et al*., 2017). In fruit plants, the anthracnose symptoms begin with the plant parts appearing shiny, followed by tissue softening, then the surface of the plant parts becoming concave and brownish, known as a lesion (Dowling *et al*., 2020; Zakaria, 2021). The pathogenic fungi in the genus of *Colletotrichum* cause anthracnose disease, which significantly affects the quality and quantity of fruit plants production globally (da-Silva *et al*., 2020; Bhunjun *et al*., 2021), such as chili (Mongkolporn and Taylor, 2018; Sudirga and Suprapta, 2021), soybean (Boufleur *et al*., 2021), apple (Khodadadi *et al*., 2020), strawberry (Chung *et al*., 2020; Dowling *et al*., 2020), mango (Li *et al*., 2019), citrus (Wang *et al*., 2021), persimmon (Carraro *et al*., 2022), and many other fruits. The *Colletotrichum* infection proves more dangerous in post-harvested fruit, particularly for the exported fruits needing storage (Chung *et al*., 2020; da-Silva *et al*., 2020). The prevention of *Colletotrichum* infection is quite challenging. The host-pathogen interaction of *Colletotrichum* can be in the form of host-specific or multiple hosts (Talhinhas and Baroncelli, 2021; Zakaria, 2021). The species in this genus also have the highest genetic variability, thus, disease management might require more complex approaches (Carraro *et al*., 2022).

Based on GenBank, about 248 species of *Colletotrichum* have been recognized (Jayawardena *et al*., 2020). These species were classified into 14 groups of monophyletic species to simplify the taxonomical features (Damm *et al*., 2019; da-Silva *et al*., 2020). Two of these species include *Colletotrichum magnum,* and *Colletotrichum gloeospiroides,* which are considered significant. These species complexes have caused much anthracnose in rapidly ripening fruits, such as, papaya. Based on the findings of Rangkuti *et al*. (2017), fungi *C. gloeosporioides*, *C. truncatun,* and *C. magnum* caused the papaya anthracnose, while Rodrigues *et al*. (2021) reported that fungus *C. gloeospiroides* is the most responsible in anthracnose disease in papaya. The papaya anthracnose has also occurred in some areas of Bali, Indonesia, causing low quality and quantity of fruits. The accurate identification of the *Colletotrichum* species needs attention for proper disease prevention and management. The characteristics of *Colletotrichum* species are phenotypically similar hence, the use of the DNA sequence approach should achieve more representative and precise data (Jayawardena *et al*., 2020; Boufleur *et al*., 2021). DNA-based identification has been widely known as the main approach to overcoming taxonomic uncertainties. For fungi, the ITS regions in ribosomal DNA proved to have the capacity to resolve taxonomic uncertainties (Jayawardena *et al*., 2020; Bhunjun *et al*., 2021). Therefore, the study aimed to identify the fungal pathogen that causes anthracnose in papaya fruits in Bali, Indonesia.

MATERIALS AND METHODS

Pathogenic fungi isolation and virulence test

The series of studies took place during July – December 2021 at the Belok Village, District of Petang, Badung Regency, and in the Laboratory of Biochemistry, Udayana University, Kabupaten Badung, Bali, Indonesia. *Carica papaya* fruits showing the symptoms of anthracnose fungi were collected from papaya fruit plants in the Village Belok, and brought to the laboratory to be cleaned in running water followed by sterile water. Then the fruits, showing the symptoms of the anthracnose disease and the healthy parts, got cut to a size of 1 × 1 cm and placed on PDA media in a petri dish. Then, setting the preparation in a dark place at room temperature proceeded until molds grew from the edges of the papaya fruits. The growing fungi were isolated, purified, and identified morphologically by observing the shape of their spores and hyphae. The Koch Postulate test proceeded by inoculating pure isolates of fungi obtained from papaya fruits, which showed symptoms of anthracnose disease in healthy fruit, to confirm that the isolated and purified fungi caused anthracnose disease in papaya fruits. If the healthy fruits show the same symptoms as the fruit infected with anthracnose, followed by reisolation of the fungi, then it is identified morphologically. If the morphological characteristics of the previous fungi exist, one can confirm the obtained fungal isolate proves the same fungus that causes anthracnose disease in papaya fruits.

Morphological characterization of pathogenic fungi

Fungal characterization used the identification guidance from Pitt and Hocking (1997) based on macroscopic and microscopic observations of morphological characters. The basis of macroscopic characteristics observations of the pathogenic fungi included colony color, shape, growth, and colony growth speed. Observing the microscopic characteristics of pathogenic fungi involved the shape and size of hyphae and spores under the light microscope (400×).

Molecular identification of pathogenic fungi

DNA extraction

Pure isolates of *Colletotrichum* spp., grown on PDA media aged three days at room temperature (±28 °C), were used for DNA extraction. Extraction of the *Colletotrichum* spp. DNA genome took place using the protocol by Parwanayoni *et al.* (2021), taking fungal hyphae from the fungal colonies and then placing them in a centrifuge bottle with 100 µL of PrepMan Ultra reagent (PrepMan Ultra Protocol, Applied Biosystem, USA). The sample was then vacuumed for 30 sec, then placed in a hot block at a temperature of 95°C–100°C for 10 min, and transferred to room temperature for 2 min. The sample was centrifuged at 10000 rpm for 2 min, taking the pellet as DNA extract and used for further analysis.

DNA amplification and electrophoresis

The DNA amplification and electrophoresis followed the method of Parwanayoni *et al.* (2021). The 18S rRNA gene got amplified by PCR using Internal Transcribed Spacer (ITS)-1 (5-TCCGTAGGTGAACCTGCGG-3) as the forward primer and (ITS)-4 (5-TCCTCCGCTT ATTGATATGC-3) as reverse primer. The total volume of the PCR mixture was 25 µL. The study used the PCR tool, Takara PCR Thermal Cycler Personal (Takara Bio, Otsu, Japan) with Ex-Tag (Takara Bio, Otsu, Japan), with the amplification parameters consisting of pre-denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 35 sec, annealing at 52°C for 55 sec, elongation at 72°C for 2 min, and post-elongation at 72°C for 10 min. The PCR product testing used electrophoresis of 1% agarose gel in 1×TBE buffer with DNA stain, PCR product as DNA template, and DNA ladder 100 bp.

Sequencing regions of ITS and DNA sequences analysis

Determining the obtained nucleotide sequences employed the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystem, Foster City, CA, USA) according to the guidelines of the tool and with the PE Applied Biosystem Automated DNA Sequencer (3130xl model, Applied Biosystem). The DNA sequence was assembled and analyzed using Genetyx 11.0 and Genetyx-ATSQ 4.0 software (Genetyx, Tokyo, Japan), then compared with the same DNA sequences from GenBank through the NCBI BLAST program. Phylogeny analysis used the MEGA 7 software (Kumar *et al*., 2016) and its construction used the Maximum Parsimony (MP) method with 1000× bootstrap.

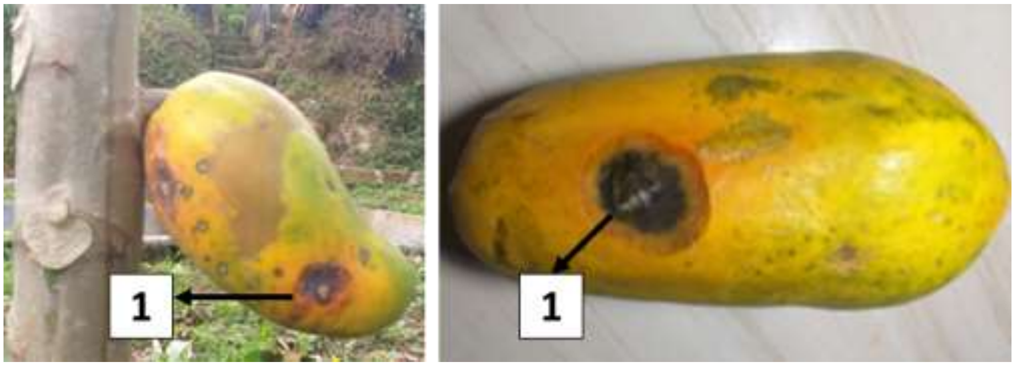
RESULTS AND DISCUSSION

Isolation of pathogenic fungi

Based on Koch's Postulate test, pure isolates of pathogenic fungi obtained from papaya fruits, displaying symptoms of anthracnose disease after being inoculated on healthy papaya fruit, showed the same symptoms as papaya infected with anthracnose disease in the field (Figure 1), where the fungi colony exhibited grey color and cylinder conidia, with a rounded tip at both ends. The use of Koch’s Postulate observed the causative relationship between a pathogen (microbes) and disease. It was first used to test the pathogenicity in microbes (fungi and bacteria) but was later used to explain viral infection (Van-de-Perre *et al*., 2021).

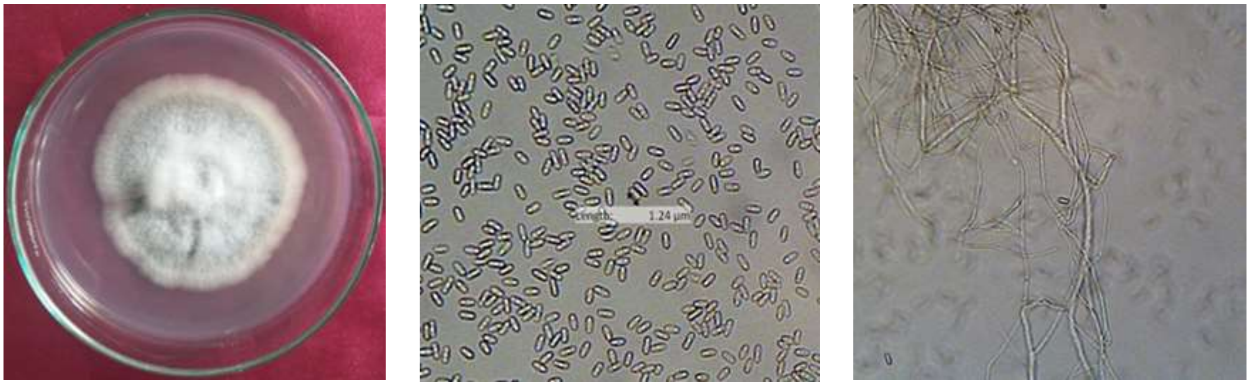
Macro- and microscopic characteristics of the pathogenic fungi

Observing the presence of fungal colonies on PDA media revealed the pathogenic fungi. The fungi *Colletotrichum* sp. grew well on PDA media at room temperature (±28 °C). The fungal growth was exponential from the third day, and the colony size reached 90 mm on



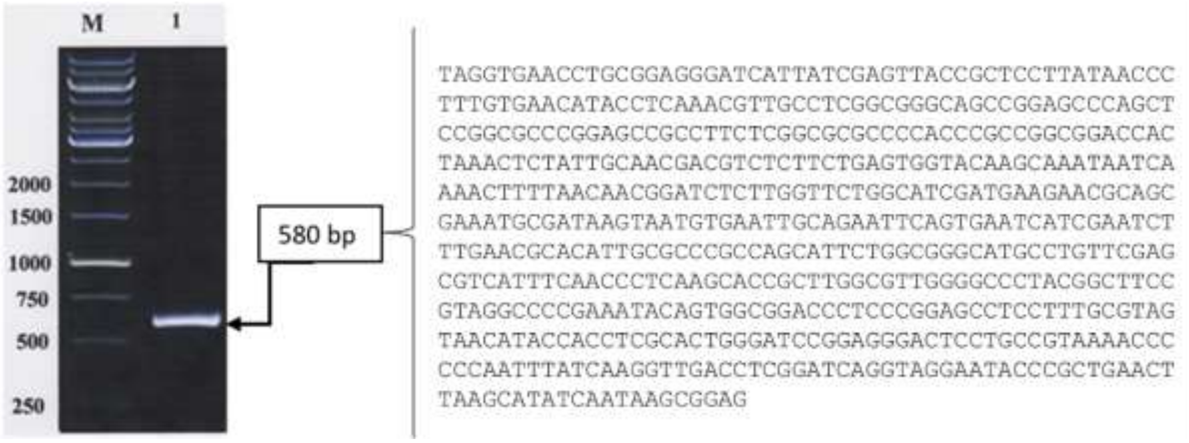
(a) (b)

Figure 1. Papaya fruits showing symptoms of anthracnose disease, a) Papaya fruits showing symptoms of anthracnose disease in the field, and b) fresh papaya fruits after being inoculated with a pure isolate of the fungus *Colletotrichum* spp. showing symptoms of anthracnose disease.



(a) (b) (c)

Figure 2. Morphology of *Colletotrichum* sp. a) Macroscopic morphology of the fungus *Colletotrichum* spp on PDA media, b) Microscopic characters of spores with 400× magnification, and c) Fungal hyphae with 400× magnification.



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TAGGTGAACCTGCGGAGGGATCATTATCGAGTTACCGCTCCTTATAACCC
TTTGTGAACATACCTCAAACGTTGCCTCGGCGGGCAGCCGGAGCCCAGCT
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TAAACTCTATTGCAACGACGTCTCTTCTGAGTGGTACAAGCAAATAATCA
AAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGC
GAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCT
TTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAG
CGTCATTTCAACCCTCAAGCACCGCTTGGCGTTGGGGCCCTACGGCTTCC
GTAGGCCCCGAAATACAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAG
TAACATACCACCTCGCACTGGGATCCGGAGGGACTCCTGCCGTAAAACCC
CCCAATTTATCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACT
TAAGCATATCAATAAGCGGAG
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Figure 3. PCR amplification of ITS genes with ITS_1F and primary ITS_4R primers; M = 1 Kb ladder marker, 1 = PCR product sample *Colletotrichum* sp. with 580 bp, and result of sequences of a DNA sample.

the seventh day after planting. The colonies displayed cream white color, then became grayish black with increasing age, with uneven colony edges. In old cultures (more than 15 days), a black spot appeared on the surface of the colony (Figure 2A). Morphological characteristics occurred, such as, colony shape, conidia shape, and the presence of setae, as well as, an appressorium shape, a size that can differentiate *Colletotrichum* species (Zakaria, 2021).

Viewing the microscopic characteristics of *Colletotrichum* sp. used a light microscope with 400× magnification. Microscopic observations included the size and shape of the hyphae and fungal spores on PDA media. *Colletotrichum* sp. had insulated and branched hyphae, while fusiform spores displayed 7–14 µm long and 3–5 µm wide. The septate spores consisted of two septa with hyaline color (Figure 2B, C). According to da-Silva *et al*. (2020), the fungi of the genus *Colletotrichum* have insulated hyphae with transparent and elongated conidia, with rounded and tapered ends, 10–16 m long and 5–7 m wide, with black conidia mass, and gray hyphae.

Molecular analysis

The genus *Colletotrichum* characterization needs a molecular approach to increase the accuracy of the identification. The common and most appropriate approach employs DNA sequences in ITS regions (Vieira *et al*., 2019; Perez-Brito *et al*., 2018; Talhinhas and Baroncelli, 2021). Based on the molecular analysis, the DNA sequences of the genus *Colletotrichum* sp. isolated from papaya fruits showed the symptoms of anthracnose disease (Figure 3). The sequence length showed 580 bp, covering the region of ITS-1, 5.8S, and ITS-2. This sequence is generally used to search for similar sequences through BLAST and to construct the phylogenetic tree to determine the species and sub-species.

Table 1 presents the highest 10 sequence similarity based on the BLAST analysis search. The genus *Colletotrichum* (sample) infecting the papaya fruits in Bali proved closely related to *Glomerella magna* strain LC-1 (accession number KC815123.1), *Colletotrichum brevisporum* isolate CATAS-CB69 (accession number MW412587.1), and *Colletotrichum brevisporum* strain CRI-N2 (accession number KT185054.1), with 100% similarity. Six accessions with 99% similarity included *Colletotrichum magnum* strain GM-1 (accession number MW295850.1), *Colletotrichum magnum* isolates LP2.1 (accession number MK530193.1), *Colletotrichum brevisporum* strain YYGXZ07 (accession number KU319458.1), *Colletotrichum* sp. 80CP/T (accession number GU066653.1), *Colletotrichum magnum* isolate LP3.2 (accession number MK530194.1), and *Glomerella magna* strain CMM1734 (accession number HM163187.1). The only accession with 98% similarity was *Colletotrichum liaoningense* strain LG9-6 (accession number MW082812.2).

Figure 4 presents the phylogenetic tree of the sample (*Colletotrichum*) using 24 species from the BLAST search with the highest affinity and Maximum Parsimony algorithm. In the recent study, the *Colletotrichum* genus sample showed close relatedness to *Glomerella magna* (accession KC815123), with a bootstrap value of 100%. The nomenclature of *Glomerella magna* is the synonym of the species *Colletotrichum magnum*. Before the molecular approach, the asexual form of this genus gained classification as *Colletotrichum,* while the sexual form got named *Glomerella*. In Bali, Indonesia, the species that caused anthracnose disease in papaya fruits seemed most likely the *Colletotrichum magnum*.

Table 1. Comparison of the similarity levels of the 18S rRNA gene from the fungal isolate *Colletotrichum* spp. with multiple sequences in GenBank using the BLAST program.

Isolates	Similarity (%)	Accession Number
Glomerella magna strain LC-1	100	KC815123.1
Colletotrichum magnum strain GM-1	99	MW295850.1
Colletotrichum brevisporum isolate CATAS-CB69	100	MW412587.1
Colletotrichum magnum isolate LP2.1	99	MK530193.1
Colletotrichum brevisporum strain YYGXZ07	99	KU319458.1
Colletotrichum sp. 80CP/T	99	GU066653.1
Colletotrichum magnum isolate LP3.2	99	MK530194.1
Colletotrichum brevisporum strain CRI-N2	100	KT185054.1
Colletotrichum liaoningense strain LG9-6	98	MW082812.2
Glomerella magna strain CMM1734	99	HM163187.1

DISCUSSION

The genus *Colletotrichum* has a wide distribution in tropical and humid regions and caused many losses in agricultural production. As an anthracnose-causing pathogen, *Colletotrichum* became one of the most studied pathogens for control mechanism formulation. In managing the disease's control mechanism, the main problem links the host association to *Colletotrichum,* which can be host-specific and multiple hosts (Talhinhas and Baroncelli, 2021; Zakaria, 2021). *Colletotrichum* also has some known mechanisms to 'hide' themselves, such as, initiating secondary conidiation without any symptoms and quiescent infections in immature fruit plants (Dowling *et al*., 2020). The other problem is that the genus *Colletotrichum* species have similar phenotypes but are quite different in phylotypes (Hyde *et al*., 2020; Jayawardena *et al*., 2020), needing molecular analysis for species identification. In comparison, before molecular analysis, only two species (*C. gloeosporoides* and *C. acutatum*) correlated with anthracnose disease in tropical fruits (Zakaria, 2021).

In this study, Koch's postulate test showed the presence of a pathogen that caused anthracnose symptoms in papaya fruits. Based on the macro- and micro-morphological characterization, the pathogen showed similar traits as that of the genus *Colletotrichum*. Deductively, this fungus reveals as an anthracnose-causing pathogen. The identification based only on morphological traits for species delimitation can be difficult due to overlapping traits in sexual and asexual forms (Bhunjun *et al*., 2021; Talhinhas and Baroncelli, 2021). Thus, to positively identify, the molecular analysis using the ITS regions took place and showed that the responsible species of this fungus is *Colletotrichum magnum*. The ITS regions have a known capacity to resolve the taxa in the genus *Colletotrichum* (Bhunjun *et al*., 2021; Perez-Brito *et al*., 2018). Based on the present results of molecular analysis, the species that caused anthracnose disease in papaya fruits indicated the *Colletotrichum magnum* in Bali, Indonesia.

Reports stated *Colletotrichum magnum* is the species that largely infects the crop plants in the family Cucurbitaceae (Tsay *et al*., 2010; Damm *et al*., 2019). However, later studies authenticated species *Colletotrichum magnum* caused the papaya anthracnose in various countries, such as, Brazil (Nascimento *et al*., 2010) and Mexico (Tapia-Tussell *et al*., 2016). The papaya anthracnose by *C. magnum* raises a new concern in tropical diseases. Perez-Brito *et al*. (2018) found the *C. magnum* that causes anthracnose in papaya in Mexico has high genetic variability and is linked to the geographic area. This factor might be related to the dispersion of *C. magnum* accession in the topology of the phylogenetic tree (Figure 4). The pattern of *C. magnum* that dispersed with the other species also resulted from the ITS sequences of *C. magnum* that tend to be identical with the other species in the *C. magnum* complex, such as *C. brevisporum, C. lobatum, C. merremiae, C. panamense, C. okinawense, C. liaoningense,* and *C. cacao* (Damm *et al*., 2019). In Bali, Indonesia, the climatic factor more likely caused the occurrence of *C. magnum* that caused anthracnose in papaya, especially during the rainy season increasing humidity, which favors infection of *C. magnum* (Rangkuti *et al.,* 2017).

The species *Colletotrichum gloeosporoides* and *Colletotrichum acutatum* cause the global anthracnose disease(Dowling *et al*., 2020). Although the disease caused by the *C. magnum* is not as high as those two other species complexes, however, this concern need not be overlooked. Based on the findings of Talhinhas and Baroncelli (2021), a lack of pathological relevance data of the species *C. magnum* requires further detailed research. Generally, water splashes and raindrops easily spread the waterborne genus *Colletotrichum*(Chung *et al*., 2020; Dowling *et al*., 2020). The recorded data on *Colletotrichum* infection showed dominated by familiar crop plants (Jayawardena *et al*., 2020). Therefore, a piercing possibility of genus *Colletotrichum* infecting other local fruit plants that have not been recorded before. Further research on the profound screening of anthracnose disease in various local fruit plants needs future undertaking.

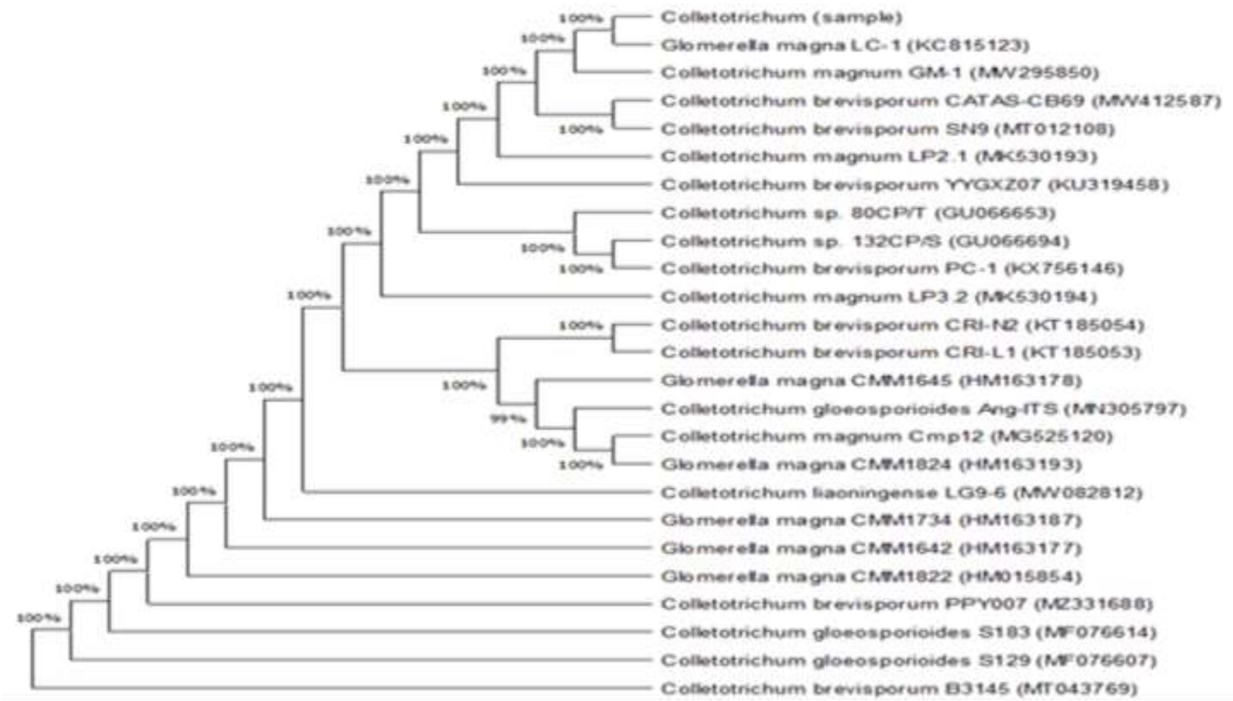

Figure 4. Phylogeny trees from the *Colletotrichum* samples were closely related to *Glomerella magna,* the same as *Colletotrichum magnum* by 100%.

CONCLUSIONS

Based on the results obtained, one can conclude that the cause of anthracnose disease in *Carica papaya* fruits in Bali, Indonesia, results from the fungi species *Colletotrichum magnum*. Therefore, the urgent need to control anthracnose disease in papaya fruits to prevent its further outbreak and reduce yield loss requires attention.

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