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Research Article
Molecular Identification of *Phytophthora* sp. From Indonesian Cocoa Using Phylogenetic Analysis

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Abstract

Background and Objective: Diseases caused by *Phytophthora* species cause widespread damage worldwide and are troubling cocoa farmers in Indonesia. The specific species causing disease in an area can be ascertained by characterizing its rDNA fragments. This study aimed to identify *Phytophthora* sp., samples from cocoa plantations in Southeast Sulawesi, Indonesia, based on phylogenetic analysis of rDNA fragments. **Materials and Methods:** Identification of rDNA fragments of *Phytophthora* sp., done by amplifying rDNA fragments using PCR (Polymerase Chain Reactions) techniques with the specific primer of *Phytophthora* (Phy-F and Phy-R) which can amplify regions of ITS1, 5.8S rRNA and ITS2. The rDNA fragments are then sequenced and analyzed using: The BLAST (Basic Local Alignment Search Tools) provided by NCBI (National Center for Biotechnology Information) via (www.ncbi.nlm.nih.gov/blast) to analyze the local alignment of DNA sequences with Genbank DNA data and Mega 7.0.26 software is used to construct the phylogenetic tree. **Results:** The DNA sequencing results showed the rDNA measuring 786 bp consisted of complete sequences of ITS 1 (210 bp), 5.8S rRNA (162 bp) and ITS 2 (414 bp). Based on phylogenetic tree analysis using the maximum likelihood method with 1000 bootstrap replications showed that the rDNA of *Phytophthora* sp., isolates and 29 comparator isolates formed 2 large groups. *Phytophthora* sp., formed a subgroup with *Phytophthora palmivora* with a bootstrap value of 99%. **Conclusion:** The type of *Phytophthora* spreading in cocoa plantations in Southeast Sulawesi, Indonesia, is 1 group with *Phytophthora palmivora*.

Key words: Cocoa, PCR, *Phytophthora palmivora*, rDNA fragments

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the main diseases that attack cocoa plants in several countries is the *Phytophthora* strain. *Phytophthora palmivora* is found in many countries and causes black pod complexes in cacao plants[1], while *P. megakarya* is found predominantly in West and Central Africa which appears to be more virulent than *P. palmivora*[2] and is becoming the dominant species in West Africa, moving from Nigeria and Cameroon into countries where it has not previously been reported. In Brazil, there are 4 *Phytophthora* species involved in cocoa crop damage, namely: *P. palmivora*, *P. capsici*, *P. citrophthora* (Smith and Smith) Leonian and *Phytophthora heveae* Thompson[3-6]. *Phytophthora capsici* is the dominant and most virulent species attacking cocoa in Brazil and other countries in Central and South America, as well as in the West Indies, Indonesia and India[6]. *Phytophthora citrophthora* is the least common in Brazil but the deadliest[3,5]. *Phytophthora heveae* Thompson has also been found to cause black pods in some countries[6].

In 2015, cocoa production in Southeast Sulawesi, Indonesia, was only 91,808 t, down from 125.079 t in the previous year. Production slightly increased in 2016, 101,835 t but still lower than in 2014 with a total planted area of 29,880 ha. This decline was due to an outbreak of the *Phytophthora* fungus, which had affected many parts of the world. Overall, it caused decreases in cocoa production ranging from 10-30% worldwide, with much higher losses in endemic areas, particularly in wet areas during the rainy season[1,7].

The infection process in cocoa fruits begins with the appearance of small spots on the fruits at each stage of fruits development, then the spots develop rapidly covering the internal tissues and the entire surface of the fruit. Observations showed that pathogens attacking the fruits cause the fruits to change colour from brown to black. Symptoms of fruit rot can be found on the upper, middle and lower ends of young and old fruit[1].

Phytophthora is a type of oomycete that attacks cocoa fruit in every region of Indonesia. It has been proposed that the main species in Southeast Sulawesi is *Phytophthora palmivora*[8]. However, this information is based on morphological identification only and it needs to be confirmed using molecular methods. Morphological methods can be influenced by environmental conditions, leading to incorrect identifications. In addition, the characters used to identify fungi, which include the type of colony, hyphae shape, production and diameter of chlamydospores and sporangiophore branching[8-10] are often subjective and hinge on the knowledge and experience of a researcher, because they often overlap between species and show substantial variation between isolates of the same species[11].

Therefore, molecular identification can help elucidate species, indeed, the methods utilized are often faster and more accurate than morphological techniques. The advances in molecular techniques, particularly PCR and DNA sequencing, have fuelled bioinformatics studies of DNA data of organisms. DNA nucleotide sequence analysis has contributed to the understanding of the phylogenetic and molecular diversity of organisms including in the *Phytophthora* genus[12]. DNA Internal Transcribed Spacer (ITS) analysis of ribosomal RNA is a common method used to determine a variation within and between *Phytophthora* species[13-16]. Sequencing of specific target regions (single and multiple) has been widely used to study the diversity of *Phytophthora*[17,18], *Pythium*[19] and other microbes such as fungi[20], phytoplasmas[21] and plants[22]. Molecular analysis of DNA sequences by Martin and Tooley[23], Kroon *et al.*[14], Cooke *et al.*[15] and Förster *et al.*[16], has increased the understanding of the phylogenetic relationships between *Phytophthora* species. Their work has been based mainly on the nucleotide sequence data of a single DNA region, the rDNA Internal Transcribed Spacer (ITS). Earlier work on the analysis of sequences to investigate genetic diversity, phylogenetic and genetic variation of *Phytophthora* and fungi were also based on this rDNA and ITS region[24].

This study can identify *Phytophthora* sp., samples from cocoa plantations in Southeast Sulawesi, Indonesia, based on phylogenetic analysis of rDNA fragments. Identification of rDNA fragments of *Phytophthora* sp., done by amplifying rDNA fragments using PCR (Polymerase Chain Reactions) techniques with the specific primer of *Phytophthora* (Phy-F and Phy-R) which can amplify regions of ITS1, 5.8S rRNA and ITS2.

MATERIALS AND METHODS

Place and time: The research was carried out at the Department of Biology, Microbiology Laboratory, Halu Oleo University, Indonesia from March-September, 2020.

Sample collection: Molecular identification starts from collecting samples from several cocoa-producing districts in Southeast Sulawesi, namely: Konawe, South Konawe, Kolaka, North Kolaka, East Kolaka and Muna. *Phytophthora* sp., samples were obtained by taking rotten fruit randomly and then isolated and rejuvenated using V8 media[25,26]. The

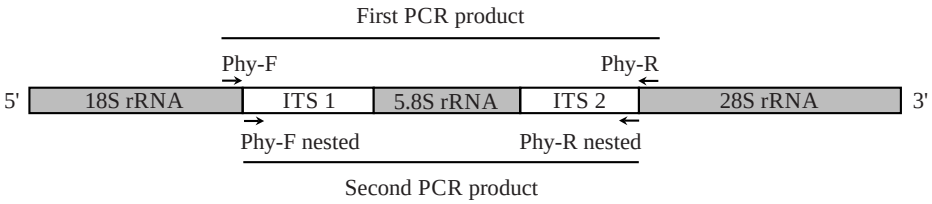

Fig. 1: Structure of rDNA and segments amplified in the PCR process

mycelium samples were inoculated into 100 mL Erlenmeyer flasks containing liquid V8 media. The isolates were incubated at 27°C for 5 days. Furthermore, DNA was extracted using the modified cetyl trimethylammonium bromide method[27].

Genomic DNA extraction: The *Phytophthora* sp., genomic DNA extraction used the modified Cetyl Trimethyl Ammonium Bromide (CTAB) method[28]. A total of 0.2 g of *Phytophthora* sp., mycelium, crushed using mortar and pestle and then put into Eppendorf 1.5 mL. Samples were added with 600 µL of lysis buffer and the solution was incubated for 30 min at 65°C while turning every 5 min. The sample was incubated into ice for 5 min then centrifuged at 10,000 rpm, at 4°C for 10 min. The supernatant is taken and added with 1× volume of Phenol Chloroform (PC). Subsequently, the sample was centrifuged at 10,000 rpm, temperature 4°C for 10 min. The supernatant was taken and added with 0.1 volume of sodium acetate 3 M pH 5.2 then added with 2× volume of 100% ethanol then incubated at -20°C for 2 hrs and centrifuged at 10,000 rpm, at 4°C for 20 min. Furthermore, the DNA pellet was washed with 0.5 mL of 70% ethanol, then dried and dissolved in 20 µL H_2O.

Specific primer design: The rDNA sequences of several species of *Phytophthora* were collected from Genbank (http://www.ncbi.nlm.gov) and were aligned using the Bioedit program. Parts of the sequences with high homology at the 5′ and 3′ ends were used as forward and reverse primers, respectively. Two specific primer pairs were used in this study shown in Fig. 1. The 1st primer pair was designed from the 3′ end of the gene encoding 18S rRNA to the 5′ end of the gene encoding 28S rRNA called Phy-F (5′-TCCGT AGGTGAACCTGCGGAA-3′) and Phy-R (5′-TCCTCCGCTTATTG ATATGCTTA-3′). The 2nd primer pair (a nested primer) was designed from the 5′ end of ITS 1 to the 3′ end of ITS 2 so that it could amplify ITS 1, 5.8S rRNA, ITS 2, Phy-F nested (5′-CC ACACCTAAAACTTTCCAC-3′) and Phy-R nested (5′-TTGAGATGC ATGCCGAAGCAT-3′).

DNA amplification using specific primers: *Phytophthora* sp., rDNA fragments were amplified via polymerase chain reaction (PCR). PCR was carried out twice, each time in a total volume of 10 µL. The 1st PCR consisted of 100 ng genomic DNA, 0.5 µM Phy-F, 0.5 µM Phy-R, 1× My Taq™ Mix (Bioline Meridian Bioscience) and dH_2O to reach 10 µL. The 2nd PCR consisted of 1µL of the 1st PCR product, 0.5 µM Phy-F nested, 0.5 µM Phy-R nested, 1× My Taq™ Mix (Bioline Meridian Bioscience) and dH_2O to reach 10 µL. The cycle consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation of 94°C for 1 min, annealing at 59°C for 30 sec, extension at 72°C for 90 sec and a final extension at 72°C for 5 min.

DNA sequencing and sequence analysis: Purification and sequencing of the 18S rDNA were done in 1st Base Singapore. Similarities of the rDNA sequences to sequences in the GenBank database were determined by using BLAST (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic analysis was performed using the maximum likelihood method, then MEGA version 7.0.26 was used to analyze kinship relationships with bootstrap tests of 1000 replicates[29-32].

RESULTS

Morphological characters of *Phytophthora* sp.: This study had characterized the colony morphology and cell morphology of *Phytophthora* sp., on cocoa plants in Southeast Sulawesi, Indonesia. There are 2 types of morphological characters of *Phytophthora* sp., isolate observed on V4 (Vegetable Juice Agar) media, namely colony morphology and cell morphology. Based on the morphological character of the colony, *Phytophthora* sp., isolates showed a round colony shape, the colour of the colony surface was white and the undersurface was brown, the colony texture was like cotton, the colony edges were uneven, there was zoning, there were radial lines and the colony diameter was 90.73 mm in Table 1 and Fig. 2. Based on cell morphology, *Phytophthora* sp., isolates has several characters, namely, the form of asexual spores are

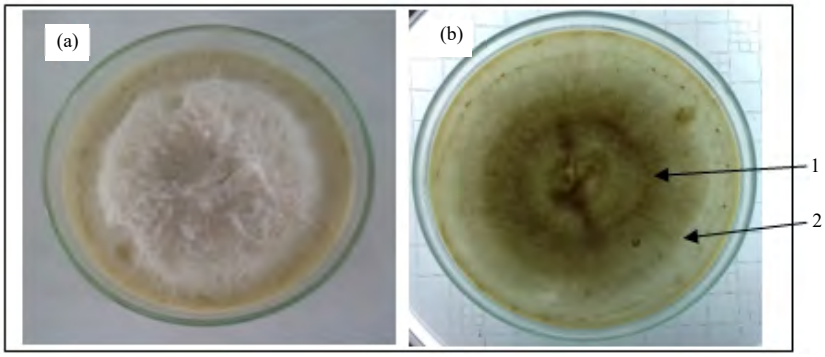

Fig. 2(a-b): Colony morphological characteristics of *Phytophthora* sp., (a) White colour on the surface and (b) Brown colour below the surface (reverse)
1: Radial line and 2: Zoning

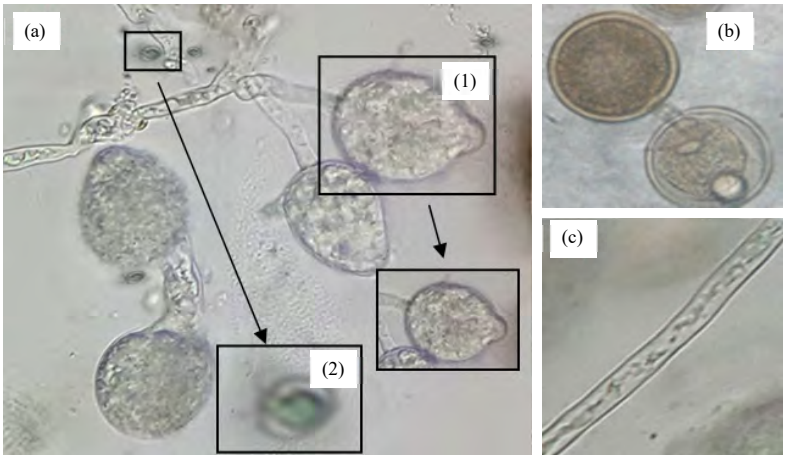

Fig. 3(a-c): Cell morphological characterization of *Phytophthora* sp., using 400× magnification, (a) *Phytophthora* cell morphology, (b) Chlamydospores and (c) Asepta hyphae
1: Sporangium and 2: Zoospores

Table 1: Colony morphological characteristics of *Phytophthora* sp.

Characters observed	Results of observations
Colony form	Round
Colony color on the surface	White
Colony color below the surface	Brown
Colony texture	Like cotton
Colony edges	Uneven
Zoning	Exist
Radial lines	Exist
Diameter (mm)	90.73

Table 2: Cell morphological characteristics of *Phytophthora* sp. on vegetable juice agar media (V4)

Characters observed	Results of observations
Asexual spora	Sporangium and chlamydospores
Hyphae type	Asepta
Zoospores	Exist
Zoospores forms	Round and has a flagellum
Zoospores colors	Black green
Sporangiophore	Exist

sporangium and Chlamydospores, the hyphae type were asepta, there are zoospores, the shape of the zoospores is round with flagella, the colour of the zoospores is greenish-black and has sporangiophore in Table 2. In the sporangium, there are zoospores with 2 flagella in Fig. 3a, chlamydospores that is round and thick-walled in Fig. 3b. The hyphae type were asepta or lacked a cell wall in Fig. 3c.

Amplification of rDNA fragment of *Phytophthora* sp.: The DNA template used in the amplification is of excellent quality which is characterized by the absence of a smear pattern along the migration path on the agarose gel in Fig. 4. The rDNA fragments amplification results show thick and firm bands and forming a single band measuring around 780 bp in Fig. 5.

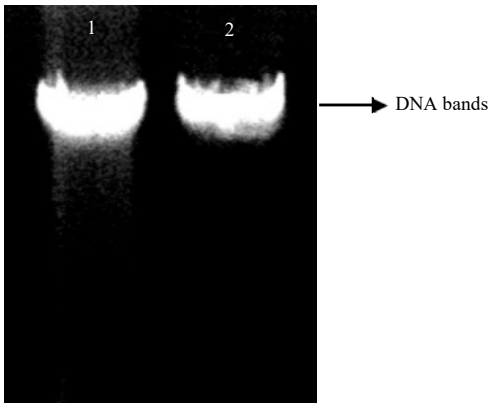

Fig. 4: Genomic DNA extraction results of *Phytophthora* sp.
1 and 2: DNA bands

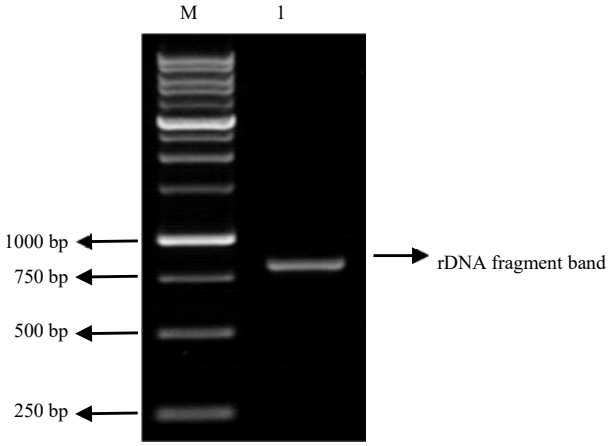

Fig. 5: Results of rDNA fragment amplification of *Phytophthora* sp., using Phy-F and Phy-R primers on agarose gel 1% (1) and 1 kb ladder marker (M)

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1   CCACACCTAAAAACTTTCCACGTGAACCGTATCAAAACTTAGTTGGGGGT 50
51  CTCTTTCGGCGGCGGCTGCTGGCTTCATTGCTGGCGGCTGCTGTTGGGAG 100
101 AGCTCTATCATGGCGAGCGTTTGGGCTTCGGTCTGAACTAGTAGCTTTTT 150
151 TAAACCCATTCTTTATAACTGATTATACTGTAGGGACGAAAGTCTCTGCT 200
201 TTTAACTAGATAGCAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGA 250
251 TGAAGAACGCTGCGAACTGCGATACGTAATGCGAATTGCAGGATTCAGTG 300
301 AGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGGGAG 350
351 TATGCCTGTATCAGTGTCCGTACATCAAACTTGGTTTTCTTCCTTCCGTG 400
401 TAGTCGGTGGTGGATGTGCCAGATGTGAAGTGTCTTGCGGCTGGTCTTCG 450
451 GATCGGCTGTGAGTCCTTTGAAATGTACTGAACTGTACTTCTCTTTGCTC 500
501 CAAAAGCGTGGCGTTGCTGATTGTGGAGGCTGCTTGCGTAGCCAGTCTGG 550
551 CGACCAGTTTGTCTGCTGTGGCGTTAATGGAGGAGTGTTCGATTCGCGGT 600
601 ATGGTTGGCTTCGGCTGAACAGACGCTTATTGAATATTTCTTCAGCTGTG 650
651 GTGGTATGATTGGTGAACCGTAGCTATGTGAGCTTGGCTTTTGAATTGGC 700
701 TTTGCTGTTGCGAAGTAGAGTGGCGGCTTCGGCTGTCGAGGGTCGATCCA 750
751 TTTGGGAACTTGTGTATGCTTCGGCATGCATCTCAA              786
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Fig. 6: Sequence of rDNA fragments of *Phytophthora* sp.
(Genbank Acc. MT644188) Light gray background: ITS 1, underline: 5.8S rRNA and dark gray background: ITS 2

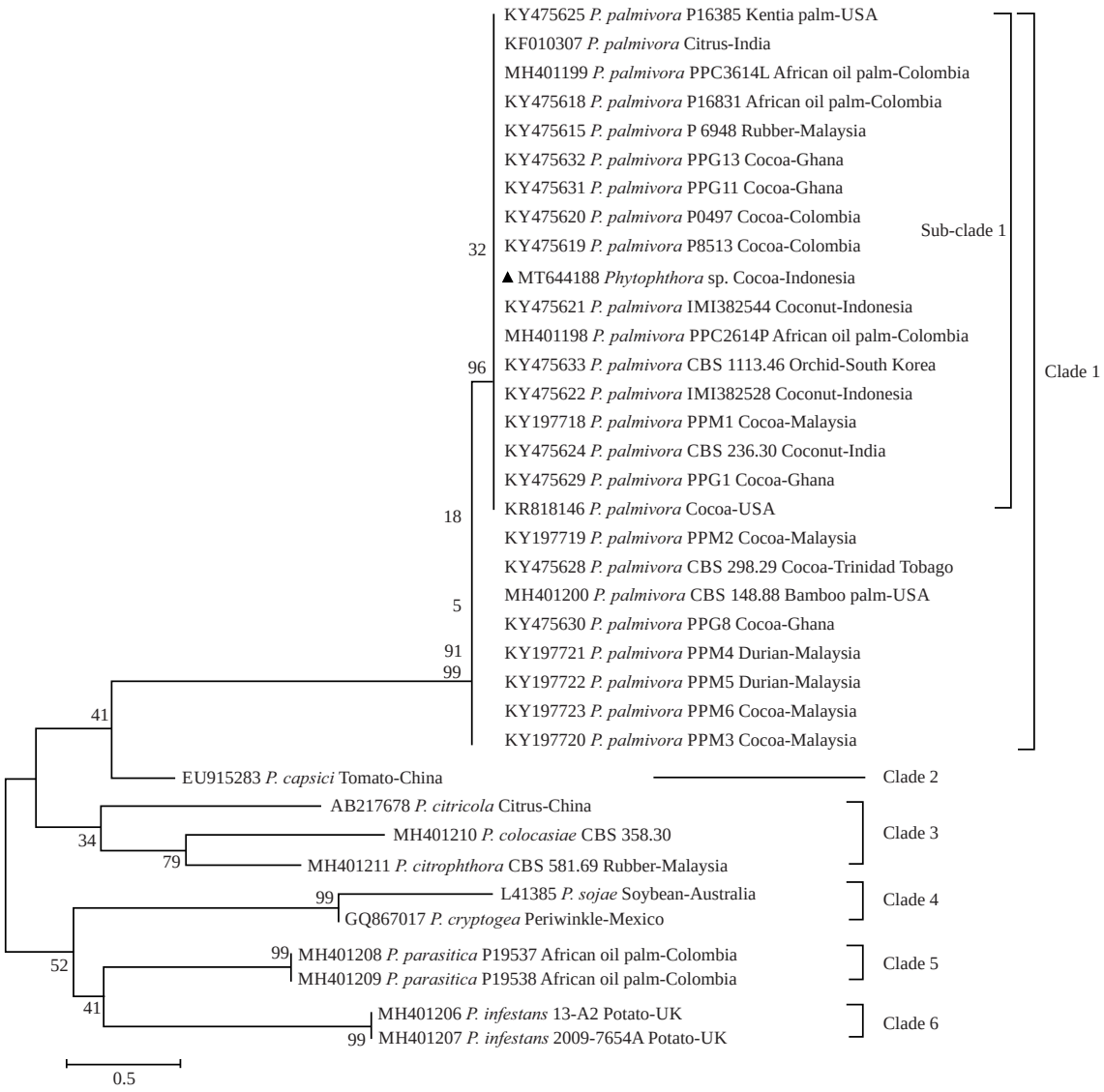

Fig. 7: Phylogenetic relationship of *Phytophthora* sp.

Phylogenetic relationship was inferred by using the maximum likelihood method32 from clustal W sequence alignment of ITS 1-5.8S rRNA-ITS 2 sequences of *Phytophthora* sp., isolates from Indonesia (▲) and 35 comparator isolates are available in GenBank (25 isolates of *Phytophthora palmivora* from various hosts and 10 isolates of other *Phytophthora*). The tree was constructed with Mega 7.0.26 software 33 with 1000 bootstrap replications, bootstrap replicates values are indicated at the nodes and scale bar represents genetic distance

Sequencing of rDNA fragments of *Phytophthora* sp.: The DNA sequencing results showed that the rDNA fragments that were successfully amplified by the Phy-F nested and Phy-R nested primers measuring 786 bp in Fig. 6 consisted of complete sequences of ITS 1 (210 bp), 5.8S rRNA (162 bp) and ITS 2 (414 bp).

Phylogenetic analysis: *Phytophthora* sp., isolates and 35 comparator isolates formed 6 clades in Fig. 7. Based on the phylogenetic relationship in Fig. 7, it shows that all *Phytophthora palmivora* isolates (26 isolates) were grouped into 1 clade (clade 1) with a bootstrap value of 99%. *Phytophthora* sp., isolates were in subclade 1 along with 17 other isolates, namely *Phytophthora palmivora* from cocoa, coconut, kentia palm, citrus, African palm oil, rubber and orchids with a bootstrap value of 96%. Based on the sequence of rDNA fragments, the *P. palmivora* group (clade 1) can be separated from *P. Capsici*-EU915283 (clade 2), *P. citricola*-AB217678, *P. colocasiae*-MH401210, *P. citrophthora*-MH401211 (clade 3), *P. sojae*-L41385, *P. cryptogea*-GQ867017 (clade 4), *P. parasitic*-MH401208, MH401209 (clade 5) and *P. infestans*-MH401206, MH401207 (clade 6).

DISCUSSION

Phytophthora is a disease that causes damage to cocoa plants which is currently troubling cocoa farmers in Southeast Sulawesi, Indonesia. The *Phytophthora* sp., was confirmed by Muzuni *et al*.[8] but still limited in terms of morphological characters, not yet supported by molecular characters. Based on the colony morphology (Table 1 and Fig. 2) and cell morphology (Table 2 and Fig. 3) showed that the isolates of mold isolated from cocoa pods were a group of the genus *Phytophthora* which were characterized by rounded colony shape, white colour, uneven colony edges, presence of zoning and there is a radial line on the mold colony. This characterization is following the results of previous characterizations[33,34], that the *Phytophthora* genus macroscopically has rounded colonies, the texture is like cotton, the edges of the colonies are uneven, there are radial lines and there is zoning. In addition, according to Barboza *et al*.[25] that the characteristics of *P. palmivora* colonies are generally round in shape with uneven edges and white.

Microscopic observations of mold isolates in Table 2 show that *Phytophthora* sp., has a morphological character of cells in the form of asexual spores, namely sporangium oval with a protrusion at the end. Based on these characters, the mold isolate was indicated to be of the genus *Phytophthora*. This is supported by the research results of Bush *et al*.[35], who stated that the genus *Phytophthora* was characterized by a septa hyphae, asexual spores in the form of papillary sporangium with a size (30-60×20-50 m). In addition, Masanto *et al*.[10] showed that the characteristics of the *Phytophthora* genus were aseptic hyphae, spherical chlamydospores and flagellated zoospores. Chlamydospores are black as a dormancy phase due to unfavourable conditions[6,9].

This study, have confirmed *Phytophthora* sp., on cocoa plants in Southeast Sulawesi, Indonesia molecularly based on phylogenetic analysis of rDNA fragments. Phylogenetic trees are illustrations of evolution that occur in certain groups of organisms that belong to the same ancestors, which are arranged based on similarities in several ways, such as genes and proteins[36]. This aimed to determine the kinship relationship between the organisms. The DNA sequence used in this analysis was 786 bp (Fig. 6), in contrast to that performed by Maizatul-Suriza *et al*.[37] was 900 bp in size. However, this experiment was able to perform a phylogenetic analysis because the sequence already contained variable regions, namely ITS 1 (210 bp) and ITS 2 (414 bp). This study constructed a phylogenetic tree of *Phytophthora* sp., isolates using the maximum likelihood method, then MEGA version 7.0.26 was used to analyze kinship relationships. The tree was based on sequences of rDNA fragments consisting of regions ITS 1, 5.8S rRNA and ITS 2 using the NJ method with 1000 bootstrap replications. The numbers on branches are bootstrap values, which indicate the level of accuracy[27].

Isolates of *P. palmivora* originating from cocoa in Indonesia (MT644188) showed a high similarity (80.64-100% identity, based on BLAST report) with other isolates and 99.24-100% identity with other *Phytophthora palmivora*. Further assessments using phylogenetic analysis showed similar results. Based on the phylogenetic relationship in Fig. 7, it shows that *Phytophthora* sp., isolates and 35 comparator isolates formed 6clades and all *Phytophthora palmivora* isolates (25 isolates) were grouped into 1 clade (clade 1) with a bootstrap value of 99%. Maizatul-Suriza *et al*.[37] has also grouped all *Phytophthora palmivora* isolates (26 isolates) originating from different hosts and demographics into 1 clade with strong bootstrap values using ITS sequences. These results indicate that *Phytophthora palmivora* has a broad host, not specific to a particular host. Other studies have shown that *Phytophthora palmivora* isolates from oil palm can infect rubber and durian leaves[37]. Scibetta *et al*.[11], explained that *Phytophthora palmivora* provides cross pathogenicity between different hosts: Coconut, cocoa, durian, rubber, bamboo palm, betel palm and orchid against durian and rubber leaves. *Phytophthora* sp., isolates are in sub-clade 1 along with *Phytophthora palmivora* from cocoa, coconut, kentia palm, citrus, African palm oil, rubber and orchid with a bootstrap value of 96%. Based on the phylogenetic tree analysis showed that *Phytophthora* sp., that is, the type of *Phytophthora* spreading in cocoa plantations in Southeast Sulawesi, Indonesia, is one group with *Phytophthora palmivora*.

CONCLUSION

This study confirms that the species of *Phytophthora* sp., that is spreading in the cocoa plantations in Southeast Sulawesi, Indonesia, is *Phytophthora palmivora*. This is indicated by the phylogenetic relationship between the *Phytophthora* sp., sample and 25 *Phytophthora palmivora* isolates with a bootstrap value of 99%. In addition, it was also shown from the BLAST report that the rDNA fragment of *Phytophthora* sp., samples has a high similarity with *Phytophthora palmivora* with 99.24-100% identity.

SIGNIFICANCE STATEMENT

This study discovered the *Phytophthora* sp., spreading in Southeast Sulawesi, namely *Phytophthora palmivora*, which was determined molecularly using rDNA as an identification target that can be beneficial for the development of plant-pathogen identification tools. This study will help researchers to uncover the exact existence of plant diseases that are not influenced by environmental conditions which are still ignored by many researchers. Thus to ensure the presence of a pathogen in plants, apart from being identified morphologically, it needs to be followed by molecular identification.

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