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Molecular Identification of Isolated Fungi from Kelantan and Terengganu Using Internal Transcribed Spacer (ITS) Region

Noor Afiza Badaluddin, Siti Noraisyah Tan Jamaluddin, Nur Shafiqah Ihsam, Mohammad Hailmi Sajili and Noor Asidah Mohamed

School of Agricultural Science and Biotechnology,
Faculty of Bioresource and Food Industry,
Universiti Sultan Zainal Abidin, Besut Campus,
22200 Besut, Terengganu Darul Iman, MALAYSIA.

Corresponding author: Noor Asidah Mohamed
School of Agricultural Science and Biotechnology,
Faculty of Bioresource and Food Industry,
Universiti Sultan Zainal Abidin, Besut Campus,
22200 Besut, Terengganu Darul Iman, MALAYSIA.
Email: norasidah@unisza.edu.my

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ABSTRACT

Fungi are morphologically, ecologically, metabolically and phylogenetically diverse. Fungi play important roles as one of the major decomposer in ecosystems, dominated by Saprophytic fungi. The identification of fungi is important to differentiate each fungi owing to their special ability in our ecosystem. However, the identification of fungi is become very challenging for those untrained mycologists. Essentially, the identification of fungi at the species-level is more problematic. Traditional approaches, based on the morphological or physiological features alone are unreliable because of the limited amount of morphological characters for fungi identification. Thus, a reliable molecular approach is required to identify the fungi at species-level. Here, we have successfully identified the species of 25 isolated fungi samples from the polluted areas in Kelantan and Terengganu by using the molecular identification utilizing the Internal Transcribed Spacer (ITS) regions. Phylogenetics tree was then constructed, using MEGA v7.0 software to illustrate the inter-relationships among the isolates. Among of 25 isolated fungi samples, 23 were identified from Ascomycota division and another two were from the Zygomycota division. From our observation, the most frequent species grew for both polluted areas were from *Lasiodiplodia* sp.

Keywords: ITS, fungi identification, phylogenetic tree, MEGA, fungi diversity

ABSTRAK

Kulat berbeza dari segi morfologi, ekologi, metabolic dan secara filogenetik. Kulat memainkan peranan yang penting sebagai pengurai utama di dalam sesuatu ekosistem, di mana kebanyakannya di dominasi oleh kulat saprofit. Pengenalpastian kulat penting untuk membezakan setiap kulat mempunyai kebolehan istimewa dalam ekosistem kita. Walau bagaimanapun, pengenalpastian kulat pada peringkat spesis sangat mencabar terutamanya kepada ahli mikologi yang tidak terlatih. Pada asalnya, pengenalpastian kulat pada peringkat spesis lebih membebankan. Pendekatan secara tadisional berdasarkan ciri morfologi dan fisiologi tidak boleh dipercayai oleh kerana karakter morfologi yang terhad untuk pengenalpastian kulat. Oleh itu, pendekatan secara molecular yang lebih dipercayai diperlukan untuk pengenalpastian kulat pada peringkat spesis. Di dalam kajian ini, sebanyak 25 sampel kulat yang telah diasingkan daripada kawasan tercemar di Kelantan dan Terengganu Berjaya dikenalpasti dengan menggunakan pengenalpastian secara molecular menggunakan bahagian *Internal Transcribed Spacer* (ITS). Pokok filogenetik kemudian dibina dengan menggunakan perisian MEGA v7.0 untuk mengilustrasikan hubungan antara kulat yang dikumpul. Daripada 25 sampel kulat yang diasingkan, 23 kulat telah dikenalpasti sebagai divisi Ascomycota dan dua lagi daripada divisi Zygomycota. Spesis yang paling kerap tumbuh di kawasan tercemar adalah *Lasiodiplodia* sp.

Kata kunci: ITS, pengenalpastian kulat, poko filogenetik, MEGA, diversiti fungi

INTRODUCTION

Fungi are among of the most abundant eukaryotes on earth's biosphere, making up the diverse microorganisms. Fungi are classified in kingdom Fungi and separated from other kingdom plant and animals due to the presence of chitin in their cell wall. They are lack of chlorophyll and do not undergo photosynthesis, but they obtain foods via absorption of dissolved molecules. The fungal kingdom encompasses an enormous diversity of taxa with variety of ecological niches, life-cycle strategies, and morphologies. Fungi biomass activity also related to the aquatic ecosystem as they play role in the uptake and sequestration of dissolved inorganic nutrients (Mulholland *et al.*, 2000). Wood and leaf is responsible as carbon and energy sources for microorganism and food web in fresh water. For example, the wood-inhabiting fungi in freshwater habitats are important owing to their abilities of making soft-rot cavities in wood by producing various enzymes that can degrade wood (Wong *et al.*, 1998). There are also a fungal community that are dominating the breakdown of leaves and other allochthonous detritus in stream and river for increasing the palatability of the substrates to detritus feeders (Barlocher, 1992).

Fungi may present in diverse habitats such as soil, stream and river, even in extreme habitat. These diverse in habitat display the diverse in fungi structure and biochemistry. This also results from difference in

substrate preference among the fungi and lead to fungi variation (Tsui *et al.*, 2000). However, the substrate that present in those polluted area may be different as they could exist in form of organic or inorganic pollution, resulting from the development and urbanization process due to inefficient management of waste products. The lack of knowledge on fungal community in river ecosystem due to a very few studies especially focused on fungal biomass on wood also is a problem that constrains the information on fungi variation being well known (Gulis *et al.*, 2008).

The identification of fungi at the species level is vital in many research areas such as health sciences and agriculture, where finding the causal agents of diseases is essential to discover suitable treatment, elucidation of outbreaks, and transmission mechanisms (Araujo, 2014). Furthermore, the understanding of the specific roles of microorganisms in an ecosystem, their abundance, and their community constitution in ecological and biodiversity studies can only be achieved through their reliable identification (Peay *et al.*, 2008). Species identification based on traditional phenotypic methods is often time consuming and laborious and is hampered by the unstable and subjective nature of phenotypic characteristics, which are readily influenced by culture conditions. Therefore, this study was carried out to molecularly identify the 25 fungal collection that were previously isolated nearby Kelantan River and Chendering industrial area by using Internal Transcriber Space (ITS) region.

MATERIAL AND METHODS

Extraction of Fungi Genomic DNA

Fungi were subcultured on nutrient-rich medium Potato Dextrose Agar (PDA). Then half of the fungal growths were scraped out from the plate (Da Silva and Clark, 2013). The genomic DNA of the fungi was extracted using FavorPrep Fungi/ Yeast Genomic DNA Extraction Mini Kit according to the manufacturer's instructions. The yielded DNA were stored at –20°C for further use. 10 µl of total DNA were loaded onto a 1% agarose gel and electrophoresed to separate the DNA.

Polymerase Chain Reaction

The sequence of ITS was amplified with the forward primer pair ITS1: 5'- TCCGTAGGTGAACCTGCGG -3' and reverse primer ITS4: 5'- TCCTCCGCTTATTGATATGC -3' (White al. 1991) with a total reaction volume of 20 µL (100 ng DNA template, 0.5 µM of reverse and forward primers, 5 µL of PCR mix (exTEN 2X PCR master Mix)). ITS regions are normally used in genotypic identification. The mixtures were amplified by the following conditions: an initial denaturation at 95°C for 2 minutes followed by 35 cycles at 95°C for 30 seconds, 48.4°C for 30 seconds, and 72°C for 1 minute, and a final elongation step at 72°C for 10 minutes in Applied Biosystems™ Veriti™ 60-well Thermal Cycler. The PCR products were detected on 1.0% agarose gel and were visualized by Fujifilm LAS-3000 Imager. PCR products were sent to 1st Base DNA Sequencing Service for sequencing.

Molecular Identification and Phylogenetic Analysis

The sequence alignment of the isolates were performed by aligned the DNA sequences of the isolated fungi using the MEGA 7.0 software. For the further verification of the most closely related sequence, the sequence were compared to the Gene Bank data using the BLAST tools available in the National Center for Biotechnology Information the website (NCBI, http://www. ncbi.nlm.nih.gov/blast/blast.cgi). Phylogenetic trees were then constructed based on the hierarchical clustering of the alignments of ITS sequences and produced by Maximum likelihood using MEGA 7 software of the bootstrap values (1000 replicates). Maximum likelihood method applied the sequence evolution model based of the highest likelihood on the observed data (Jill *et al.*, 2006; Egan *et al.*, 2007; Roy *et al.*, 2014). This is an ideal model for building a phylogeny using sequence data and often employed for the publications of evolutionary pylogenetics tree analysis studies (James *et al.*, 2006; de Hoog *et al.*, 2013; Godoy *et al.*, 2016)

RESULTS AND DISCUSSION

Identification of Fungi Collection

Table 4.1 showed the summary of the 25 fungal isolates, 2 were from Zygomycota division and 23 were from Ascomycota division. The most frequent isolates found were *Lasiodiplodia* spp. with 12 isolates (48%), *Trichoderma*

spp. with 5 isolates (20%) and *Pestalotiopsis* spp. with 3 isolates (12%). *Mucor indicus, Gibberella moniliformis, Sordaria fimicola* and *Clonostachys rosea* were only found once.

Table 4.1 Fungal isolates substrates, frequency per whole sample and percentage of isolates that were collected from Kelantan and Terengganu polluted areas.

Isolate	Substrate	Frequency	Frequency (%)
Pestalotiopsis microspore	Leaf	2	8
Pestalotiopsis oxyanthi	Leaf	1	4
Lasiodiplodia theobromae	Leaf	5	20
	Stem	5	20
Lasiodiplodia pseudotheobromae	Stem	1	4
Lasiodiplodia brasiliensis	Stem	1	4
Trichoderma asperellum	Leaf	1	4
	Stem	4	16
Rhizopus microspores	Leaf	1	4
Mucor indicus	Stem	1	4
Gibberella moniliformis	Leaf	1	4
Sordaria fimicola	Stem	1	4
Clonostachys rosea	Stem	1	4

Table 4.1 showed the substrates preferences, frequencies and percentages and closest relative identified for fungal ITS sequences against NCBI database using BLAST (NCBI, http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Here, *Lasiodiplodia* sp. produced the greatest number of occurrences, with seven isolated fungi sample were observed in the samples taken from the stem and five isolated fungi sample were taken from the leaf. However, *Mucor indicus, Gibberella moniliformis, Sordaria fimicola* and *Clonostachys rosea* were observed once in the samples taken from stem and leaf. The most prevalent species to grow on both polluted areas in Kelantan and Terengganu were *Lasiodiplodia theobromae* (found in 10 samples), followed by *Trichoderma asperellum* (found in 5 samples) then *Pestalotiopsis microspora* (found in 2 samples). The random selection of fungal isolates revealed a total of 8 fungal genera with 11 fungal species.

The amplified fungal sequences were used as BLAST queries against the NCBI database for the sequence similarity searching. Table 4.2 shows the fungi isolated from the polluted Kelantan River and Terengganu industrial area were aligned to the most closely related fungi in available in GenBank (Benson *et al.*, 2008). Most of the our samples shown an excelent sequence similirity scored with 99–100% identical to the nucleotide sequences of the ITS1-5.8S-ITS2 region of isolated fungal species in GenBank database provided by the accession numbers given in Table 4.2.

Most Frequent Isolated Fungi Species

Lasidiplodia sp.

In this study, five isolates were form leaf and another seven isolates were from stem and this species were found in both polluted areas. A study by Mohali *et al.*, in 2009 revealed a very high gene flow of *Lasiodiplodia* spp. between populations of isolates from different hosts. About 83.3% of Lasiodiplodia sp. obtained from this study comprised of *Lasidodiplodia theobrome. L. theobromae*, are known to have a cosmopolitan distributions with wide host ranges (Punithalingam 1976). Thus, the relationship of *L. theorbromae* with both hosts was not unexpected. Previous studies have reported that, Botryosphaeria spp. were seed-borne pathogens and caused losses in seed germination (Sultana and Ghaffar, 2009; Owolade *et al.*, 2009). Seed-borne disease pathogens actively attack seeds and may be harmful. Seeds can be infected by pathogens that colonise the seeds both externally or internally. *Lasiodiplodia theobromae* grew upon several hosts pathogen that commonly infects tropical and subtropical woody plants and fruit plants (Ismail *et al.*, 2012).

Trichoderma sp.

Similar to the *Lasiodiplodia* sp., *Trichoderma* species are also classified as cosmopolitan fungi that can be found in various habitats (Rahman *et al.*, 2011). This species can be easily isolated from soils, decaying woods and other

plant organic matter. *Trichoderma* sp. responds to their environment by regulation of growth, conidiation, enzyme production, and hence adjusts their lifestyle to current conditions which gives significant impact to the human, environment, agriculture and industry. This is relevant to the *Trichoderma* sp. found in polluted river in Kelantan. Evidences have suggested that *Trichoderma* sp. exhibit considerable tolerance for metals and accumulated high amounts of metals from polluted habitats. In this study, by using ITS region, five isolates of *Trichoderma asperallum* were identified where one isolate came from leaf and four isolates were from stem. Hoseinzadeh *et al.*, (2017) proposed that *Trichoderma asperellum* can be used in bioremediation for metal remediation in water and heavy metal-contaminated soils.

Pestalotiopsis sp.

Most of *Pestalotiopsis* species in this study were isolated from the leaves. Based on the sequence alignment of the ITS1-4 sequence againts the GenBank database, 2 species were closely identical to the sequence. These isolates were referred to *P. microspore* and *P. oxyanthi*. A series of recent studies has indicated that *Pestalotiopsis* sp. occurs as generalist endophytic fungi (Reddy *et al.*, 2016). *P. microspora* reported as a potential bioremediation agent in degrading polyester polyurethane (PUR) (Russell *et al.*, 2017). However, not many studies had reported that any of the *Pestalotiopsis* species exhibit biodegradation activity. We argue that previous literature suffers from certain weaknesses because microspore has robust activity in PUR degradation. Further study in screening for polyurethenase should be done.

Table 4.2 Identification of Fungal Species Isolated from Kelantan and Terengganu by DNA Sequencing of the ITS Region

Isolate	% Identification	Identification	Accession Number	No. of ITS matches/no. of identified in GenBank
KL1	99	*Pestalotiopsis microspora*	KU720061.1	528/521
KL2	100	*Lasiodiplodia theobromae*	KX022498.1	523/571
KL3	99	*Rhizopus microsporus*	KM527221.1	680/672
KL4	100	*Lasiodiplodia theobromae*	KP998517.1	525/545
KL5	100	*Pestalotiopsis oxyanthi*	KP900246.1	525/519
KL6	100	*Lasiodiplodia theobromae*	KX022498.1	523/571
KL7	99	*Trichoderma asperellum*	KU497722.1	578/568
KL8	99	*Pestalotiopsis microspora*	KU720061.1	525/521
KL9	100	*Lasiodiplodia theobromae*	KX022498.1	523/571
KS2	100	*Trichoderma asperellum*	MF061791.1	578/560
KS3	99	*Trichoderma asperellum*	LC158827.1	580/964
KS4	100	*Trichoderma asperellum*	KU497723.1	586/570
KS5	99	*Lasiodiplodia theobromae*	KM278132.1	522/517
KS6	100	*Trichoderma asperellum*	KT876619.1	576/574
KS7	99	*Lasiodiplodia theobromae*	JX275790.1	522/506
KS8	99	*Lasiodiplodia brasiliensis*	KY655212.1	648/604
KS9	99	*Mucor indicus*	KY425744.1	423/759
TL4	100	*Lasiodiplodia theobromae*	KX022498.1	524/571
TL6	100	*Gibberella moniliformis*	GU723435.1	524/513
TS1	100	*Lasiodiplodia pseudotheobromae*	EU860391.1	523/617
TS2	99	*Sordaria fimicola*	KC895516.1	561/563
TS3	99	*Lasiodiplodia theobromae*	KX022498.1	522/571
TS5	99	*Lasiodiplodia theobromae*	KX022498.1	521/571
TS7	100	*Lasiodiplodia theobromae*	KX022498.1	522/571
TS9	99	*Clonostachys rosea*	KJ540094.1	521/518

Phylogenetics Tree Analysis

Ribosomal DNA has contains the most conserved region in genome. Table 4.3 showed conserved site composition in length, AT content and GC content of ITS sequence in fungi collection

Table 4.3 Variation in length, AT content, and GC content on ITS sequences on fungi collection

Isolate		T	C	A	G	Total (bp)	%AT	%GC
Lasiodiplodia theobromae	KL2	23.7	27.2	23.7	25.4	523.0	47.4	52.6
Lasiodiplodia theobromae	KL4	23.8	26.7	24.0	25.5	525.0	47.8	52.2
Lasiodiplodia theobromae	KL6	23.7	27.2	23.7	25.4	523.0	47.4	52.6
Lasiodiplodia theobromae	KL9	23.9	27.3	23.5	25.2	523.0	47.7	52.5
Lasiodiplodia theobromae	KS5	23.9	27.0	23.8	25.3	522.0	47.7	52.3
Lasiodiplodia theobromae	KS7	23.4	27.0	23.9	25.7	522.0	47.3	52.7
Lasiodiplodia theobromae	TL4	23.7	27.1	23.7	25.6	524.0	47.4	52.7
Lasiodiplodia theobromae	TS3	23.4	27.4	23.9	25.3	522.0	47.3	52.7
Lasiodiplodia theobromae	TS5	23.2	27.3	24.0	25.5	521.0	47.2	52.8
Lasiodiplodia theobromae	TS7	23.9	26.6	23.9	25.5	522.0	47.8	52.1
Lasiodiplodia pseudotheobromae	TS1	24.1	27.0	23.7	25.2	523.0	47.8	52.2
Lasiodiplodia brasiliensis	KS8	25.0	25.5	25.8	23.8	648.0	50.8	49.3
Trichoderma asperellum	KL7	20.4	29.8	23.9	26.0	578.0	44.3	55.8
Trichoderma asperellum	KS2	20.7	29.5	23.8	26.0	576.0	44.5	55.5
Trichoderma asperellum	KS3	20.2	30.0	24.1	25.7	580.0	44.3	55.7
Trichoderma asperellum	KS4	20.1	29.4	24.6	25.9	586.0	44.7	55.3
Trichoderma asperellum	KS6	20.5	30.0	24.0	25.5	576.0	44.5	55.5
Pestalotiopsis microspora	KL1	32.4	22.0	24.4	21.2	528.0	56.8	43.2
Pestalotiopsis microspora	KL8	32.2	21.0	25.0	21.9	525.0	57.2	42.9
Pestalotiopsis oxyanthi	KL5	31.6	21.0	25.1	22.3	525.0	56.7	43.3
Gibberella moniliformis	TL6	22.6	27.6	25.9	23.9	522.0	48.5	51.5
Clonostachys rosea	TS9	23.6	26.5	26.9	23.0	521.0	50.5	49.5
Sordaria fimicola	TS2	23.2	28.0	23.2	25.7	561.0	46.5	53.7
Rhizopus microsporus	KL3	32.1	19.3	28.4	20.3	680.0	60.5	39.6
Mucor indicus	KS9	32.9	17.0	32.4	17.7	423.0	65.3	34.7
Average		24.7	26.2	24.7	24.4	543.2	48.2	51.7

Conserved region in a nucleotide sequence indicated that the species are remained unchanged. Variable sites demonstrate considerable sequence diversity among different organisms. Table 4.3 showed the variable site composition in length, AT content and GC content of ITS sequences in the fungi collection. The percentages of each base for all the fungi species were used to determine to compare and classify all the isolates.

The visualization of PCR products using 1% agarose showed single band which means that sequence of ITS1-5.8S-ITS2 was successfully amplified with forward and reverse primer. The alignment results showed a gap in the sequence caused by insertions and deletions. Results of alignment showed that the three species of genus *Lasiodiplodia* have a high degree of homology (99-100%). The average frequency of nucleotide in the ITS sequences was 24.7%, (T) 26.2%, (C) 24.7% (A) and 24.4% (G). These sequences were rich in TA which was equal to 48.2% while in GC was 51.7% (Table 4.3). This was consistent with a study by of Leskinen *et al.*, (1997) which suggested that the most composition of nucleotides in ribosomal ITS sequences is Guanine and Cytosine. Sumida *et al.*, (2004) also had shown that there was higher GC content in ITS segments. On the other hand, the ration of transition/transvertion ratio was quite high (R=0.73). The sizes of the ITS regions varied among strains from 423bp to 680bp (Table 4.3). The length of ITS regions for *Rhizopus microscopus* was found to be considerably longer (680 bp) than those of other isolated fungi. *Mucor indicus* consisted the least nucleotide length. The total GC content varied from 34.7% to 55.8%. The GC content *in Mucor indicus* and *Rhizopus microscopus* were quite similar which was 34.7% and 39.6% respectively and the AT content was high (65.3% and 60.5% respectively).

Lasiodiplodia theobrome, Lasiodiplodia pseudotheobrome and *Lasiodiplodia brasiliensis* have almost similar AGTC content and the total nucleotide sequences for all the species under *L. theobrome and L.pseudotheobrome* ranged from 521-525bp which put them under the same genera, *Lasiodiplodia* sp. *L. theobrome* and *L. pseudotheobrome* exhibited higher GC content (52.1% to 52.8%) than their AT content. However, among the three species, *L.brasiliensis* showed higher AT% content than other *Lasiodiplodia* species. To our knowledge, no previous studies have examined the difference of nucleotide composition that focus on *Lasiodiplodia* sp. Meanwhile, *Trichoderma* species were greatly differ in the AGTC content than other fungi species with total 576-578bp.

There are some potentially open questions about the validity of using ITS sequence in *Trichoderma* speciation. Huzefa *et al.* (2017) claimed that identification *of Trichoderma sp.* using ITS region can be intriguing, as it lacks variation for differentiating among species. This factor could well be responsible for the identification of *Trichoderma asperellum* of all *Trichoderma sp.* isolates in this study. It has been suggested for species-level identification of *Trichoderma*, combination of translation elongation factor 1-alpha, *tef-1* region and RNA polymerase II, RPB2 seems to be a reliable approach in successful *Trichoderma* species identification (Nagy *et al.*, 2007). The AGTC content of fungi isolates from *Pestalotiopsi*s also highlighted similar nucleotide composition (525-528bp).

When the non-coding regions of Zygomycete *Mucor miehei* were analyzed (Maicas *et al.*, 2000), ITS (198 bp) and ITS2 (255 bp) are AT rich (66% and 77%, respectively) and ITS1 is longer than the corresponding sequence of other related zygomycota (Maicas *et al.*, 2000). But, in this study, ITS sequences of *M. indicus* are still shorter than *R.microscopus*. The GC content ITS region in *M. indicus* and *R. microscopus* were very similar; 34.7% for *M. indicus* and 53.7% GC for *R. microscopus.*

Figure 4.2 shows the phylogenetic tree of the fungal collection species that were isolated from Kelantan and Terengganu. The sequences of the PCR amplicons were found to be 99–100% similar to the sequences of ITS1-5.8S-ITS2 region of the respective genera and species of closely related fungi documented in the GenBank. These finding allow us to infer homology from the degree of similarity found between our isolated and those within the GenBank database, which we concluded, were very similar. The phylogenetic tree, which presents the inter-relationships among the isolates, was built with maximum likelihood (Kumar et el., 2016) and 1000 replicates of bootstrap method. The phylogenetics analysis shows two basic nodes, one ascending for Ascomycota division and the other descending for Zygomycota division. The tree shows more detail for division Ascomycota as 92% of the fungal isolates belonged to this division. The tree with the highest likelihood is shown. The percentage of trees where the associated taxa clustered together is shown next to the tree branches. The tree was drawn to scale with branch lengths measured in the number of substitutions per site. As shown in Figure 4.2, Maximum-likelihood analysis of ITS sequences placed *M. indicus, R. microsporus* and a large clade consisted of the *Lasiodiplodia* sp. outside a large clade comprised of *Pestalotiopsis, S. fimicola, G. moniliformis, C. rosea* and *Trichoderma* sp.which was supported with a bootsrap value of 100%. All the isolates from leaf and stem could be classified as Sordariomycetes, Dothideomycetes or Mucoromycotina. Dothideomycetes mainly included Botryosphaeriales (Subclade I). Sordariomycetes included Xylariales (Subclade II), Sordariales (Subclade III)

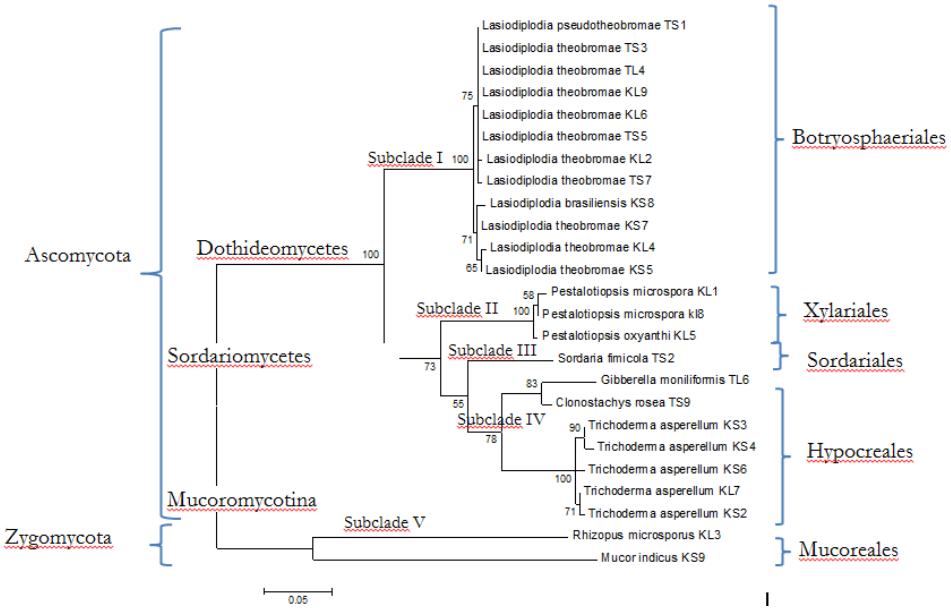

Figure 4.2: Maximum likelihood tree demonstrating the relationship of 25 isolates generated from the analysis of ITS with 95% confidence level. The bootstrap values (1000 replication) are shown next to branches.

and Hypocreales (Subclade IV). Mucorales (Subclade V) was included in Mucoromycotina. Ingroup of divison Ascomycota was separated from outgroup with 100% bootstrap value.

CONCLUSION

In this study, the utilization of molecular approach using ITS region to identify fungi to their species level is a reliable method. The strains in this study reflect the fungal diversity existed in polluted areas in Kelantan and Terengganu. In this works, *Lasiodiplodia* sp. was identified as the most frequent species observed for both polluted areas. Here, we also recognized some of the species identified are belong to potent fungi that act as the bioremediation agents which are *Trichoderma asperellum* and *Pestalotiopsis micropora*. The screenings for the fungi with bioremediation potencial at the polluted areas are promising for further studies.

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REFERENCES

Araujo, R. (2014). Towards the genotyping of fungi: methods, benefits and challenges. *Current Fungal Infection Reports* **8**(3), 203-210.

Bärlocher, F. (1992). Research on aquatic hyphomycetes: historical background and overview. In *The Ecology of Aquatic Hyphomycetes* (p. 1-15). Springer Berlin Heidelberg.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Wheeler, D. L. (2008). GenBank. *Nucleic Acids Research* **36**(Database issue): D25.

Da Silva, W. L., and Clark, C. A. 2013. Infection of sweetpotato by Fusarium solani and Macrophomina phaseolina prior to harvest. *Plant Dis*. **97**:1636-1644.

de Hoog, G. S., Ahmed, S. A., Najafzadeh, M. J., Sutton, D. A., Keisari, M. S., Fahal, A. H., & van de Sande, W. W. (2013). Phylogenetic findings suggest possible new habitat and routes of infection of human eumyctoma. *PLoS Neglected Tropical Diseases* **7**(5): e2229.

Egan, A. N., & Crandall, K. A. (2006). Theory of Phylogenetic Estimation. *Evolutionary Genetics: Concepts and Case Studies* **1**: 426-436.

Godoy, P., Reina, R., Calderón, A., Wittich, R. M., García-Romera, I., & Aranda, E. (2016). Exploring the potential of fungi isolated from PAH-polluted soil as a source of xenobiotics-degrading fungi. *Environmental Science and Pollution Research* **23**(20): 20985-20996.

Gulis, V., Rosemond, A. D., Suberkropp, K., Weyers, H. S., & Benstead, J. P. (2004). Effects of nutrient enrichment on the decomposition of wood and associated microbial activity in streams. *Freshwater Biology* **49**(11): 1437-1447.

Hoseinzadeh, S., Shahabivand, S., & Aliloo, A. A. (2017). Toxic metals accumulation in Trichoderma asperellum and T. harzianum. *Microbiology* **86**(6): 728-736.

Ismail, A. M., Cirvilleri, G., Polizzi, G., Crous, P. W., Groenewald, J. Z., & Lombard, L. (2012). Lasiodiplodia species associated with dieback disease of mango (Mangifera indica) in Egypt. *Australasian Plant Pathology* **41**(6): 649-660.

James, T. Y., Kauff, F., Schoch, C. L., Matheny, P. B., Hofstetter, V., Cox, C. J., & Lumbsch, H. T. (2006). Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* **443**(7113): 818-822.

Jill Harrison, C., & Langdale, J. A. (2006). A step by step guide to phylogeny reconstruction. *The Plant Journal* **45**(4): 561-572.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology And Evolution* **33**(7): 1870-1874.

Maicas, S., Adam, A. C., & Polaina, J. (2000). The ribosomal DNA of the zygomycete Mucor miehei. *Current Genetics* **37**(6): 412-419.

Mohali, S., Burgess, T. I., & Wingfield, M. J. (2005). Diversity and host association of the tropical tree endophyte Lasiodiplodia theobromae revealed using simple sequence repeat markers. *Forest Pathology* **35**(6): 385-396.

Mulholland, P. J., Tank, J. L., Sanzone, D. M., Wollheim, W. M., Peterson, B. J., Webster, J. R., & Meyer, J. L. (2000). Nitrogen cycling in a forest stream determined by a 15N tracer addition. *Ecological Monographs* **70**(3): 471-493.

Nagy, V., Seidl, V., Szakacs, G., Komoń-Zelazowska, M., Kubicek, C. P. & Druzhinina, I. S. (2007). *Applied Environmental Microbiolology*, **73**, 7048□ 7058.

Owolade, B. F., Fawole, B., & Osikanlu, Y. O. K. (2001). Fungi associated with maize seed discolouration and abnormalities in South Western Nigeria. *African Crop Science Journal* **9**(4): 693-697.

Peay, K. G., Kennedy, P. G., & Bruns, T. D. (2008). Fungal community ecology: a hybrid beast with a molecular master. *AIBS Bulletin*, **58**(9): 799-810.

Punithalingam, E. (1976). Botryodiplodia theobromae.[Descriptions of Fungi and Bacteria]. *IMI Descriptions of Fungi and Bacteria*, **52**:519.

Rahman, A., Begum, M. F., Rahman, M., Bari, M. A., Illias, G. N. M., & Alam, M. F. (2011). Isolation and identification of Trichoderma species from different habitats and their use for bioconversion of solid waste. *Turkish Journal of Biology* **35**(2): 183-194.

Huzefa, A., Miller, A. N., Pearce, C. J. & Oberlies, N. H. (2017). Fungal identification using molecular tools: a primer for the natural products research community. *Journal of Natural Products,* **80**(3): 756-770.

Reddy, M. S., Murali, T. S., Suryanarayanan, T. S., Rajulu, M. G., & Thirunavukkarasu, N. (2016). Pestalotiopsis species occur as generalist endophytes in trees of Western Ghats forests of southern India. *Fungal Ecology* **24**: 70-75.

Roy, S. S., Dasgupta, R., & Bagchi, A. (2014). A Review on Phylogenetic Analysis: A Journey through Modern Era. *Computational Molecular Bioscience* **4**(03): 39.

Russell, J. R., Huang, J., Anand, P., Kucera, K., Sandoval A. G., Dantzler, K. W., Hickman, D., Jee, J., Kimovec F. M., Koppstein, D., Marks, D. H., Mittermiller, P. A., Núñez, S. J., Santiago, M., Townes, M. A., Vishnevetsky, M., Williams, N. E., Vargas, M. P. N., Boulanger, L., Bascom-Slack, C. & Strobel, S. A. (2011). Biodegradation of polyester polyurethane by endophytic fungi. *Applied And Environmental Microbiology* **77**(17): 6076-6084.

Sultana, N. & Ghaffar, A. (2009). Seed-borne fungi associated with bottle gourd [Lagenaria siceraria (Mol.) Standl]. *Pak. J. Bot* **41**(1): 435-442.

Tsui, C. K., Woodhall, J., Chen, W., Andrélévesque, C., Lau, A., Schoen, C. D., & de Hoog, S. G. (2011). Molecular techniques for pathogen identification and fungus detection in the environment. *IMA Fungus* **2**(2): 177-189.

Wong, K. M. K., Goh, T. K., Hodgkiss, I. J., Hyde, K. D., Ranghoo, V. M.,Tsui, C. K. M., Ho, W. H., Wong, S. W. & Yuen, T. K., (1998). Role of fungi in freshwater ecosystems. *Biodivers. Conserv*, **7**(1): 1187-1206.