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SHORT COMMUNICATION

A potential mating-type biomarker to detect pathogenic *Ganoderma* species

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ABSTRACT

Aims: The basal stem rot disease in oil palm is caused by the pathogenic *Ganoderma boninense*, which is infectious after mating and forming dikaryotic hyphae. This study was aimed to generate a mating-type biomarker for the detection of pathogenic *Ganoderma* species.
Methodology and results: Mating-type region of *Ganoderma* was amplified using polymerase chain reaction (PCR) and primers flanking the mating-type region of other basidiomycetes. Amplified fragments were sequenced and were identified as the *Ganoderma* pheromone receptor gene of *mat*B locus called the *gprb2* gene. Using this biomarker, the pheromone receptor gene was detected in a total of 107 pathogenic *Ganoderma* spp. while the gene was not detected in the non-pathogenic *Ganoderma lucidum*. Phylogenetic tree analyses of the gene fragment encoding the partial amino acid sequence of *gprb*2 showed clades of close evolutionary relationship among the 107 pathogenic *Ganoderma* spp. Phylogenetic analyses using deduced amino acid sequences of the *Ganoderma* pheromone receptor b2 gene, *gprb*2 with homologous pheromone receptors of other basidiomycetous fungi revealed high conservation of this pheromone receptor within their respective taxonomy.
Conclusion, significance and impact of study: A potential mating-type biomarker was successfully identified that could detect pathogenic *Ganoderma* spp. The research findings will be helpful in oil palm screening to detect pathogenic *Ganoderma* spp. and gain further insight into the role of the mating-type loci of *Ganoderma* towards its pathogenesis in causing the basal stem rot disease of oil palm.

Keywords: Pheromone receptor, *Ganoderma boninense*, basal stem rot disease, oil palm

INTRODUCTION

Cultivation of the African oil palm (*Elaies guineensis* Jacq.) in Malaysia has been practiced for the commercial production of palm oil. The palm oil industry has contributed significantly to the Malaysian economy, with an estimated gross domestic product (GDP) of MYR 36.87 billion in the year 2020. However, oil palms are under the threat of basal stem rot (BSR) disease caused by the white-rot fungus of the *Ganoderma* species, also known as *G. boninense* (Ho and Nawawi, 1985; Idris *et al.*, 2000; Wong *et al.*, 2012; Bharudin *et al.*, 2022). The high incidence of BSR severely decreases the palm oil yield and threatens the Malaysian oil palm industry, causing an estimated loss of up to USD 500 million a year (Arif *et al.*, 2011; Ommelna *et al.*, 2012).

The pathogenic basidiomycete *G. boninense* causes BSR in oil palms by colonizing the cortex, endodermis, pericycle, xylem, phloem and pith of the palm (Rees *et al.*, 2009). The emergence of basidiomata on the palm trunk base indicates long-term dissemination and is usually too late to save the palm (Siddiqui *et al.*, 2021). As a basidiomycete, *Ganoderma* undergoes the sexual life cycle to reproduce. The mating system of *G. boninense* is determined to be heterothallic and tetrapolar (self-incompatible for mating) with multiple alleles at both mating type loci (Pilotti *et al.*, 2002; 2003). Two mating loci, known as *mat*A and *mat*B, are responsible for

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regulating mating specificity. Successful mating requires both parental strains to differ in at least one allele at both the A and B loci. Mating in the basidiomycete plant pathogen such as *Ustilago maydis* and other smut fungi is directly related to virulence or pathogenicity, whereby successful mating or sexual reproduction is required to form infectious dikaryotic hyphae before being able to infect a host plant (Bakkeren *et al*., 2008). Likewise, *G. boninense* in its monokaryotic mycelial stage was not able to cause infection but reconstituted dikaryotic mycelia could initiate basal stem rot infection on oil palm through pathogenicity tests (Chan *et al.*, 2011). Currently, however, there is little known about the relation of the *Ganoderma* mating system to its pathogenicity in causing BSR in oil palm. Previous reports have largely been focused on other control strategies, such as in-field detection, bio-control and omics approaches (Isaac *et al*., 2018; Madihah *et al*., 2018; Ramzi *et al*., 2019; Yusoff *et al*., 2021). Therefore, this study was conducted to generate a marker based on the mating-type locus of *Ganoderma,* which can be used for the detection of pathogenic *Ganoderma* species.

MATERIALS AND METHODS

Fungal cultures and growth conditions

A total of 106 isolates of *Ganoderma* spp. (Table 1) were obtained from ACGT Sdn. Bhd. in Kuala Lumpur, Malaysia. These fungal cultures were isolated from fruiting bodies grown from BSR infected oil palms of Genting Plantation Estates, Malaysia and were pathogenic towards oil palm seedlings after being subjected to pathogenicity tests (unpublished data) according to the method described by Sariah *et al.* (1994). One pathogenic *Ganoderma* culture, *G. boninense* PER71 was obtained from the Malaysian Palm Oil Board (MPOB) of Bandar Baru Bangi in Selangor, Malaysia, while a non-pathogenic *Ganoderma lucidum* culture, GANOD-0000030 was obtained from ACGT Sdn. Bhd and was not pathogenic towards oil palm seedling after being subjected to a pathogenicity test (unpublished data). All the cultures were grown on Potato Dextrose Agar (PDA) plates. For DNA extraction purpose, plugs of *Ganoderma* sp. cultures grown on PDA were sub-cultured into Potato Dextrose Broth (PDB) and incubated for a week at 30 °C without agitation until the isolates grew as a mycelial mat on the surface of the broth. The mycelial mats were dried using C-fold paper towels, weighed and frozen at –80 °C for further use.

Polymerase chain reaction (PCR) amplification of *Ganoderma* mating-type loci

Genomic DNA was isolated from the *Ganoderma* spp. cultures by first lysing the frozen *Ganoderma* mycelia into powder with Qiagen TissueLyser II (Germany) followed by genomic DNA extraction according to the methods described by Raeder and Broda (1985). A total of 20 primer pairs that flank the mating-type genes of other basidiomycetes such as *Serpula lacrymans*, *Phaenerochaete chrysosporium*, *Pleurotus djamor*, *Volvariella volvacea*, *Coprinellus disseminatus* and *Flammulina velutipes* (Table 2) were synthesized. The genomic DNA of *Ganoderma boninense* GANOD-0000009 was used as a proxy for PCR amplification work in order to test the primers (Table 2). Each PCR reaction contained 2.5 µL of 10× Taq buffer, 0.75 µL of 50 µM $MgCl_2$, 0.5 µL of 10 µM dNTPs, 0.2 µL of Taq DNA Polymerase (5 U μL^{-1}, Invitrogen™), 2 µL of 2.5 µM forward and reverse primers each and 10 ng of DNA template, brought up to a final volume of 25 µL with ultra-pure water. Gradient PCR was performed to determine the optimal annealing temperatures for the primers in an Eppendorf 5331 Mastercycler® Gradient (Germany) thermal cycler under these conditions: 5 min at 95 °C, followed by 30 cycles of 45 sec at 95 °C, 45 sec at 40-60 °C and 45 sec at 72 °C and a final extension step at 72 °C for 10 min. Aliquots of 5 µL of each reaction were analyzed on 1% (w/v) agarose gel in TAE buffer. Positively PCR-amplified DNA products from the mating-type primers were purified using ExoSAP-IT™ and sequenced using Applied Biosystems 3730 XL DNA analyzer (USA) at Genting Laboratory Services Sdn. Bhd. (Malaysia). The sequences were identified using the Basic Local Alignment Search Tool (BLAST) program of the National Center for Biotechnology Information, NCBI (https://blast.ncbi.nln.nih.gov/), GenBank (NCBI) and the ACGT's proprietary *Ganoderma* database of ACGT Sdn. Bhd. in Kuala Lumpur, Malaysia.

Table 1: Pathogenic *Ganoderma* spp. isolated from BSR infected oil palm in Malaysia.

Location of plantation	Number of *Ganoderma* samples
Kedah	18
Melaka	31
Selangor	5
Johor	44
Sabah	8
Total	106

Primer design of the target biomarker

The *G. boninense* specific primer pair, designated as GPRB2 forward and reverse, was designed using the Primers-BLAST program by NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the annotated mating-type sequences from ACGT's proprietary *Ganoderma* database (GPRB2-F: '5-TGT AAA ACG ACG GCC AGT GCC GCG AGC ACC TTC TTG TA-3' and GPRB-R: 5'-CAG GAA ACA GCT ATG ACC TTC GTC GCA CTC TCA GGC AC-3').

PCR screening for the mating-type locus in pathogenic *Ganoderma* spp.

A PCR screening using the GPRB2 primers as markers for amplification of the mating-type locus was carried out

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Table 2: Primers used to amplify the mating-type loci of *Ganoderma* spp.

No	Primers	Primer sequence (5' to 3')	Reference
1	MATA11-F	GCC TCT TGG TTG TTT TTA TTG	Skrede *et al.* (2013)
	MATB1-R	TCG TAG GAC GGC ATC CAA AGC	
2	PcMIP3-F	AGT GCT TCT GTG CGA GTT CA	James *et al.* (2011)
	PcMIP3-R	ATT GCC CGA TCG AAG AGA TA	
3	PcMIP4-F	ACT ACG CGC CCT CAG TCT AC	James *et al.* (2011)
	PcMIP4-R	AAC AGC GGA CGG TGT AGT G	
4	PdMIP1-F	CGA GTA CAC CTT CGT CAG CA	James *et al.* (2004)
	PdMIP1-R	GGC ATG GAA GAC TTG ACT CC	
5	PYd21MIP-F	CAA CTG CCA CTC CAA TAA TCC	Chen *et al.* (2012)
	PYd21MIP-R	CCC CTC TAA AAC TTT CCC CTC	
6	MIP1-F	CCA TYC TMA TGG ARC ACT TCC TCA	James *et al.* (2004)
	MIP1-R	ACC TCY YKC TTG TAC YKY TCG CCC	
7	MIP2-F	TTC CAY GAR ATG GGN CAY GCN AT	James *et al.* (2004)
	MIP2-R	RTA NCC RAA NAR RTG NCC RAA	
8	CdMIP-F	CTG CGG GCA ACT GGR AAC AA	James *et al.* (2006)
	CdMIP-R	GAA GGA CGT CTC TGG CAC ATA	
9	matAa-F	TCA GGT GGA AGT TTG GGT A	Wang *et al.* (2016)
	matAa-R	GGG CAA TGC GTA GGG TAT	
10	matAb1-F	TGA CAC GCC TGG TCT CCT G	Wang *et al.* (2016)
	matAb1-R	GCG AGT GCC CTC TGC TAC AT	
11	matAb2-F	ACG CAG CAC GCT TGA GAA	Wang *et al.* (2016)
	matAb2-R	GGA AGA TGG TGG CGA AGA	
12	Fl matA 1.1-F	GAG GAG GTG ATC CAA ATA GC	van Peer *et al.* (2011)
	Fl matA 1.1-R	TCT GCC GCA CCT CTG TGT TG	
13	Fl matA 2.1-F	CTC TCA GCC TTC GCC ATA TC	van Peer *et al.* (2011)
	Fl matA 2.1-R	TTT CGT CCT CCA CGA CTA CC	
14	matA HD2.2-F	TTG TCG GGC CAA TAT CAA GG	van Peer *et al.* (2011)
	matA HD2.2-R	TTC AGG CAG CGC TTA AAG TC	
15	MATB1-F	TCC TTC GCA CCT CAT GGC AGC	Skrede *et al.* (2013)
	MATB1-R	TCG TAG GAC GGC ATC CAA AGC	
16	PcSTE3.1-3F	TTC GCA AAC GTT GTC TAT GG	James *et al.* (2011)
	PcSTE3.1-3R	GAG CGC AAG AAG TGC AAA TA	
17	PdCLA4-F	CTT CGC ACA GAA ACC GAA AT	James *et al.* (2004)
	PdCLA4-R	GGT GCG TTG ACG GAC ATA AT	
18	CdSTE3.1-F	CAT CGC TCC TGT ATG GTG TG	James *et al.* (2006)
	CdSTE3.1-R	CTG GAG AAT AGG GAC GCA AA	
19	CdSTE3.2-F	TCG ATC GTA TGG AAC GRT AA	James *et al.* (2006)
	CdSTE3.2-R	AKC GTC TAG GYG TGA GGT TC	
20	CdSTE3.3-F	CCC ATT TGG TGT GAC ATC TG	James *et al.* (2006)
	CdSTE3.3-R	GGT CAA GAG CTG GCT GAA CT	

on the 106 pathogenic *Ganoderma* spp., including *G. boninense* PER71 *(*Sundram *et al.*, 2011) as the positive control (pathogenic) and *G. lucidum* GANOD-0000030 as the negative control (non-pathogenic). PCR mixtures and conditions were the same as mentioned above.

Bioinformatics analyses

Amplicon sequences of 106 *Ganoderma* spp. and *G. boninense* PER71 derived from the PCR amplification with GPRB2 primers were aligned using ClustalW. A phylogenetic tree was constructed with a bootstrap value of 1000 by the neighbour joining method using MEGA6 software (Tamura *et al.*, 2013) using the nucleotide sequences of 107 amplicons from the pathogenic *Ganoderma* spp. Selected nucleotide sequences of amplicons were also translated into amino acid sequences using the ExPASy server (https://web.expasy.org/translate/). Multiple sequence alignments were then conducted with the homologous mating-type proteins of other basidiomycetes downloaded from GenBank (https://www.ncbi.nlm.nih.giv/genbank) using ClustalW. Phylogenetic tree was constructed with a bootstrap value of 1000 by neighbour joining method using MEGA6 software.

RESULTS AND DISCUSSION

Amplification of *Ganoderma* mating-type locus

PCR amplicons were detected for two sets of primer pairs out of the 20 pairs of mating-type primers tested, which

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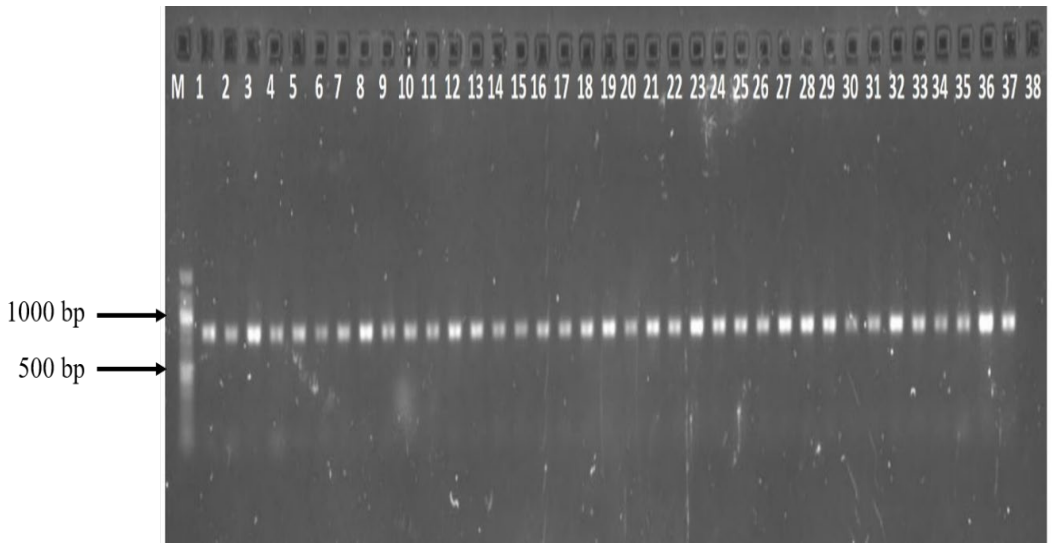

Figure 1: Agarose gel electrophoretic profile of PCR products amplified using primer pair GPRB2 and genomic DNA templates of 38 representative *Ganoderma* species. Lane M: DNA marker 1kb ladder (NEB); Lane 1-35: Pathogenic *Ganoderma* spp.; Lane 36: *G. boninense* GANOD-0000009; Lane 37: *G. boninense* PER71; Lane 38: *G. lucidum* GANOD-0000030.

were CdMIP-F and CdMIP-R and CdSTE3.3-F and CdSTE3.3-R. These amplicons were subsequently purified and sequenced. BLASTn analysis using nucleotides amplified by CdMIP primers and BLASTx analysis using translated nucleotides for the *G. boninense* GANOD-0000009 sample did not show any similarity with mating-type regions of fungi, whereas BLASTn analysis for the sequence amplified by CdSTE3.3 primers indicated a 98% sequence similarity with a 78% coverage to *G. boninense Q9C1Q9* gene for pheromone receptor CPRa1p (GenBank LR727589.1). Analysis of these sequences using ACGT's proprietary *Ganoderma* database revealed that the sequences were annotated as the *mat*B mating-type pheromone receptor gene. Therefore, a more targeted new primer pair, GPRB2 Forward and GPRB2 Reverse, was designed (annealing temperature: 65.7 °C) that produced amplicons with a fragment length of 850 bp.

Pheromone signaling plays a vital role in mating and it is the *mat*B locus that encodes for pheromones and pheromone receptors. Pheromones are only able to activate receptors from a different allele which is to promote outbreeding (Jones and Bennett, 2011). The amplified fragment by GPRB2 primers was also identified as *G. boninense Q9C1Q9* gene for pheromone receptor CPRa1p (GenBank LR727589.1) through BLASTn search with 92% sequence similarity and a 91% coverage. BLASTx search was then performed using the translated nucleotides, resulting in 96% sequence similarity to *G. boninense* pheromone receptor CRPa1p (GenBank VWO99365.1) with 98% coverage. The GPRB2 primers were able to amplify the pheromone receptor gene encoded by the *mat*B locus in *Ganoderma* which would be a potential mating-type biomarker to identify and detect pathogenic *Ganoderma* species.

PCR assay for amplification of mating-type region in pathogenic *Ganoderma* spp.

All 106 pathogenic *Ganoderma* spp. and the pathogenic *G. boninense* PER71 produced PCR bands at the length of 850 bp using GPRB2 primers while no amplification was detected for *G. lucidum* GANOD-0000030 (Figure 1). *G. lucidum* is found to cause BSR in coconut trees only but is not pathogenic towards oil palms (Bhaskaran, 2000; Idris *et al*., 2000). The *Ganoderma* pheromone receptor gene facilitates the pheromone signaling for sexual reproduction (Madihah *et al*., 2019; Bharudin *et al*., 2022) and could be crucial for the mating of monokaryotic *Ganoderma* to the pathogenic dikaryotic phase that enables the fungus to infect and cause BSR in oil palm host. As such, the GPRB2 marker could be used to detect pathogenic from non-pathogenic *Ganoderma* species.

Bioinformatic analyses

The phylogenetic tree constructed using the pheromone receptor gene amplified by GPRB2 marker for 107 pathogenic *Ganoderma* spp. (Figure 2) displayed a topology of 3 main clades (Clade 1, 2 and 3) with a strong bootstrap value of over 70%. Out of the 107 pathogenic *Ganoderma* spp., 101 or about 94% formed the biggest clade (Clade 1) with a strong bootstrap value of 99%. The pheromone receptor gene encoded by *Ganoderma mat*B locus was shown to be highly conserved and could have evolved monophyletically from a common ancestor. To further understand the evolutionary relationship of *mat*B in *Ganoderma*, the pheromone receptor gene was translated into protein sequences and a phylogenetic tree was constructed with pheromone receptor proteins of other basidiomycetes (Figure 3) using MEGA6.

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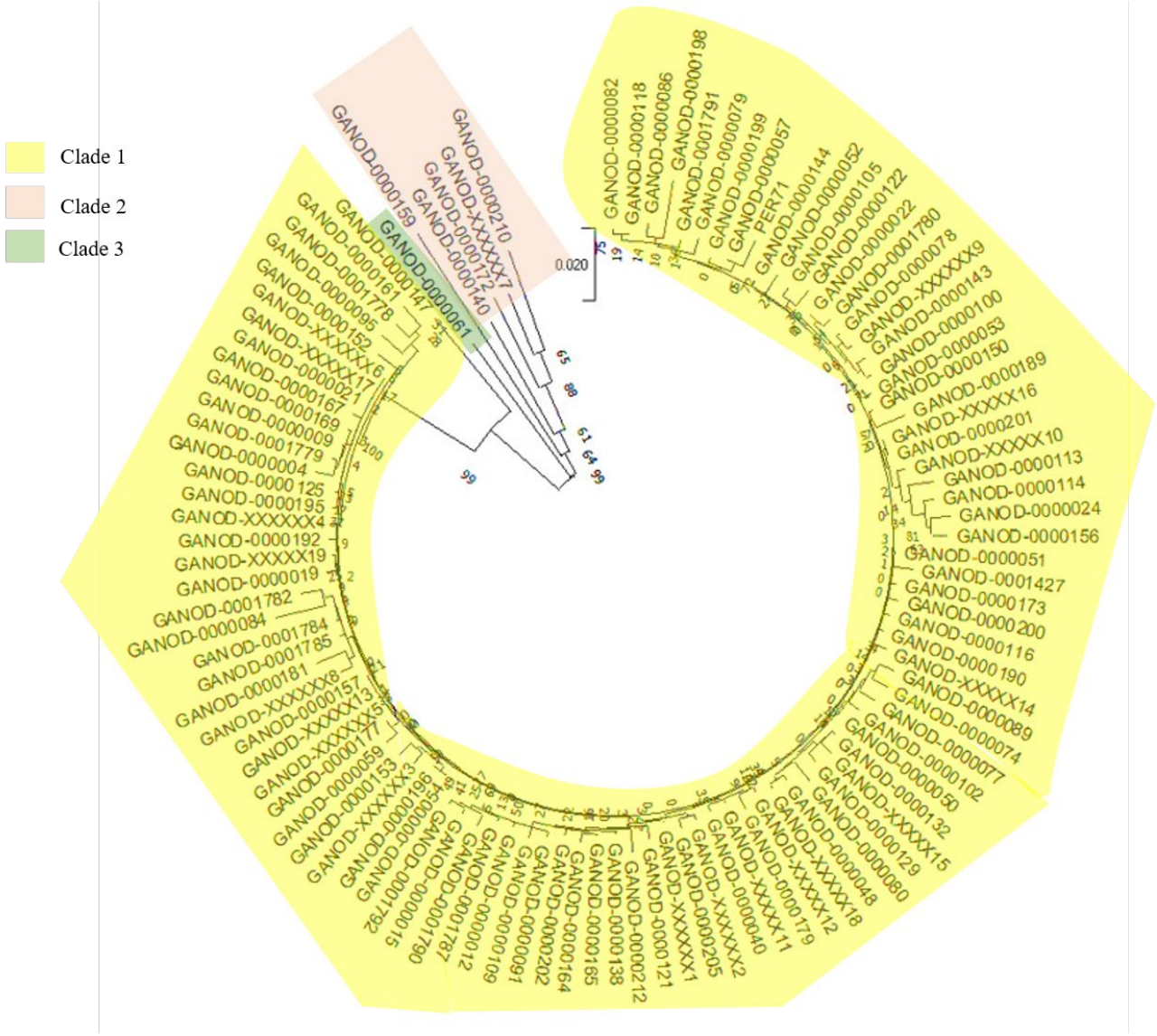

Figure 2: Phylogenetic tree constructed using the pheromone receptor gene of *mat*B locus of 107 *Ganoderma* spp. that are pathogenic towards oil palm. The tree was constructed by neighbour-joining with bootstrap confidence value for 1000 replicates as indicated at the nodes. The scale bar represents 0.02 substitutions per nucleotide position.

Pheromone receptor from the yeast *Saccharomyces cerevisiae* (GenBank CAA82019.1) was used as an outlier for the construction of the phylogenetic tree. It can be seen from the constructed tree that the pheromone receptor proteins of the basidiomycetes were grouped according to the taxonomic classification. The *Ganoderma* pheromone receptor is grouped with the proteins of fungi from the Agaricomycetes class, such as *Trametes coccinea*, *Trametes cinnabaria*, *Coprinellus disseminatus* and *Pleurotus djamor*. Fungi that belong to the Agaricomycetes class are species that produce basidiocarps. The pheromone receptor of *Ganoderma* was related the closest to *Trametes* species and they belonged to the order Polyporales, which consists of wood-rotter species. Pheromone receptors of smut fungi such as *Ustilago maydis* (GenBank AAA34228.1) and *U. hordei* (GenBank CAJ41875.1) formed the second clade of the phylogenetic tree. They are from the Ustilaginomycetes class and are smut fungi that cause the smut disease in grasses, maize and wheat (Money, 2016; Martínez-Soto *et al.*, 2020). Basidiomycetous fungi identify compatible partners by means of mating pheromones and recognition by pheromone receptors, which are highly conserved within their taxonomy (Raudaskoski and Kothe, 2010; Coelho *et al.*, 2017).

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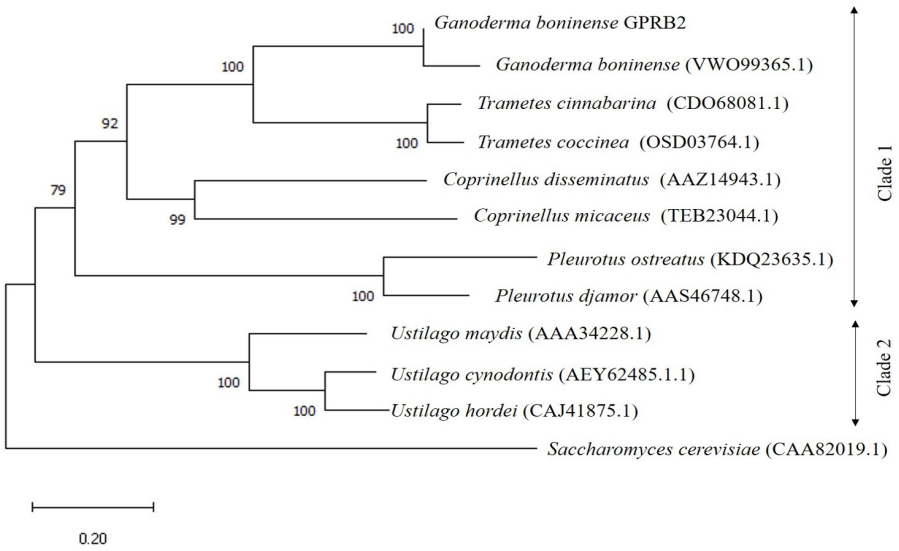

Figure 3: Phylogenetic tree constructed using *Ganoderma* pheromone receptor protein GPRB2 with pheromone receptors of other basidiomycetes. The tree was constructed by neighbour-joining with bootstrap confidence value for 1000 replicates as indicated at the nodes. The scale bar represents 0.20 substitutions per nucleotide position.

CONCLUSION

A potential mating-type biomarker GPRB2 has been identified based on the *Ganoderma* pheromone receptor gene of the *mat*B locus. The discovery of this marker may lead to more insights to the relationship and the role of *Ganoderma* mating-type locus with regards to its pathogenesis to oil palm. The potential marker discovered in this study, however, requires further validation to investigate its robustness in discriminating between pathogenic and non-pathogenic *Ganoderma* species in screening oil palm. Nonetheless, the finding of this research would facilitate in formulating early detection measures via rapid screening assays such as the Loop-Mediated Isothermal Amplification (LAMP) to detect the *Ganoderma* pathogens that cause devastating BSR disease in commercial oil palm plantations in Malaysia.

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