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Designing primers for loop-mediated isothermal amplification (LAMP) for detection of *Ganoderma boninense*

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Abstract

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Basal Stem Rot (BSR) caused by *Ganoderma boninense* is one of the most serious diseases of South East Asia's oil palm industry. The losses due to this disease were reported up to RM 1.5 billion a year in Malaysia. Typical methodologies currently used for detection of BSR infection, usually involved visual observation followed by detection of the pathogen using invasive and/or time consuming and expensive instruments. This includes the use of molecular DNA based technique, *Ganoderma* Selective Media (GSM), molecular techniques. However, most of these methods cannot be performed *in situ*. Samples need to be sent to the laboratory for testing. In this paper, a diagnostic tool using loop-mediated isothermal reaction (LAMP) is presented for detection of *G. boninense*. LAMP reaction which consist of a set of four primers, two outer and two inner, was designed specifically to recognize the manganese superoxide gene (MnSOD) obtained from NCBI Genbank (Accession no: U56128) of *G. boninense,* the causal pathogen of BSR. The assay was conducted in the thermal block with temperature 65°C for 50 min and the LAMP products were viewed on agarose gel electrophoresis. This technique removes the need to perform the reaction in thermal cycler as it can be done in a heat block. Results show the ladder-like pattern of bands sizes from 683 bp specifically to the gene MnSOD was amplified. Thus, the chosen set of primers can be used for detection of *G. boninense* in oil palm estates subjected to sensitivity and specificity.

Keywords: oil palm; basal stem rot; *Ganoderma boninense*; detection; LAMP; MnSOD

Introduction

The oil palm industry especially in two major producers in the world – Malaysia and Indonesia, has been increasingly threatened by a disease called Basal Stem Rot (BSR) which is caused by white rot fungus named *Ganoderma boninense* (Rees et al., 2012). The pathogen causes serious damage to large number of crops with a significant negative impact to the economy. This fatal disease caused a total loss up to RM 1.5 billion a year in Malaysia (Arif et al., 2011). The loss is mainly because the planters failed to detect the infection earlier to allow any quick action to be taken to avoid further losses.

To date, there is no reliable detection method which is applicable in field. Several methods were used to detect *Ganoderma* infection such as using *Ganoderma* selective medium (GSM), biochemical methods or molecular DNA based technique are currently not available in the field. Most of the methods are expensive, required trained specialist and appropriate laboratory facilities.

Molecular test for rapid and specific identification called loop-mediated isothermal amplification (LAMP) invented by Notomi et al. (2000) is a molecular technique that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. The technique removes the need to per-

form the reaction in thermal cycler (Tomlinson and Boonham, 2008). LAMP is based on the principle of autocycling strand displacement DNA synthesis performed by the *Bacillus stearothermophilus* (*Bst*) DNA polymerase, for the detection of a specific DNA sequence (Notomi et al., 2000). This technique uses four to six primers that recognize six to eight regions of the target DNA and thus provides high specificity (Nagamine et al., 2002). LAMP produces large amounts of DNA (Notomi et al., 2000) and shows high tolerance to biological contaminants (Kiatpathomchai et al., 2008) thereby simplifying sample preparation. This paper presents the design of LAMP primers potentially to be used in detecting *G. boninense* using LAMP assay.

Materials and Methods

Isolation of *G. boninense* from collected fruiting bodies

Fruiting bodies were collected from Basal Stem Rot oil palm of Sawit Kinabalu Langkon estates, Sabah, Malaysia. Internal tissues of fruiting bodies were excised and cultured on *Ganoderma* selective media (GSM). The medium was prepared as described by Ariffin and Idris (1992). The fungi which successfully grew on the GSM after 5 days were isolated, transferred and maintained in potato dextrose agar (PDA) at 37°C.

DNA extraction

Approximately 500 µl of lysis buffer (10% of Cetyltrimethyl ammonium bromide or CTAB, 5 M of NaCl with pH 8.0, 1 M of Tris-Cl with pH 8.0, polyvinylpyrrolidone (PVP), β-Mercaptoethanol and water) was transferred into 1.5 mL microcentrifuge tube, mix with scrapped off fungal mycelium from PDA and then grinded using micropestle to distrupt the fungi tissues for 60 seconds before adding 6 µl of RNase. The tube was then incubated in thermomixer for 1 hour at 65°C without any agitation. Before protein precipitation step using phenol:chloroform:isoamyl alcohol (PCIA) with ratio 25:24:1 , 6 µl of Proteinase K was added. After adding 600 µl of PCIA, the tubes were inverted for 2 minutes for mixing and then centrifuged at 13000 rpm for 10 minutes at 4°C. The upper layer of the solution was transferred into new tube with extra careful to avoid sucking the precipitated protein or the PCIA solution. The DNA precipitation was done using equal volume of isopropanol and incubated overnight in -20°C in refrigerator for higher yield. After an overnight incubation, the tubes were centrifuged at 13000 rpm for 10 minutes at 4°C and then the supernatant was discarded. Washing step was using 70% of ethanol for 4 times and dry the ethanol residue in a dessicator (Eppendorf) for 10 minutes at 45°C. Elution buffer (QIAGEN) was added to elute the DNA and stored in -20°C for further use. There were 2 total tubes containing DNA extracted label as 28/7 S8 and 31/7 S3. The concentration and purity of genomic DNA was checked using Nanovue Spectrophotometer (GE brand) and its integrity was viewed using 1% of agarose gel electrophoresis.

Polymerase chain reaction (PCR) and Sanger sequencing

PCR protocol used was as described by Boyle et al. (2008). The primers used were ITS 1 and ITS 4. Each master mix tube contains the components for a PCR reaction of 25 µl. The reaction began with an initial 94°C denaturation for 3 min, followed by 30 cycles of annealing, 96°C for 15 s, 60°C for 1.5 min, 72°C for 2 min. A final extension step at 72°C for 5 min allowed all amplicons to be fully extended. After getting the PCR products with expected size and no contamination from other organisms, PCR products are sent to First Base laboratories for sequencing. BLAST search was conducted to obtain sequences for closest matches in the NCBI Genbank database.

Designing LAMP primers

LAMP primers were designed according to the manganese superoxide dismutase (MnSOD) gene obtained from NCBI Genbank (Accession no: U56128). The primers were designed using Primer Explorer V5. There were 5 sets of primers generated by the software which was 1 set consist of 4 primers (2 outer primers and 2 inner primers). Several key factors need to be considered in order to choose the best set of primers. After considering all the key factors; melting temperature (Tm), stability at the end of the primers, guanine cytosine (GC) content, secondary structure and distance between primers, ID 47 was chosen and synthesis in Integrated DNA Technology (IDT) laboratory in Singapore. The sequences of the primers are shown in Table 1.

Table 1
LAMP primers for ID 47

Primers	Sequences	Length of the sequences
F3	5’-AGCGATGGCCCCCTCA-3’	16
B3	5’-GATCGTAATCGCTTCTCCGG-3’	20
FIP	5’-TGAATGGCGGCGGTCGTCGAGTCCGCGATCGAGCAGAA-3’	38
BIP	5’-GCTGGGGCTGGCTCGTAAGACGCGAAGCACCCGTAT-3’	36

LAMP assay

The LAMP assay was carried out in a reaction mixture (final volume 25 µl) containing 32 pmol each of inner primers (FIP, BIP), 8 pmol each of outer primers (F3, B3), 1.4 mM of deoxyribosenucleotide triphosphate (dNTPs), 0.8 M betaine (Sigma-Aldrich), 20 mM Tris HCl, 10 mM of $(NH_4)_2SO_4$, 8 mM of $MgSO_4$, 320 U/ml *Bst* DNA polymerase (NEB) and 80-100 ng of genomic DNA from sample 28/7 S8 and 31/7 S3. LAMP reaction was performed using heating block at 65°C for 50 min and 85°C for 10 minutes for deactivation step. The LAMP products were viewed using 1.5 % of agarose gel electrophoresis and stained using ethidium bromide (EtBr) under UV lamp.

Results

Isolation of *G. boninense* from collective fruiting bodies

The concentration and purity of the *G. boninense*'s DNA extracted at absorbance 260/280 and 260/230 is shown in Table 2 while the genomic DNA viewed in 1% agarose gel electrophoresis is showed in Figure 1.

Table 2
Concentration and purity of genomic DNA extracted from *Ganoderma boninense*

Sample	Concentration (ng/µl)	260/280 wavelength	260/230 wavelength
28/7 S8	278.5	2.025	2.102
31/7 S3	221.5	1.934	1.952

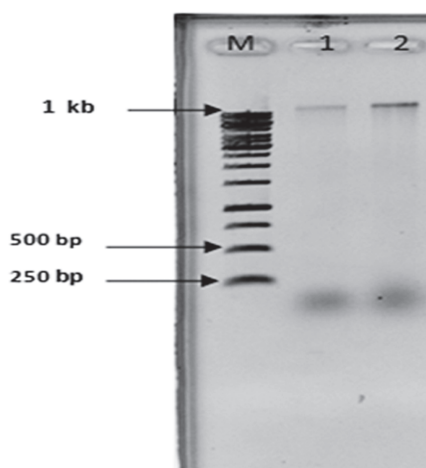

Fig. 1. Extraction of genomic DNA (inverted picture). Lane M: DNA ladder 1kb (Fermentas); Lane 1: 1 µl of genomic DNA from sample 28/7 S8; Lane 2: 1 µl of genomic DNA from sample 31/12 S4

Identification of *G. boninense* using polymerase chain reaction (PCR) and Sanger sequencing

The identification of *G. boninense* was performed using PCR and Sanger sequencing. PCR product with the expected size of PCR product as 670 base pairs (bp) is shown in Figure 2.

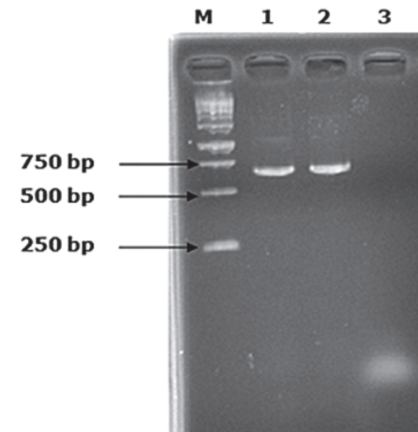

Fig. 2. PCR reaction at regions ITS 1 and ITS 4 for DNA of *G. boninense* (inverted picture). Lane M: 1 kb GeneRuler; Lane 1: PCR product of sample 28/7 S8; Lane 2: PCR product of sample 31/7 S3; Lane 3: Negative control

The sequences of ITS 1 and ITS 4 regions sequenced using Sanger sequencing for sample 28/7 S8 and 31/7 S3 were done in First Base laboratories as shown in Figure 3.

BLAST search was performed to compare the assembled sequences (ITS 1 and ITS 4) obtained with the sequences that are available in Genbank. Table 3 shows the most homologous five microorganisms from NCBI Genbank in comparison with chosen isolates, that are sample 28/7 S8 and 31/7 S3.

Analysis of LAMP products

The final products of LAMP reactions were analyzed using 1.5 % agarose gel electrophoresis. LAMP products that had been amplified after 50 min incubation at 65°C and denatured at 85°C is shown in Figure 4. The ladder-like pattern shows a positive result which means the amplification successfully done by the chosen set of LAMP primers.

Discussion

Molecular techniques such as polymerase chain reaction (PCR) had been widely used to differentiate and identify species of *Ganoderma* (Moncalvo et al., 1995; Chong et al., 2011). Nucleotide sequence information from relatively conserved genes

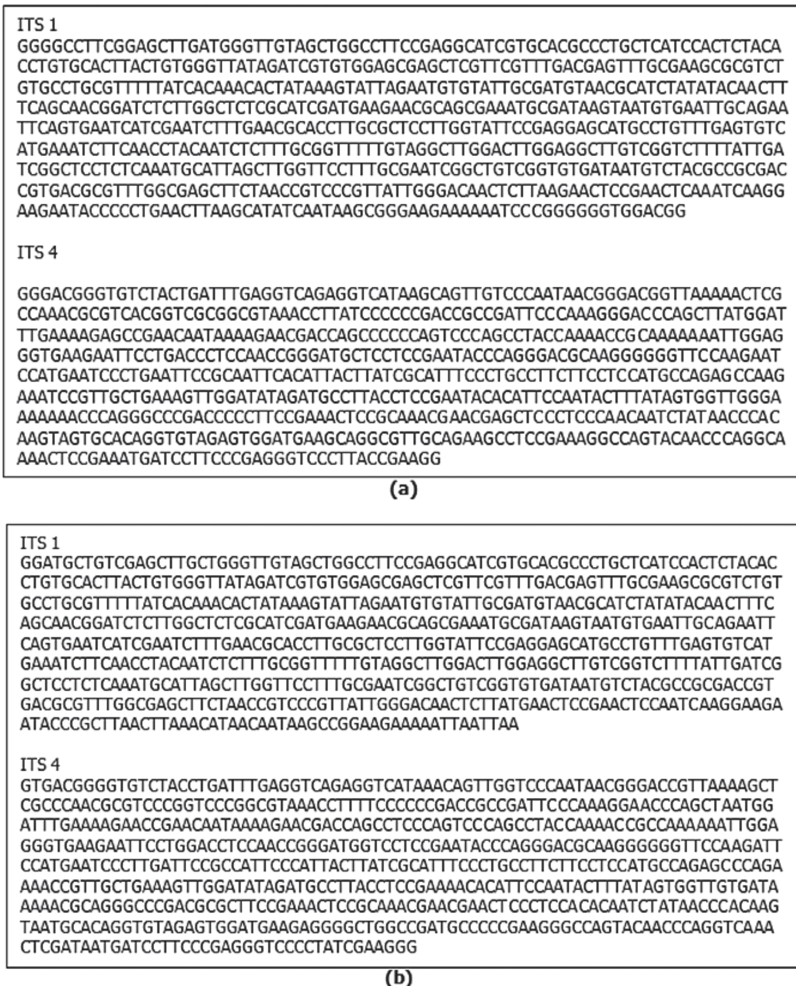

Fig. 3. The ITS 1 and ITS 4 sequences from extracted DNA samples: a) Sequences of ITS 1 and 4 for sample 28/7 S8, b) Sequences of ITS 1 and 4 for sample 31/7 S3

or DNA segments such as internal transcribed spacer (ITS) regions has been selected as specific targets for PCR detection of *Ganoderma* (Utomo and Niepold, 2000). This is because the variability of ITS regions provides sufficient resolution at various taxonomic levels (Muthelo, 2009). BLAST search of the ITS sequence data indicated the isolates of 28/7 S8 and 31/7 S3 are closely related to *Ganoderma* species BRIUMS, which are believed from genus *Ganoderma* (Chong et al., 2012). Both biological samples sequenced in this study were considered from species *G. boniniense* since the closest species of *Ganoderma* that listed from NCBI database matches results (Table 3a and 3b) is *Ganoderma boninense* strain GLBS from Nottingham University Campus Malaysia, located in West Malaysia. *Ganoderma* BRIUMS isolates from this study possibly have a slight genetic variation compared to any *G. boninense* strain that have been deposited in NCBI database. The alteration of genetic content was because of different geographical location (Kok et al., 2013) since *Ganoderma* BRIUMS isolates were obtained from oil palm estates in Sabah, which located in East Malaysia. The fungus need to adapt to new hosts and improve the resistance towards diverse environment condition. Thus, insertion, deletions or base substitutions are likely to happen because of the change in evolution and contribute to the variation of genetic sequences in the pathogenic fungal isolates presented in this study.

Loop-mediated isothermal amplification (LAMP) of the target DNA is an amplification technology with the high diagnostic potential than conventional PCR-based methods. It is reported that LAMP is currently used in many fields such as clinical point of care diagnostics, phytoquarantine, and food safety (Niessen, 2014). LAMP technology has been successful-

Table 3
BLAST search results for NCBI Genbank from two DNA samples: a) 28/7 S8; b) 31/7 S3

No	Access no.	Description	Max score	Total score	Query value	Max ident
1	JN234428.1	*Ganoderma sp*. BRIUMSb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene; complete sequence; and internal transcribed spacer 2, partial sequence	1074	1704	94%	98%
2	JN234427.1	*Ganoderma sp*. BRIUMSa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1068	1704	94%	98%
3	KF164430.1	*Ganoderma boninense* strain GBLS internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1066	1671	94%	98%
4	JN234429.1	*Ganoderma sp*. BRIUMSc internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1064	1686	96%	98%
5	KX092000.1	*Ganoderma boninense* isolate GB001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1062	1680	94%	98%

(a)

No	Access no.	Description	Max score	Total score	Query value	Max ident
1	JN234428.1	*Ganoderma sp*. BRIUMSb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1061	1654	97%	97%
2	KF164430.1	*Ganoderma boninense* strain GBLS internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1055	1616	95%	97%
3	JN234427.1	*Ganoderma sp*. BRIUMSa internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1055	1654	97%	97%
4	JN234429.1	*Ganoderma sp*. BRIUMSc internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	1051	1647	97%	97%
5	KX092000.1	*Ganoderma boninense* isolate GB001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	1050	1630	97%	97%

(b)

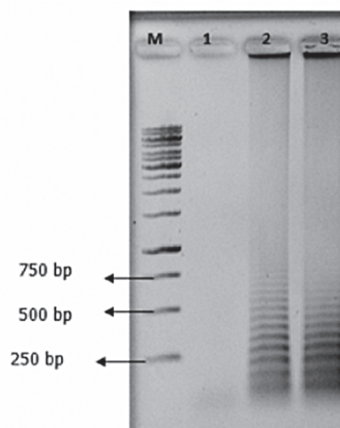

Fig. 4. LAMP detection of *G. boninense* (inverted picture). Lane M: 1 kb ladder (Fermentas); Lane 1: Negative control; Lane 2: LAMP products from DNA sample 28/7 S8; Lane 3: LAMP products from DNA sample 31/7 S4

ly applied to detect *Meloidogyne enterolobii* (Niu et al., 2012), *Erwinia amylovora* (Moradi et al., 2012) and citrus bacterial canker (Rigano et al., 2010). However, this technology has yet been applied to detect manganese superoxide (Mn-SOD) gene from *Ganoderma boninense*.

Mn-SOD is an enzyme related to the antioxidant defence mechanism of the cell. Fréalle et al. (2006) sequenced 20 partial superoxide dismutase (SOD) from 19 pathogenic fungi and found all sequences encoded tetrameric manganese (Mn)-containing SODs and showed higher variability than small subunit (SSU) ribosomal RNA (rRNA) and similar to internal transcribed spacer (ITS) rRNA. This analysis suggesting that Mn-SOD could be used to identify closely related fungal species.

This study aimed to develop LAMP primers for detection of *G. boninense* in Malaysia. The ability of chosen set of primers shows positive results in detecting *G. boninense*. Detection of amplified product in LAMP has originally been accomplished by agarose gel electrophoresis which typically reveals a ladder

like pattern of DNA fragments as shown in Figure 4. However, due to massive production of DNA during LAMP, the risk of cross contamination of samples by aerosolized product is very high and may compromise analytical results (Ohori et al. 2006; Yarita et al., 2007). Many researchers proposed of using calcein-$MnCl_2$ as the visualization method but it is found the colour change of calcein-$MnCl_2$ is less sensitive compared to hydroxynaphthol blue (Wastling et al., 2010). SYBR Green I is the most available and sensitive general nucleic acid fluorescence dyes because it can be excited by both UV and visible light. This makes the SYBR Green I is suitable for detection of *G. boninense* using LAMP in the oil palm plantation. The colour of this dye is changes from orange to bright green fluorescence when conjugated into DNA (Tao et al., 2011). Thus, more works need to be done using LAMP primers and SYBR Green I for developing a detection tool of *G. boninense* in the field.

References

Ariffin, D., & Idris, S. (1992). The *Ganoderma* selective medium (GSM). PORIM Information Series.

Arif, M. S., Roslan, A., Idris, A. S., & Ramle, M. (2011, November). Economics of oil palm pests and Ganoderma disease and yield losses. In *Proceedings of the Third MPOB-IOPRI International Seminar: Integrated Oil Palm Pests and Diseases Management*. Kuala Lumpur Convention Centre Kuala Lumpur.

Boyle, S. A., Yarwood, R. R., Bottomley, P. J., & Myrold, D. D. (2008). Bacterial and fungal contributions to soil nitrogen cycling under Douglas fir and red alder at two sites in Oregon. *Soil Biology and Biochemistry*, *40*(2), 443-451.

Chong, K. P., Lum, M. S., Foong, C. P., Wong, C. M. V. L., Atong, M., & Rossall, S. (2011). First identification of *Ganoderma boninense* isolated from Sabah based on PCR and sequence homology. *African Journal of Biotechnology*, *10*(66), 14718-14723.

Chong, K. P., Abdullah, S., & Ng, T. L. (2012). Molecular fingerprint of Ganoderma spp. from Sabah, Malaysia. *International Journal of Agriculture and Biology*, *15*(6), 1112-1118.

Fréalle, E., Noël, C., Nolard, N., Symoens, F., Felipe, M. S., Dei-Cas, E., Camus, D., Viscogliosi, E. & Delhaes, L. (2006). Manganese superoxide dismutase based phylogeny of pathogenic fungi. *Molecular Phylogenetics and Evolution*, *41*(1), 28-39.

Kiatpathomchai, W., Jaroenram, W., Arunrut, N., Jitrapakdee, S., & Flegel, T. W. (2008). Shrimp Taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. *Journal of Virological Methods*, *153*(2), 214-217.

Kok, S., Wong, W. C., Tung, H., Goh, Y. K., & GoH, K. J. (2013). In vitro growth of Ganoderma boninense isolates on novel palm extract medium and virulence on oil palm (*Elaeis guineensis*) seedlings. *Malaysian Journal of Microbiology*, *9*(1), 33-42.

Moncalvo, J. M., Wang, H. H., & Hseu, R. S. (1995). Phylogenetic relationships in Ganoderma inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia*, *87*(2), 223-238.

Moradi, A., Nasiri, J., Abdollahi, H., & Almasi, M. (2012). Development and evaluation of a loop-mediated isothermal amplification assay for detection of *Erwinia amylovora* based on chromosomal DNA. *European Journal of Plant Pathology*, *133*(3), 609-620.

Muthelo, V. G. (2009). *Molecular characterisation of Ganoderma species* (Doctoral dissertation, University of Pretoria, South Africa).

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, *16*(3), 223-229.

Niessen, L. (2014). Current state and future perspectives of loop-mediated isothermal amplification (LAMP)-based diagnosis of filamentous fungi and yeasts. *Applied Microbiology and Biotechnology*, *99*(2), 553-574.

Niu, J. H., Jian, H., Guo, Q. X., Chen, C. L., Wang, X. Y., Liu, Q., & Guo, Y. D. (2012). Evaluation of loop-mediated isothermal amplification (LAMP) assays based on 5S rDNA-IGS2 regions for detecting *Meloidogyne enterolobii. Plant Pathology*, *61*(4), 809-819.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, *28*(12), e63-e63.

Ohori, A., Endo, S., Sano, A., Yokoyama, K., Yarita, K., Yamaguchi, M., Kamei, K., Miyaji, M. & Nishimura, K. (2006). Rapid identification of *Ochroconis gallopava* by a loop-mediated isothermal amplification (LAMP) method. *Veterinary Microbiology*, *114*(3-4), 359-365.

Rees, R. W., Flood, J., Hasan, Y., Wills, M. A., & Cooper, R. M. (2012). Ganoderma boninense basidiospores in oil palm plantations: evaluation of their possible role in stem rots of *Elaeis guineensis*. *Plant Pathology*, *61*(3), 567-578.

Rigano, L. A., Marano, M. R., Castagnaro, A. P., Do Amaral, A. M., & Vojnov, A. A. (2010). Rapid and sensitive detection of Citrus Bacterial Canker by loop-mediated isothermal amplification combined with simple visual evaluation methods. *BMC Microbiology*, *10*(1), 176.

Tao, Z. Y., Zhou, H. Y., Xia, H., Xu, S., Zhu, H. W., Culleton, R. L., Han, E. T., Lu, F., Fang, Q., Gu, Y. P., Liu, Y. B., Zhu, G. D., Wang, W. M., Li, J. L., Cao, J. & Gao, Q. (2011). Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of *Plasmodium vivax* infection. *Parasites & Vectors*, *4*(1), 115.

Tomlinson, J., & Boonham, N. (2008). Potential of LAMP for detection of plant pathogens. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *3*(066), 1-7.

Utomo, C., & Niepold, F. (2000). Development of diagnostic methods for detecting ganoderma-infected oil palms. *Journal of Phytopathology*, *148*(9-10), 507-514.

Wastling, S. L., Picozzi, K., Kakembo, A. S., & Welburn, S. C. (2010). LAMP for human African trypanosomiasis: a comparative study of detection formats. *PLoS Neglected Tropical Diseases*, *4*(11), e865.

Yarita, K., Sano, A., Murata, Y., Takayama, A., Takahashi, Y., Takahashi, H., Yaguchi, T., Ohori, A., Kamei, K., Miyaji, M., Nishimura, K. (2007). Pathogenicity of *Ochroconis gallopava* isolated from hot springs in Japan and a review of published reports. *Mycopathologia*, *164*(3), 135-147.