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Morphological and molecular identificationof fungal pathogens causing gummosis disease of *Citrus* spp. in Indonesia

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Abstract. *Henuk JBD, Sinaga MS, Hidayat SH. 2017. Morphological and molecular identificationof fungal pathogens causing gummosis disease of* Citrus *spp. in Indonesia. Biodiversitas 18: 1100-1108.* Gummosis is one of the most important diseases of citrus. The disease is caused by several species of *Phytophthora*, i.e. *P. nicotianae*, *P. palmivora*, *P. citrophthora*; as well as *Lasiodiplodia theobromae* (synonyms: *Botryodiplodia theobromae* and *Diplodia natalensis*; teleomorph *Botryosphaeria rhodina*). The objective of this study was to identify the isolates of fungal pathogens, i.e. *Phytophthora* spp. and *B. theobromae* and/or *D. natalensis* from several different areas of citrus central production in Indonesia based on their morphological and molecular characteristics. This study included two activities, i.e. (i) collection, isolation, and identification of pathogenic fungi from infected tissues, basal stem roots, and soil surface and rhizosphere; and (ii) extraction of genomic DNA and amplification of DNA fragments using PCR technique for molecular characterization. Results of morphological characterization and DNA sequence analysis revealed that gummosis was caused by *Botryodiplodia theobromae* Pat. (Teleomorph *Botryosphaeria rhodina* (Cooke) Arx.)), and *P. citrophthora*.

Keywords: Citrus, gummosis, morphology and molecular characteristics

INTRODUCTION

Gummosis is one of the most important citrus disease. It potentially causes the financial loses for farmers due to the reduction of citrus production in both quantity and quality. Gummosis is the second most significant disease of citrus after *citrus vein phloem degeneration* (CVPD). This disease can infect in both seedlings and adult citrus plant causing the total loss of citrus production and the inhibition of current wide spreads of citrus in many areas in Indonesia.

Gummosis disease was mostly caused by *Phythoptora*, such as *P. nicotianae*, *P. palmivora*, *P. citrophthora*, but the latest findings showed that another fungal species, i.e. *Botryodiplodia theobromae* and/or *D. natalensis* can also induce gummosis (Wang et al. 2011). The comprehensive reports related to the identification of fungal pathogens associated with gummosis are, however, still limited. Therefore, an accurate identification of fungus for early detection of plant pathogen is very important. This can also provide basic and accurate information, which is required for a successful plant disease control management (Ma and Themis 2007). The pathogen can be conventionally identified based on morphological characteristics followed by molecular method such as *polymerase chain reaction* (PCR) for further confirmation.

The development of molecular methods has a high positive impact on plant pathology researches. The use of PCR methods to detect pathogens in plant tissues or other samples is an effective method to complement with conventional methods in the identification of pathogen morphological characteristics. This is because the identification of pathogen morphology using conventional method is mostly time consuming, affected by environmental condition, labor-intensive and requiresan extensive knowledge in taxonomy (Silvar et al. 2005). PCR is a powerful tool which has been successfully reported in the identification and detection of different fungal plant pathogens. This tool has high specificity, sensitivity,rapidity and can be operated automatically (Ippolito et al. 2002).

PCR method has been used in many applications, including detection of plant pathogens and is generally regarded as the most sensitive test available. For example, the PCR assay was able to detect *P. infestans* in the absence of visible symptoms and also unidentified the pathogen species via plating on selective agar medium (Hussain et al. 2016). PCR method could be also used as an alternative method in the visual evaluation of potato seed infected by several levels of late blight disease (Tooley et al.1998). However, the species level identification of *Phytophthora* in terms of morphology, up to now, is often complicated due to the lack of diagnostic tool of morphological characters (Ippolito et al. 2002). Of the reason, the application of PCR methods in order to identify *Phytophthora* and other plant pathogens, i.e. *B. theobromae* and/or *D. natalensis*is a highly promising method. Therefore, this research was aimed to identify the *Phytophthora* spp. and *B. theobromae* and/or *D. natalensis* isolated from several different areas of citrus central production in Indonesia in terms of the morphological and molecular characteristics.

MATERIALS AND METHODS

Collection of infected plant samples

Infected plant samples were collected from 11 location of citrus central production in Indonesia, i.e. Berastagi (Karo), Kampar, Muaro Jambi, Tulang Bawang Barat, Garut, Jember, Batu-Malang, Bangli, Timor Tengah Selatan (TTS) (Figure 1), Banjarmasin, and Banjarbaru. A purposive sampling method was employed to ensure that samples were only taken from citrus plants showing the disease symptoms and infection signs of gummosis causing fungal pathogens. In each location, samples were collected from five diagonal intersection points.

Isolation of the pathogen

The pathogens were isolated from the infected tissues, basal stem root, and from soil surface and rhizosphere. Pathogen isolation from infected stem was conducted by washing the stem in running water, followed by surface sterilization using 0.5% chlorox for 30 seconds. The sample was then rinsed using sterile aquadest and was incubated in a PDA medium. The fungi cultures were then re-cultured in separate PDA plates to produce single colonies. The fungi culture shown as *Phytophthora* spp. colony characteristics was re-cultured in V8 media plates to stimulate their sporulation. Isolation of pathogens from soil samples were performed using an apple bait method for three days incubation period. Part of spot symptom that was furthest apart from soil attached to the apple bait was taken and cultured in V8 media. Pathogen isolation was also conducted using a flooding method, where a hollowed healthy fresh citrus fruit was placed on soil flooded with sterile water, and the growing pathogen on the surface of hollowed fruit tissue was isolated for further analysis.

Morphological identification

Identification of the pathogens based on morphological characteristics was carried out by both macroscopic and microscopic observations. Species identification of *Phytophthora* spp. was performed following identification key of Erwin and Ribeiro (1996) while that of *Botryodiplodia* sp. was based on identification key of Barnett and Hunter (1998). Observed morphological characteristics were described and presented in tables and figures.

Molecular identification

Identification of the pathogens using molecular method was conducted by amplifying the internal transcribed spacer (ITS) area of the ribosomal DNA (rDNA) using PCR with a pair of universal primers, ITS4 and ITS5 (White et al. 1990).DNA extraction of the pathogenic fungi was conducted based on a modified method from Abd-Elsalam et al. (2003).The PCR mixture was prepared as follows: 15.3 µL of ddH_2O; 2.5 µL of buffer 10x + Mg^{2+} ; 2.5 µL of sucrose cresol (10x); 0.5 µL of dNTP, 10 mM; forward and reverse primers, 1 µL each; 0.2 µL of recombinant *Taq* DNA polymerase (5U μL^{-1}); 2 µL of DNA sample. The total volume of the mixture was 25 µL. DNA amplification was performed using a thermocycler (GeneAmp PCR System 9700) in a 35 cycles consisting of three main steps, i.e. initial denaturation at 94°C for 5 minutes, followed by denaturation at 94°C for 1 minute; annealing process at 52°C for 1 minute; and extension process at 72°C for 2 minutes. After 35 cycles, the PCR was set at 72°C for 10 minutes and the temperature was lowered to 4°C. Amplified DNA fragments were separated using gel electrophoresis in a 1% TBE agarose gel, added with 1 µL of ethidium bromide (0.5 µL10 mL^{-1} agarose), and the DNA samples were then run in a 70 V electric current for 30 minutes.

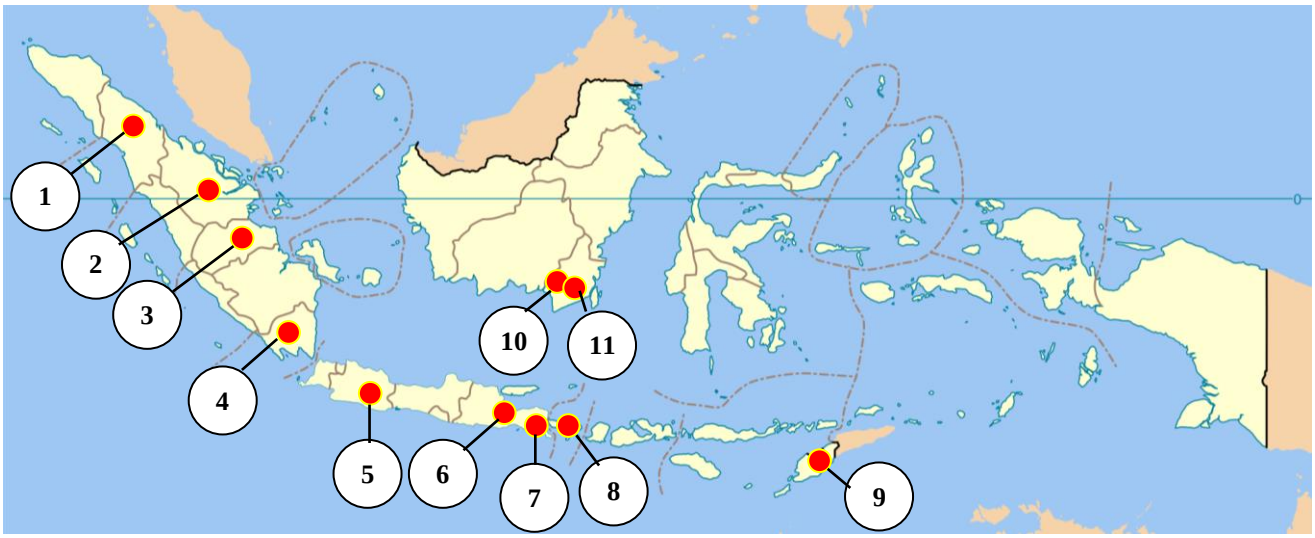

Figure 1. Sample sites of gummosis disease of *Citrus* spp. in citrus central production of Indonesia. 1. Berastagi (Karo), 2. Kampar, 3. Muaro Jambi, 4. Tulang Bawang Barat, 5. Garut, 6. Batu-Malang, 7. Jember, 8. Bangli, 9. Timor Tengah Selatan, 11. Banjarmasin, 10. Banjarbaru

The separated DNA was the visualized under a Transilluminator UV. Fragment DNA of PCR product used for sequencing. DNA sample and primers were sent to Macrogen Inc. South Korea for purification by using *QIAquick gel extraction kit* (Qiagen) and sequencing. Result of sequencing were edited using Genetix Win version 4.0, Clustal X (Thompson et al. 1997) and Mega version 4.0 (Tamura et al. 2007). Homology analysis was conducted by *basic local alignment search tool* (BLAST). For phylogenetic analysis, each nucleotide sequence wasfound from this research and nucleotide sequences selected from Genbank (http://www.ncbi.nlm.nih.gov) were aligned using Clustal W software (http: //www.ebi.ac.uk).Then,result of nucleotide alignment was transferred to Mega version 4.0 to obtain final alignment for construction of phylogenetic tree. Reconstruction of phylogeny was based on maximum parsimony (MP) method with bootstrap 1000 times. Calculation of matrix identity was conducted to know contiguity of Indonesian isolate with other isolates taken from the Genbank.

RESULTS AND DISCUSSION

Morphological identification of the pathogens

Field survey carried out in several central production areas of citrus in Indonesia revealed that either seedlings or adult citrus stands infected by the gummosis fungi exhibited unique symptoms and signs. In infected seedlings, the surface of the infected stem skin was sunken and produced gummosis in the form of limpid structure in wet conditions and turned to golden brown in dry condition. The roots were rotten followed by death of the seedlings. These symptoms and signs are in line with that described by Ploetz (2003). The plants would die if the spot had encircled the stem skin. Generally, the disease could only attack to the top of the plants until the length of 60 cm above the soil surface, while from the roots, the disease could develop and reach to the entire roots (Lutz and Menge 1986). On the adult citrus plant, the symptoms appeared on the stem skin with many spots or wounds nearly exfoliated. In the wounded area, gum appeared to be already dried out. Severe infection could kill the whole infected plant.

When the dried out gum collected from infected seedlings and adult citrus plants were plated on PDA medium, no pathogen was observed. This was in line with Naqvi (2004) who stated that gum was produced asthe plant reaction against pathogen infection but it did no talways contain pathogens. Gum is produced to localize pathogens to prevent further spreads to healthy plant tissues. The gum produced from a plant skin tissue indicates an advanced infection level (Agrios 2005).

As many as 12 isolates were collected from the fields included one *Phytophthora* isolate collected from Oehala village, Timor Tengah Selatan (East Nusa Tenggara) and 11 *Botryodiplodia* isolates collected from Berastagi (Karo, North Sumatra), Kampar (Riau), Muaro Jambi (Jambi), Tulang Bawang Barat (Lampung), Garut (West Java), Jember and Malang (East Java), Bangli (Bali), Timor Tengah Selatan (East Nusa Tenggara), Banjarmasin and Banjarbaru (South Kalimantan).

In PDA media, colony of *Phytophthora* isolate was white and *rosaceous* while in V8 media, it was *stellate* and *cottony* at juvenile and old stages, respectively. Growth of the isolates covered the entire PDA cultured media (Ɵ 9 cm) within 21 days after isolation. Meanwhile, when it was cultured in a V8 media, colony of the fungus covered the entire plate just within 10 days after isolation. Macroscopic observation revealed that hyphae of this fungus are non-septate, branched, *catenulate*, *corraloid*, hyalin, smooth to rough, swollen, round to ovoid, and non-regular (Figure 2.A-C). Globose chlamydospore was formed intercallarly (Figure 2.D). Several different types of sporangia were observed (Figure 3), i.e. globose (Figure 3.A, E-G), ellipsoid (Figure 3.B), ovoid (Figure 3.C), limoniform (Figure 3.D), and some distortic and asymmetric shape (Figure 3.H-J). Each sporangium type exhibited unique size, with an average of 15.00-28.12 µm x 9.37-15.75 µm (Table 1).

Morphological characteristics of isolate from Oehala (Timor Tengah Selatan) observed in the present study were similar with identification key off Erwin and Ribeiro (1996). Therefore, it was concluded that the isolate was *Phytophthora citrophthora.*

In contrast to *phytophtora* isolate, *Diplodia* sp. and/or *Botryodiplodia* sp. isolate showed a faster growth rate. The isolate covered the entire PDA media between 3 and 7 days after isolation. Aerial mycelium of the fungus was white at the beginning, but it turned to black greenish to grey from 4 to 5 days. Interestingly, itturned to black in 10 days after inoculation. Macroscopically, the hyphae are septate; hyalin at the beginning, and then turned to brown (Figure 4.A). The chlamydospore was formed intercallarly (Figure 4.B, C). The pycnidia grew faster when it was cultured in a WA medium streaked with a piece of sterile rice straw (± 2 weeks after isolation) (Figure 5.A) than that in a PDA medium (± 4 weeks after isolation). The pycnidia were formed in cluster inside stroma (Figure 5.B), with single conidiophores, and conidia produced inside the pycnidia. Conidia consisted of young conidia (Figure 5.C) and mature conidia (Figure 5.D), with the shape of them were *ovoid* and *ellipsoid*, respectively. Young conidia were hyaline, possessed a two layer wall, granular, and non-septate while mature conidia were brown with one layer cell wall and possessed one septum that forms two cells. The conidia size varied from 18.75 to 31.87 µm in length, and 11.25 to18.75 µm in width, with an average length and width of 25.31 µm x 15.00 µm, respectively (Table 2).

Results indicated that the observed fungi possessed characters which similar to the unique identity of *Botryodiplodia theobromae* Pat. Interestingly, *B. theobromae* (Pat.), also known as the synonym of *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. and *Diplodia natalensis* Pole-Evans, is the teleomorph of *Botryosphaeria rhodina* (Cooke) Arx) (Timmer et al. 2000).

Molecular identification of the pathogens

Molecular identification of the pathogens was carried out using PCR technique employing a pair of universal primers, ITS4 and ITS5. These primers amplified the ITS region of rDNA ineach of the collected isolates, i.e. 11 *B. theobromae isolates* from 11 locations and one *P. citrophthora* isolate from Oehala village (Timor Tengah Selatan, East Nusa Tenggara). PCR amplification product of *B. theobromae* isolates was ± 550 bp in size while that of *P. citrophthora* isolate was ± 700 bp (Figure 6). The employed primers were universal primers designed to amplify the small sub-unit 18S, the entire region of ITS4 and ITS5, as well as 5,8S and some part of large sub-unit 28S rDNA in many different fungal species (White et al. 1990). Size of the PCR product matched to the expected size previously reported by other workers (Slippers et al. 2005; Begoude et al. 2009; and Ippolito *et al* .2002). The different band size between PCR amplified fragment of *B. theobromae* and *P. citrophthora* was caused by size difference of rDNA ITS regions of the two fungal species. ITS is a low-conserved region in the fungal rDNA but another regions, i.e. the small sub-unit, 18S, 5,8S, and large sub-unit 28S and 5S are highly conserved region of fungal rDNA. These regions have high similar sequence among different organisms (Darmono et al. 2006). Despite the differences, identification with PCR technique should be still continued with a sequencing analysis to confirm the identity of the amplified fungal DNA.

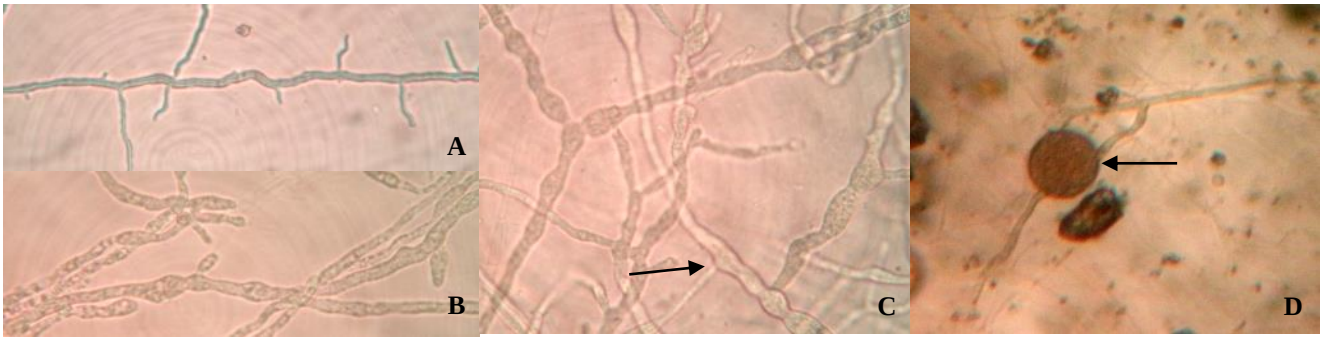

Figure 2. Morphological appearance of Hyphae, Mycelia, and Chlamydospore. A. Young hypha was hyalin not swollen yet, non-septate and branched; B. Corraloid mycelia; C. The swollen hypha; D. Intercallar form of the chlamydospore. Magnification of 400x

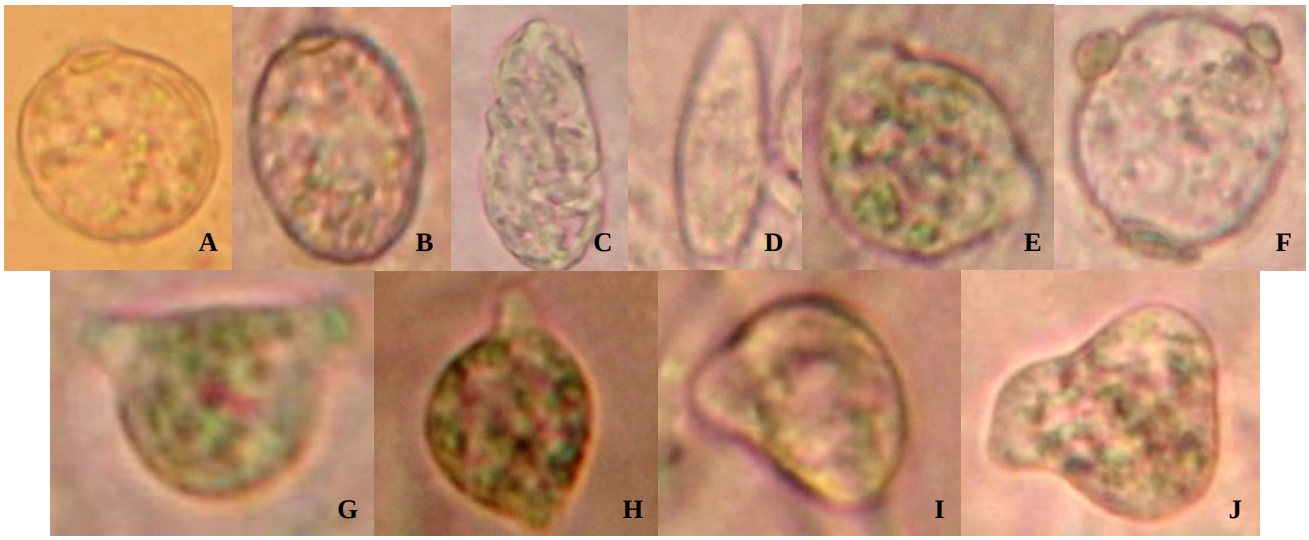

Figure 3. Sporangia shape and papilla of *P. citrophthora*. A. *Globose*, semipapillate; B. *Ellipsoid*, semipapillate; C. *Ovoid*, non-papillate; D. *Limoniform*, non-papillate; E-G. G*lobose*, bipapillate; H-J. Distortic or asymmetric form; H-I. Papillate sporangium; J. *Bilobed* (2 *apices*), non-papillate. Magnification of 1200x.

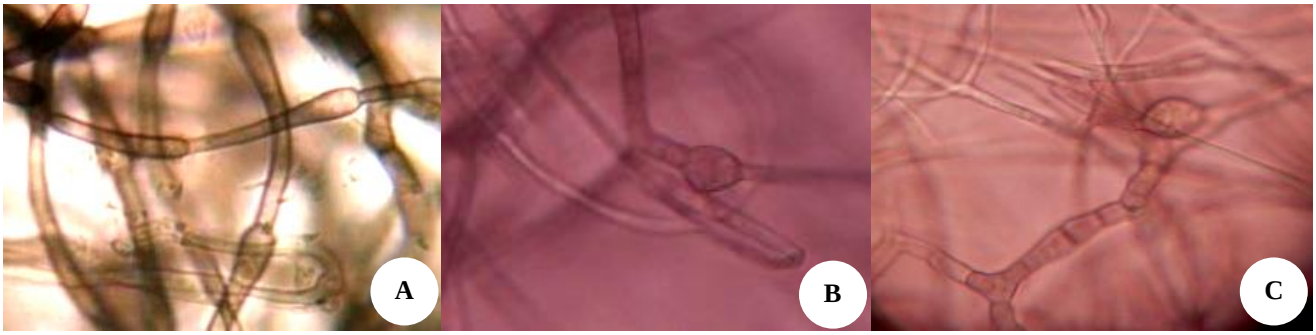

Figure 4. Formation of hypha (A) and chlamydospore as intercallar (B, C) (400x magnitude)

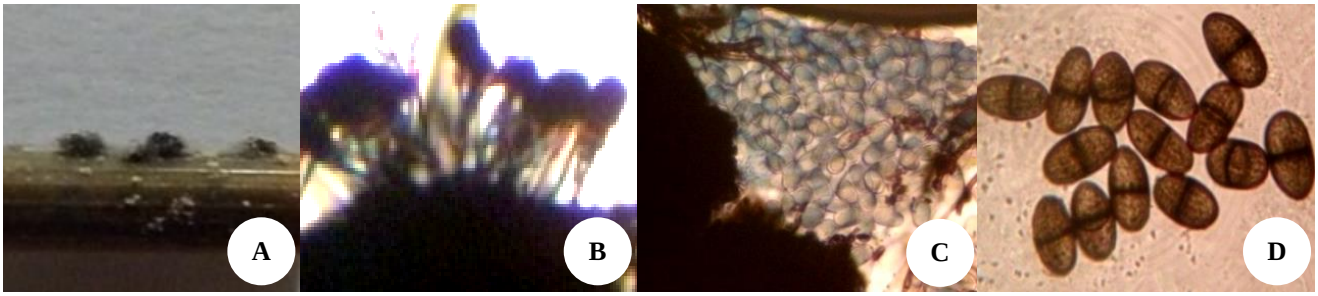

Figure 5. Morphology of pycnidia and the conidia of *B. theobromae.* A. The pyicnidia cluster in rice straw; B. Pycnidia (40x magnitude); C. Young conidia f released from pycnidia (100x magnitude); D. Mature conidia (400x magnitude)

Table 1. Sporangia size of *P. citrophthora* observed under inverted microscopes

Shape	Size (µm)	Average (µm)	Ratio (l/w)
Globose	15.00-18.75	15.75	1.00
Ellipsoid	13.12-18.75 x 11.25-16.87	17.19 x 15.00	1.15
Ovoid	26.25-30.00 x 9.37-13.12	28.12 x 11.25	2.50
Limoniform	22.50 x 9.37	22.50 x 9.37	2.40
Globose, bipapillate	15.00	15.00	1.00
Globose, bipapillate	18.75	18.75	1.00
Globose, bipapillate	15.00	15.00	1.00
Distortic (asymetric)	22.50 x 15.00	22.50 x 15.00	1.50
Distortic (asymetric)	18.75 x 15.00	18.75 x 15.00	1.25
Distortic (asymetric)	26.25 x 18.75	26.25 x 18.75	1.40

Table 2. Conidia size of *Diplodia* spp. and/or *Botryodiplodia* spp.

Origin of isolate	Size of conidia *in vitro* (µm)	Average	Ratio (l/w)
Berastagi (Karo)	26.26-30.00 x 13.12-15.00	27.75 x 14.25	1.95
Kampar	26.25-28.12 x 13.12-16.87	27.37 x 14.62	1.87
Jambi	24.37-28.12 x 13.12-16.87	26.25 x 15.00	1.75
Lampung	24.37-28.12 x 15.00-16.87	26.25 x 15.37	1.71
Garut	26.25-30.00 x 15.00-16.87	27.75 x 16.12	1.72
Jember	28.12-30.00 x 15.00-18.75	28.50 x 16.87	1.69
Malang	22.50-26.25 x 13.12-15.00	24.75 x 14.25	1.74
Kintamani	18.75-24.37 x 11.25-15.00	24.75 x 14.25	1.66
Timor Tengah Selatan	18.75-26.25 x 11.25	22.50 x 11.25	2.00
Banjarbaru	24.37-26.25 x 15.00	25.50 x 15.00	1.70
Banjarmasin	28.12-31.87 x 15.00-18.75	29.62 x 16.50	1.79

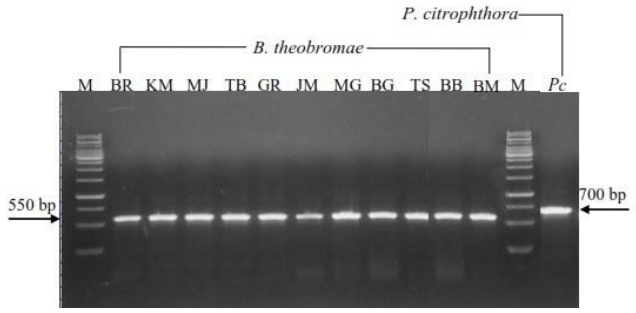

Figure 6. PCR amplified regions of 11 *B. theobromae* and one *P. citrophthora* isolates using ITS4 and ITS5 universal primers. 1 kb marker (M), Berastagi (Karo) (BR), Kampar (KM), Muaro Jambi (MJ), Tulang Bawang Barat (TB), Garut (GR), Jember (JM), Batu-Malang (MG), Bangli (BG), Timor Tengah Selatan (TS), Banjarbaru (BB), Banjarmasin (BM) and *P. citrophthora* isolate from Timor Tengah Selatan (*Pc*)

Sequencing analysis of PCR amplified regions

Sequencing results of PCR amplified regions of 10 *B. theobromae* isolates are presented in Table 3. Nucleic acid sequence of these isolates was then subjected to BLASTN alignment to determine the identity of *B. theobromae*. BLASTN alignment indicated that sequences of the 10 isolates had a high similarity (more than 90%, *e-value* 0.0) with *B. rhodina* and *L. theobromae* isolates (Table 4). Clavarie and Notredame (2003) proved that two genes or DNA fragments are homolog if 70% of the nucleotide sequence or 25% of amino acid sequence of those two genes or DNA fragments is identical to each other, with a minimal sequence length of 100. Based on BLASTN alignment results, it was confirmed that PCR products of the 10 isolates collected from infected citrus plants from different areas belonged to *B. theobromae* (synonym *L. theobromae*; teleomorph *B. rhodina*).This confirmed the results of morphological identification in this study. The nucleotide sequence of *B. theobromae* published in *GenBank* was that of its teleomorph name, i.e. *B. rhodina* and *L. theobromae* (synonym of *B. theobromae*). These published sequences of the isolate samples were mostly collected from sub-tropical countries in where the environmental condition might enable the pathogenic fungi forming its sexual phase (teleomorph). This sexual phase is very useful to survive instressful conditions including extremely high or low temperature, extreme drought, or in the absence of host plants.

A phylogenetic analysis was then done by comparing the alignment results of nucleotide sequences of isolates and several isolates of Botryosphaeriaceae family (selected from *GenBank*) as references using *ClustalW* software (Table 5) (http://www.ebi.ac.uk). The alignment results were transferred to Mega version 4.0 (Tamura et al. 2007) to produce a final alignment to generate a phylogenetic tree. Alignment results revealed that the 10 isolates employed in this study had no similarity with another species group and the nine fungal isolates of Botryosphaeriaceae family selected from *GenBank*. However, each of 10 isolate samples shared a similarity of nucleotide sequences. This could be seen from the unconcentrated nucleotide columns.

Results of phylogenetic analysis indicated that the isolates of *B. theobromae* from 10 different areas of citrus production in Indonesia were different and separated from 10 different isolates selected from *GenBank* as shown in two different phylogenetic groups/clusters (Figure 7). Isolates from Indonesia were clustered in the first group while *B. parva*_Australia_citrus [EF173922.1], *Sphaeropsis sapinea*_North Sumatra-Indonesia_pine [AY253294.1], *Lasiodiplodia* sp._East Kalimantan-Indonesia_meranti [AB297716.1], *Lasiodiplodia* sp._Malaysia_citrus [GU066721.1], *L. theobromae*_Malaysia_soursop [GU066603.1], *B. rhodina*_China_jackfruit [EU407235.1], *Botryosphaeria* sp._Malaysia_citrus [GU066640.1], *L. theobromae*_Taiwan_manggo [GQ502461.1] and *L. pseudotheobromae*_Suriname_citrus [EF622081.1] were clustered in the second group. Isolates from Indonesia alone were further sub-divided into two sub-groups. The first sub-group included Muaro Jambi, Jember dan Kampar, and the second sub-group included Bangli, Timor Tengah Selatan, Batu-Malang, Banjarbaru, Berastagi (Karo), Garut, and Tulang Bawang Barat. Results of matrix analysis revealed a high similarity among Indonesian isolates, with a score of >90%. However, when these isolates were compared with isolates from *GenBank*, the similarity score was very low (<50%).

Table 3. Nucleotide sequence size of isolates of infected citrus plants employed in the present study

Isolate	Location	Fragment size (bp)
Botryodiplodia theobromae	Berastagi (Karo, North Sumatra)	542
B. theobromae	Kampar (Riau)	602
B. theobromae	Muaro Jambi (Jambi)	537
B. theobromae	Tulang Bawang Barat (Lampung)	535
B. theobromae	Garut (West Java)	570
B. theobromae	Jember (East Java)	420
B. theobromae	Batu-Malang (East Java)	527
B. theobromae	Bangli (Bali)	559
B. theobromae	Timor Tengah Selatan (East Nusa Tenggara)	513
B. theobromae	Banjarbaru (South Kalimantan)	555
B. theobromae	Banjarmasin (South Kalimantan)	-*)
P. citrophthora	Oehala (Timor Tengah Selatan, East Nusa Tenggara)	-**)

Note: *) Nucleotide data was not available due to a low DNA quality. **)No amplified PCR fragment due to a possible contamination from other fungi

Table 4. BLASTN analysis results of the sequence of the 10 isolates employed in this research with other sequences stored in Genbank

Accession no.	Species	Accession origin	Host	% homology*	Origin of isolate
EU938329.1	*B. rhodina*	Brazil	Manggo (*Mangifera indica*)	100	Banjarbaru
EU938329.1	*B. rhodina*	Brazil	Manggo (*M. indica*)	100	Bangli
EU938329.1	*B. rhodina*	Brazil	Manggo (*M. indica*)	100	Berastagi (Karo)
EU938329.1	*B. rhodina*	Brazil	Manggo (*M. indica*)	99	Tulang Bawang Barat
FJ904838.1	*L. theobromae*	Kenya	Margosa (*Azadirachta indica*)	100	Muaro Jambi
FJ904838.1	*L. theobromae*	Kenya	Margosa (*A. indica*)	99	Kampar
EU938331.1	*B. rhodina*	Brazil	Manggo (*M. indica*)	100	Batu-Malang
EU938332.1	*B. rhodina*	Brazil	Manggo (*M. indica*)	98	Timor Tengah Selatan
GQ502460.1	*L. theobromae*	Taiwan	Manggo (*M. indica*)	100	Garut
HM008598.1	*L. theobromae*	Taiwan	Avocado (*Persea americana*)	99	Jember

Note: *The accessions were selected based on the highest homological sequence

Table 5. The list of nucleotide sequence of selected Family Botryosphaeriaceae used for phylogenetic analysis

Out-group and Family Botryosphaeriaceae	Accession no.	Origin of accession (year)	Host	Fragment size (bp)
Polymyxagraminis	EU244488.1	Switzerland (2007)	Wheat (*Triticum aestivum*)	610
Lasiodiplodia sp.	GU066721.1	Malaysia (2010)	Citrus (*Citrus* spp.)	545
Botryosphaeria parva	EF173922.1	Australia (2007)	Citrus (*Citrus* spp.)	515
Botryosphaeria sp.	GU066640.1	Malaysia (2009)	Citrus (*Citrus* spp.)	548
L. pseudotheobromae	EF622081.1	Suriname (2008)	Citrus (*Citrus* spp.)	542
Lasiodiplodia sp.	AB297716.1	Kaltim-Indonesia (2007)	Meranti (*Shorea*sp.)	539
Sphaeropsis sapinea	AY253294.1	Sumut-Indonesia (2003)	Pine (*Pinus*sp.)	504
B. rhodina	EU407235.1	China (2008)	Jackfruit (*Artocarpusheterophyllus*)	542
L. theobromae	GQ502461.1	Taiwan (2009)	Manggo (*M. indica*)	533
L. theobromae	GU066603.1	Malaysia (2009)	Soursop (*Annona muricata*)	545

The grouping of *B. theobromae* isolates from 11 locations in Indonesia might correlate with the origin of the seedlings. For example, in Kampar and Muaro Jambi production areas, the seedlings were obtained from Jember; in Banjarbaru, Berastagi (Karo) dan Tulang Bawang Barat, the seedlings were obtained from Garut; in Timor Tengah Selatan, the seedlings were obtained from Malang. However, in Bangli, seedlings were obtained from both surrounding areas and other seedlings came from outside Bangli. Different groups of isolates in Indonesia and *GenBank* indicated a possible evolution in a relatively short period. This might happen due to *B. theobromae* belongs to Deuteromycetes class, which might change its genetic characters resulting in a new race. Generally, the evolution and genetic changes depend on the generation period, number of reproduction unit in each generation, the mechanism of genetic variation, and the extreme pressure of fungal growth and development (Agrios 2005). Changes in pathogens can cause an increase or decrease of their pathogenicity. These changes can produce a new race, which may be more virulent and enable to attack the variety of resistant host in the future, or vice versa, it may become a new avirulent race (Sinaga 2006).

In conclusion, 12 fungal isolates were successfully collected from 11 location of citrus central production in Indonesia included one *Phytophthora* isolate from Oehala village, Timor Tengah Selatan (East Nusa Tenggara) and 11 *Botryodiplodia* isolates from Berastagi (Karo, North Sumatra), Kampar (Riau), Muaro Jambi (Jambi), Tulang Bawang Barat (Lampung), Garut (West Java), Jember and Malang (East Java), Bangli (Bali), Timor Tengah Selatan (East Nusa Tenggara), Banjarmasin and Banjarbaru (South Kalimantan). The morphological characterization and molecular analysis of fungal isolates triggering gummosis in the citrus samples was done using universal primers designed from DNA sequences obtained from internal transcribed spacer regions (ITS4 and ITS5). It showed that gummosis was mostly caused by *Botryodiplodia theobromae* Pat. (Teleomorph *Botryosphaeria rhodina* (Cooke) Arx.) and *P. citrophthora*. Result of phylogenetic analysis also indicated that the isolates of *B. theobromae* from 10 different citrus production areas in Indonesia were different and separated from the 10 different isolates selected from *GenBank* as shownin two distinguished phylogenetic groups/clusters. Isolates from Indonesia were clustered in the first group, which were further sub-divided into two sub-groups. The first sub-group included Muaro Jambi, Jember dan Kampar, and the second sub-group included Bangli, Timor Tengah Selatan, Batu-Malang, Banjarbaru, Berastagi (Karo), Garut, and Tulang Bawang Barat. Results of matrix analysis revealed a high similarity of nucleotide sequences among Indonesian isolates, with a score of >90%.

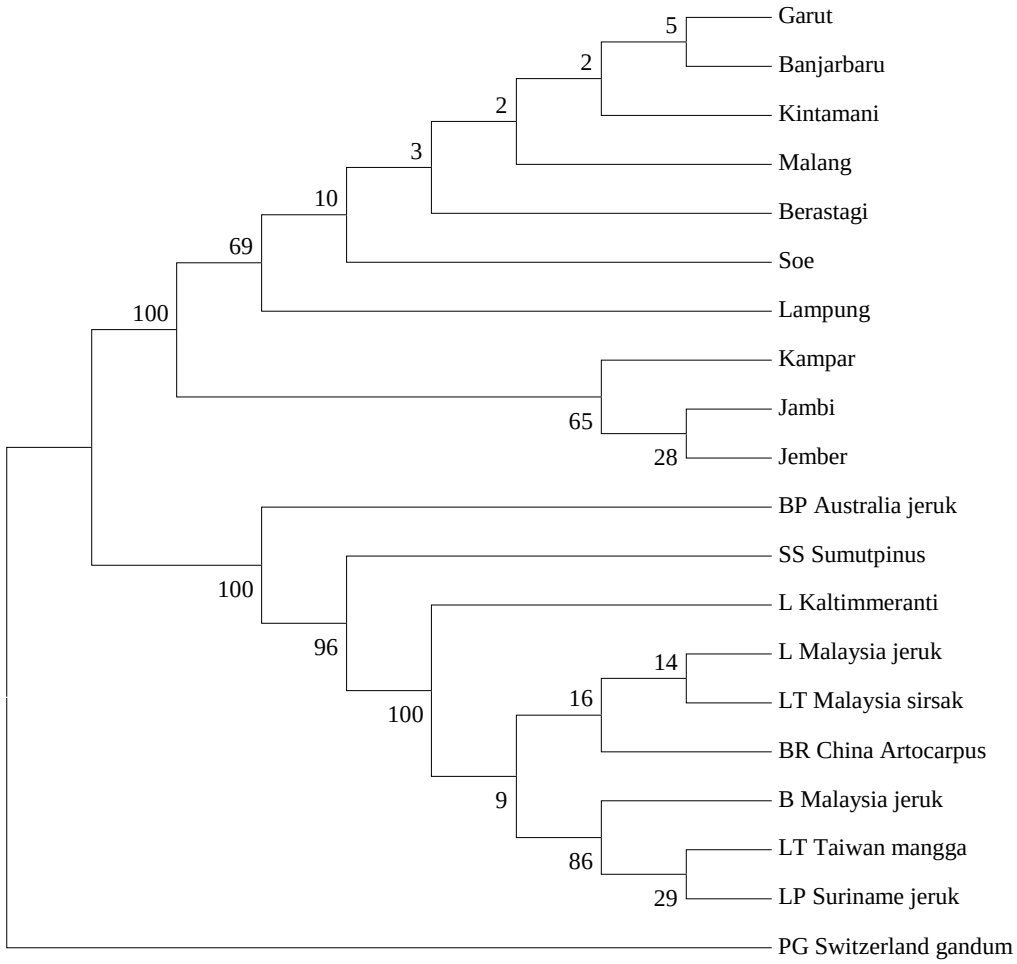

Figure 7. Phylogenetic sequence of observed samples of *B. theobromae* isolates compared to that of several different Botryosphaeriaceae family infecting other host plants from different regions in Asia. BP Australia jeruk (*Botryosphaeria parva* from *citrus* spp.), SS Sumut pinus (*Sphaeropsis sapinea*from *Pinus* sp.), L Kaltim Meranti (*Lasiodiplodia* sp. from *Shorea* sp.), L Malaysia jeruk (*Lasiodiplodia* sp. from *Citrus* spp.), LT Malaysia sirsak (*Lasiodiplodia theobromae* from *Annona muricata*), BR China Artocarpus (*Botryosphaeria rhodina* from *Artocarpusheterophyllus*), B Malaysia jeruk (*Botryosphaeria* sp. from *Citrus* spp.), LT Taiwan mangga (*Lasiodiplodia theobromae* from *Mangifera indica*), and LP Suriname jeruk (*Lasiodiplodia pseudotheobromae* from *Citrus* spp.). *Polymyxa graminis* from Wheat (*Triticum aestivum*) with an accession number EU244488.1 was used as out-group. The numbers on the branches show the confidence level of the branch separation

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