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Sequence analysis of *Ganoderma boninense* isolates from oil palm

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Abstract. Basal Stem Rot (BSR) is a prevalent oil palm disease caused by *Ganoderma* fungus. Many oil palm plantations suffered losses due to BSR disease, which caused deaths of oil palm crops. *Ganoderma* has host plants from the Palmae family such as oil palm, coconut, Nipah, aren, areca nut, papyrus, and also can be found in Industrial Plantation Forest (HTI) like *Acacia*, even in the forest wood can also be encountered *Ganoderma*. *Ganoderma* has high genetic diversity. Fruiting bodies which isolated from oil palm tree located in different locations is one of the factors causing genetic diversity. The *Ganoderma* isolates analysed in this study were isolates derived from different oil palm plantation. *Ganoderma* isolates collection of PT Socfindo is used in a screening test to obtain oil palm material which has resistance to *Ganoderma* attack. The present study confirmed through DNA sequences that *Ganoderma* derived from oil palm has been defined as a *Ganoderma boninense*, that is very virulent for the appearance of BSR disease.

1. Introduction

Ganoderma boninense species cause basal Stem Rot (BSR) disease in oil palm. However, this disease is known to be caused by different *Ganoderma* pathogens in each country, in Malaysia, the causes of BPB are identified as *G. boninense, G. zonatum, G. miniatocintum* and *G. tornatum* [1]. However, in the 1900s, using artificial inoculation techniques through root and rubber woods was able to prove that *G. boninense* is a dominant pathogenic *Ganoderma* species to oil palm based on the Koch Postulat concept [2]. The causes of palm oil BPB disease in some countries are reported to vary, for example, some species of saprophytic *Ganoderma* from the *Basidiomycota* group. In West Africa, the causes of

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BSR are identified as *G. lucidum*, in Nigeria identified as *G. zonatum, G. encidum, G. colossus,* and G. *applanatum* [3]. *G. boninense* found in Indonesia has a molecular difference. *G. boninense* from some regions of Indonesia does not have a very close relationship [5].

The phenotypic diversity of *Ganoderma* is also found in the PT Socfin Indonesia plantation of oil palm plantations afflicted with BSR disease. There are differences in color, surface shape, sticking pattern on the palm oil rod and basidiocarp edge pattern. But its genetic diversity is not well understood. Therefore, this study was conducted to determine sequence analysis of selected isolates of *G. boninense* causes BSR existing in PT Socfin Indonesia and outside PT Socfin Indonesia.

2. Materials and Method

The materials used in this study were 138 isolates of *Ganoderma* (13 isolates has been selected for the DNA sequencing), distilled water, peeled potato, agar powder, dextrose/sucrose, chloramphenicol C0378 Sigma-Aldrich, streptomycin S6501 Sigma-Aldrich, PDA (Potato Dextrose Agar) media, WA media(Water Agar), *Ganoderma* DNA extract, 70% ethanol, 0.1 g nitrogen liquid, polyvinylpyrrolidone (PVPP), CTAB buffer, TAE buffer, TE buffer, NaCl, isopropanol, chloroform, specific primers, H_2O, agarose gel, GelRed, ladder DNA, loading lye. The tools used in this research are tissue, aluminium foil, paper label, permanent marker, pen, scalpel blade, scalpel handle, beaker glass volume 500 cc, magnetic bar, rubber gloves, hair cap, mask, Wheaton tube, incubator Liebhr, micro pipette size 1-50 µl, 100-500 µl, pipet tips (white, yellow and blue), peeler, pot, gas stove, gas cylinder, Cimarex hot plate, Mednif horizontal autoclave, tape for sterilization indicator, sterile plastic petrix with 9 cm diameter, glass plate with 9 cm diameter, needle for spinal anaesthesia Spinocan size 0,53 x 88 mm, parafilm, laminar airflow Esco, bunsen lamp, refrigerator, particle counter Handilaz, mortar, tweezers, Memmert oven, freezer , Eppendorf tube, micro tube 2.0 ml and 1.5 ml, PCR device, spectrometer, electrophoresis device, electrophoresis (Power PAC 3000, Bio-Rad), PCR (Thermal cycler) applied biosystem, Gel Doc, analytical scales Sartorius TE313S, Erlenmeyer, spatula, centrifuge (Eppendorf 5415), spectrophotometer, fume hood, microwave, pincers, camera, notebooks, computers.

2.1. Sample collection

Identification of sample location was chosen by the highest rate of *Ganoderma* infection in the estate. The samples as minimum 3 unit of each block were collected, from live palm with high infection symptom (score 3–4). The fresh fruiting body and not too old with symptom on the upper part still smooth and lower portion with white colour, the thickness of mycelium at the centre part more than 5 mm. Survey and select a fruiting body on an infected oil palm tree were done.

The sample location (estate, block, row number, tree number) and notes into *Ganoderma* sampling form were recorded. The picture of the sample tree and sample of the fruiting body were taken. Fruiting body from palm trunk and cut at the middle of the fruiting body to identify that size of mycelium is enough for the sample, then cover with aluminium foil. Plastic clip was used to identity of an example (sample code) and put into the plastic box then include it and bring to Pathology Laboratory. Total DNA was extracted from *Ganoderma* isolate using cetyl trimethyl ammonium bromide (CTAB) method with minor modification to increase yields as earlier reported [6]. The total DNA was used for sequence analysis.

2.2. Source of sequenced isolates

The origin of isolates was taken for sequencing are from different places. They are from North Sumatera Province (Asahan, Simalungun, Serdang Bedagai, Batu Bara, Tebing Tinggi, Labuhanbatu Utara), South

Sumatera Province from Sumatera Candi Kencana plantation and West Sumatera Province from Pangian Barat plantation as depicted in Table 1. Meanwhile, the isolates were same isolated from oil palm tree and seedlings.

Table 1. Source of sequenced isolates.

No	Isolates	Country	Region	Plantation	Source of Isolate
1.	006PTN3	Indonesia	Asahan	Sei Dadap	Fruiting Body Basal Stem Rot (BSR)
2	I5	Indonesia	Simalungun	Bah Lias	Tissue Upper Stem Rot (USR)
3.	J	Indonesia	Serdang Bedagai	Bangun Bandar	Fruiting Body Basal Stem Rot (BSR)
4.	CS7	Indonesia	South Sumatera	SCK	Fruiting Body Basal Stem Rot (BSR)
5.	I202	Indonesia	Asahan	Aek Loba	Fruiting Body Basal Stem Rot (BSR)
6.	Paya Pinang	Indonesia	Tebing Tinggi	Paya Pinang	Fruiting Body Basal Stem Rot (BSR)
7.	Isolate 192	Indonesia	Serdang Bedagai	Matapao	Fruiting Body Basal Stem Rot (BSR)
8.	NJ72	Indonesia	Batu Bara	Tanah Gambus	Fruiting Body (nursery)
9.	CS1	Indonesia	West Sumatera	Pangian Barat	Fruiting Body Basal Stem Rot (BSR)
10.	Isolate 191	Indonesia	Serdang Bedagai	Bangun Bandar	Fruiting Body BSR (fallen dead trunk)
11.	NJ3	Indonesia	Batu Bara	Tanah Gambus	Fruiting Body (nursery)
12.	001MJIR	Indonesia	Labuhanbatu Utara	MJIR	Fruiting Body Basal Stem Rot (BSR)
13.	NJ54	Indonesia	Batu Bara	Tanah Gambus	Fruiting Body (nursery)

2.3. Sequence and data analysis

Thirteen nucleotide partials named as isolates: 006PTN3, 25, 3J, CS7, 202, Payah Pinang, 192, NJ72, CS1, 191, NJ3, 001MJR, and NJ54. The functional assignment of DNA sequences of 13 isolates was performed based on a similarity search of the sequences against the Genbank non-redundant (nr) peptide database of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using BLASTX [7].

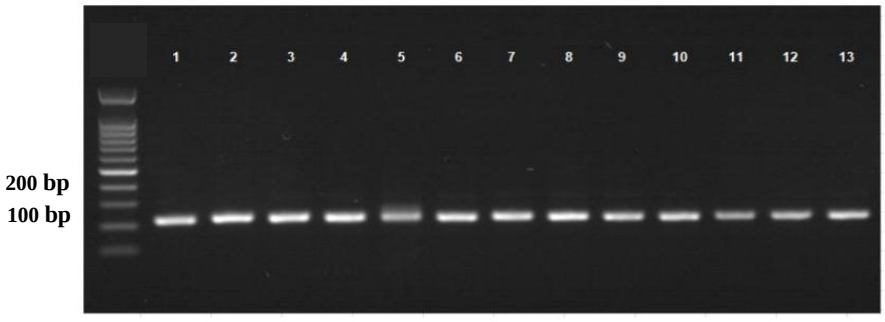

Figure 1. DNA polymorphism of *Ganoderma* isolates using selected SSR primer KT124394

2.4. Phylogenetic analysis of predicted Ganoderma

The DNA sequences were aligned, and similarity scores were obtained using the FASTA version 3.4t26 [8] of the DNA Data Bank of Japan (Mishima, Shizuoka, Japan). The best score of results is shown in Table 3. Phylogenetic analysis of 13 isolates DNA sequences was conducted with CLUSTAL W version 1.83 [9] of the DNA Data Bank of Japan followed by drawing with TreeView, ver. 1.6.6 [10] based on a neighbor-joining method. Bootstrap analysis with 1000 replications was used to assess the strength of the nodes in the tree [11].

3. Results and Discussion

Diseases of BSR have been reported to appear in some areas where oil palm crops are located, covering from Africa such as Congo, Cameroon, Ghana, Nigeria, America such as Honduras, Oceania such as Papua New Guinea and regions in Southeast Asia such as Indonesia and Malaysia [1-5]. Many infections occur with the appearance of the fruiting body at the base of the stem of the oil palm tree, the base of the stem is hollow, the spear leaves do not open, yellowing canopy. BSR can result in huge losses [1-2].

The DNA sequence between 13 isolates of *Ganoderma* from *Elaeis guineensis* shared 89-99% among themselves (Table 1), showing Ganoderma boninense strain NJ3 microsatellite 17b sequence/KT124394.1. The total score varied among the isolates from 228 to 333 (Table 2). Similarly E-value show diversity among the isolates investigated.

Table 2. Description isolates of *G. boninense* using Blastx [8]

Isolates	Description/Accession	Identity (%)	Total Score	E-value
1. 006PTN3	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	95	287	5e-75
2. I5	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	89	228	3e-57
3. J	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	99	327	3e-87
4. CS7	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	99	326	1e-86
5. I202	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	98	326	1e-86
6. Paya Pinang	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	99	333	7e-89
7. Isolate 192	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	99	327	3e-87
8. NJ72	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	99	333	7e-89
9. CS1	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	96	300	7e-79
10. I191	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	98	316	7e-84
11. NJ3	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	94	285	2e-74
12. 001MJIR	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	98	327	3e-87
13. NJ54	*Ganoderma boninense* strain NJ3 microsatellite 17b sequence/KT124394.1	99	327	3e-87

The genotypic diversity of *Ganoderma* spp. on palm oil is quite high. This may be explained by the nature of the fruit body which is the result of different heterokaryon marriages between hyphae. To produce the fruit body, *Ganoderma* spp. must perform sexual reproduction with different hyphae. Thus,

DNA recombination that occurs during sexual reproduction contributes to high genetic diversity [12]. Two types of *Ganoderma* grow on oil palm (dead and alive palm oil) and dead coconut.

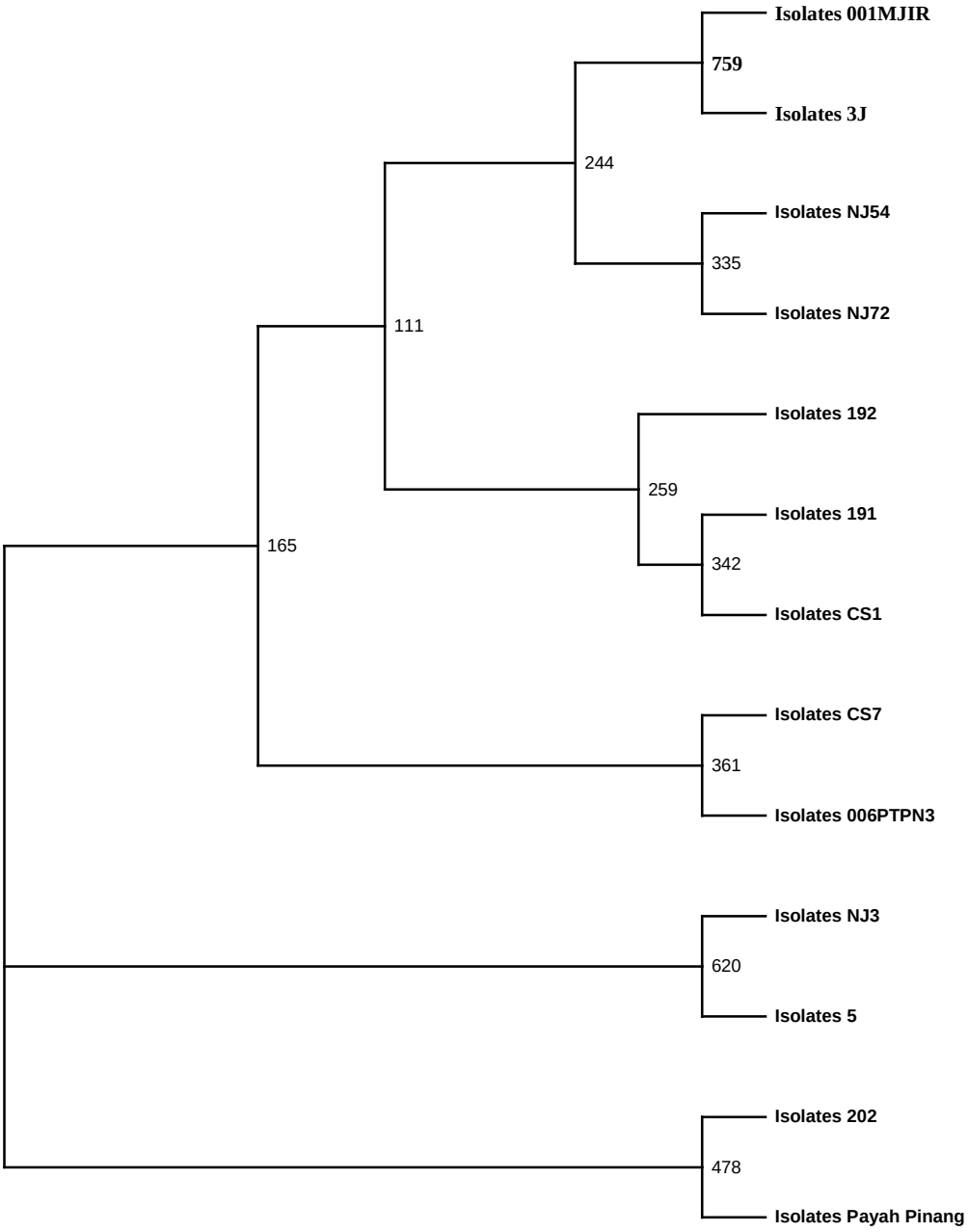

Figure 2. The dendrogram is depicting the genetic relationship of Isolates *Ganoderma* using selected SSR primer KT124394. The indicated scale corresponds to 0.1 DNA sequence substitutions per site. Numbers indicate bootstrap value from 1000 replicates.

Type *Palmae*, *Ganoderma boninense*, is the primary pathogen in oil palm and is usually found in large quantities in dead coconut trunk. The 'forest' type, *Ganoderma tornatum*, usually grows as a saprophyte on the trunk of oil palm and coconut and stump. Also, it is widespread to grow on hardwood [13-14].

To control BSR, knowledge of the characteristics, properties, and behaviour of *Ganoderma* spp. is indispensable. One of the early stages of characterization is molecular identification. Conventional classification methods have limitations in discrimination because of the morphological characteristics of *Ganoderma* spp. may change depending on environmental conditions. This has in some aspects confused the identity of *Ganoderma* species that attack oil palm in Malaysia [4-5].

To confirm the relationship among the isolates, a clustering was carried out as previously described [15]. Figure 2 shows that there are three branches of a phylogenetic tree, namely main branch consisted of 9 isolates: 001MJIR, 3J, NJ54, NJ72, 192, 191, CS1, CS57, and 006PTPN3. The second branch comprised only two isolates: NJ3 and 5. The last branch had only two isolates: 202 and payah pinang.

To confirm the homology among the isolates, the DNA sequence was performed. The isolates show high similarity among the strains (more than 90% similarity each other) as displayed in Table 3. It is noteworthy that the utilization of primer KT124394 was powerful to detect the possibility the existence of *G. boninense* in the oil palm plantation.

Table 3. DNA sequence similarity between isolates

Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13
1. 006PTN3	100												
2. I5	90	100											
3. J	93	90	100										
4. CS7	93	92	94	100									
5. I202	90	90	96	94	100								
6. Paya Pinang	93	92	96	96	95	100							
7. Isolate 192	93	92	97	97	95	97	100						
8. NJ72	91	89	96	97	95	98	95	100					
9. CS1	93	92	94	94	93	95	96	94	100				
10. Isolat 191	90	92	95	96	94	96	98	95	96	100			
11. NJ3	90	90	95	93	93	94	94	93	93	93	100		
12. 001MJIR	92	90	98	96	94	96	98	98	96	98	94	100	
13. NJ54	92	91	96	97	94	96	95	97	95	97	93	98	100

The selected SSR primer KT124394 for sequencing gives the best result, the base pair of all isolates locates on the band 200 bp. To compare with the referenced paper [16] show size (bp) of primer KT124394 is 234-243. Therefore, to ensure the biomolecular *Ganoderma* species present in the collection, this study was conducted using Simple Sequence Repeats (SSR) method for the next study.

4. Conclusions

All the isolates which taken from a different location on this research are *Ganoderma boninense*, usually found as a main virulent pathogen on oil palm tree is known as a causal agent for BSR disease. The present study clarified through DNA sequences that *Ganoderma* from oil palm has been defined as a *G. boninense,* which is identical virulent for the appearance of BSR disease.

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