

A RAPID AND EFFICIENT DNA EXTRACTION PROTOCOL FOR *Ganoderma zonatum*, A BASAL AND UPPER STEM ROT PATHOGEN OF OIL PALM IN MALAYSIA

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ABSTRACT

Ganoderma zonatum is associated with both the basal and upper stem rot diseases in oil palm. Despite the severity of these diseases, there is only limited information on the molecular characteristics of this oil palm pathogen. Most of the studies on *G. zonatum* related to oil palm are focused on the epidemiology and genetic diversity of the organism. In other palm species, *G. zonatum* has also been identified as the causal agent of bud rot disease. To further characterise the organism using molecular techniques, the ability to isolate good quality DNA samples is important. In this study, seven DNA extraction protocols were evaluated and the best protocol, Boehm protocol, had the highest yield of good quality DNA. The protocol was able to yield $208.95 \pm 4.52 \mu\text{g}$ DNA per gram of sample with purities above 1.80 for $A_{260/280}$ and 2.0 for $A_{260/230}$. This extraction protocol is a rapid and efficient protocol that employs cetyl trimethylammonium bromide (CTAB), sodium dodecyl sulphate (SDS), β -mercaptoethanol and proteinase K in the lysis buffer. The Boehm protocol was further tested on three other *Ganoderma* species found in the oil palm plantations and a medicinal fungus, *G. lucidum*. It was noted that the protocol was efficient, with high yields for *G. zonatum* when compared to the other four species. This is probably due to the fact that extraction protocols for each organism requires specific optimisation to obtain optimal yield and purity. In conclusion, the Boehm protocol was best suited for genomic DNA extraction of *G. zonatum* and found suitable for downstream applications such as PacBio sequencing.

Keywords: basal stem rot, DNA extraction, *Ganoderma zonatum*, mycelium, upper stem rot.

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INTRODUCTION

Ganoderma zonatum is a fungus that belongs to the phylum Basidiomycota, class Agaricomycetes, order Polyporales and family Ganodermataceae (Steyart, 1967). *Ganoderma zonatum* has been associated with basal stem rot (BSR) disease of oil palm in South-east Asia, a disease that has threatened the Malaysian oil palm industry with an export revenue of RM 65.12 billion (Kushairi *et al.*, 2019). It is a well-known fact that BSR in Malaysia, Indonesia and Papua New Guinea (PNG) is caused predominantly by the pathogen *Ganoderma boninense*. Another *Ganoderma*

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species that was reported to cause BSR was *G. miniatocinctum*, while *G. tornatum* is a saprophyte that is found in oil palm plantations (Idris, 1999).

Idris (1999) reported the presence of both *G. boninense* and *Ganoderma miniatocinctum* was associated with the presence of *Ganoderma zonatum* in the oil palm plantations. *Ganoderma zonatum* was also reported to cause other diseases such as upper stem rot (USR) disease in Malaysia (Hassan *et al.*, 2005; Rakib *et al.*, 2014; 2017), Indonesia (Rees *et al.*, 2012) and PNG (Pilotti, 2005). Rakib *et al.* (2015) found that *G. zonatum* from USR-infected palms tend to be more aggressive than *G. zonatum* from BSR-infected palms in Malaysia. On the contrary, *G. boninense* from BSR-infected palms were more aggressive than *G. boninense* from USR-infected palms. Here the different degree of aggressiveness was evaluated based on criteria such as area under disease progress curve (AUDPC), epidemic rate, severity of foliar symptoms, disease severity index (DSI), stem bole and primary root necrosis. As an example, the mean DSI recorded for *G. zonatum* of USR-infected palms (6.84%) was significantly higher compared to BSR-infected palms (2.39%) at 12 weeks after infection. In the USA, *G. zonatum* is also a lethal pathogen that causes butt rot in over 60 palm species (Elliott *et al.*, 2018) and this is of great concern in Florida. The only way to curb the spread of butt rot is through prevention, as commonly practised in the USA (Elliott and Broschat, 2000).

Recently, Elliott *et al.* (2018) examined the genetic variability of 25 *G. zonatum* isolates infecting 12 different palm species in Florida and an additional 17 more isolates from eight different countries. The DNA were extracted using the Gentra® Puregen® Cell Kit (Qiagen) (Elliott *et al.*, 2018). As for *G. zonatum* in BSR (Rakib *et al.*, 2017; Wong *et al.*, 2012) and USR (Rakib *et al.*, 2017) studies in oil palm, the DNA were extracted using DNeasy Mini Plant Kit (Qiagen). Numerous studies have highlighted that cell lysis efficiency, DNA yield and quality depend largely on sample type and protocols used (Gul *et al.*, 2017; Nagi *et al.*, 2013; Natarajan *et al.*, 2016; Perry *et al.*, 2014; Umesha *et al.*, 2016). Most of the fungi studied to date require modifications to existing extraction protocols to produce good quality and high yield of nucleic acids (Motkova and Vytrasova, 2011; Nagappan *et al.*, 2018; Verant *et al.*, 2016). It is pertinent to establish the best extraction protocol for each fungus that is studied in depth. Moreover, it would be an added advantage if one DNA extraction protocol is feasible to be used across most fungi species or on closely related species.

It would be beneficial to be able to go a step further to study any organism at the nucleotide level (Choi and Kim, 2017; Li *et al.*, 2018). In order for any sequencing project to proceed, such as PacBio sequencing, large amount of good purity DNA is required to meet this requirement. Usage of

extraction kit is not suitable as it yields low amount of DNA and is not cost-effective (Pipan *et al.*, 2018; Psifidi *et al.*, 2015; Umesha *et al.*, 2016). Thus, the current study explored and recommend a time efficient and cost-effective DNA extraction protocol of *G. zonatum* found in the oil palm plantations in Malaysia. Here, we presented a rapid and suitable DNA extraction protocol with minimal amount of tissues required to produce high yields of good purity DNA.

MATERIALS AND METHODS

Biological Materials

Ganoderma zonatum, *G. boninense*, *G. miniatocinctum* and *G. tornatum* stocks were obtained from the GanoDROP Unit, Biology Division, Malaysian Palm Oil Board (MPOB), Malaysia. The fruiting body of *G. lucidum* was purchased from the Cepaul Mushroom Nursery, Kajang, Selangor, Malaysia. Actively growing eight day old mycelia were transferred into 250 ml of Potato Dextrose Broth and incubated at 27±1°C for 14 days. The cultures were harvest and washed with sterilised distilled water and frozen in liquid nitrogen prior to DNA extraction.

Genomic DNA Isolation of *Ganoderma* Species

A total of seven protocols were tested for genomic DNA isolation of *G. zonatum*. The first and second protocol used cetyl trimethylammonium bromide (CTAB) (Voigt *et al.*, 1999) and sodium dodecyl sulphate (SDS) (Moslem *et al.*, 2010) extraction buffers, respectively. The third protocol tested, as described by Kim *et al.* (1997) used polyvinylpyrrolidone (PVP) and SDS, whereby, the subsequent protocol also applied SDS as one of the components in the extraction buffer (Weiland, 1997). The fifth protocol was an online protocol by Vilgalys (2018), whereby he applied a 2 X CTAB extraction buffer. The second last extraction protocol investigated, employed Triton X-100 and SDS as the lysis buffer (Extraction method 1: Van Burik *et al.*, 1998). The final protocol employed a combination of CTAB, SDS, β-mercaptoethanol and proteinase K (Boehm, 2004). RNAse was added in all the protocols evaluated. All the protocols explored were described using the respective author's name for easy identification. The quantity and quality of extracted nucleic acids were measured using Nanodrop™ 1000 Spectrophotometer.

Analysis of DNA by Agarose Gel Electrophoresis

The quality and size of DNA was determined on 0.8% (w/v) agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1mM EDTA), which was

precast with 3 μl of EtB 'Out' nucleic acid staining solution (Yeastern Biotech Co., Ltd). Two microlitres (50 $\text{ng } \mu\text{l}^{-1}$) of sample were loaded into each lane. Electrophoresis was performed at 80 V for 40 min and the agarose gel was post-stained with the same staining solution for 10 min. The agarose gel was visualised using the G:BOX Chemi XX9 (Syngene).

Statistical Analysis

All extractions were performed in triplicates to account for variability. One way analysis of variance (ANOVA) was used to determine statistical differences of DNA yield between protocols using a significance level of $P < 0.05$. A T-test was used to compare mean DNA yield between two protocols. Data are shown as the mean \pm SD.

Polymerase Chain Reaction (PCR) Amplification

To verify the quality of the extracted DNA, the DNA was used as a template in a PCR experiment to amplify a microsatellite region, as described by Merciere *et al.* (2015). The size of DNA was determined on 4.0% (w/v) Super Fine Resolution (SFR) agarose gel in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, and 1mM EDTA), which was precast with 12.5 μl of EtB 'Out' nucleic acid staining solution (Yeastern Biotech Co., Ltd). Six microlitres of PCR product were loaded into each lane. Electrophoresis was performed at 100 V for 3 hr and the SFR agarose gel was post-stained with the same staining solution for 10 min. The agarose gel was visualised using the G:BOX Chemi XX9 (Syngene).

RESULTS AND DISCUSSION

In the process of identifying the best DNA extraction protocol for *G. zonatum*, seven protocols were evaluated on this pathogen that is associated with BSR, USR and butt rot. Six protocols that were evaluated have been used on fungi from different phylum such as Basidiomycota, Ascomycota, Zygomycota, Deuteromycota and Oomycota. The seventh protocol (Kim protocol) is a simple and rapid protocol used to extract DNA from fruit trees and conifers that have high levels of polyphenols. This protocol was selected as high level of polyphenols have been reported in *Ganoderma lucidum* (Dong *et al.*, 2019) and was thought to be suitable for *G. zonatum*. We also included a commonly used protocol, CTAB as a positive control in this study.

The starting mycelial weight used in these seven protocols was 0.1 g and the amount of DNA recovered ranged from ~ 13 - ~ 260 $\mu\text{g DNA g}^{-1}$ of sample (Table 1). The integrities of the extracted DNA were separated and observed in 0.8% agarose

gel (Figure 1). All the seven protocols were able to extract intact high molecular weight genomic DNA. The bands from the Moslem and Kim protocols in lanes 2 and 3, respectively appeared to be brighter with a slight smear, indicating some levels of DNA contamination has occurred. The protocol that produced the highest yield recorded from this study was with the Van Burik protocol that used a combination of two components in the lysis buffer. In this protocol, the application of Triton X-100 and SDS were capable of disrupting the fungal cell wall and membrane wall producing a yield of 259.77 ± 6.67 $\mu\text{g DNA g}^{-1}$ of sample, which is significantly higher ($P < 0.05$) compared to the other six protocols. However, the average purity for $A_{260/230}$ was way below the standard absorbance ratio of 2.0 and above. The average absorbance ratio of $A_{260/230}$ measured by Nanodrop was 0.89 ± 0.05 , which indicates high levels of polysaccharides detected in the extracted DNA.

The protocols by Kim, Vilgayls, Weiland and Moslem also had high levels of polysaccharide contamination, with low absorbance ratios between 0.26 ± 0.01 to 1.14 ± 0.04 for $A_{260/230}$. These are indications of the presence of contaminant biomolecules. These protocols also had exceptionally low yields, between 12.67 ± 0.03 to 65.17 ± 2.00 $\mu\text{g DNA g}^{-1}$ of sample. The results demonstrated that the lysis buffers employed were not sufficient in breaking down the cell and membrane walls of *G. zonatum*. On the contrary, the Boehm protocol with a mixture of several components such as CTAB, SDS, β -mercaptoethanol and proteinase K in the buffer was able to remove contaminated biomolecules such as proteins, carbohydrates, lipids or other nucleic acids, producing absorbance ratios of 2.14 and 2.22 for $A_{260/280}$ and $A_{260/230}$ respectively. The expected ratios of $A_{260/280}$ and $A_{260/230}$ for 'pure' DNA are commonly within the range of 1.8 - 2.0 and 2.0 - 2.2 (Seo *et al.*, 2011; Lucena-Aguilar *et al.*, 2016). It was notable that this protocol produced a yield of 208.95 ± 4.52 $\mu\text{g DNA g}^{-1}$ of sample, which was only a reduced yield of ~ 50 $\mu\text{g DNA g}^{-1}$ of sample when compared to the van Burik protocol. The combination of components in this buffer were extremely efficient in the lysis of cell wall and cell membrane of *G. zonatum*. Due to the anionic and cationic conditions of SDS and CTAB, these buffers work well with each other in solubilising proteins and lipids (Tripathy *et al.*, 2017). Generally, *Ganoderma* species contains β -glucans as the major active polysaccharides (Obodai *et al.*, 2017) and are rich in phenolic compounds from fruiting bodies and mycelia (Mishra *et al.*, 2018). The inclusion of β -mercaptoethanol with SDS and CTAB aided in the breaking of disulfide bonds between the cysteine residue present in the crude extract (Wingfield, 2001; Winther and Thorpe, 2014). Furthermore, β -mercaptoethanol is an antioxidant that is commonly used to address issues related to

phenolics (Calderon-Cortes *et al.*, 2010; Sahu *et al.*, 2012). Meanwhile, proteinase K which works better in the presence of SDS, inactivates DNases and leaves the DNA complete and intact. This suggests that the DNA extracted by the Boehm protocol was able to produce good quality DNA for *G. zonatum*. The Boehm protocol was reported by Gracia *et al.* (2014), where it was applied as a modification to the existing Moller protocol to produce good quality DNA from two fungal pathogens of alfalfa, *Phoma medicaginis* and *Colletotrichum trifolii* that was suitable for internal transcribe spacer sequencing.

The commonly used Voigt (CTAB) protocol also produced good quality DNA in terms of absorbance ratio of $A_{260/280}$ and $A_{260/230}$ (Table 1). Nevertheless, the yield was 2.4x lower with $87.32 \pm 5.21 \mu\text{g DNA g}^{-1}$ of sample when compared to the Boehm protocol. Albeit, the Voigt protocol is still viable but the drawback to this protocol is that additional material is required to achieve higher yields of DNA and it is labour intensive. The duration required for the Voigt protocol is two days, in contrast to the Boehm protocol which takes approximately 4 hr from the beginning of the process until DNA is ready for use. Time is of the essence especially when a large number of samples are needed to be tested or sequenced.

To further assess the reliability of the Boehm extraction protocol and attest on the viability of one protocol used across closely related fungi species, the three *Ganoderma* species that were found in oil palm plantations and one reference species, *G. lucidum* were evaluated. It was noted that *G. boninense*, *G. miniatocinctum*, *G. tornatum* and *G. lucidum* produced rather low yields compared to *G. zonatum*, ranging from 33.40 ± 1.25 to $89.83 \pm 1.26 \mu\text{g DNA g}^{-1}$ of sample (Table 2). Nevertheless, the *G. miniatocinctum* DNA was of good quality, with absorbance ratios of 2.04 ± 0.02 and 2.00 ± 0.02 for $A_{260/280}$ and $A_{260/230}$, suggesting that the sample is free from proteins and polysaccharide compounds. In Figure 2, *G. boninense* and *G. lucidum* are shown to have bands with higher intensity compared to the other *Ganoderma* species. This could be due to the presence of carbohydrates, lipids, salts or phenol that absorb strongly at 230 nm (Lucena-Aguilar *et al.*, 2016). The results correlate well with the $A_{260/230}$ absorbance ratio as presented in Table 2. Kuhad *et al.* (2004) concluded that the selection of extraction buffers such as CTAB or SDS is fungus-specific when tested with six species of basidiomycetes. Hence, with further optimisation, the Boehm protocol could be suitable for use as a standard extraction protocol for closely related species though the yield produced is low.

TABLE 1. GENOMIC DNA YIELD AND QUALITY OF *G. zonatum* EXTRACTED FOLLOWING SEVEN PROTOCOLS

Protocols	Duration	Parameters	Replicate 1	Replicate 2	Replicate 3	Average
Voigt	~2 days	A260/280	1.90	1.92	1.91	1.91 ± 0.01
		A260/230	1.93	1.77	1.85	1.85 ± 0.08
		$\mu\text{g DNA g}^{-1}$ sample	81.50	91.55	88.90	87.32 ± 5.21 ^a
Moslem	~2 hr	A260/280	1.75	1.71	1.76	1.74 ± 0.03
		A260/230	1.18	1.10	1.15	1.14 ± 0.04
		$\mu\text{g DNA g}^{-1}$ sample	49.59	53.28	50.49	51.12 ± 1.92 ^b
Kim	~4 hr	A260/280	1.11	1.09	1.12	1.11 ± 0.02
		A260/230	0.27	0.26	0.25	0.26 ± 0.01
		$\mu\text{g DNA g}^{-1}$ sample	12.69	12.63	12.69	12.67 ± 0.03 ^c
Weiland	~3 hr	A260/280	1.65	1.64	1.65	1.65 ± 0.01
		A260/230	1.14	1.13	1.12	1.13 ± 0.01
		$\mu\text{g DNA g}^{-1}$ sample	58.55	58.40	59.20	58.72 ± 0.43 ^d
Vilgalys	~2 days	A260/280	1.99	1.98	1.97	1.98 ± 0.01
		A260/230	1.28	1.17	1.15	1.20 ± 0.07
		$\mu\text{g DNA g}^{-1}$ sample	63.15	65.20	67.15	65.17 ± 2.00 ^d
Van Burik	~4 hr	A260/280	1.83	1.78	1.80	1.80 ± 0.03
		A260/230	0.94	0.86	0.86	0.89 ± 0.05
		$\mu\text{g DNA g}^{-1}$ sample	267.30	254.60	257.40	259.77 ± 6.67 ^e
Boehm	~4 hr	A260/280	2.14	2.14	2.14	2.14 ± 0.00
		A260/230	2.26	2.17	2.22	2.22 ± 0.05
		$\mu\text{g DNA g}^{-1}$ sample	204.70	213.70	208.45	208.95 ± 4.52 ^f

Note: Means ± standard deviation (S.D.) in a column between protocols with different superscripts differ significantly ($P < 0.05$). Data are presented as mean ± S.D. of three biological replicates.

Next, we also assessed the integrity of the extracted DNA of *G. zonatum* across the seven protocols and also on the selected Boehm protocol on the five *Ganoderma* species. This was performed with PCR using Simple Sequence Repeat (SSR) primer pair that has an amplicon size of 150 bp. Firstly, we conducted experiments that showed PCR amplification of the extracted DNA from all the seven protocols (Figure 3). Despite disparity between the purity and concentrations of DNA, PCR amplification was successful from the seven protocols evaluated (Pipan *et al.*, 2018). Apart from DNA quality and concentration, there are many factors to consider when assessment is

based on PCR, such as suitability of primers, quality of reagents or even the thermal cycler used. Nevertheless, it is important to note that the Boehm protocol was able to produce the highest yield of highly pure DNA, making it suitable for most molecular biology applications. DNA from the five *Ganoderma* species extracted using the Boehm protocol was also suitable for PCR amplification (Figure 4). Nevertheless, the SSR band from the *G. lucidum* DNA was less intense compared to the amplicons from the other species. This is probably due to the lower quality of DNA produced from *G. lucidum* (Table 2).

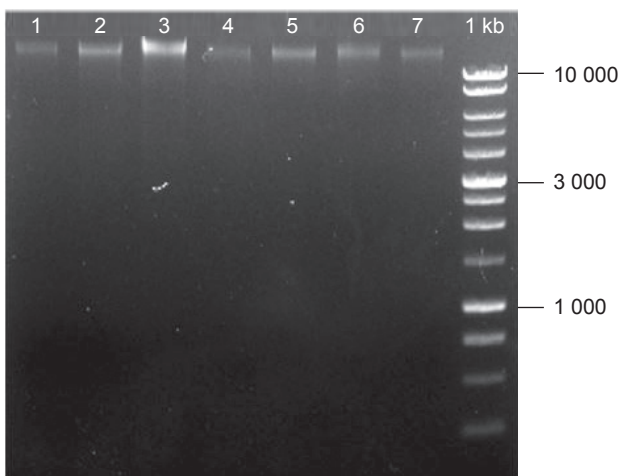


Figure 1. Agarose gel electrophoresis of extracted genomic DNA. Two microlitres of sample ($50 \text{ ng } \mu\text{l}^{-1}$) were loaded into each lanes. *Ganoderma zonatum* extracted with seven published protocols: 1) Voigt, 2) Moslem, 3) Kim, 4) Weiland, 5) Vilgalys, 6) Van Burik and 7) Boehm.

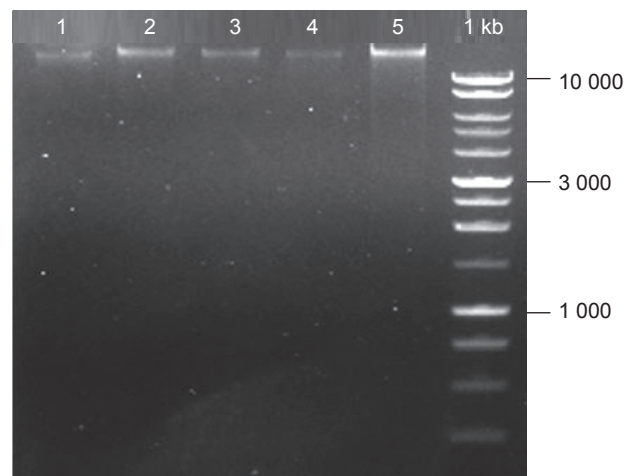


Figure 2. Agarose gel electrophoresis of extracted genomic DNA. Two microlitres of sample ($50 \text{ ng } \mu\text{l}^{-1}$) were loaded into each lanes. *Ganoderma* species tested with the Boehm protocols were: 1) *Ganoderma zonatum*, 2) *G. boninense*, 3) *G. miniatocinctum*, 4) *G. tornatum* and 5) *G. lucidum*.

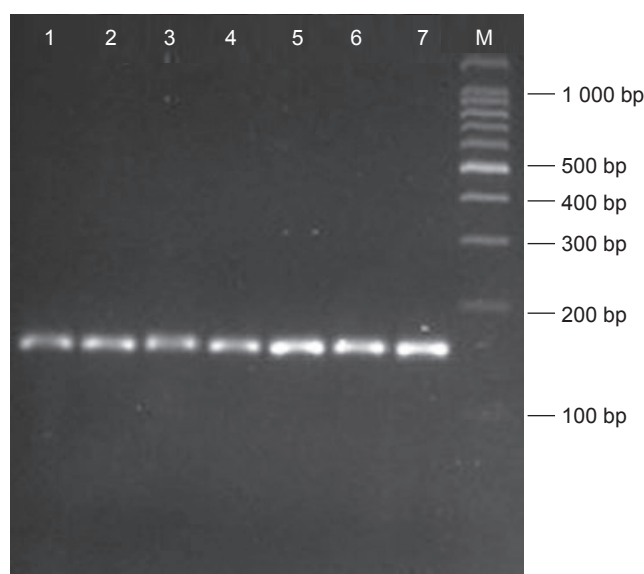


Figure 3. Agarose gel electrophoresis of genomic DNA. Polymerase chain reaction products of amplified microsatellite regions of *Ganoderma zonatum* extracted with seven published protocols: 1) Voigt, 2) Moslem, 3) Kim, 4) Weiland, 5) Vilgalys, 6) Van Burik, 7) Boehm and M-100 bp DNA ladder.

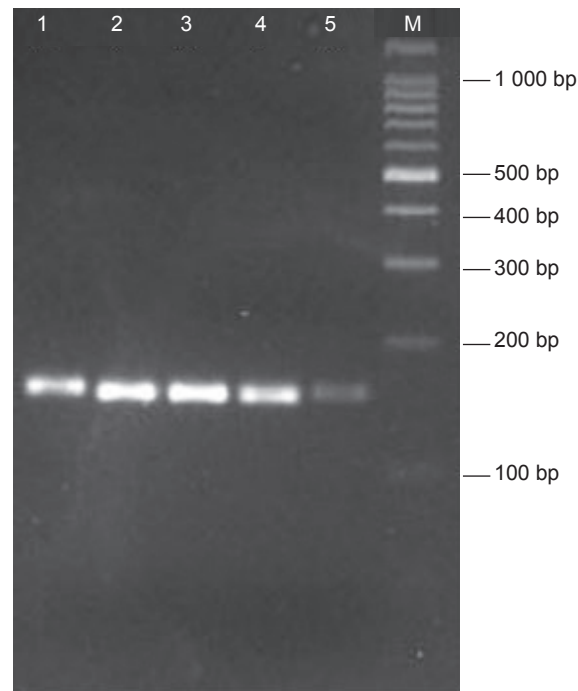


Figure 4. Agarose gel electrophoresis of genomic DNA. Polymerase chain reaction products of amplified microsatellite regions of *Ganoderma* species extracted via the Boehm protocol. Lane 1-5: *Ganoderma zonatum*, *G. boninense*, *G. miniatocinctum*, *G. tornatum*, *G. lucidum* and M-100 bp DNA ladder.

TABLE 2. GENOMIC DNA YIELD AND QUALITY OF FIVE *Ganoderma* SPECIES EXTRACTED VIA BOEHM (2004) PROTOCOL

Species	Parameters	Replicate 1	Replicate 2	Replicate 3	Average
<i>G. zonatum</i>	$A_{260/280}$	2.14	2.14	2.14	2.14 ± 0.00
	$A_{260/230}$	2.26	2.22	2.17	2.22 ± 0.05
	$\mu\text{g DNA g}^{-1}$ sample	204.70	208.45	213.70	208.95 ± 4.52
<i>G. boninense</i>	$A_{260/280}$	1.99	1.97	2.09	2.02 ± 0.06
	$A_{260/230}$	1.69	1.60	1.55	1.61 ± 0.07
	$\mu\text{g DNA g}^{-1}$ sample	41.35	41.40	41.70	41.48 ± 0.19
<i>G. miniatocinctum</i>	$A_{260/280}$	2.02	2.05	2.05	2.04 ± 0.02
	$A_{260/230}$	2.02	2.00	1.98	2.00 ± 0.02
	$\mu\text{g DNA g}^{-1}$ sample	65.00	65.00	66.05	65.35 ± 0.61
<i>G. tornatum</i>	$A_{260/280}$	2.03	2.01	2.03	2.02 ± 0.01
	$A_{260/230}$	1.80	1.73	1.69	1.74 ± 0.06
	$\mu\text{g DNA g}^{-1}$ sample	88.65	89.70	91.15	89.83 ± 1.26
<i>G. lucidum</i>	$A_{260/280}$	1.90	1.83	1.81	1.85 ± 0.05
	$A_{260/230}$	1.46	1.35	1.21	1.34 ± 0.13
	$\mu\text{g DNA g}^{-1}$ sample	34.40	32.00	33.80	33.40 ± 1.25

Note: Data are presented as mean \pm S.D. of three biological replicates.

CONCLUSION

The Boehm protocol was the most efficient, simple and reliable DNA extraction protocol tested on the mycelium of *G. zonatum*. This protocol eliminates the use of phenol and requires very low amount of tissue to produce high yield and good quality DNA. It takes about 4 hr to complete the procedure compared to the commonly used

CTAB protocol which takes approximately two days. The evaluation of protocols was aimed at obtaining good quality DNA for downstream applications, such as PacBio sequencing. Therefore, for approaches such as PacBio or Southern blot analysis which normally require large quantity and good quality DNA (Nagappan *et al.*, 2018), the short duration of the Boehm protocol was able to achieve that.

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