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Morphological Variation and Molecular Characteristics of *Phytophthora palmivora* Isolates from Several Areas of Cocoa Plantations in South Sulawesi and Their Virulence on Sulawesi 2 Cocoa Clone

Variasi Morfologi dan Karakteristik Molekuler Isolat *Phytophthora palmivora* dari Beberapa Area Pertanaman Kakao serta Virulensinya Pada Kakao Klon Sulawesi 2

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ABSTRACT

The morphology, molecular, and virulence characterization of *Phytophthora palmivora* isolates obtained from different locations can be used to provide useful information to control rot disease in cocoa pods. Therefore, this study was conducted to characterize morphological variation and molecular characteristics of *P. palmivora* isolates from different cocoa plantations affected by pod rot, and to evaluate their virulence on Sulawesi 2 cocoa clone. A total of 21 isolates of *P. palmivora* were obtained from infected cocoa plantations in Luwu, Gowa, and Pinrang Districts for this purpose. Morphological characteristics of *P. palmivora* isolates on V-8 media were found to be dominated by stellate or cottony form and terminally or intercalary chlamydospores. The results also showed that sporangia were varied among isolates from different sources and one isolate was discovered as unpapillate and ellipsoid sporangium. Moreover, the phylogenetic analysis conducted based on the ITS rDNA region showed that all sequences from this study were nested in *P. palmivora* clade. The results further showed that a total of 10 *P. palmivora* isolates including PA4, PA5, PI1, PI2, PI3, PI7, G2, G4, G6, and G8 exhibited highly virulent activity, 9 isolates as virulent, and only 1 isolate as less virulent.

Keywords: cocoa pod rot, *Phytophthora palmivora*, molecular characteristics, virulence test

ABSTRAK

Karakterisasi morfologi, molekuler, dan virulensi isolat *P. palmivora* yang diperoleh dari lokasi berbeda dapat memberikan informasi yang berguna dalam upaya pengendalian penyakit busuk buah kakao. Penelitian ini bertujuan untuk mengkarakterisasi variasi morfologi dan karakteristik molekuler isolat *P. palmivora* dari berbagai tanaman kakao yang terkena penyakit busuk buah, serta mengevaluasi virulensinya pada klon kakao Sulawesi 2. Sebanyak 21 isolat *P. palmivora* diperoleh dari perkebunan kakao yang terinfeksi di Kabupaten Luwu, Gowa, dan Pinrang. Ciri morfologi isolat *P. palmivora* pada medium V-8 didominasi oleh bentuk stelata atau kapas, dan klamidospora terminal atau interkalar. Sporangium bervariasi antara isolat dari sumber yang berbeda, dan satu isolat memiliki sporangium *unpapillate* dan *ellipsoid*. Analisis filogenetik berdasarkan wilayah rDNA ITS menunjukkan bahwa semua sikuens dari penelitian ini bersarang di *clade P. palmivora*. Sebanyak 10 isolat *P. palmivora* (PA4, PA5, PI1, PI2, PI3, PI7, G2, G4, G6, G8) menunjukkan aktivitas virulensi yang tinggi, 9 isolat bersifat virulen dan hanya 1 isolat yang kurang virulen.

Kata kunci: busuk buah kakao, *Phytophthora palmivora*, karakteristik molekuler, uji virulensi

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INTRODUCTION

Phytophthora cocoa pod rot caused by *Phytophthora palmivora* is still a significant problem in Indonesia despite several control efforts developed and implemented over the past few years. This disease has the potential to inflict complete crop losses of up to 100% in tropical regions, primarily due to the presence of disease conducive environmental conditions or inadequate management practices. The risk was identified to be particularly significant in smallholder cocoa plantations (Guest 2007). *Phytophthora* has the capability to infect cocoa at all stages of development. An effective strategy to control this disease in the field is by understanding the life cycle, virulence, and other characteristics of *P. palmivora* in addition to planting resistant or tolerant cocoa clones. This means it is important to consider variations in pathogen populations in the process of developing disease management strategies.

The current morphological-based methods to identify *P. palmivora* include asexual characteristics such as the colony type, hyphal swelling, chlamydospore production and diameter, sporangiophore branching, sporangia shape and size, caducity, pedicel, and papilla length as well as the sexual characteristics such as size of antheridia, oogonia, and oospore (Waterhouse 1974a; 1974b; Erwin and Ribeiro 1996; Appiah *et al* 2003; Rodríguez-Polanco *et al.* 2020). These methods have several limitations due to high morphological variation among isolates existing in the same population. Therefore, the efforts to improve identifying and diagnosing *Phytophthora* genus require a better understanding of their biology, diversity, and taxonomic relationships. This indicated the importance of additional information on molecular identification (Kroon *et al.* 2012). The advancement of molecular techniques and recent comprehensive multigene phylogenetic analysis of the genus (Blair *et al.* 2008) have provided sequence databases to aid the identification of unknown species. For example, polymerase chain reaction (PCR)—based techniques such as DNA sequence analysis—have been widely used for the phylogenetic analysis of *Phytophthora* species (Cooke *et al.* 2000). The DNA analysis was applied as an alternative to morphological characterization of *Phytophthora* species. Furthermore, several fungal species have been classified using an internal transcribed spacer (ITS) rDNA, mitochondrial cytochrome c oxidase subunit 1 (COX1), and elongation factor 1 (EF-1) regions (Martin *et al.* 2012).

Several studies have also been conducted to characterize and identify the causative agent of black pod disease, but none found a clear relationship between morphology, virulence, and genetic groups. Meanwhile, disparate results have been reported around the world on the aggressiveness of *P. palmivora* isolates from different hosts and countries (Surujdeo-Maharaj *et al.* 2001; Torres 2016; Fuzitani *et al.* 2018). Therefore, this study was focused on analyzing the morphology, molecular characterization, and pathogenicity variation of *P. palmivora* isolates from different locations in South Sulawesi.

MATERIALS AND METHODS

Isolates

The infected cocoa pod was collected from three cocoa plantations in the Districts of Luwu, Pinrang, and Gowa in South Sulawesi, Indonesia. The parts used as samples were cut at 0.5 cm × 1 cm between the boundary of the healthy and infected areas. Moreover, the surface of the samples was sterilized for one minute using 70% ethanol, rinsed three times with sterile distilled water, and air-dried for one minute on sterile filter paper to isolate *P. palmivora*. The pieces were later placed on a V-8 juice agar medium produced with 15 g bacto agar, 1 g $CaCO_3$, 50 mL of V8® Juice, and 950 mL of distilled water, and incubated at 25 °C in the dark. Furthermore, a single colony was purified and subcultured for further analysis.

Morphological Characterization

Isolates were characterized using 7 days old *P. palmivora* culture on V8 medium with a focus on the colony morphology, sporangia shape, chlamydospores, pedicles, and papillae in line with the guidelines stipulated by Stamp *et al.* (1990). A compound microscope (Carl Zeiss, Primo Star) was used for the observation at 400× magnification. Moreover, the morphology of the sporangium and zoospore was analyzed by immersing the 21 isolates colony in drops of sterile water on object glass for 8–12 hours at 10 ℃.

DNA Extraction

DNA was prepared by modifying the *hexadecyltrimethylammonium bromide* (CTAB) method described by Doyle and Doyle (1990). The process involved homogenizing a 50–200 mg mycelium (wet weight) in a mortar under liquid nitrogen and transferring the powder produced to a solution, preheated to 65°C, containing 20 g L^{-1} CTAB, 100 mM Tris-Cl (pH 8.0), 20 mL EDTA and NaCl 1.4 M. After incubating for 30 minutes at 65 °C, the solution was extracted with one volume of chloroform. Moreover, the water phase was transferred to a new tube after which the DNA was precipitated for 30 minutes on ice with one volume of precooled 2-propanol and centrifuged in the cold for 15 minutes at 12000 g. The pellets were washed once using 70% ethanol, dried, and then redissolved in 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA.

Amplification Using Specific Primer for *Phytophthora palmivora*

A total of 20 isolates were selected for further molecular analysis using a specific primer for *P. palmivora* including Pal1s (5'-CACGTGAACCGTATCAAAACT-3') and Pal2a (5'-CAATCATACCACCACAGC TGA-3') (Chirapongsatonkul *et al.* 2015). The PCR was conducted in a 25 µL reaction containing 10 ng of DNA template, 1.25 µM of each oligonucleotide primer (Pal1s and Pal2a), 0.2 mM dNTP, 1× PCR buffer, 1 U of DyNazymeTM II DNA polymerase (Finnzymes, Espoo, Finland), and nuclease-free water. Moreover, the amplification was initiated by denaturation at 94 ℃ for 5 min followed by 25 cycles at 95 ℃ for 30 sec, 58 ℃ for 30 sec, and 72 ℃ for 1 min as well as a 10 min final extension at 72 ℃ in a thermocycler (GeneAmp PCR System 2400, Perkin Elmer, Foster City, CA, USA). The PCR amplification products were later separated in 15 g L^{-1} agarose gel through electrophoresis in 1× TAE and stained using a Quantifast SYBRGreen PCR kit (Qiagen).

Amplification of ITS rDNA Region

The DNA ITS sequence was amplified using ITS4 and ITS5 primers (5'-TCCTCCGCTTATTGATATGC-3' and 5'-GGAAGTAAAAGTCGTAACAAG-3') (Ristaino *et al.* 1998). The process involved preparing a 25 µL total volume of PCR reagent consisting of 2.5 µL 10× PCR buffer, 1.5 µL $MgCl_2$ 0.5–2.5 mM, 0.5 µL dNTP 200 µM, 1 µL ITS4 primer 0.4 µM, 1 µL ITS5 primer 0.4 µM, 0.2 µL Taq DNA Polymerase, 2 µL DNA template 50 ng, and 16.3 µL ddHO. Meanwhile, the DNA was amplified using Gene Amp PCR System 9700 at the cycling conditions of 96 ℃ for 5 min followed by 35 cycles of 96 ℃ for 1 min, 47 ℃ for 1 min, 72 ℃ for 1 min, and then a final step of 72 ℃ for 10 min according to Trout *et al.* (1997). The PCR amplification products were also separated in 15 g L^{-1} agarose gel through electrophoresis in 1× TAE and stained using a Quantifast SYBRGreen PCR kit (Qiagen). Furthermore, the DNA bands were observed over transilluminator UV and photographed under UV gel documentation.

Sequencing

The purified fragments were subjected to forward and reverse sequencing in accordance with the procedures of the company (First BASE, Malaysia). BioEdit software was used to assemble, clean, and modify the sequences obtained manually. The Clustal W method—included in BioEdit software—was used to align the sequences (Larkin *et al.* 2007). Moreover, the homologies were compared to databases using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/blast.cgi).

The alignment method was also applied to select the optimum substitution model and to generate the dendrogram while the maximum likelihood approach with a bootstrap of 1000 iterations was used to produce phylogenetic reconstructions. These computations were conducted using the MEGA X computational tool (Kumar *et al.* 2018).

Virulence Test on Cocoa Pod

Virulence of *P. palmivora* isolates was tested using a 2 month old healthy cocoa pod of Sulawesi 2 clone with the surface sterilized using 70% ethanol and perforated through a Ø 5 mm cork borer. The selection of the Sulawesi 2 clone was based on its high productivity, resistance to cocoa pod rot *P. palmivora*, and moderate resistance to vascular streak dieback pathogen as described by ICCRI (https://iccri.net/product/klon-sulawesi-02/).

Twenty *P. palmivora* isolates were used in this virulence test by using pieces of mycelium. G5 isolate was exempted from molecular and virulence assays due to its different sporangia morphology and its very slow growth. The assay included all *Phytophthora* isolates with three replicates (pod) while distilled water was used to inoculate control pod with four duplicates. Each pod was cleansed in running tap water and surface disinfected in 75% ethanol for 1 minute. The inoculation was performed in a laminar flow chamber using a 12 mm^3 plug of 5 day old *Phytophthora* isolates culture on a corn meal agar (CMA). The plug was placed in a 1 cm^2 deep cut made in the middle of the skin of each cocoa pod using a cork borer to position the mycelial disc (Wael Alsultan *et al.* 2022). The symptoms of virulence were observed every day for a period of 7 days after the inoculation (dpi) based on the criteria developed by Waterhouse (1975) in Rubiyo (2008).

RESULTS

A total of 21 isolates were obtained from three different cocoa plantations (Tabel 1) and the colony morphology of each is presented in Table 2. The results showed that all isolates have hyphal swellings containing several nuclei and non-septate. Chlamydospores were also formed between the hyphae known as the intercalary or the terminal chlamydospores formed at the end of the hyphae. The sporangia were found to be globose or ovoid in shape with short to medium pedicles and the papillate had simple sympodial branched sporangiophores (Figure 1). However, one isolate from Gowa (G5) was observed to have an ellipsoid sporangial shape without papillae,

Table 1 Origin of *Phytophthora palmivora* isolates from three districts of South Sulawesi

List of isolates and ecological conditions	Origin of isolates		
	Luwu	Pinrang	Gowa
Isolates	PA1, PA2, PA3, PA4, PA5, PA6, PA7	PI1, PI2, PI3, PI4, PI5, PI6	G1, G2, G3, G4, G5, G6, G7, G8
Location	Sampeang Village, Sub-district Bajo Barat	Tapporang Village, Sub-district Batulappa	Batangkaluku Village, Sub-district Sumba Opu
Elevation (m asl)	1500–2000	1007	25–100
Rainfall (mm/year)	1673	1860	2152
Cocoa clone	45 and Buntu Batu	Sulawesi 1 and Sulawesi 2	Sulawesi 1, Sulawesi 2, Panimbu, THR Jeni, MO7, MO5, M01, PCK MO6,
Shade	Coconut tree	Coconut tree and black pepper	Coconut tree
Sanitation	Abundant plant residues	Abundant plant residues	No plant residue
Fungicide treatment	Hexaconazole	Hexaconazole	Mancozeb and Paraquat Dichloride

Table 2 Morphological characteristics of 21 isolates *Phytophthora palmivora* from three different cocoa plantations on V8 juice agar media at 7 dpi

Isolate	Hyphal swellings	Sporangia				Chlamydospore	
		Form	Papilla	Pedicle	Branch type	Form	Annotation
PI1	Loops	Ovoid	SP	Short	SS	TC, Cli	Abundant
PI2	Torulose	Ovoid	SP	Short	SS	TC, Cli	Abundant
PI3	Coralloid	Ellipsoid	SP	Short	SS	TC, Cli	Abundant
PI4	Torulose	Ovoid	P	Short	SS	TC, Cli	Abundant
PI5	Loops	Limoniform	P	Short	SS	TC, Cli	Abundant
PI6	Torulose	Ovoid	P	Short	SS	TC, Cli	Abundant
PI7	Coralloid	Ovoid	SP	Short	SS	TC, Cli	Abundant
PA1	Coralloid	Ovoid	P	Short	SS	TC	Few
PA2	Coralloid	Ovoid	P	Short	SS	TC	Few
PA3	Coralloid	Limoniform	P	Medium	SS	TC	Few
PA4	Coralloid	Limoniform	P	Medium	SS	TC	Abundant
PA5	Coralloid	Ovoid	SP	Short	SS	TC	Abundant
PA6	Coralloid	Ovoid	SP	Short	SS	TC	Abundant
G1	Coralloid	Ovoid	P	Short	SS	TC	Few
G2	Coralloid	Ovoid	P	Short	SS	TC	Few
G3	Irregular	Ovoid	P	Short	SS	TC, Cli	Present
G4	Loops	Ovoid	SP	Short	SS	TC	Few
G5	Coralloid	Globose	NP	Medium	SS	TC	Few
G6	Loops	Limoniform	P	Short	SS	TC	Few
G7	Coralloid	Limoniform	SP	Short	SS	TC	Few
G8	Coralloid	Ovoid	P	Short	SS	TC	Few

Note: SP, Semi papillate; P, Papillate; NP, Non-papillate; SS, Simple sympodium; Cli, Chlamydospore intercalary; and TC, Terminal chlamydospore.

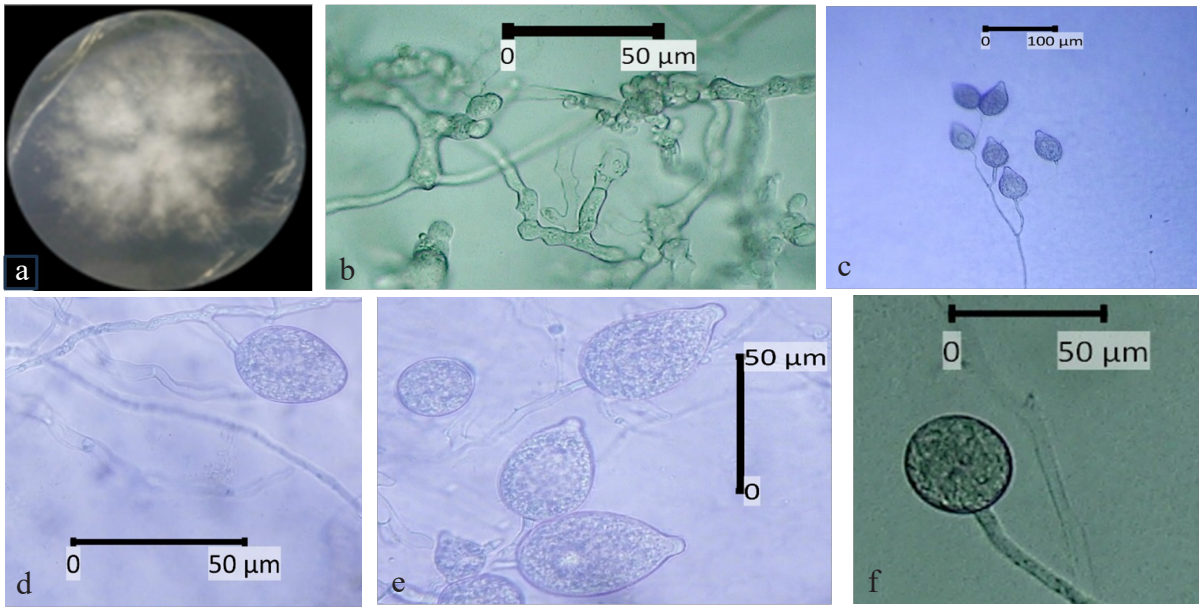


Figure 1 Colony morphology variation *Phytophthora palmivora* on V8 juice agar media. a, Stellate type colony; b, Coralloid hyphae; c, Simple sympodium of sporangiophore; d, Ellipsoid non-papillate sporangium; e, Ovoid papillate sporangium; and f, Terminal chlamydospore.

long pedicles, and slow growth on V8 media so that was excluded from molecular and virulence tests.

The amplification of 20 *Phytophthora* isolates from infected cocoa pods using *P. palmivora* specific primers Pal1s and Pal2a showed positive results with band 650 bp (Figure 2). Furthermore, molecular identification of 20 isolates conducted using ITS4 and ITS5 primers produced a 900 bp amplicon. The results of blast nucleotide analysis conducted using the ITS database also showed a 100% match to *P. palmivora* LT670914 (Figure 3). Moreover, the alignment of the samples from three areas in South Sulawesi showed that their cluster was 100% similar to *P. palmivora* LT670914. The comparison of *P. quercetorum, P. litchi, P. megakarya, P. cactorum, P. capsici, P. citricola*, and *P. citrophthora* with *Phytophthora*

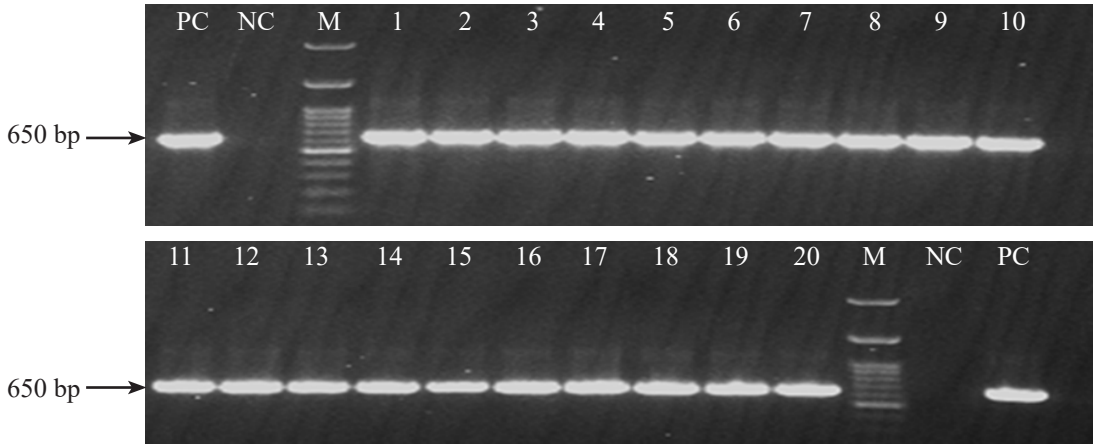


Figure 2 Amplification of *Phytophthora palmivora* using primer Pal1s and Pal2a generated a 650 bp band (left). Separating with 1.2 % agarose gel was stained with Quantitec SYBRGreen (Qiagen). PC, Positive control; NC, Negative control; M, Marker; 1-6, Pinrang isolates (PI1-PI6); 7-13, Palopo isolates (PA7-PA13); and 14-20, Gowa isolates (G1-G4, and G6-G8).

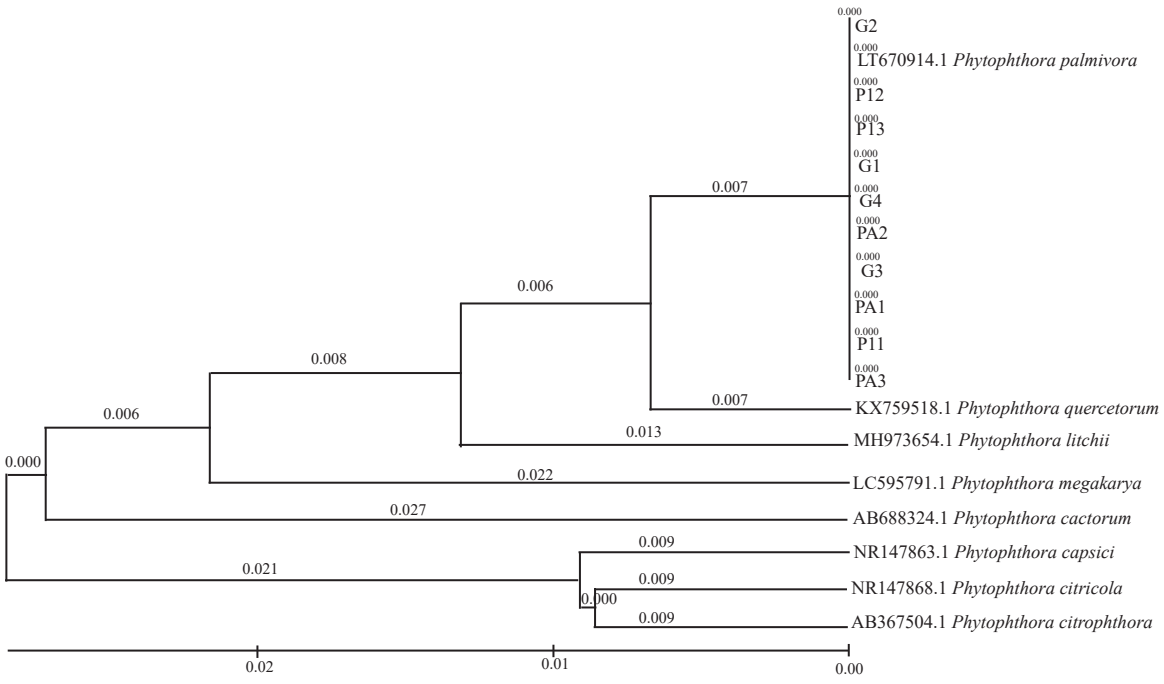


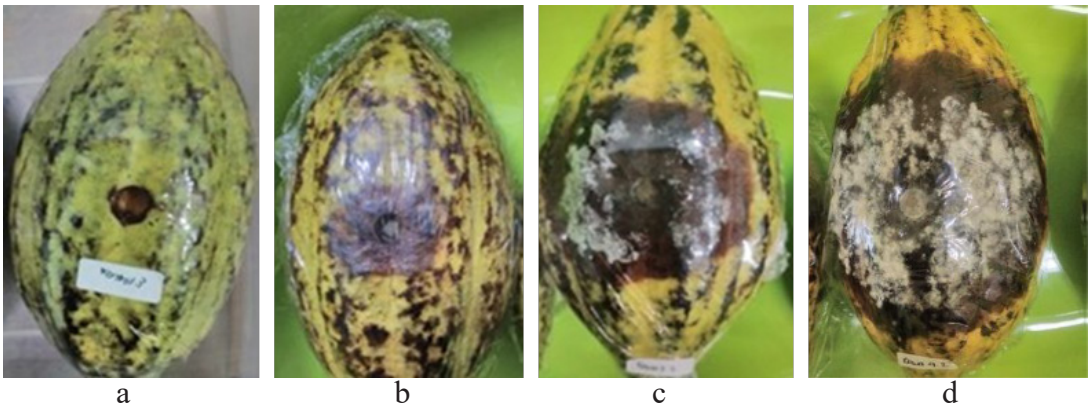
Figure 3 Relationships of *Phytophthora* species on cocoa based on neighbor-joining analysis of rDNA-ITS region sequences. Cluster analysis of 10 cocoa pod rot isolates compared with various *Phytophthora* species and strains.

isolates tested indicated significant nucleotide sequence variation ranging from 7 to 27 base pairs difference for every 100 base pairs.

The virulence of *P. palmivora* isolates was characterized based on their ability to produce necrotic symptoms on inoculated cocoa pods. The resulted show that 10 isolates of *P. palmivora* as highly virulent, 9 as virulent, and only 1 as less virulent (Figure 4; Table 3).

DISCUSSION

The appearance of *P. palmivora* colony on the medium was generally found to have a star-like or stellate pattern even though *P. capsici* colony commonly has a rosaceous form (Appiah *et al.* 2003; Andrews *et al.* 2015). Morphological variation of *Phytophthora* species had been previously associated with the usage of different mediums for isolation (Scott *et al.* 2009).



a b c d

Figure 4 Pod rot symptom on Sulawesi 2 clone after seven days of inoculation and incubated at room temperature. a, Control inoculated with sterile water; b, Less virulent isolates; c, Virulent isolates; and d, Highly virulent isolates.

Table 3 Virulence Grouping of *Phytophthora palmivora* isolates cocoa pod of clone Sulawesi 2 after seven days of inoculation

Isolates	Latent period (days)	Necrotic areas (%)	Category
PA1	2.7	26.70	Virulent
PA2	4.3	21.70	Less virulent
PA3	2.0	26.70	Virulent
PA4	2.6	56.14	Highly virulent
PA5	2.0	56.33	Highly virulent
PA6	2.3	33.37	Virulent
PI1	2.0	66.70	Highly virulent
PI2	2.0	66.70	Highly virulent
PI3	2.0	65.00	Highly virulent
PI4	2.0	26.18	Virulent
PI5	3.0	45.11	Virulent
PI6	3.1	42.81	Virulent
PI7	2.5	56.14	Highly virulent
G1	3.0	39.33	Virulent
G2	2.0	72.30	Highly virulent
G3	3.0	38.30	Virulent
G4	2.3	65.00	Highly virulent
G6	2.4	73.20	Highly virulent
G7	2.6	39.69	Virulent
G8	2.0	71.21	Highly virulent

Microscopic observation showed that all isolates had similar characteristics with *P. palmivora* on the V8 media. This was indicated by their round colony with a flat segmented edge, white colony color, and stellate form. The results showed that only 2 isolates from Pinrang had different appearances by growing slowly with the tiny colony on the V8 media. Appiah *et al.* (1999) had previously reported the possibility of differentiating *Phytophthora* species based on the type of their colony. This was due to the fact that *P. palmivora* was identified with a stellate form, *P. capsici* with rosaceous, and *P. megakarya* with cottony. Moreover, the shapes of *P. palmivora* sporangia ranged from ovoid-ellipsoid to obpyriform and were papillate and caducous, indicating they have short pedicels (Saul Maora 2008). The results also showed that there was no correlation between the origin of isolates and the morphological characteristics observed. This was indicated by the fact that differences were found in both isolates from the same area and those from other locales. According to Drenth and Sendall (2001), several morphological characteristics can be used to identify *Phytophthora* species and these include the colony morphology, chlamydospore, sporangia, and hyphal swelling, as well as the sexual structure. It was further noted that *P. palmivora* has different forms of hyphal swelling including torulose, coralloid, and loop. These hyphal swellings usually form a branch point followed by globular swelling with a thick wall called chlamydospores which can be developed between, in the end, or in addition to the hyphae. The chlamydospore morphology of several species is not usually significantly different and this is the reason it is rarely used for identification purposes. Previous studies have described and differentiated morphological features of 80 cocoa isolates of *Phytophthora* from Africa, America, Pacific regions, and Southeast Asia originally designated as *P. palmivora*. The relationships between these isolates were also reported based on morphological forms of *P. palmivora*. Moreover, the results of this study showed that the sporangial stalk or pedicel length of isolates varied significantly, thereby, leading to their separation into four groups (Kaosiri *et al.* 1978; Drenth and Sendall 2001). There was a need to ensure the accuracy of the preliminary identification of *Phytophthora* species by using morphological traits. However, it was important to note that molecular identification methods were quicker, more specific, and more sensitive than morphological methods (Capote *et al.* 2012).

Phytophthora sp. isolates were characterized using the DNA sequence of ITS and this led to the generation of a uniform 900 bp. Umayah and Purwantara (2006) had previously identified isolates from cocoa using ITS region from ribosomal DNA (rDNA) as well as PCR with primer pairs ITS 5 and ITS 4 or ITS 1 and ITS 4 followed by the digestion of amplicon using restriction enzymes Alu 1 and Msp 1. The amplification of *P. palmivora* in a single band or amplicon was estimated at 900 bp. The results further showed that *P. palmivora* isolates formed the same group *P. palmivora strains* LT670914. Moreover, the blast nucleotide analysis conducted with the ITS database also indicated a 100% match to *P. palmivora* LT670914. This means all the 10 representative isolates tested belonged to the *P. palmivora* species.

Test conducted for virulence also showed that the 20 fungal isolates considered typical *P. palmivora* spores caused necrotic symptoms on the cocoa pod of the Sulawesi 2 clone. The first symptom was a small dark spot on the inoculated pod which was observed between 2–4 days after inoculation while the most apparent symptoms were rotten or blackening surrounding cocoa fruit as indicated in Figure 4. The small spots were observed to have started like dirty spots and later became thicker on every part as the fruit developed (Bowers *et al.* 2001; Guest 2007). The results showed that only PA2 isolates induced the first symptom on day 4 of inoculation, thereby, causing a necrotic area under 25% and this was classified as less virulent isolates. Meanwhile, the other 19 isolates, from both Gowa and Pinrang districts produced the first symptoms on day 2 which was very fast and reached the

necrotic area between 26.15%–73.2%. They were subsequently classified into virulent and highly virulent isolates. This means the dominance of these *P. palmivora* isolates can provide a significant challenge to cocoa farms in this region. Meanwhile, Rubiyo *et al* (2020) grouped the Sulawesi 2 into a moderately resistant clone. The direct aggressiveness and pathogenicity of the high virulence clone of *P. palmivora* from Sulawesi in influencing the severity of infections and diseases mean there was a need to prevent their spread. However, the acquisition of knowledge regarding the distribution of the virulent *P. palmivora* isolates in South Sulawesi requires conducting further analysis using a larger number of isolates from other different cocoa growing centers as well as the application of several cocoa clones with different resistant levels for inoculation test.

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