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RESEARCH PAPER

Morphological and molecular identification as well pathogenicity of the causal agents of fruit rot disease of apple manalagi (*Malus sylvestris*) in Pujon, East Java

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ABSTRACT

Apple fruit rot disease is one of the major apple diseases in Indonesia. It has been caused by several species of pathogenic within the genus *Colletotrichum*. This research aims to identify species of the pathogen causing apple fruit rot disease in Pujon, East Java. Suspected fungal isolates were identified based on morphological (macroscopic and microscopic) and molecular characteristics. Based on the morphological observation, five isolates were identified as *Colletotrichum* spp. These isolates have similar morphological characteristics such as white-greyish colony color, texture colony velvety, zonation conidia concentric, round cylindrical conidial end, and conidia with 10.4–12.8 µm in length and width 3.1–3.52 µm, respectively. Isolates M1 showed the highest pathogenicity, therefore selected for molecular identification. Molecular identification was conducted using ITS1 and ITS4 primer, AM1 was identified as *C. gloeosporioides* with 99.57% similarity to *C. gloeosporioides* JX-19 variety from China.

Key words: apple, *Colletotrichum*, fruit rot, pathogenicity

INTRODUCTION

Apple is one of the most important agricultural commodities in Malang Regency that is commonly grown in the Pujon Subdistrict. Recently, this commodity is hampered by fruit rot disease that causes a major decline in overall production and marketability. Production of apples decreased from 176,350 kg in 2018 to 129,100 kg in 2019. Apple trees that produced also decreased from 100,000 trees to 79,400 producing trees in the Pujon Subdistrict (BPS-Statistics Indonesia, 2019). Several cases led to a decrease in the number plants that produce apples besides climate and the growing number of old plants, too because apple plants are attacked by pests and diseases, one of which is rotten apples (Ratnawati & Sulistyaningrum, 2019). In apples, fruit rot disease is mainly caused by the infection of pathogenic fungus *Colletotrichum* spp. is a widespread fruit disease occurring in most countries where apples are cultivated (Shi et al., 1996). Particularly in Pujon Subdistrict, this fungus infestation is commonly found in the Manalagi variety, although it is also found in other popular varieties such as Anna and Rome Beauty. The incidence of apple rot disease in Batu City some areas of the Manalagi variety reached 80–90%, while the Anna and Rome Beauty varieties only reached 10–15% (Fauziah et al., 2021). Currently, the spread of this fungus is controlled by cultivating the resistant varieties (Nuryanto, 2018; Shishido, 2011; Suharsono, 2011).

Pervasive *Colletotrichum* spp. spread in the Pujon area is allegedly exacerbated by several environmental factors, such as temperature and growing conditions. Pujon is known to experience daily fog and has a relatively moderate daily temperature, ranging from 22–30 ºC. These conditions alongside constant rain in the rainy season make the perfect germination and growing conditions for *Colletotrichum* spp. In addition, temperatures ranging from 20–30 ºC also could prompt the formation of appressorium of *C. gloeosporiodes* isolates in the surrounding area (Arauz, 2000). Furthermore, Pujon's high altitude means higher humidity, which also contributes to the rapid growth of *Colletotrichum* in the area (Rubiyo et al., 2011; Suhendi et al., 2005).

The common species that have been reported causing fruit rot are *Colletotrichum acutatum*, *C. gloeosporioides*, and *Glomerella cingulata* (González & Sutton, 2004; González et al., 2006). The symptom of fruit rot usually resembles other diseases and varies depending on external factors namely temperature and

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humidity. Frequently, disease identification is based on morphological and microscopically identification of the pathogen (González et al., 2006). Currently, the molecular approach is also used to better identify the pathogen (Freeman et al., 1998) and the combination of both molecular and morphological approaches has been set as the gold standard in identifying *Colletotrichum* spp. (Brown et al., 1996).

The fruit rot disease pathogen of apples in Pujon Subdistrict has not been properly identified and characterized. This research aims to identify the species-causing agent of fruit rot disease in Pujon Subdistrict, Malang Regency, East Java. Furthermore, it can provide a better understanding of the pathogen of fruit rot disease in Pujon.

MATERIALS AND METHODS

Research Site. This research was performed from February to September 2021. The laboratory works were conducted in the Mycology Laboratory at the Indonesian Citrus and Subtropical Fruits Research Institute. Field observations of the symptom and sample collection were conducted in Wiyurejo Village, Pujon Subdristict, Malang Regency, East Java, Indonesia.

Sample Collection. Infected apple fruit was taken from several apple plantations in Wiyurejo Village, Pujon Subdristict, Malang, East Java. In this research, the Manalagi variety was selected due to its high susceptibility to fruit rot disease compared to Rome Beauty and Anna.

Pathogen Isolation. The pathogen was isolated by cutting a 1 × 1 cm area in between the asymptomatic and symptomatic areas. The sample surface was sterilized with alcohol 70% solution for 1 min and rinsed thrice using sterile distillate water. The sterile sample was then placed in a PDA medium (Himedia, India) supplemented with 0.25 mL Terramycin (New York, U.S.A) and incubated for 72 hours.

Morphological Identification. Morphological identification was performed on culture fungi 1 to 12 days after inoculation. The observation was performed on the colony color and colony shape as well as the shape and size of conidia. Microscopic identification was carried out by taking the culture fungi using the preparatory needles and placing them on glass objects that had been dripped with distilled water and then closed using a glass deck in lactophenol. The observation was conducted under a compound microscope (Olympus BX51, Tokyo, Japan) with 40× magnification. The images obtained were compared to the literature image (Barnett & Hunter, 1998).

Pathogenicity Test. The cleaned apples were perforated with a diameter of 5 mm and a depth of 3 mm using a cork borer on the side of the fruit surface. Then, in the wound, a suspension of the apple rot pathogen was inoculated. The apples that have been injured are placed in a plastic tub and covered with clear plastic. The four corners of the tub are placed with wet cotton to maintain moisture, then incubated for 7 days at 28 ºC. Observations were made every day until the 7th day of incubation after inoculation, by measuring the diameter of the lesions on apples. The level of pathogenicity is determined based on the size of the diameter of the lesion, namely the diameter of the lesion is 0.8–1.8 cm, the level of pathogenicity is low, 1.9–3.0 cm is moderate, and > 3.0 is high. From the results of the pathogenicity test, one isolate with the highest pathogenicity value was taken to be further identified molecularly until sequencing.

Molecular Identification

DNA Extraction. A representative fungi was cultivated in potato dextrose broth medium (PDB) at room temperature for 7 days. After 7 days, fully grown mycelium was taken, filtrated, washed with sterile aquadest, and air-dried for 10 min. The dried mycelium was then used for DNA extraction according to the protocol described by Sambrook & Russel (1989) with modification. As much as 0.2 g of dried mycelium is crushed with a sterile pestle. Then, 1 mL of extraction buffer and 10 µL β-mercaptoethanol were added to the solution. The solution was then incubated at 65 ºC for 30 min. A mount 750 µL of chloroform: isoamyl alcohol (CI) (24: 1) were added and centrifuged together at 11,000 rpm for 10 min. Afterward, the supernatant was taken and mixed with 1 mL isopropanol and stored at 4 ºC for 1 hour to allow DNA precipitation. After the incubation, the solution was centrifuged (microcentrifuge survival legend 17, Germany) at 12,000 rpm for 10 min and the pellet was washed with ethanol 70% for 10 min. Ethanol was removed and the pellet DNA was air-dried overnight before being resuspended in a 100 µL TE buffer (1.67 mL Tris 1.5 M, 0.5 mL EDTA 0.5 M, 25 mL distillate water) pH 8 for long-term usage. DNA was kept at -20 ºC for long-term storage.

PCR Amplification. Chromosomal DNA was amplified using the primer of ITS 1 (5-TCCGTAGGTGAACCTGCGG-3) dan ITS 4

(5-TCCTCCGCTTATTGATATGC-3) (White et al., 1990) under the program of pre-denaturation at 94 ºC (4 min), followed by 35 cycles of denaturation at 94 ºC (35 s), annealing at 52 ºC (55 s), elongation 72 ºC (2 min) and post elongation at 72 ºC for 10 min (Nishizawa et al., 2010). The PCR-cocktail for this reaction consists of 12.5 µL of PCR kit, 1 µL of each primer, 1 µL of template DNA, and 8.5 µL of ddH_2O (Nishizawa et al., 2010). The PCR product was sent to PT Genetika Science Indonesia for sequencing. The sequencing result was analyzed for sequence similarities using Basic Local Alignment Tools (BLAST) in the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was constructed using MEGA6 (Tamura et al., 2013) under the Neighbor-Joining algorithm with 1000× bootstraps.

RESULTS AND DISCUSSION

The Pathogen of Apple Fruit Rot Disease. Infected fruits were easily recognized based on visual symptoms (Figure 1). *Colletotrichum* is always found associated with the symptomatic apple fruits in Pujon Subdistrict. The symptom on the young and fully-grown fruits is visually similar to bitter rot and fruit rot disease. Infected fruits usually developed red patchy marks that expanded as the infection proceeded. Furthermore, these red patchy marks became brown and the spore of the pathogen was present in the symptomatic area. The symptoms are similar to the apple rot disease in Kentucky plantations, in the US that has brown patchy marks and also present the necrotic zone under the marks (Munir et al., 2016). *Colletotrichum* is a genus of cosmopolitan fungus that consists of more than 189 species all over the world (Freeman & Rodriguez, 1995; Weir et al., 2012). They were found in tropical and subtropical regions and were known to cause anthracnose disease in host plants (Dean et al., 2012; Bailey & Jeger, 1992), such as apples (Sutton, 2014).

Five isolates were obtained from symptomatic apples that have the characteristics of *Colletotrichum* spp. namely AM1, AM2, AM3, AM4, and AM5. Isolates showed the characteristics of *Colletotrichum* spp. such as white-grayish colonies, cotton-like mycelia, long cylindrical conidia, and spores with a concentric zone (Figure 2). These characteristics were in line with *Colletotrichum* characteristics described by (Khodadadi et al., 2020), which also found white-greyish mycelia and round-cylindrical spores.

Morphological Identifications of the Pathogen of Apple Fruit Rot Disease. *Colletotrichum* sp. obtained from the field grew well in the PDA medium. It takes approximately 12 days for the fungus to fully cover the medium in a 9 mm petri dish. The morphological characteristics of isolates were in-line with the five suspected pathogens that were found. The morphological characteristics of isolates found shown in Table 1. Bajpai et al. (2009) reported that the *Colletotrichum* colonies usually grow well at room temperature and fill the whole Petri dish in 7 days. A similar finding was also reported by Munir et al. (2016) that found *Colletotrichum* has

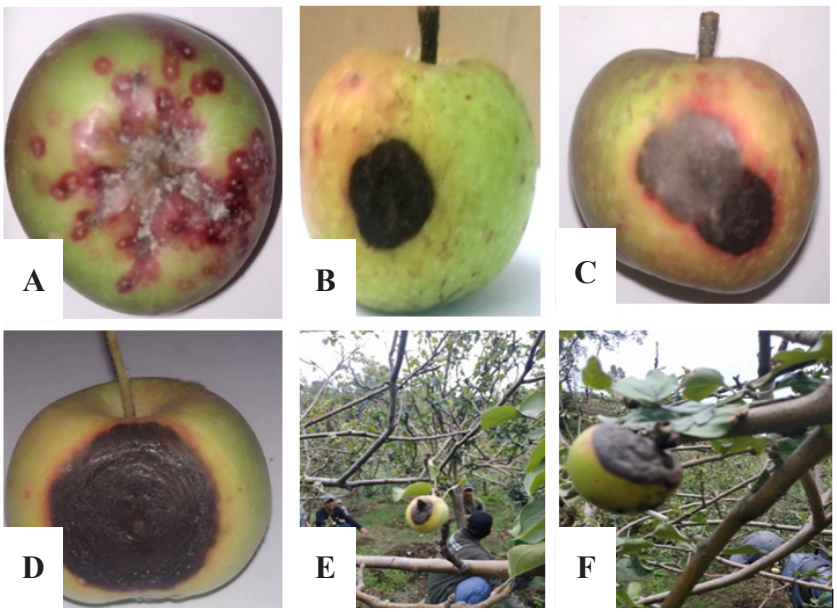

Figure 1. Symptoms of fruit rot disease on apple Manalagi on Pujon. A. Small irregular red spots; B. Brown spots on the surface there are black spots of fungal colonies; C. Brown spots; D. Brown spots forming a concentric circle; E & F. Blackish brown spots on young fruit.

white-grayish colonies and round cylindrical conidia (Table 2).

During the observations, all *Colletotrichum* isolates showed a steady and similar growth rate. The largest colony observed during the 12 days of observations was 9 cm in diameter (Figure 3). On the second day, the diameter of isolates expanded to 0.5–0.8 cm. After 12 days, black spots appeared on the top of the colony. Based on the microcospically observations, all isolates have long cylindrical spores with round edges. The spore's length ranges from 10.4–12.8 µm and the width ranges from 3.1–3.52 µm (Table 3). These isolates have non-insulated appressorium with 11.19 × 4.64 µm and have phialide conidiophores. Among 5 isolates, the largest spore was found in AM1 with 12.8 µm in length and 3.52 µm in diameter. Furthermore, isolates also visualized ring-like radial growth, similar to the *C. gloeosporioides*. Sharma & Kulshrestha (2015) also

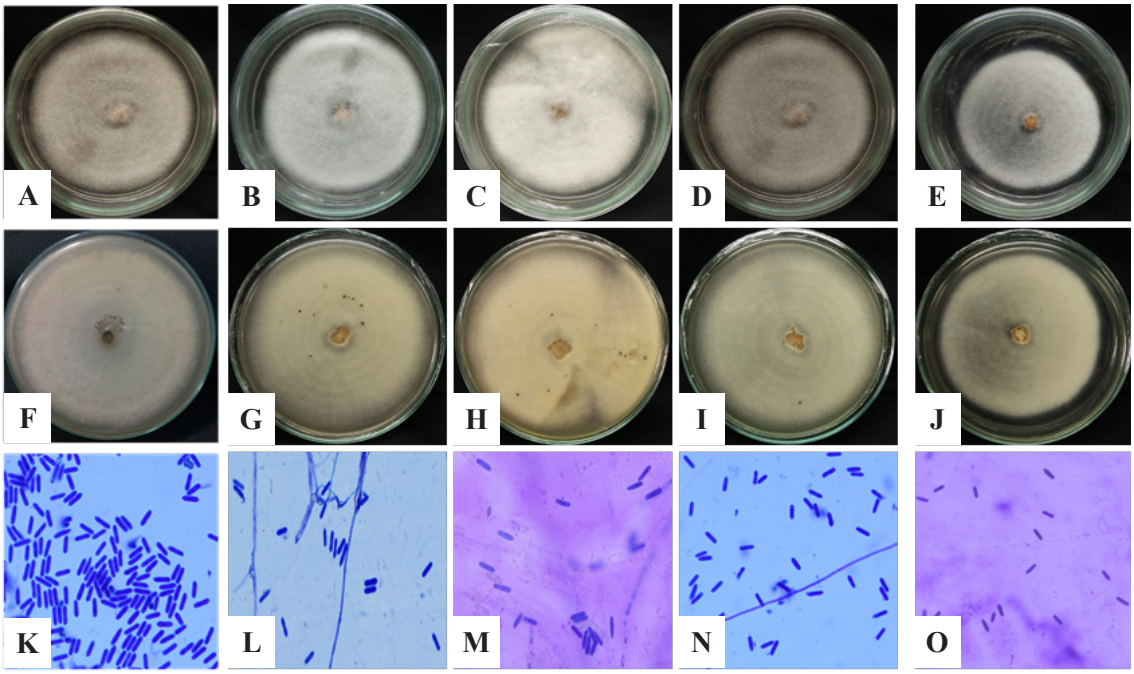

Figure 2. Characterization fungi *Colletotrichum* AM1, AM2, AM3, AM4, AM5. Colony color of *Colletotrichum* on front PDA: A. AM1; B. AM1; C. AM3; D. AM4; E. AM5. Colony color of *Colletotrichum* on reverse PDA: F. AM1; G. AM1; H. AM3; I. AM4; J. AM5. Conidia on PDA: K. AM1; L. AM1; M. AM3; N. AM4; O. AM5. All scale bars = 20 µm.

Table 1. Characteristic of field symptoms of fruit rot disease in Pujon Subdistrict, Malang Regency, East Java

Number samples	Apple Varieties	Symptoms
1	Manalagi	Brown spots on the surface there are black spots of fungal colonies
2	Manalagi	Brown spots
3	Manalagi	Brown spots forming a concentric circle
4	Manalagi	Blackish brown spots on young fruit
5	Manalagi	Small irregular red spots

Table 2. Cultural and morphological characteristics of the *Colletotrichum gloeosporioides* on PDA

Isolates	Colony colour		Texture	Zonation	Conidial shape
	Upper side	Lower side			
AM1	Grayish white	Black Grey	Velvety	Concentric	Cylindrical and straight
AM2	Grayish white	Black Grey	Velvety	Concentric	Cylindrical and straight
AM3	Grayish white	Black Grey	Velvety	Concentric	Cylindrical and straight
AM4	Grayish white	Black Grey	Velvety	Concentric	Cylindrical and straight
AM5	Blackish gray	Black Grey	Velvety	Concentric	Cylindrical and straight

reported similar findings on *C. gloeosporioides* which have 10.3–18.2 µm.

Fungal characteristics were important for initial differentiating among *Colletotrichum* species causing fruit rot diseases with unique conidial size and shape could be easily distinguished. Previous studies have revealed that *C. capsici* (syn. *C. truncatum*) can be differentiated from *C. gloeosporioides* based on its conidial size and shape (Weir et al., 2012). In the current study was observed significant differences in conidial size, with the lengths and widths of the species. Crouch et al. (2009) state that the shapes and sizes of mycelial appreciation in combination with the host range are useful for characterizing the grass-associated *Colletotrichum* species complex.

Pathogenicity Test. The origin of the isolate greatly affects the ability of each isolate to infect apples. The difference in the onset of symptoms is influenced by environmental conditions, pathogen isolates, genetics of plants, method of inoculation, and plant physiology. Pathogenicity tests showed that five isolates were capable of causing fruit rot disease in apples, even though each isolate showed varying virulence. The most severe symptoms were observed in isolated AM1. The most severe symptoms were observed in the AM1 isolate. The symptom was visible 2–3 days after inoculation. Meanwhile, on the other isolates, the symptoms were visible on the fourth or fifth day after inoculation. All isolates showed moderate virulence levels, however, the highest lesion diameters were produced by AM1.

Symptoms that appeared on fruit inoculated with AM1 isolate had a higher lesion diameter of 2.8 cm with moderate virulence. Whereas isolates had a lower lesion diameter of 1.9–2.2 cm on the seventh day after inoculation (Table 4). According to Munir et al. (2016), compared to the other *Colletotrichum* spp. species, *C. gloeosporiodes* has the highest virulence and pathogenicity potency.

Phylogenetic Identification. Morphological characteristics and host preference were not reliable criteria for *Colletotrichum* identification (Weir et al., 2012). Molecular identification using ITS was conducted to confirm morphological identification. AM1 isolate was representative to phylogenetically

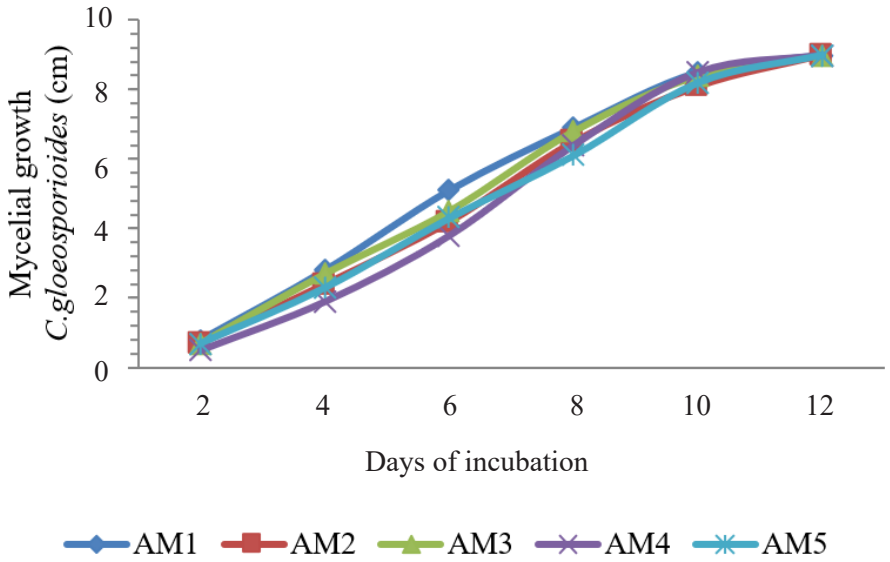

Figure 3. Daily mycelial growth of *Colletotrichum gloeosporioides* isolates

Table 3. The mean width and length of spores produced by 10-day-old *Colletotrichum gloeosporioides* isolates

Isolates	Width (µm)	Length (µm)
AM1	3.5	12.8
AM2	3.1	10.4
AM3	3.1	11.1
AM4	3.3	12.1
AM5	3.3	12.2

identification based on the sequence of ITS regions with ITS1 and ITS4 primers (Nishizawa et al., 2010). ITS primers are considered the gold standard in identifying *Colletotrichum* spp. (Chen et al., 2006; Martínez et al., 2000; Peres et al., 2002; Tapia-Tussell et al., 2008). Amplification with these primers will produce 500 bp DNA fragment amplicons (Figure 4). The BLAST search result showed that the AM1 isolate was close to *C. gloeosporioides*. The percentage of similarity from BLAST and the accession number of the sequences are listed in Table 5. The percentage of similarity obtained from BLAST ranged from 97.35% to 99.57% for ITS regions. The AM1 isolates showed their highest homology with *Colletotrichum gloeosporioides* JX-19 from China is 99.57%, followed by *C. gloeosporiodes* from the USA at 98.47%. Phylogenetic tree analysis showed that the AM1 isolate was placed within a group of *Colletotrichum gloeosporiodes* (Figure 5).

This finding confirms that *Colletotrichum gloeosporiodes* is the main pathogen attacking apples in Pujon Subdistrict, East Java. Previously, several species of *Colletotrichum* were known attacking apples in other plantations within Malang Regency, East Java. Pradana et al. (2013) reported that *Colletotrichum* spp. was infecting apples in the Poncokusumo area. Prakoso et al. (2019) found that this species was identifiable with ITS primers at 480 bp. In Malang's neighboring city, Batu, fruit rot disease caused by *Gleosporium* sp. is also found infecting Manalagi variety with 90% disease intensity (Fauziah et al., 2021). According to Kim et al. (2016) and Haskarini et al. (2019) fruit rot disease in apple fruit is mainly caused by *Gleosporium* sp. and *Colletotrichum gloeosporioides*.

Table 4. Severity scores of representative *Colletotrichum* isolates obtained from apple samples with fruit rot disease in Pujon Subdistrict, Malang Regency, East Java, Indonesia

Isolates	Lesion diameter (cm)	Virulence level
AM1	2.8	Moderate
AM2	2.1	Moderate
AM3	2.2	Moderate
AM4	1.9	Moderate
AM5	2.0	Moderate

Lesion diameters: Low= 0.8–1.8 cm; Moderate= 1.9–3.0 cm; Higth ≥ 3.0 cm.

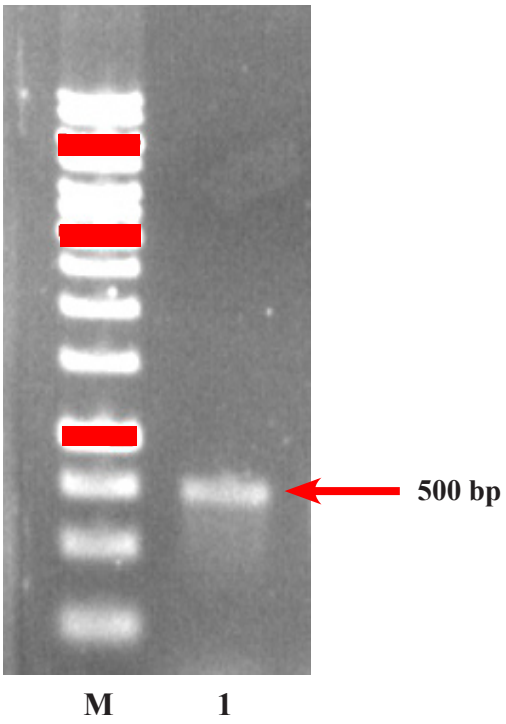

Figure 4. Amplification of PCR used ITS1 and ITS4 primer. M= Leader 1 Kb (fermentas); 1= The result of *Colletotrichum* fungi spp. AM1 isolate.

CONCLUSION

Based on spore morphology, colony characteristics, and ITS gene sequence characteristics, fruit rot disease in Pujon Subdistrict, Malang Regency, East Java is mainly caused by *Colletotrichum gloeosporiodes* that are closely related to *Colletotrichum gloeosporioides* strain JX-19 from China 99.57%.

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Table 5. Similarity of ITS sequences of *Colletotrichum* species isolated from apple plants with fruit rot disease in Pujon Subdistrict, Malang Regency, East Java compared to that of the accessions in the GenBank database

Isolat	Similarity (%)	Accession
C. gloeosporioides strain JX-19 China	99.57	HQ645082.1
C. gloeosporioides USA	98.47	MN565959.1
C. gloeosporioides strain CR-5 India	98.47	KC493156.1
C. gloeosporioides South Africa	98.47	MW080959.1
C. gloeosporioides strain JX-1 China	98.47	HQ645079.1
C. clidemiae USA	98.39	JX010274.1
C. arecicola China	98.21	NR_171191.1
C. aenigma New Zealand	98.21	JX010244.1
C. aotearoa New Zealand	98.21	JX010205.1
C. horii New Zealand	97.86	NR_119754.1
C. conoides China	98.03	NR_152284.1
C. aotearoa New Zealand	98.03	JX010220.1
C. cigarro New Zealand	97.86	NR_120138.1
C. cordylinicola Thailand	97.68	JX010226.1
C. asianum Australia	98.03	JX010192.1
C. asianum Philippines	98.03	JX010195.1
C. asianum Panama	98.03	JX010193.1
C. alatae India	97.68	JX010190.1
C. alatae Nigeria	97.50	JX010191.1
C. clidemiae New Zealand	98.39	NR_120142.1
C. gloeosporioides UK	98.39	EU371022.1
C. alienum New Zealand	98.21	NR_120141.1
C. alienum New Zealand	98.21	JX010246.1
C. alienum Australia	98.03	JX010217.1
C. aeschynomenes New Zealand	98.57	NR_120133.1
C. fructicola Australia	98.57	JX010166.1
C. fructicola Israel	98.57	JX010167.1
C. fructicola Japan	98.57	JX010174.1
C. fructicola Thailand	98.57	JX010165.1
C. fructicola Brazil	98.57	JX010164.1
C. salsolae Hungary	98.03	JX010242.1
C. queenslandicum New Zealand	98.39	JX010276.1
C. yulongense China	97.35	NR_171186.1
Kylindria aquatica	60.37	NR_169932.1

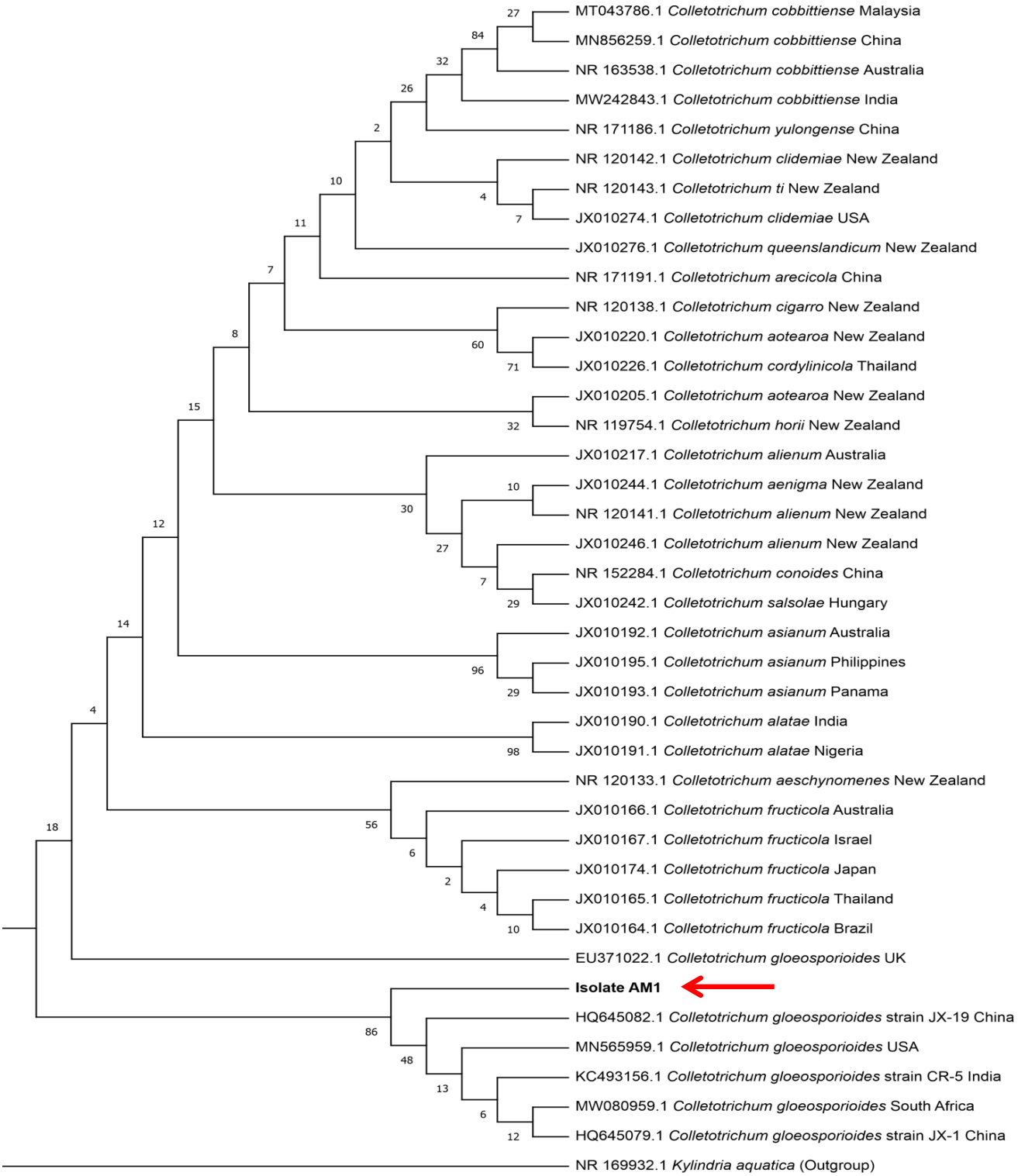

Figure 5. Phylogenetic analysis of *Colletotrichum* used UPGMA (p-distance model; transition+ transversions substitutions; uniform rates patterns; 1000× bootstrapping).

facilities.

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AUTHORS' CONTRIBUTIONS

UT is the main contributor who plays a role in conducting research, observing, and compiling scientific papers. ALA, AM, and SW is who acts as a mentor, validation, and preparation of scientific papers.

COMPETING INTEREST

No competing interests.

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