

Fungal community associated with mixed infection of anthracnose and stem end rot diseases in Chokanan Mango

ANI WIDIASTUTI¹*, SURYANTI¹, ALVINA CLARA GIOVANNI¹, ISLAMINATI ANNA SANTIKA¹,
NIKEN RASMI PARAMITA²

¹Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora No. 1, Bulaksumur, Sleman 55281, Yogyakarta, Indonesia.
Tel./Fax.: +62-274-6492599, *email: aniwidiastuti@ugm.ac.id

²PT. Polowijo Gosari Indonesia Holding. Jl. Raya Daendels, Doudo, Panceng Sub-district, Gresik District 61154, East Java, Indonesia

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Abstract. Widiastuti A, Suryanti, Giovanni AC, Santika IA, Paramita NR. 2023. Fungal community associated with mixed infection of anthracnose and stem end rot diseases in Chokanan Mango. *Biodiversitas* 24: 2163-2170. Anthracnose and stem end rot are the top two prevalent diseases causing losses in mango fruit worldwide. Both diseases often appear together in ripened fruits. The aim of this research was to evaluate the fungal community associated with mixed infection of anthracnose and stem end rot diseases in Chokanan mango based on metagenomics analysis through amplicon targeted next generation sequencing. The results showed that approximately 152,000 sequences were observed. The average total tags from the observed OTUs number were 146,485, of which 126,230 were taxon tags. The ten most common occurring fungal genera were *Colletotrichum*, *Penicillium*, *Diaporthe*, *Purpureocillium*, *Aspergillus*, *Cunninghamella*, *Neofusicoccum*, *Mortierella*, *Rhizopus*, and *Kazachstania*. Of these, genus *Colletotrichum* showed 77% dominance of based on Krona display value. Based on the number of OTUs, seven species, such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Penicillium simplicissimum* (Oudem.) Thom, *Neofusicoccum cordaticola* Pavlic, Slippers & M.J.Wingf., *Diaporthe arengae* R.R.Gomes, Glienke & Crous, *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson, *Alternaria alternata* (Fr.) Keissl., and *Fusarium oxysporum* Schldtl., showed dominance in mixed infection. These findings reveal the major status of post-harvest pathogens in mango fruits that should be controlled. This is the first report on *N. cordaticola* and *D. arengae* associated with mixed infection of anthracnose and stem and rot disease on post-harvest mango in Indonesia.

Keywords: *Colletotrichum gloeosporioides*, *Diaporthe arengae*, fungal diversity, mango, metagenomics, *Neofusicoccum cordaticola*

Abbreviations: OTUs: Operational Taxon Units; NGS: Next Generation Sequencing; QC: Quality Control; DNA: Deoxyribonucleic Acid; ITS: Internal Transcribed Spacer; ACE: Abundance-based Coverage Estimators; PD: Phylogenetic Diversity; TEF-1 α : Translation Elongation Factor-1 α ; β tub: β tubulin

INTRODUCTION

Anthracnose and stem end rot diseases are the most devastating diseases in mango (*Mangifera indica* L.) worldwide, including Indonesia (Widiastuti 2013; Widiastuti et al. 2015; Galsurker et al. 2018; Benatar et al. 2021). These diseases are difficult to control due mainly to their disease symptoms are not visible in the field or immature fruits. However, when the fruits start to ripen disease development occurs very rapidly and often occurs in the retailers and consumers chain causing consumers' complaints against producers.

Numerous studies have been conducted on each disease, however, no studies have been conducted on the mixed infection caused by anthracnose and stem end rot pathogens. Previous studies mainly focused on a single disease either anthracnose or stem end rot, not discussing the interaction between complex pathogens causing postharvest diseases in mango fruit. The use of the Chokanan mango cultivar, as the fruit model variety, has great potential to be developed because of its ability to flower in the off-season (Bejo and Kamaruddin 2014). Although Chokanan mango is one of the most prospective

mango varieties to export with high demand in the Indonesian premium market, but the anthracnose and stem end rot diseases become foremost constraints. This research is essential to comprehend the complex pathogens causing the disease in the postharvest stage and to determine proper postharvest handling to improve fruit qualities both for the domestic and export markets.

Colletotrichum spp. was reported to be the main pathogen causing anthracnose (Li et al. 2019; Alvarez et al. 2020; Tovar-Pedraza et al. 2020; Benatar et al. 2021; Lu et al. 2022). Galsurker et al. (2018) reported that complex pathogens that caused stem end rot disease in the Botryosphaeria group, such as *Dothiorella dominicana* Petr. & Cif., *Dothiorella mangiferae* Syd. & P.Syd., *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Neofusicoccum* spp., *Phomopsis mangiferae* S.Ahmad, *Cytosphaera mangiferae* Died., *Pestalotiopsis* sp. and *Alternaria alternata* (Fr.) Keissl. To target complex species of pathogens and fungal communities in the interaction of anthracnose and stem end rot mixed infection of mango, high throughput analysis using Next Generation Sequencing (NGS) through metagenomic analysis is very powerful which provide the data of the complex microbial

community. Culture dependent study is a main constrain in the research of microbial communities because some microbes are un-culturable on the media (Kellenberger 2001). The current use of metagenomic analysis in the valuation of microbial communities in their natural habitat, without culturing individually in media, has significantly improved the study of the taxonomic structure of microbial consortia. Lately, researchers have utilized metagenomic analysis concerning plant pathology, disease management and plant-microbes ecology research (Pinto et al. 2014; Taylor et al. 2014). Metagenomics analysis has been used widely to study the microbial diversity in environments and living organisms, including humans, animals, and plants to comprehend the variety of microorganisms in specific circumstances (Taylor et al. 2014; Navitasari et al. 2015). It is also well developed to understand the microbial diversity in the environment of healthy and sick plants to comprehend the microbes' key between the conditions (Effendi et al. 2019). This research aimed to study the fungal community on the mango with mixed infection of anthracnose and stem end rot disease.

MATERIALS AND METHODS

Sample collection and storage

Samples of Chokanan Mango with physiologically ripe fruit conditions (15 weeks) were received from the plantation of PT. Polowijo Gosari Indonesia Holding, Sukodono, Panceng, Gresik District, East Java (Figure 1) and brought to the Laboratory of Control Technology, Faculty of Agriculture, Universitas Gadjah Mada,

Yogyakarta, Indonesia. In the laboratory, mangos were incubated in air ventilated room for two weeks until reaching the consumption ripe fruit phase. Mangos with mixed infection of anthracnose and stem end rot diseases were collected as samples for this research. Mango peel samples from the symptomized area were taken as much as 50-100 g, put into a collection tube, and stored at -80°C for DNA metagenomic extraction.

Genomic DNA extraction and amplicon metagenomic sequencing

Genomic DNA was extracted from the peel of the Chokanan mango fruit using the Plant Genomic DNA Mini Kit (Geneaid, Taiwan) according to the protocol. Five mango samples were used for DNA isolation as replications. Quality control (QC) for the NGS process was performed by using Nanodrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE) with a QC standard of $20\ \mu\text{L}/\text{ng}$ for minimum genomic deoxyribonucleic acid (DNA) concentration and 1.8 for minimum genomic DNA purity. Three QC passed samples were pooled into one tube for amplicon metagenomic sequencing and sent to Novogene Co. Ltd. Service Company (Beijing, China). The library preparation and metagenomic sequencing was conducted using Illumina Novaseq 6000. The Internal Transcribed Spacer (ITS) regions of fungal species were amplified using primers ITS5-1737F (GGAAGTAAAAGTCGTAACAAGG) and ITS2-2043R (GCTGCGTTCATCGATGC) targeting ITS 1 region with a fragment length of 200-400 bp (Deng et al. 2019).

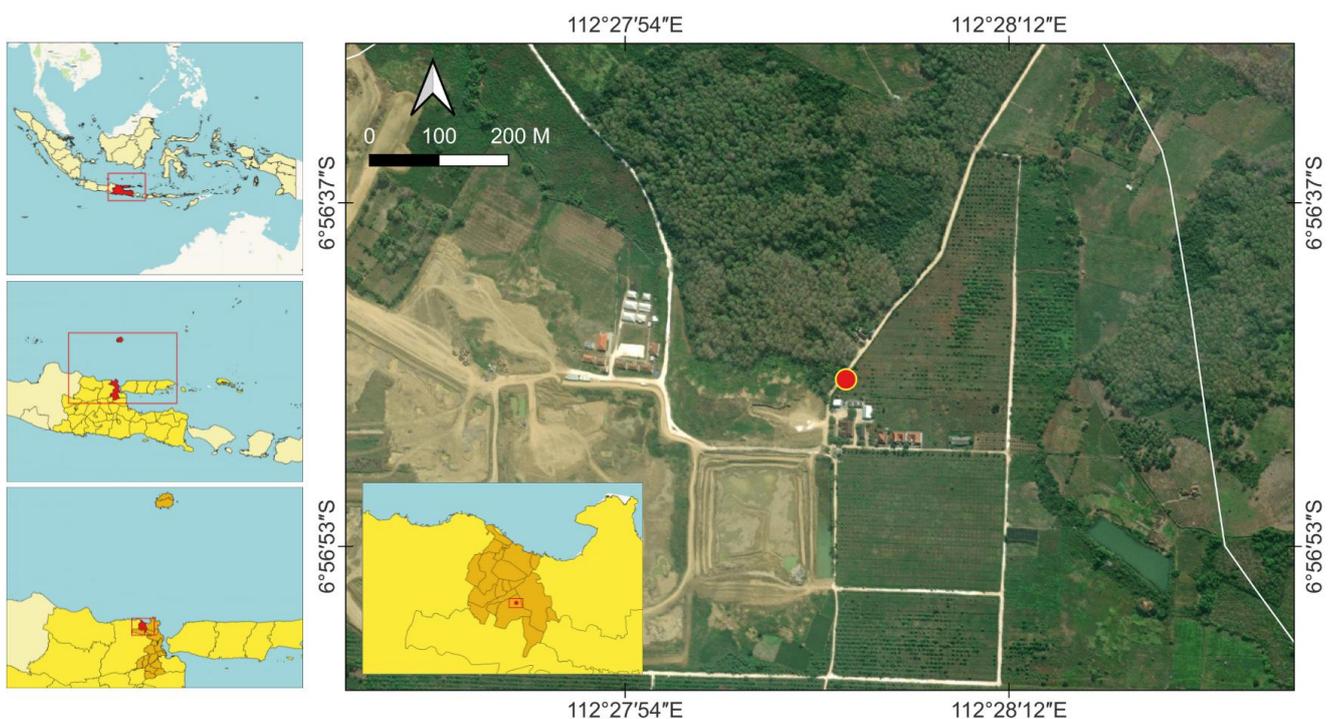


Figure 1. Sampling location of Chokanan mango. The blue dot showed the location of Chokanan mango plantation in Sukodono, Panceng, Gresik District, East Java (Map coordinate: $-6.945811793200989, 112.46787900738526$). The blue color in inserted map showed Gresik District. Source: <https://goo.gl/maps/rLmfavJNLrx1oKwD6>

Bioinformatics analysis

Sequencing data processing

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. FLASH (V1.2.7) was used to combine paired-end readings (Magoč and Salzberg 2011). According to the quality-controlled procedure, quality control on the raw tags was done under certain filtering settings to locate the high-quality clean tags (Bokulich et al. 2013). The tags were compared to the reference database (Unite V8.2 database using the UCHIME algorithm) to identify the chimera sequences, and eliminate them (Edgar et al. 2011; Haas et al. 2011) and obtain the effective tags.

Operational taxonomic units (OTUs) cluster and taxonomic annotation

UPARSE software (Edgar 2013) was used to perform sequence analysis using all effective tags. Sequences with less than 97% were assigned to the same OTUs. For each OTU, a representative sequence was evaluated for additional annotation. For species annotation at each taxonomic rank (kingdom, phylum, class, order, family, genus, species), sequences were analyzed using blast using blastall (Version 2.2.25) and the Unite V8.2 database (Köljalg et al. 2013). The MUSCLE program (Edgar 2004) was applied to determine the phylogenetic connection of all OTU representative sequences. The abundance information for OTUs was normalized using a conventional sequence number matching to the sample with the fewest sequences. Next, alpha and beta diversity were calculated based on these normalized output data.

Alpha diversity

Alpha diversity was applied to analyze the complexity of biodiversity for a sample through 6 indices, including Observed-species, Chao1, Shannon, Simpson, Abundance-based coverage estimators (ACE), and Good-coverage. All these indices in our samples were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

RESULTS AND DISCUSSION

Mixed symptoms of anthracnose and stem end rot diseases on Chokanan mango

In the present study, mixed infections of anthracnose and stem end rot in Chokanan mango were shown by the mixed symptoms of fruit rot started from the stem with brown color and a black spot of anthracnose (Figure 2). An early symptom of anthracnose disease was observed as a black spot and spread into some areas in mango peel. The spots were dry and sunken, and their size gradually increased and spots coalesced. Different from anthracnose, stem end rot disease symptoms begin from the fruit stem, brown color, and rapidly spread to a wider area of mango peel into an extensive lesion. Consequently, the fruit turned rotten and soft.

Symptoms of both the diseases were observed in the present investigation. The mixed infection occurred together late, so it is difficult to distinguish. Both of the disease symptoms did not appear on 15-week age when they arrived in the laboratory as they were not 70% mature. The symptoms gradually emerged during the ripening process and observed at 17 weeks of optimum ripening. Apart from this, the symptoms of disease were clearly visible on the yellow bright Chokanan mangoes.

Metagenomics analysis of Chokanan mango associated with mixed infection of anthracnose and stem end rot diseases

The quality of the metagenomics data was first shown in the rarefaction curve (Figure 3A). The flatter shape at the end of curve showed that a credible number of samples had been taken. Results revealed that approximately 152,000 sequences were observed, with the total average number of Operational Taxon Units' (OTUs) of 146,485 tags, consisting of 126,230 taxon tags (Figure 3B). On the basis of taxa relative abundance, ten major fungal genera were observed associated with mixed infection of anthracnose and stem and rot diseases in Chokanan mango. The ten fungal genera were *Colletotrichum*, *Penicillium*, *Diaporthe*, *Purpureocillium*, *Aspergillus*, *Cunninghamella*, *Neofusicoccum*, *Mortierella*, *Rhizopus*, and *Kazachstania* (Figure 4).

Colletotrichum was found to be the dominant fungal genera causing anthracnose and stem end rot mixed infection in Chokanan mango as much as 77% (Figure 5). This data supported the result of alpha diversity indices, as shown in Table 1. Based on the Shannon index with a value of 1.586, it was found that fungal species were relatively diverse in the sample of Chokanan mango after being infected by anthracnose and stem end rot mixed infection. However, the Simpson index value was 0.411 indicating relatively low evenness or high dominance, which was dominated by the *Colletotrichum* genus. The numbers of OTUs on Chao1 and ACE index and good coverage indicated that all species in the sample were observed.



Figure 2. Mixed infection of anthracnose and stem end rot diseases in Chokanan mango (blue arrow: anthracnose symptom; red arrow: stem end rot disease symptom)

The alignment of OTUs representative sequences to the database reference identified some pathogens species, such as *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc, *A. alternata*, *Diaporthe arengae* R.R.Gomes, *Neofusicoccum cordaticola* Pavlic and *Fusarium oxysporum* Schldtl in the mixed infection of anthracnose and stem end rot on

Chokanan mango (Figure 6). Other fungal species, such as *Penicillium simplicissimum* (Oudem.) Thom, *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson, *Cunninghamella elegans* Lendn., *Rhizopus arrhizus* A.Fisch. and *Aspergillus* spp. were also found as predominant species in the mixed infection.

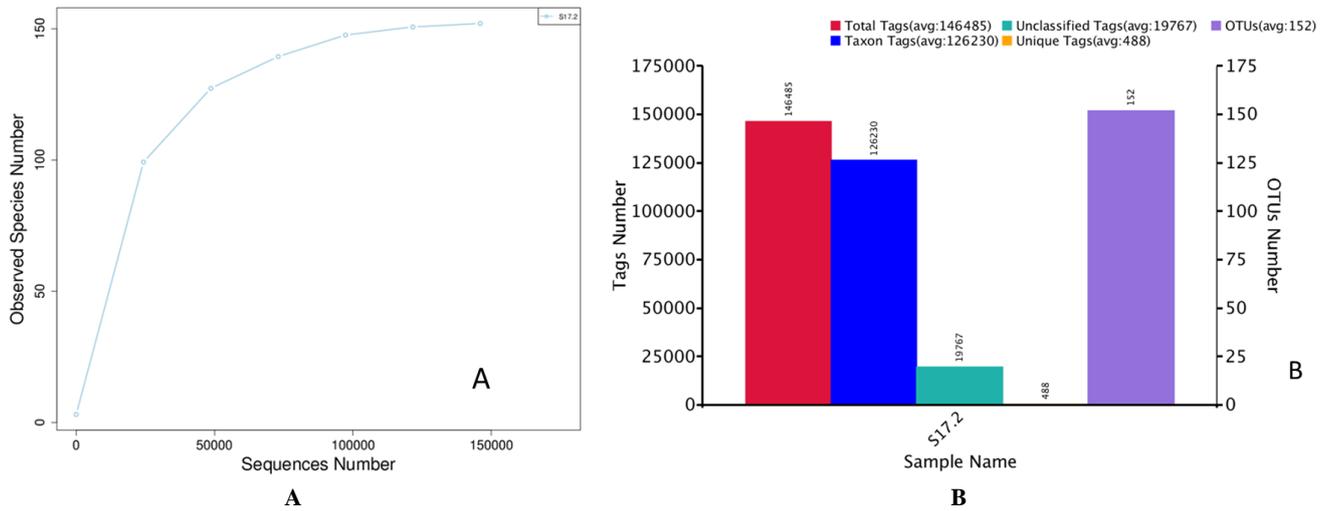


Figure 3. A. Rarefaction curve of fungal community’s composition and; B total taxon of Operational Taxon Units (OTUs) of anthracnose and stem end rot mixed infection in Chokanan mango

Table 1. Alpha diversity indices of a fungal community of anthracnose and stem end rot mixed infection in Chokanan mango

Sample	Observed_species	Shannon	Simpson	Chao1	ACE	Goods_coverage	PD_whole_tree
S17.2	152	1.586	0.411	152.000	152.000	1.000	36.645

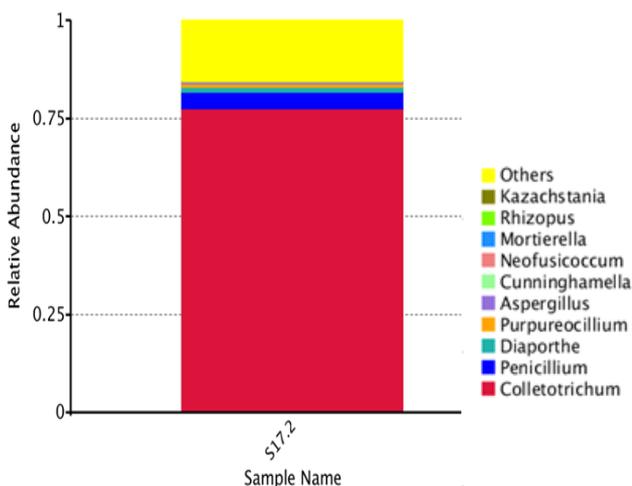


Figure 4. Taxa relative abundance of ten fungal genera in Chokanan mango with mixed infection of anthracnose and stem end rot disease

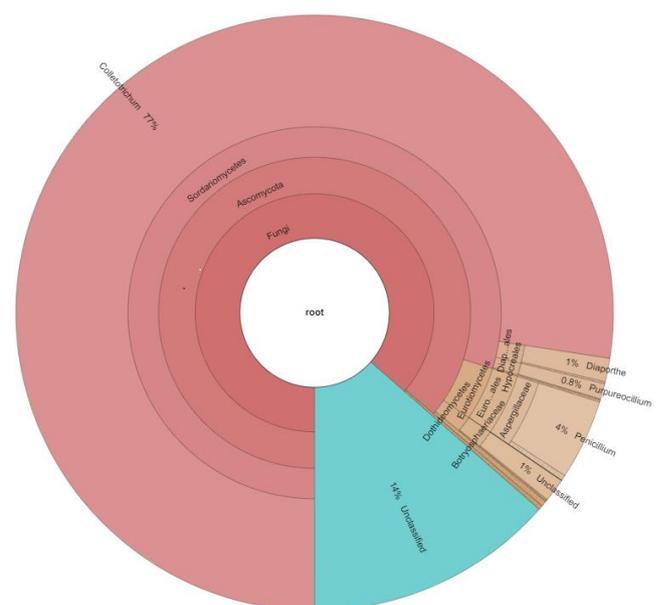


Figure 5. Krona analysis showed different fungal community causing anthracnose and stem end rot mixed infection in Chokanan mango

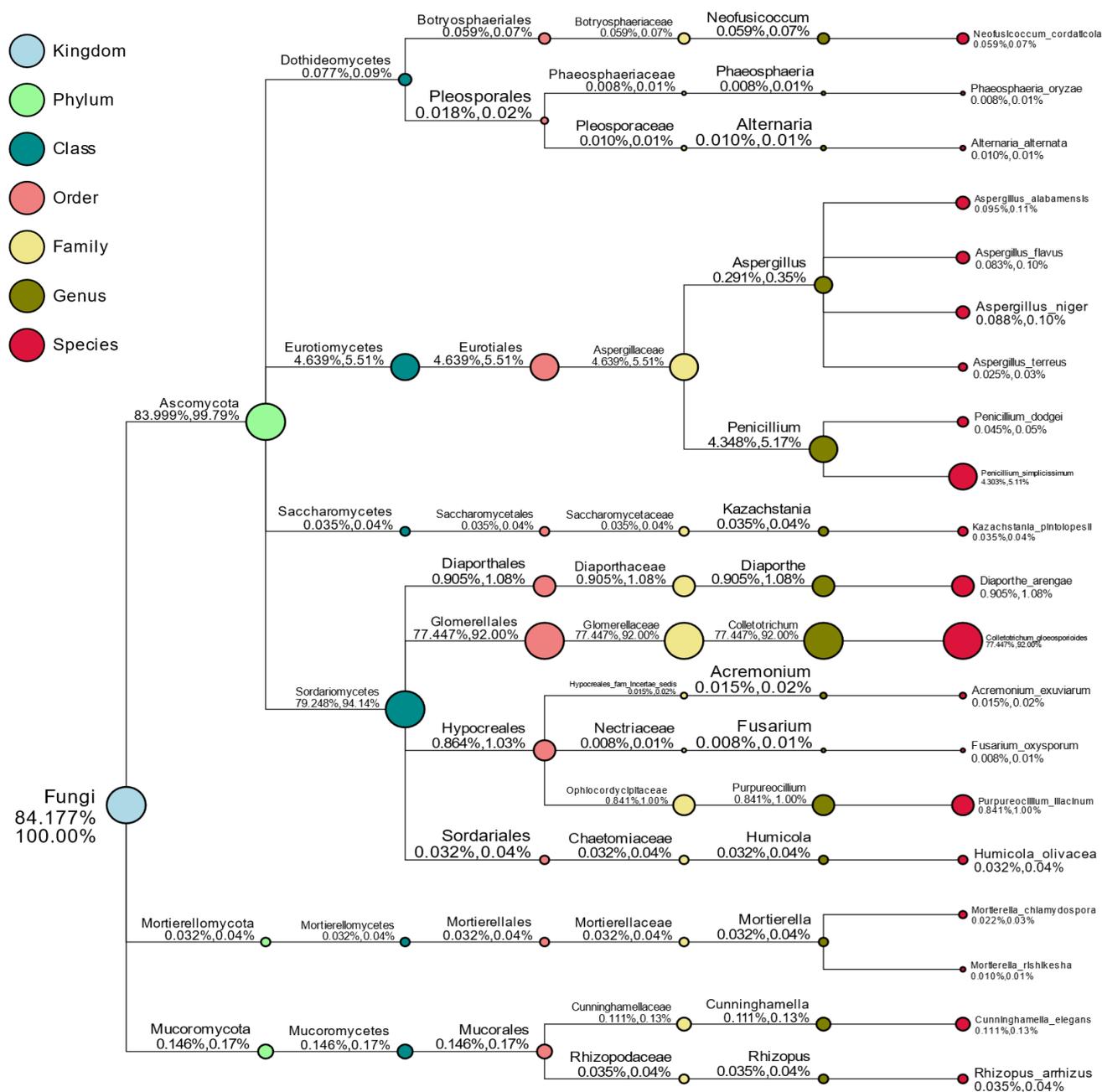


Figure 6. Phylogenetic relationship based on OTUs representative sequences in the fungal community of anthracnose and stem end rot mixed infection in Chokanan mango

Discussion

Mango, the King of Fruit, is one of Indonesia's most important superior fruit commodities because it has exotic appeal and high economic value both domestically and for export. Nationally, mango production ranks third after bananas and oranges. Indonesian Statistics Agency (BPS 2023) showed that mango production in 2021 reached 2.83 million tons. There are many varieties of mangoes in Indonesia, but no research has been found on the Chokanan variety of mangoes. Chokanan is a superior variety from Thailand which is currently being developed in Indonesia. The variety has an oval-shaped character with medium fruit

size (250-350 g), golden yellow skin, orange-yellow flesh, sweet taste (TSS=14-16%), pleasant aroma, slightly fibrous and high yield (TSS=14-16%). 60-100 fruits per tree (Azhar et al. 2013). It has great potential to be developed due to its ability to flower off-season, therefore it is able to bear fruit throughout the season (Bejo et al. 2014). Therefore, it has a great opportunity to strengthen Indonesia's export commodity. In Indonesia, Chokanan has begun to be developed both among hobbyists and large plantations. Due to the company's note, adjacent to the important export potency, Chokanan mango also has a great demand from Indonesian consumers because of its

distinctive color, aroma and taste. However, anthracnose and stem end rot diseases are the main mango postharvest disease problem during transportation and marketing which were difficult to be managed.

PT Polowijo Gosari Indonesia through its subsidiary has developed Chokanan Mango in Gresik, East Java, provided the samples in the present study. Symptom of both diseases did not occur when fruits are on trees or unripe, yet it emerged along with the fruit ripening and senescence processes. Chokanan mangoes ripen in 15 weeks and can be stored for about two weeks for consumption. However, anthracnose and stem end rot diseases emerged during the storage and ripening process. Galsurker et al. (2018) reported that some plant pathogens grow endophytically in the stem during fruit development in the orchard, they remain dormant until the onset of fruit ripening. When the fruits ripened, they change their stage into necrotrophic pathogens and infect fruits. A climacteric process on mango postharvest, which increases the respiration, ethylene, and sugar content, is presumably related to the activity of complex pathogens to develop their infection (Liu et al. 2022).

Many studies related to understanding the anthracnose and stem end rot disease in postharvest mango, which conducted individually, have been reported. As a result, types of pathogens and how to develop a strategy for controlling the diseases can be identified. However, to our best knowledge, there is no study reported the interaction between the diseases. Regardless of preliminary observations, we found that the two diseases could co-occur in one mango fruit, indicated by the appearance of disease symptoms caused minimally by the two pathogens. It is unknown whether anthracnose and stem end rot pathogens collectively infect fruits during ripening or compete each other to perform their dominancy.

Recent advances in metagenomic studies have contributed to revealing microbiome communities in various environmental samples. Next-generation sequencing (NGS) for metagenomics is a method that allows identification of existing microbial communities without culturing. NGS technology can generate more sequences per sample, thus enabling the analysis of a large number of detailed taxonomic profiles (An et al. 2021). In metagenomics, the entire genomic DNA is obtained from a sample. The resulting DNA fragments, reads or sequence contigs, genome sequences are mapped to the taxonomic group database through sophisticated bioinformatic methods. The results will provide an overview of species composition in metagenomic samples based on nucleotide sequence composition, comparison of conserved protein domain-coding sequences, and identification of 16S rRNA or ITS sequences in samples (Fricke et al. 2011). By reading all the DNA sequences of all species of organisms in one ecosystem, scientists are able to find out the various types of microbes in the micro-ecosystem, as well as their interactions (Bodor et al. 2020). Metagenomics analysis is considered a useful tool to elaborate the fungal community in a specific circumstance, including comprehending the microbiomes in diseased organ plants. As the dependent culture method could not cover some fastidious fungi,

metagenomics provides a database to comprehend the fungal community, which is very useful for further research. Since there is no previous study reported on how the mixed infection of the anthracnose and stem end rot against mango plant. The present study disclosed the fungal community and diversity of such pathogens which were responsible for the mixed infection in Chokanan mango.

The rarefaction curves can directly indicate the rationality of sequencing data volume and indirectly indicate the richness of microbial community in the samples. In this regard, a steep curve means that many species need to be discovered, while a flatter curve referred to a credible number of samples that had been taken, or only the scarce species remain to be sampled (Lundberg et al. 2013). The shape of the curve means that all data obtained in this analysis are valid for analysis. Most of the tags with the value of 126,230 taxon tags identified in the present study reflected the top ten fungal genera which were dominated by the *Colletotrichum* genus. The number of unclassified tags recorded was categorized as small value, of which only approximately about 10% in comparison to the total tags, indicating that the data provided was reliable.

Many reports comprehended that various species of *Colletotrichum* were associated with anthracnose disease in mango, such as *Colletotrichum asianum* Prihast., L.Cai & K.D.Hyde, *Colletotrichum cliviicola* Damm & Crous, *Colletotrichum cordylinicola* Phouliv., L.Cai & K.D.Hyde, *Colletotrichum endophyticum* Manamgoda, Udayanga, L.Cai & K.D.Hyde, *Colletotrichum fructicola* Prihast., L.Cai & K.D.Hyde, *Colletotrichum gigasporum* Rakotonir. & Munaut, *C. gloeosporioides* (Penz.) Penz. & Sacc, *Colletotrichum karsti* You L.Yang, Zuo Y.Liu, K.D.Hyde & L.Cai, *Colletotrichum liaoningense* Y.Z.Diao, C.Zhang, L.Cai & Xi L.Liu, *Colletotrichum musae* (Berk. & M.A.Curtis) Arx, *Colletotrichum scovillei* Damm, P.F.Cannon & Crous, *Colletotrichum siamense* Prihast., L.Cai & K.D.Hyde and *Colletotrichum tropicale* E.I.Rojas, S.A.Rehner & Samuels (Li et al. 2019). *Colletotrichum gloeosporioides* has long been reported to be associated with mango anthracnose (Prakoso et al. 2019; Benatar et al. 2021) but other species gained their important status recently. *Colletotrichum asianum* was first reported to be a pathogen of mango anthracnose in Indonesia in 2021 (Benatar et al. 2021) as well as in other countries (Alvarez et al. 2020; Tovar-Pedraza et al. 2020). *Colletotrichum plurivorum* was first reported in China as a causal agent of mango anthracnose (Lu et al. 2022). *Colletotrichum* showed 77% dominance among the fungal community even though in the mixed infection with stem end rot disease, therefore attention to *Colletotrichum* spp. as the foremost postharvest pathogen in mango is always obliged. The dominance pattern was also supported by the Simpson alpha diversity index, as much as 0.411, which shows a dominancy in the fungal community rather than evenness. Simpson's diversity index is used to measure diversity by taking into account the number of species present, as well as the relative abundance of each species ranging from 0 to 1. As species richness and evenness increase, so does diversity (Tucker et al. 2017).

Some species of *Neofusicoccum* and *Diaporthe* genera were reported as a pathogen of stem end rot and dieback disease in mango and other fruit trees (Marques et al. 2013; Serrato-Diaz et al. 2014; Lim et al. 2019). *Neofusicoccum* is a genus in the family Botryosphaeriaceae, a fungal family whose many species were reported as stem end rot pathogens (Marques et al. 2013; Galsurker et al. 2020). *Neofusicoccum parvum* (Pennycook & Samuels) Crous, Slippers & A.J.L. Phillips, *Neofusicoccum mangiferae* (Syd. & P.Syd.) Crous, Slippers & A.J.L. Phillips and *Neofusicoccum batangarum* Begoude, Jol.Roux & Slippers had been reported to cause stem end rot and dieback diseases in mango (Marques et al. 2013; Puig and Winterstein 2021), but not yet reported to affect mango in Indonesia. Apart from on mango tree, a group of Botryosphaeriaceae was commonly reported as a pathogen on citrus gummosis (Aloi et al. 2021), including in Indonesia (Henuk et al. 2017). Up to date in Indonesia, limited report of *Neofusicoccum* spp. was found, and it was reported in *Eucalyptus* and rubber trees to cause *Fusicoccum* fall leaf disease (Sakalidis et al. 2013; Sarsono et al. 2020). However, based on the phylogenetic relationship map, *N. cordaticola* was found in this study, which was never reported in Indonesia. Lim et al. (2019) identified some species of *Diaporthe*, namely *Diaporthe arecae* (H.C.Srivast., Zakia & Govindar.) R.R.Gomes, Glienke & Crous, *Diaporthe eugeniae* R.R.Gomes, Glienke & Crous, *Diaporthe pascoei* R.G.Shivas, Jacq.Edwards & Y.P.Tan, *Diaporthe perseae* (Zerova) R.R.Gomes, Glienke & Crous, and *Diaporthe ueckeriae* which were responsible to cause stem end rot in mango in Malaysia based on ITS, Translation Elongation Factor-1 α (TEF-1 α), and β tubulin (β tub) sequences. This study found *D. arengae* associated with the mixed infection of anthracnose and stem end rot disease, which have also never been reported in Indonesia. Newly identified pathogens grabbed the concern of plant pathologists to be more aware of the pathogen threats along with the higher possibility of global trading which contributes to the spread of pathogens in wider areas.

This research revealed *C. gloeosporioides* was the dominant species based on the OTUs data, and it plays important role as the main pathogen causing anthracnose on the Chokanan mango. The finding is related to some reports of the important status of *C. gloeosporioides* causing mango anthracnose (Diedhiou et al. 2007; Prakoso et al. 2019; Benatar et al. 2021; Sharma et al. 2021). Other dominant pathogens, such as *A. alternata*, *D. arengae*, *N. cordaticola* and *F. oxysporum*, are assumed to be associated with the incidence of stem end rot disease Diskin et al. (2017). Some other species found in the present research, such as *P. simplicissimum*, *P. lilacinum*, *C. elegans*, *Rhizopus arrhizus* A.Fisch., and *Aspergillus* spp. were reported to have a different role in the fungal community. *P. simplicissimum* was reported as a plant growth-promoting fungi (Hossain et al. 2007), while *C. elegans* was a natural enemy of some plant nematodes (CABI 2019). On the other hand, other species were known as ubiquitous fungi which can grow in many environments. *Aspergillus* spp. is known as a fungal group which is commonly found on weakened or damaged

(Satitmunnaithum et al. 2022). In addition, *Aspergillus* spp. is also one of the spoilage fungi which take the opportunity to grow in damaged fruits and thus, proper postharvest handling is crucial to produce high-quality products.

Metagenomic analysis revealed pathogens and beneficial fungal species associated with mixed infection of mango anthracnose and stem end rot disease. The metagenomic results showed the significant status of some postharvest pathogens in mango and biosecurity, which some of them were not yet reported in Indonesia. This is the first report on *N. cordaticola* and *D. arengae* associated with mixed infection of anthracnose and stem and rot disease on post-harvest mango in Indonesia.

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