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Research Article
Morphometric Variation of *Phytophthora palmivora* Causing Black Pod Rot Disease on Cocoa (*Theobroma cacao* L.) in Indonesia

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Abstract

Background and Objective: Black pod rot disease caused by *Phytophthora palmivora* is one of factors contributing in decrease of cocoa production in Indonesia. This research was conducted to study the variation of *P. palmivora* causing black pod rot disease in Indonesia based on morphological characteristics and sexual reproduction. **Materials and Methods:** Pathogen was isolated from cocoa pods showing black rot symptoms in Indonesia for morphological and sexual characterization as well as molecular confirmation. Quantitative data of sporangial and sexual features were recorded and arranged in multivariate as well as calculated using phonetic method with hierarchical cluster. Those characters were clustered through average linkage and reconfirmed with principal component analysis (PCA) and dendrogram using NTsys 2.10e program. **Results:** Fifty-five isolates of pathogenic agents had been successfully collected from 38 regencies (23 provinces) in Indonesia. They had various sporangial shape, i.e., distorted, ellipsoid, globose, obpyriform and ovoid as well as and spherical chlamydospores. These features were recognized as the characteristics of *Phytophthora palmivora*. Molecular analysis confirmed that all isolates were positively detected with multiplex PCR using species-specific primers. Sexual reproduction was characterized with the formation of amphigynous antheridia as well as spherical oogonia and oospores in which A2 type occurred more frequently thanA1 type. **Conclusion:** Those isolates were classified into 8 clusters which were independent with geographical area and mating types.

Key words: *Phytophthora palmivora*, morphological characteristics, sexual features

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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

Indonesia is the 3rd cocoa producing country in the world after Ivory Coast and Ghana and represents Asian region in top ten rank of cultivating cocoa countries which are dominated by African and south American countries. Previously, Indonesia was listed as the second leading country in cocoa production which contributed about 19.50 and 15.43% to the world in 2010 and 2011, respectively[1]. However, it was recorded that the acreage and production of cocoa in Indonesia decreased for last five years (2012-2016)[2].

The occurrence of pests and diseases were reported as one of factors taking part in decrease of cocoa production in Indonesia, in addition to farmers' behavior for adopting cultivation technology and limitation of superior planting materials[3]. McMahon and Purwantara[4] documented three main pests and diseases affecting cocoa production in Indonesia, such as cocoa pod borer (*Conopomorpha cramerella*), vascular-streak dieback (VSD) disease (caused by *Oncobasidium theobromae* and then identified as *Ceratobasidium theobromae*) and black pod rot disease caused by *Phytophthora palmivora*.

The last disease impacted on the largest losses[4] since it could directly affect pod production by degrading the quality of harvested cocoa beans[5]. Actually, *P. palmivora* could infect and caused the various symptoms on all parts of cocoa plant such as pods, beans, leaves, chupons and flower cushion[4,6]. This disease had been reported causing average losses about 10-30% of cocoa production in the world[4], while the losses around 10-20% or equal to more than USD 788 million was recorded in Indonesia[5]. Recently, Purwantara *et al.*[7] reported that the incidence of black pod rot on cocoa in Indonesia could reach up to 70-80%.

An accurate identification of pathogen provides the basic knowledge to develop proper strategies for integrated plant disease management. The understanding of variability in *Phytophthora* was also important improvement of control measures[8-9].

It is required the characteristics of several distinct features such as sporangial shape and dimension, pedicel, papillate, chlamydospores as well as sexual structures to differentiate *P. palmivora* with other species of *Phytophthora*. Despite of many morphological features were stable, the range size of certain sexual characters were often too great to be of consistent use for identification[8]. This experiment, therefore, was conducted to study morphometric variation of *P. palmivora* isolates causing black pod rot disease on cocoa in Indonesia according to their morphological characteristics and sexual reproduction.

MATERIALS AND METHODS

The current experiments were conducted in Indonesia and Japan during the year 2017-2018. The isolation and culture of isolates were carried out in Department of Plant Pests and Diseases, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta (Indonesia), while morphological identification, sexual characterization and molecular detection were performed in River Basin Research Center, Gifu University, Gifu (Japan).

Samples collection: Samples of cocoa pod showing black rot symptoms were collected from cocoa growing areas (representing each of main islands such as Sumatera, Java, Kalimantan, Sulawesi, Nusa Tenggara and Papua) in Indonesia using method of purposive random sampling. Each symptomatic pod was packed using dry paper for further isolation and identification in the laboratory.

Isolation and culture of pathogen: Symptomatic samples were cleaned using tap water, air-dried, surface-sterilized using 70% alcohol and then aseptically continued with the isolation in the laminar air flow. Pathogen was isolated by culturing the small pieces ($\pm$5 mm^2) of samples (border of healthy and diseased region after peeled) on semi-selective (Corn Meal Agar (CMA) (17 g L^{-1}) plus STAR Agar L-grade 01 (Rikaken Co., Ltd., Nagoya, Japan) (5 g L^{-1}) added with 200 µL of antibiotics (Merck, Darmstadt, Germany) i.e., nystatin (0.05 g L^{-1}), ampificin (1.25 g L^{-1}), rifampicin (0.05 g L^{-1}) and miconazole (0.005 g L^{-1}) for volume 200 mL of media). Cultures were incubated for 3-5 days at ambient temperature, then sub-cultured into slant CMA of glass tube for storage.

Morphological characterization: The isolates were cultured on V8 juice agar medium (163 mL L^{-1}) (Campbell Soup Company, New Jersey, USA) and incubated for 5-7 days in growth chamber at 25°C. Their morphological characteristics (such as type of hyphae, shape of sporangia and chlamydospores) were observed and documented under optical microscope (Olympus CKX53) (Olympus Corporation, Tokyo, Japan). Sporangial quantitative data such as length (l), breadth (b), l/b ratio, length of pedicel and length of pappillate as well as diameter of chlamydospores were measured.

DNA extraction: DNA was extracted using Prepman Ultra Reagent under protocol of manufacturer (Applied Biosystems, Foster city, CA, USA). A small number of aerial mycelia from one week old-cultures on V8 juice agar medium was

aseptically collected using a sterile needle and then put into 1.5 mL tube containing 100 µL of Prepman Ultra Reagent (2 times dilution with SDW by adding 50 µL of Prepman Ultra Reagent into 50 µL of SDW). They were incubated at 100°C and room temperature for 10 and 3 min, consecutively. The step was continued with centrifugation at 15,000 rpm in fixed-angle rotor (rotor number AF2724A) using Kubota 3740 instrument (Kubota Corporation, Tokyo, Japan) for 3 min. As much of 80 µL of supernatant was transferred into a new tube and then diluted with 100 µL of TE Buffer. This DNA solution was then used for molecular identification.

Molecular identification: The multiplex PCR using universal primers, i.e., 18S-69F (CTGCGAATGGCTCATTAAATCAGT) and 18S-1118R (GGTGGTGCCCTTCCGTCAA) as well as species-specific primers for *P. palmivora*, namely GUPal6fw (CTTCAGCTGTGGTGGTATGATT) and GUPal8rv (CATGCCGAAGCATACACAAG) was conducted under conditions of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. Each 25 µL of PCR reaction containing SDW, 50 ng μL^{-1} of DNA template, 4 mg mL^{-1} of BSA, 2.5 µL of 10×PCR Buffer, 25 mM of $MgCl_2$, 10 mM of dNTPs, 25 µM of forward and reverse species-specific primers, 10 µM of forward and reverse universal primers and 5 U μL^{-1} of FastStart *Taq* DNA polymerase (Merck) was performed using T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). PCR product was visualized with electrophoresis in 3% agarose gel at 100 Volt for 45 min using Mupid®-exU (Mupid, Tokyo, Japan). Prior to documentation under High Performance UV Transilluminator UVP (UVP, Upland, CA, USA) and capture of digital camera (Canon Powershot G16) (Canon, Tokyo, Japan), the agarose gel was stained with GelRed solution (Gene Target Solutions Pty., Ltd., Sidney, Australia) by immersing for 45 min.

Characterization of sexual reproduction: The tested and standard (A1 or A2) isolates of *P. palmivora* were cultured on 60 mm Petri dish containing V8 juice agar media (163 mL L^{-1}) and incubated in growth chamber at 25°C for 5 days. The active growing mycelia of those isolates were plugged using 5 mm borer and then put oppositely on the margin of a new V8 agar medium (163 mL L^{-1}) on 60 mm Petri dish. Each tested isolates of *P. palmivora* were dual-cultured with different standard isolates of *P. palmivora* separately. The culture of same (A1 vs A1 and A2 vs A2) and different (A1 vs A2) standard isolates of *P. palmivora* on one plate were considered as control. All cultures were incubated in growth chamber at 20°C for 2 weeks. The formation of sexual features (antheridia, oogonia and oospores) were observed and their shape and dimension were noted.

Data analysis: Prior to analysis using phonetic method for clustering the isolates based on the variation in morphological features, sexual structures and mating type, those characters were set up in the matrix of operational taxonomic unit (OTU) to be quantified as multivariate data. The OTU was notified as name of isolates and characters are scored as 1 (present) or 0 (absent) as well as multivariate. Data were analyzed with hierarchical cluster using SPSS Program 16.0 version (SPSS Incorporation, Chicago, Illinois, USA)[10].

The presence or absence of morphological characters in clustering were figured out by determining similarity index using coefficient of simple matching. These characters were then clustered through average linkage and reconfirmed with principal component analysis (PCA), while coefficient of agglomerative were used to recognize the relationship among isolates through dendrogram using NTsys 2.10e program (Exeter Software, Setauket, New York, USA)[10-12].

RESULTS

Identification of isolates: Fifty-five isolates of pathogenic agents had been successfully collected from 38 regencies (23 provinces) in Indonesia with the major host plants were cocoa pod (52 isolates), chayote (2 isolates) and papaya (1 isolate) (Table 1). Those isolates produced clumsy, coenocytic and hyaline hyphae, pappillate sporangium with various shape, i.e., distorted, ellipsoid, globose, obpyriform and ovoid, while the spherical chlamydospores were terminally and intercalary generated (Fig. 1). These features were recognized as the characteristics of *Phytophthora palmivora*. Then, molecular analysis confirmed that all isolates were positively detected with the multiplex PCR using species-specific primers (Fig. 2a-c).

Morphological characteristics: The sporangium of these isolates had length (l) about 34.85-68.35 µm in range, range of breadth (b) between 25.64-43.68 µm, l/b ratio around 1.17-1.89 as well as length of pappillate and pedicel ranging between 1.93-8.05 and 1.92-9.38 µm, respectively; while the chlamydospores were characterized with range of 25.27-44.95 µm in diameter (Table 2).

Sexual characteristics: It was found that there were 44 isolates which were detected as A2 types and 10 isolates

Table 1: Isolates of *Phytophthora palmivora* used in this study

Isolates no.[a]	Collection code	Geographical location	Host plant	Isolation date
I1	LBP1	Deli Serdang, north Sumatera	Cocoa pod	2017
I2	BKN1	Kampar, Riau	Cocoa pod	2017
I3	BSK1	Tanah Datar, west Sumatera	Cocoa pod	2017
I4	CRP1	Rejang Lebong, Bengkulu	Cocoa pod	2017
I5	MRD1	Ogan Komering Ulu Selatan, south Sumatera	Cocoa pod	2017
I6	KOT1	Tanggamus, Lampung	Cocoa pod	2017
I7	GDT1	Pesawaran, Lampung	Cocoa pod	2017
I8	KBA1	Central Bangka, Bangka Belitung	Cocoa pod	2017
I9	PDG1	Pandeglang, Banten	Cocoa pod	2017
I10a	CJR1	Cianjur, West Java	Cocoa pod	2017
I10b	CJR2	Cianjur, West Java	Cocoa pod	2017
I10c	CJR3	Cianjur, West Java	Cocoa pod	2017
I11a	SMD1	Sumedang, West Java	Cocoa pod	2017
I11b	SMD2	Sumedang, West Java	Cocoa pod	2017
I12	PWT1	Purwokerto, Central Java	Cocoa pod	2017
I13a	BTG1	Batang, Central Java	Cocoa pod	2017
I13b	BTG2	Batang, Central Java	Cocoa pod	2017
I14	WSB1	Wonosobo, Central Java	Cocoa pod	2017
I15	TMG1	Temanggung, Central Java	Chayote	2017
I16	UNR1	Semarang, Central Java	Cocoa pod	2017
I17a	WAT1	KulonProgo, D.I. Yogyakarta	Cocoa pod	2017
I17b	WAT2	KulonProgo, D.I. Yogyakarta	Cocoa pod	2017
I17c	WAT3	KulonProgo, D.I. Yogyakarta	Cocoa pod	2017
I17d	WAT4	KulonProgo, D.I. Yogyakarta	Chayote	2017
I18a	SMN1	Sleman, D.I. Yogyakarta	Cocoa pod	2017
I18b	SMN2	Sleman, D.I. Yogyakarta	Papaya	2017
I19a	WNO1	GunungKidul, D.I. Yogyakarta	Cocoa pod	2017
I19b	WNO2	GunungKidul, D.I. Yogyakarta	Cocoa pod	2017
I19c	WNO3	GunungKidul, D.I. Yogyakarta	Cocoa pod	2017
I19d	WNO4	GunungKidul, D.I. Yogyakarta	Cocoa pod	2017
I19e	WNO5	GunungKidul, D.I. Yogyakarta	Cocoa pod	2017
I20a	JMR1	Jember, East Java	Cocoa pod	2017
I20b	JMR2	Jember, East Java	Cocoa pod	2017
I21	BYW1	Banyuwangi, East Java	Cocoa pod	2017
I22	SAG1	Sanggau, West Kalimantan	Cocoa pod	2017
I23	SMR1	Samarinda, East Kalimantan	Cocoa pod	2017
I24	PKY1	Mamuju Utara, West Sulawesi	Cocoa pod	2017
I25	MKS1	Makassar, South Sulawesi	Cocoa pod	2017
I26	LSS1	Kolaka Utara, Southeast Sulawesi	Cocoa pod	2017
I27a	KKA1	Kolaka, southeast Sulawesi	Cocoa pod	2017
I27b	KKA2	Kolaka, southeast Sulawesi	Cocoa pod	2017
I28	RMB1	Bombana, southeast Sulawesi	Cocoa pod	2017
I29	KDI1	Kendari, southeast Sulawesi	Cocoa pod	2017
I30	BNG1	Buton Utara, Southeast Sulawesi	Cocoa pod	2017
I31	KTG1	Kotamobagu, north Sulawesi	Cocoa pod	2017
I32	MME1	Sikka, Nusa Tenggara Timur	Cocoa pod	2017
I33	AMB1	Ambon, Maluku	Cocoa pod	2017
I34a	SML1	Saumlaki, Maluku	Cocoa pod	2017
I34b	SML2	Saumlaki, Maluku	Cocoa pod	2017
I35	TTE1	Ternate, north Maluku	Cocoa pod	2017
I36	MNK1	Manokwari, West Papua	Cocoa pod	2017
I37a	TIM1	Mimika, Papua	Cocoa pod	2017
I37b	TIM2	Mimika, Papua	Cocoa pod	2017
I37c	TIM3	Mimika, Papua	Cocoa pod	2017
I38	JAP1	Jayapura, Papua	Cocoa pod	2017

[a]Capital letter indicated the origin countries of isolates (I for Indonesia), the number represented geographical areas from which the isolates were collected, small letter described the number of isolates collected from different locations in same geographical areas

were detected as A1 types. One isolate (I20a) from cacao in east Java was positively reacted with both standard isolates (A1 and A2 types). However, it could not produce sexual organ under single culture so that the mating

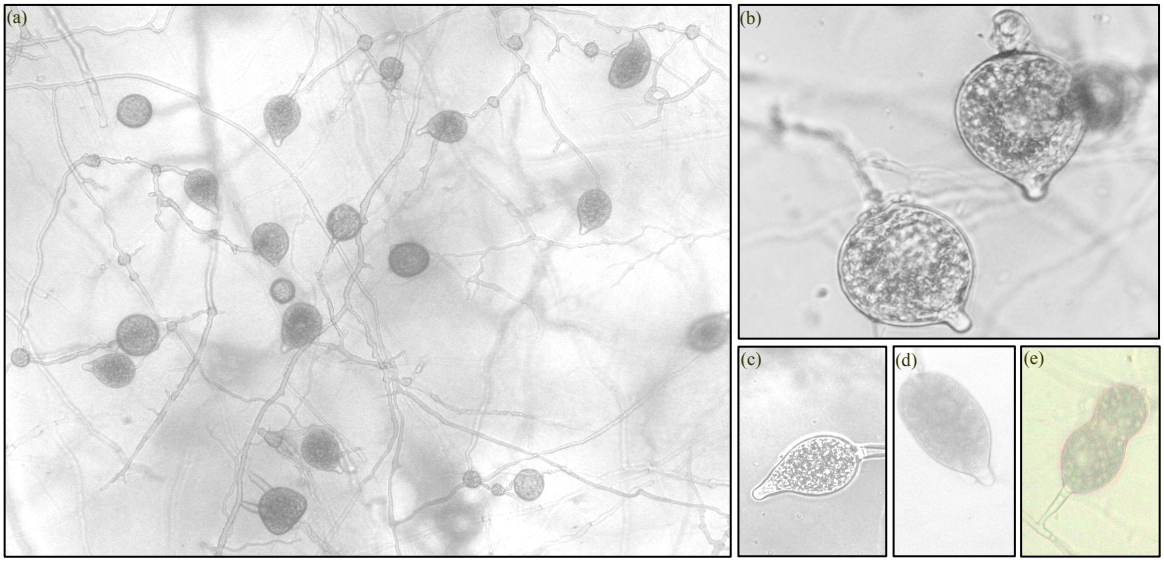

Fig. 1(a-e): Characteristics of morphological features of pathogenic agents isolated from symptomatic cocoa pod rot in cacao growing areas of Indonesia, (a) Appearance of hyphae, sporangia and chlamydospores on V8 agar medium, (b) Globose- (top) ovoid- (bottom), (c) Obpyriform, (d) Ellipsoidal and (e) Distorted-shaped sporangia

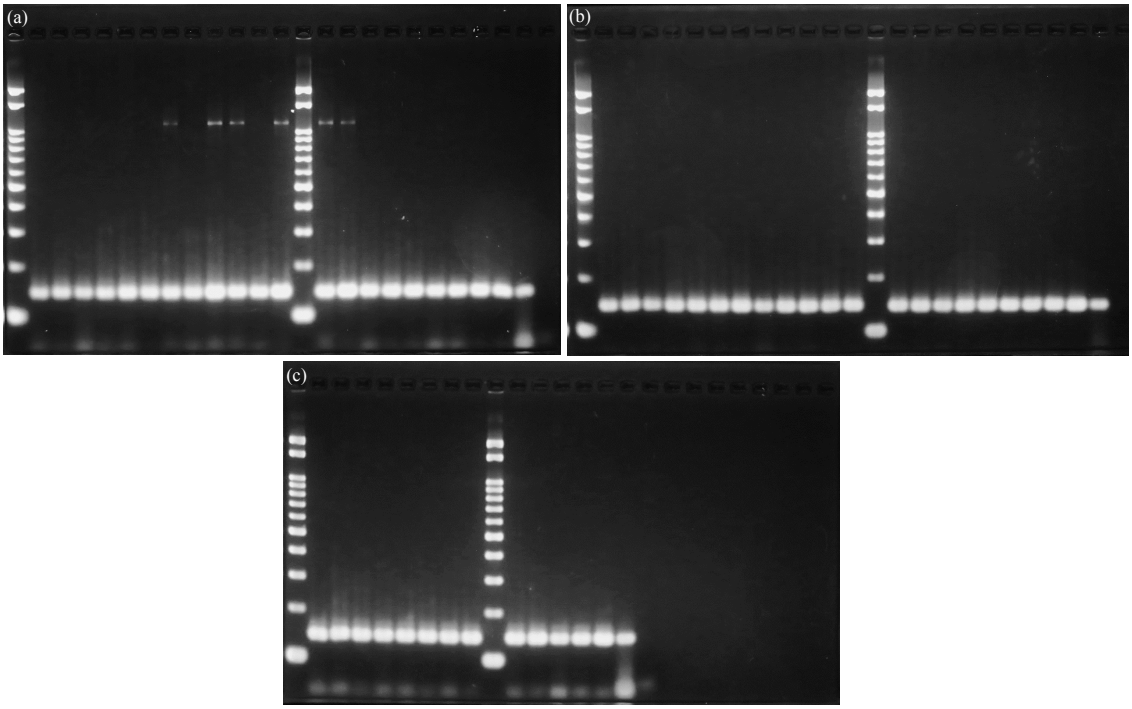

Fig. 2(a-c): Performance of DNA bands from all isolates after molecular assay using multiplex PCR method with species-specific primers at 150 bp in size, (a) Isolates no. 1-21, (b) Isolates no. 22-42 and (c) Isolates no. 43-55 with +ve and -ve controls were isolate of P0633 and SDW, respectively

type of this isolate could not determine yet. Those isolates could produce amphigynous antheridia (range of size about 10.64-14.68×7.02-12.20 μm) as well as spherical oogonia (ranging 24.53-33.76 μm in diameter) and oospores (range of diameter 20.85-27.43 μm) (Table 3, Fig. 3a, b).

Table 2: Morphological features of isolates collected from cocoa pod rots on cocoa growing areas in Indonesia

Isolates	Sporangium					Length of pedicel	Diameter of chlamydospore
No.[a]	Type	Length/L (µm)	Breadth/B (µm)	LB ratio	Length of papillate (µm)	(µm)	(µm)
I1	Distorted, obpyriform, ovoid	50.50	31.76	1.57	2.77	2.37	29.52
I2	Distorted, obpyriform, ovoid	45.13	26.00	1.72	5.65	5.17	39.17
I3	Distorted, ovoid	52.09	37.08	1.40	7.79	5.27	39.11
I4	Ellipsoid, obpyriform, ovoid	53.26	33.90	1.57	4.11	3.09	36.53
I5	Globose, obpyriform, ovoid	56.13	33.64	1.67	7.11	3.74	34.21
I6	Distorted, globose, obpyriform, ovoid	57.94	40.40	1.43	6.00	3.18	44.95
I7	Globose, obpyriform, ovoid	48.68	37.08	1.32	5.22	6.49	43.91
I8	Distorted, obpyriform, ovoid	46.47	32.28	1.44	4.07	2.57	32.65
I9	Ovoid	54.27	38.52	1.40	4.67	5.16	28.22
I10a	Distorted, ovoid	48.42	32.52	1.48	4.11	4.63	34.32
I10b	Globose, ovoid	45.96	33.79	1.35	2.42	5.65	33.02
I10c	Globose, ovoid	44.49	30.60	1.45	5.91	3.09	29.70
I11a	Distorted, globose, ovoid	47.41	29.43	1.60	3.68	3.84	33.76
I11b	Globose, ovoid	39.75	29.87	1.32	5.27	4.95	31.37
I12	Globose, obpyriform, ovoid	50.11	33.80	1.47	2.05	2.89	31.73
I13a	Distorted, globose, ovoid	48.37	32.63	1.48	6.17	3.89	32.17
I13b	Distorted, ovoid	52.00	33.40	1.56	6.25	2.70	34.31
I14	Globose, ovoid	55.34	43.68	1.26	3.86	5.29	40.97
I15	Globose, obpyriform, ovoid	47.07	33.72	1.40	8.05	2.07	30.73
I16	Globose, ovoid	57.37	42.10	1.36	3.95	3.84	35.61
I17a	Distorted, ovoid	51.90	34.72	1.49	4.15	5.37	36.90
I17b	Distorted, obpyriform, ovoid	51.76	35.23	1.47	4.31	5.11	35.24
I17c	Distorted, obpyriform, ovoid	47.56	33.82	1.40	7.51	4.45	35.79
I17d	Globose, obpyriform, ovoid	62.92	39.45	1.58	5.80	3.06	38.56
I18a	Ellipsoid, globose, ovoid	56.60	38.67	1.47	3.63	4.12	43.87
I18b	Ellipsoid, obpyriform, ovoid	52.98	35.15	1.50	3.37	3.37	35.42
I19a	Distorted, ellipsoid, obpyriform	59.15	35.14	1.68	6.35	5.35	37.73
I19b	Ellipsoid, obpyriform, ovoid	52.75	37.48	1.40	4.89	6.58	38.00
I19c	Ovoid, ellipsoid, distorted	63.79	37.50	1.70	6.11	9.38	41.33
I19d	Ellipsoid, globose, obpyriform, ovoid	68.35	36.23	1.89	4.70	3.73	41.05
I19e	Globose, obpyriform, ovoid	65.89	36.27	1.81	3.33	4.11	43.17
I20a	Distorted, obpyriform, ovoid	45.57	31.75	1.42	6.14	2.18	30.88
I20b	Ellipsoid, obpyriform, ovoid	47.61	31.03	1.56	4.47	3.21	34.17
I21	Distorted, obpyriform, ovoid	47.66	32.70	1.46	5.77	5.67	25.27
I22	Distorted, ellipsoid, obpyriform, ovoid	61.41	36.63	1.69	6.05	6.02	35.42
I23	Ovoid	51.26	36.41	1.41	5.28	3.00	32.84
I24	Obpyriform, ovoid	42.54	30.98	1.37	5.63	1.92	31.00
I25	Ovoid	45.11	31.76	1.41	3.77	4.67	34.87
I26	Globose, Ovoid	41.84	29.83	1.40	4.61	5.19	33.02
I27a	Globose, obpyriform, ovoid	45.46	38.43	1.17	5.66	3.86	39.11
I27b	Distorted, globose, ovoid	40.50	30.04	1.34	1.93	3.94	32.48
I28	Globose, ovoid	41.41	33.06	1.24	5.33	2.75	37.67
I29	Globose, obpyriform, ovoid	50.36	32.37	1.55	5.84	3.05	34.68
I30	Globose, ovoid	41.77	34.48	1.20	4.14	4.86	38.14
I31	Distorted, globose, ovoid	46.06	37.91	1.21	5.18	6.11	40.77
I32	Globose, obpyriform, ovoid	40.63	31.19	1.30	3.61	2.90	34.68
I33	Globose, ovoid	44.76	37.26	1.19	5.65	4.97	40.03
I34a	Ovoid	45.31	31.37	1.44	4.29	3.50	33.58
I34b	Globose, ovoid	42.39	30.62	1.39	4.26	3.45	41.32
I35	Globose, ovoid	42.07	34.84	1.41	5.74	3.71	36.53
I36	Ellipsoid, globose, obpyriform, ovoid	43.68	33.59	1.30	5.13	3.34	31.55
I37a	Globose, ovoid	34.85	25.64	1.35	2.43	4.02	33.39
I37b	Globose, ovoid	37.26	28.44	1.30	1.98	4.11	32.28
I37c	Globose, ovoid	44.01	32.51	1.35	4.85	3.19	31.73
I38	Globose, obpyriform, ovoid	38.98	30.24	1.29	5.97	2.21	33.01

[a]Capital letter indicated the origin countries of isolates (I for Indonesia), the number represented geographical areas from which the isolates were collected, small letter described the number of isolates collected from different locations in same geographical areas

Table 3: Sexual structures of isolates collected from cocoa pod rots on cocoa growing areas in Indonesia

Isolates No.[a]	Compatibility		Mating type	Size of antheridium (µm)	Diameter of oogonium (µm)	Diameter of oospore (µm)
	A1	A2				
I1	-	+	A1	11.34×7.55	24.53	21.40
I2	+	-	A2	12.29×9.70	32.88	26.24
I3	-	+	A1	13.61×8.93	26.17	21.47
I4	+	-	A2	10.91×9.71	32.38	25.69
I5	+	-	A2	13.20×11.74	32.75	26.98
I6	-	+	A1	12.23×12.10	28.39	22.97
I7	-	+	A1	12.17×12.11	27.77	22.60
I8	+	-	A2	12.28×11.47	33.76	27.26
I9	+	-	A2	13.81×10.28	32.56	27.31
I10a	+	-	A2	13.73×8.54	27.30	22.87
I10b	+	-	A2	11.19×9.45	27.21	23.26
I10c	+	-	A2	11.38×8.66	28.09	23.75
I11a	+	-	A2	13.06×11.11	28.37	23.98
I11b	+	-	A2	13.46×7.85	29.06	24.68
I12	+	-	A2	13.20×9.26	33.16	26.56
I13a	+	-	A2	13.21×10.56	32.47	26.24
I13b	+	-	A2	11.52×8.88	30.63	25.09
I14	+	-	A2	13.50×11.31	29.20	23.84
I15	+	-	A2	10.64×12.20	32.10	25.50
I16	+	-	A2	10.74×9.64	27.30	21.72
I17a	+	-	A2	12.92×9.91	32.51	25.46
I17b	+	-	A2	13.04×9.29	33.02	27.12
I17c	+	-	A2	11.72×8.33	31.64	24.72
I17d	+	-	A2	13.04×10.54	31.23	26.03
I18a	+	-	A2	11.36×9.38	31.87	27.43
I18b	+	-	A2	11.96×8.24	26.80	22.14
I19a	-	+	A1	14.04×7.02	29.42	23.98
I19b	-	+	A1	13.91×9.96	29.38	23.80
I19c	-	+	A1	14.68×8.93	27.53	22.87
I19d	-	+	A1	14.65×8.98	26.37	21.77
I19e	-	+	A1	11.60×9.66	30.39	25.51
I20a	-	+	ND[b]	12.86×9.30	27.63	23.34
	+	-	ND	13.27×8.80	31.13	24.07
I20b	+	-	A2	11.05×9.90	29.61	24.31
I21	+	-	A2	14.23×10.13	33.67	27.05
I22	-	+	A1	13.27×9.77	24.82	20.85
I23	+	-	A2	12.70×9.69	31.32	25.45
I24	+	-	A2	14.49×9.10	27.90	23.75
I25	+	-	A2	12.47×8.38	27.54	23.32
I26	+	-	A2	10.99×11.82	26.75	22.60
I27a	+	-	A2	12.54×9.50	26.61	22.55
I27b	+	-	A2	12.71×7.77	28.13	23.89
I28	+	-	A2	12.27×8.58	29.01	24.21
I29	+	-	A2	12.82×9.49	33.25	26.47
I30	+	-	A2	12.49×9.56	26.33	22.60
I31	+	-	A2	13.75×7.85	28.23	24.35
I32	+	-	A2	12.26×11.80	27.90	23.52
I33	+	-	A2	13.64×11.66	26.61	21.86
I34a	+	-	A2	11.71×8.53	27.49	23.93
I34b	+	-	A2	11.13×8.21	29.47	24.77
I35	+	-	A2	13.60×9.48	26.38	21.68
I36	+	-	A2	13.45×10.03	25.78	21.68
I37a	+	-	A2	12.94×9.54	29.75	22.41
I37b	+	-	A2	12.21×10.63	29.66	24.63
I37c	+	-	A2	12.07×10.87	27.53	23.94
I38	+	-	A2	12.09×9.26	28.78	24.21

[a]Capital letter indicated the origin countries of isolates (I for Indonesia), the number represented geographical areas from which the isolates were collected, small letter described the number of isolates collected from different locations in same geographical areas. [b]ND: Not determined due to its positive reaction with both standard isolates (A1 and A2 types) without any formation of oospores in single culture

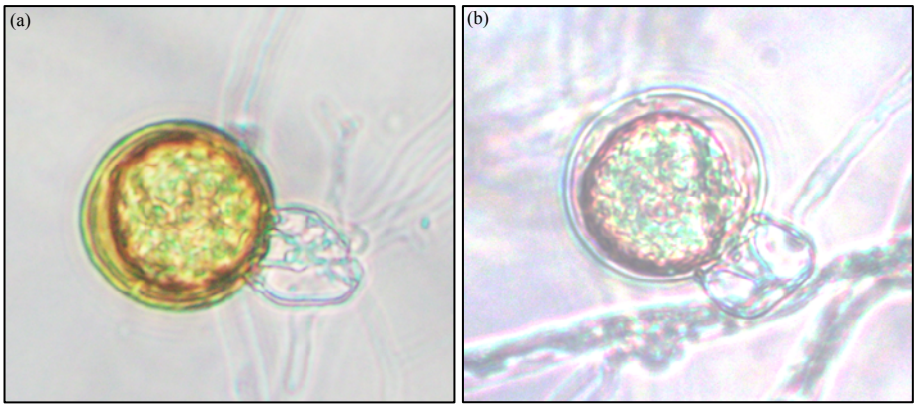

Fig. 3(a-b): Sexual structures produced by representative isolates from different mating types and countries. Oospores of (a) A1 and (b) A2 types

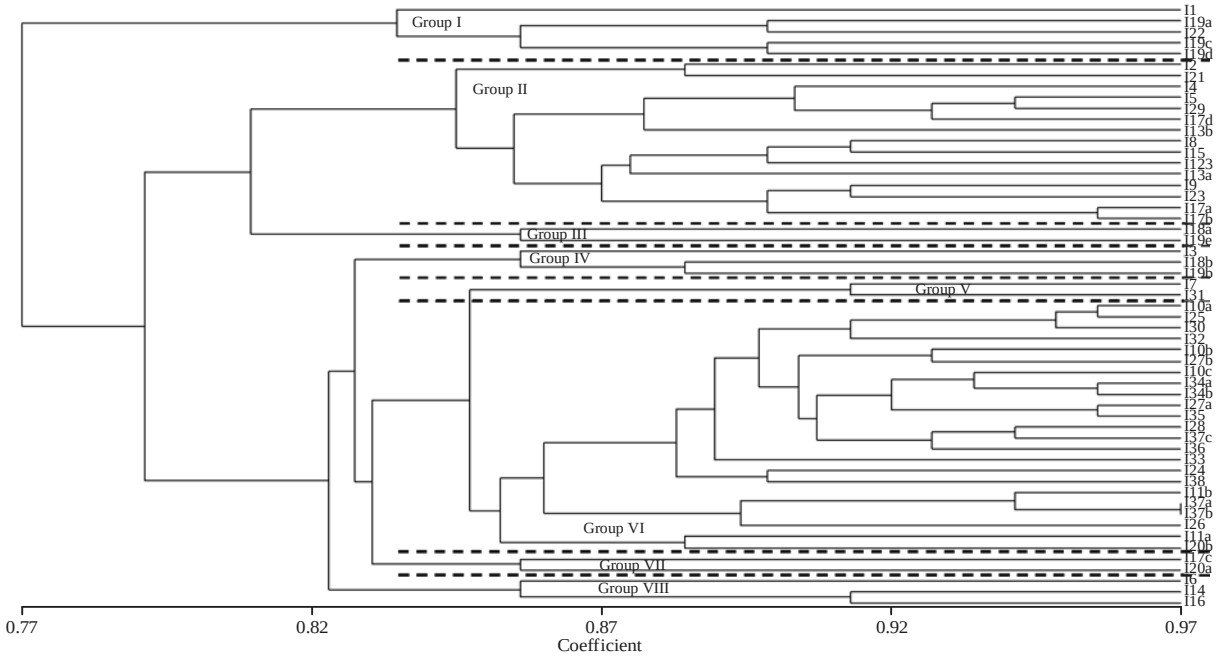

Fig. 4: Dendrogram of clustering the *Phytophthora palmivora* isolates based on variation of morphological features and sexual structures

Morphometric variation: These isolates were clustered into 8 groups with range of similarity level around 83-95% in which 3 groups were main clusters and others were scattered as small groups (Fig. 4). Three large clusters and similar scatter phenomenon of small groups were also illustrated by graph of principle component analysis (PCA) (Fig. 5).

DISCUSSION

This research could be considered as further study on diversity of *P. palmivora* causing cocoa black pod rot disease in Indonesia. Previously, some researchers had investigated the diversity of this pathogen either using morphological, physiological or molecular characteristics which were isolated from various host plants[9,13-22].

In general, the quantitative data of morphological and sexual features in this study was parallel with some previous experiments[15-16,19,22-24], but certain characters, like length and breadth of sporangium were larger than former study[9]. Santoso[9] also reported similar sporangial shapes (i.e., globose, irregular, ovoid, obpyriform) of this pathogen isolated from durian in west Java (Indonesia). However, several

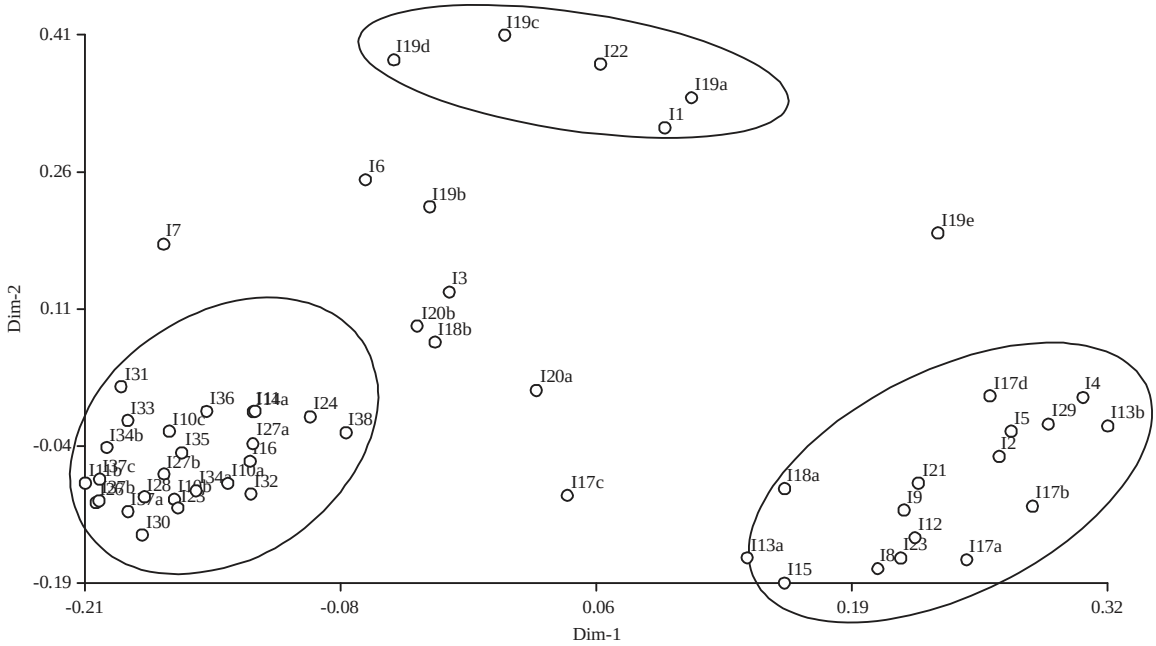

Fig. 5: Principle component analysis (PCA) clustering the *Phytophthora palmivora* isolates based on variation of morphological features and sexual structures

morphological (sporangial length and breadth) and sexual (diameter of oogonia and oospores as well as size of antheridia) structures of these findings were smaller than preceding researches[15,19,23-25]. Such phenomenon illustrated various dimension of morphological and sexual characters of this pathogen in the world.

Present findings were less than the investigation of Motulo *et al*.[18] isolated from coconut analyzed using Randomly Amplified Polymorphic DNA (RAPD) technique. It was assumed that a great number of variation groups indicated the most various relationship among isolates[18]. High variation was also reported on *P. palmivora* isolates from cocoa growing areas in world[19]. On contrary, the restricted variation in *P. palmivora* isolates was documented on former experiments of Forster *et al*.[14], Sudheesh and Sreekumar[21] and Mohammed *et al*.[17].

High similarity level of this research was almost equal to genetic similarity finger printed using RAPD method reported by Motulo *et al*.[18] and Umayah *et al*.[22] as well as using Amplified Fragment Length Polymorphisms (AFLP) by Purwantara and Umayah[20]. It was assumed that the possibility on development of new strain of *P. palmivora* would be low in the future. On the other hand, small similarity distance was obtained between *P. palmivora* isolates from coconut and cocoa in Indonesia[19].

The specific cluster of A1-mating type isolates in current investigation was also reported by Maora *et al*.[15] on *P. palmivora* from cocoa in Papua New Guinea between locations, farms within locations, trees within farms and within individual trees. However, the categorization of all isolates based on morphological and sexual features was generally not consistent. Those clusters were independent on host plant, mating types and geographical areas as well.

Comparable results were also reported by some researchers Maora *et al*.[15], Mchau and Coffey[16], Mohammed *et al*.[17], Purwantara and Umayah[20] and Umayah *et al*.[22]. Wide variation of *P. palmivora* were found among various type of cocoa and source of isolation on infected plant parts[22]. However, close relationships among isolates according to geographical regions and host plants were revealed by researches of Forster *et al*.[14], Mohammed *et al*.[17] and Sudheesh and Sreekumar[21]. Such condition described uncertainty clustering of this pathogen in the world.

Recent experiment did not discover the consistency of sporangial shape for certain clusters. This feature could affect the length, breadth and l/b ratio of sporangium. Erwin and Ribeiro[26] explained that sporangia were variable in shape depending on the isolates.

It was supposed that the close relationship among population of *P. palmivora* in Indonesia due to its ability in infecting wide range of host plants and introduction of cacao seedlings assisting its migration from one to other areas in Indonesia. It was reported that transportation of cacao

seeds/seedlings in Indonesia for last 5 years (2013-2017) was mostly originated from Java and Sulawesi to other cocoa growing areas[27].

CONCLUSION

Current findings on classification and relationship among *P. palmivora* isolates might due to its ability in infecting wide range of host plants and introduction of cacao seedlings which assisted its migration from one to other areas in Indonesia. The implementation of quarantine measure was suggested for restricting the movement of cacao seedlings in Indonesia so that the dispersal of existing pathogen, the possibility of hybridization and the formation of new strain of pathogen could be prevented.

SIGNIFICANCE STATEMENT

This study discovered the great number of isolates from throughout cocoa growing areas in Indonesia which were not only characterized with morphological and sexual structures, but also reconfirmed with molecular analysis using newly developed species-specific primers in River Basin Research Center at Gifu University, Japan. It could be beneficial for accurate and appropriate morphological and molecular identification method of this pathogen. The outcomes would also help the researchers to uncover the critical areas of *P. palmivora* on cocoa that many researchers were not able to explore. Thus, a new theory on morphometric variation and identification of this pathogen might be arrived at.

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