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EVALUATION OF ITS AND LSU LOCI IN DNA BARCODING OF SELECTED MEDICINAL MACROFUNGI FROM CALABARZON REGION, PHILIPPINES

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ABSTRACT

Macrofungi are considered one of the diverse groups of organisms that are recognized for their ecological, economical and medicinal uses. CALABARZON, or Region IV-A in the Philippines is known to have high macroscopic fungal diversity due to its cool climate and rich vegetation. Documentation of medicinal macrofungi in the Philippines using molecular data is not yet fully utilized. Hence, DNA barcoding of selected medicinal macrofungi namely: *Auricularia auricula-judae* (Mont.) Sacc., *Auricularia mesenterica* (Dicks) Pers., *Auricularia polytricha* (Mont.) Sacc., *Dictyophora indusiata* (Vent. Desv., *Ganoderma applanatum* (Pers.) Pat., *Ganoderma lucidum* (Leys.) Karst., *Schizophyllum commune* Fr. and *Volvariella volvacea* (Bull.) Sing. were collected to established their molecular identity. The efficiency of nuclear ribosomal DNA

internal transcribed spacer (*ITS*) and large sub-unit (*LSU*) were evaluated for molecular identification of the collected samples in terms of universality and discriminatory ability. Results for universality revealed that *ITS* yielded higher Polymerase Chain Reaction (PCR) rates with 78.57% than *LSU* with 64.29%. While in DNA sequencing rates, provided a higher accounting to 88.89% than 81.82% of *ITS.* The discriminatory power based on species identification and resolution, both barcode markers were able to identify all the taxa, resolving 100% identity. Moreover, sequence divergence of inter- and intraspecific in the *ITS* and *LSU* barcode loci were significantly different from each other ($\rho =< 0.01$) when compared in Wilcoxon Mann-Whitney Test.

Keywords: Medicinal Macrofungi, DNA barcoding, Large sub-unit (LSU), nuclear ribosomal internal transcribed spacer (ITS)

INTRODUCTION

Macrofungi are best described with unique fruiting bodies that are visible to the naked eyes. They appeared as umbrella like the fleshy mushroom, stinkhorns, bracket macrofungi, puffballs and bird's nest [1]. These eukaryotic organisms are heterotrophic, thus they depend on decaying organic matter for nutrition through extracellular enzyme production. They are classified into Ascomycetes or Basidiomycetes which are spore–bearing and grow on different substrates like decomposing plants and animals, leaf litter, animal dung, inside the body of insect, twigs, tree trunks or branches [2].

Macrofungi, specifically mushrooms have been exploited by mankind since time immemorial for their culinary and medicinal properties. Bioactive compounds from mushrooms, which include polysaccharides, triterpenoids, lectins, steroids, and proteins, have been discovered. These compounds are known for their contributions to the improvement of human health. Recent studies revealed several health benefits including antitumor, antioxidant, hypoglycemic and anti–human papilloma virus [3], [4], [5], [6]. Many mushrooms are also recognized as good sources of nutraceutical compounds [1], [7], [8].

The CALABARZON or Region 4-A in the Philippines, which is composed of the provinces of Cavite, Laguna, Batangas, Rizal and Quezon, is abundant with several species of mushroom that are still unidentified. Comprising a total land area of 1,636,303 hectares, this region is distinguished as one of the most biodiverse

landscapes in the country. It's 74,378 ha. from the total land area are classified as protected [9].

Nowadays, DNA barcoding that employs short genetic sequence obtained from a segment of a genome is a globally accepted method to confirm the identity of organisms [10]. Thus, DNA barcoding was applied in this study in order to facilitate and ensure a more precise identification [11] of some medicinal macroscopic fungi and to evaluate the efficiency of the nuclear ribosomal DNA-internal transcribed spacer (*ITS*) and large sub-unit (*LSU*) capability as DNA barcode for medicinal macroscopic fungi. The evaluations were done in terms of universality with Polymerase Chain Reaction (PCR) and DNA sequencing success rate. Another is discriminatory power for each species mean inter – and intraspecific divergence and resolution of species.

MATERIALS AND METHODS

Study Site and Sample Collection

Purposive sampling was employed to collect eight medicinal macrofungi (Figure 1& 2 and Table 1) in five sites namely: Site 1, Mt. Palaypalay-Mataas na Gulod Protected Landscape in Cavite, Site 2, Mt. Maculot within the Taal Volcano Protected Landscape in Batangas, Site 3, Quezon Protected Landscape in Quezon, Site 4, Mt. Banahaw San Cristobal Protected Landscape in Laguna and Site 5, the Hinulugang Taktak in Rizal. The preparation of samples was based on the works of Hosaka, 2008 [12].

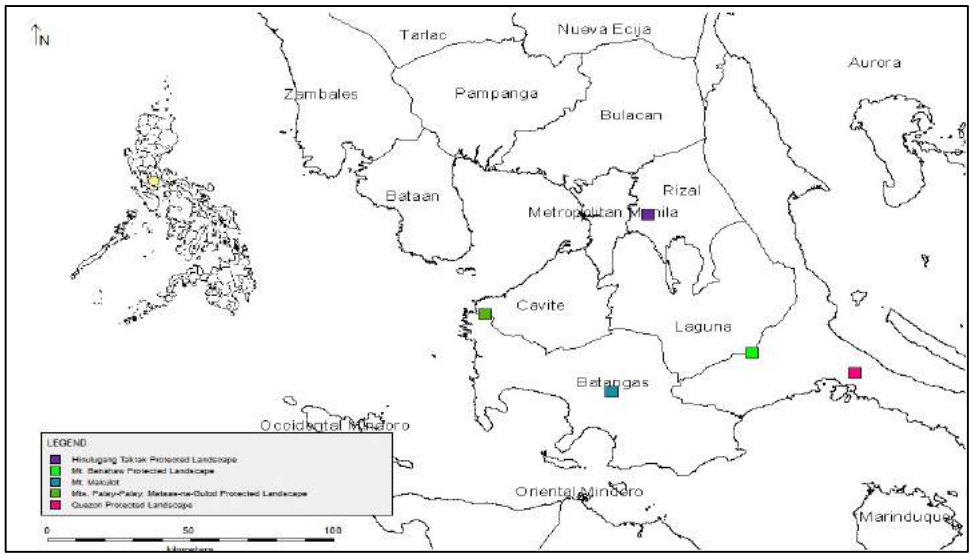

Fig.1. Map of CALABARZON pointing the location of five Protected Areas

Table 1: List of medicinal macroscopic fungi collected in CALABARZON and subjected for molecular identification		
Taxa	**Medicinal Property**	**Author**
***Auricularia auricula-judae* (Mont.) Sacc.**	**anti-inflammatory and antitumor**	**Choudhury,M.P. & Sarma, T.C., 2014**
***Auricularia mesenterica* (Dicks) Pers.**	**Antioxidant**	**Ferreira,I.C.F.R. *et al., 2009***
***Auricularia polytricha* (Mont.) Sacc.**	**hypoglycemic effect**	**Wu,N.J. *et al.*, 2014**
Dictyophora indusiata (Vent.) Desv.	**antioxidant and anti-microbial**	**Oyetayo *et al.*2009**
***Ganoderma applanatum* (Pers.) Pat.**	**antitumor, cardiovascular, antimicrobial**	**Rai, M. *et al 2005***
***Ganoderma lucidum* (Leys.) Karst.**	**antitumor and anti immunomodulating**	**Kozarki *et al.*,2011**
***Schizophyllum commune* Fr.**	**Antimicrobial**	**Mirfat *et al.*, 2014**
***Volvariella volvacea* (Bull.) Sing.**	**antioxidant and antitumor**	**Ramkumar, *et al.,2012***
		Roy *et al.*, 2014

Fig. 2. Photographs of collected medicinal macrofungi: (A) *Auricularia auricula* (Mont.). Sacc.; (B) *Auricularia mesenterica* (Dicks.) Pers.; (C) *Auricularia polytricha* (Mont.) Sacc.;(D) *Dictyophora indusiata* (Vent.)Desv.; (E) *Ganoderma applanatum* (Pers.) Pat.; (F) *Ganoderma lucidum* (Leys.) Karst.; (G) *Schizophyllum commune* Fr.; (H) *Volvariella volvacea* (Bull.) Sing.

2.2 DNA Extraction, Amplification Purification and Sequencing

DNA extraction was accomplished following the manufacturer's instructions of DNeasy Plant Mini Kit (Qiagen, Germany).Figure 3 and 4 show the position of *ITS 4 and ITS 5* primers; *LROR* and *LR5* primers utilized in the amplification and sequencing of internal transcribed spacer (*ITS*) and large sub-unit (*LSU*) nuclear ribosomal DNA. PCR products were purified by QIAquick PCR Purification Kit (Qiagen, Germany). The purified PCR products were forwarded to MACROGEN, South Korea for sequencing.

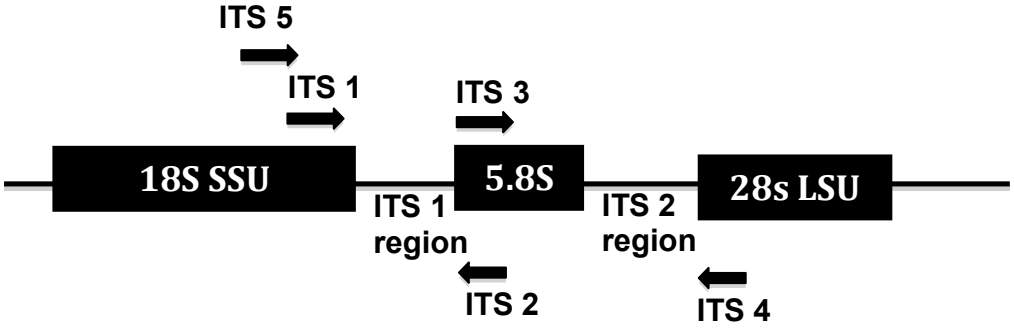

Figure 3: Position of *ITS 4 & ITS* 5 primers in nuclear ribosomal DNA which are used in amplification *ITS* region.

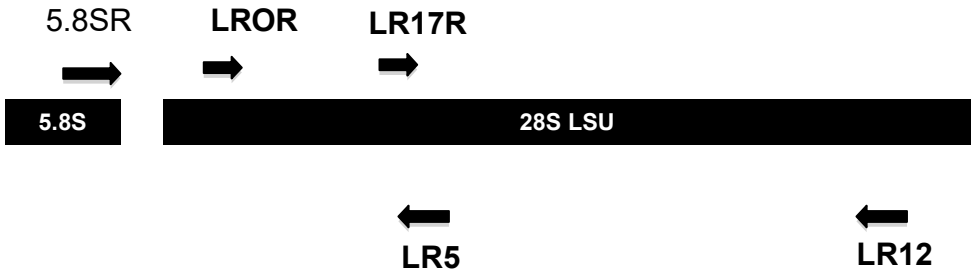

Figure 4: Position of *LROR* forward primer and *LR5* reverse primers in nuclear ribosomal DNA used in the study for the amplification of 28S *LSU* region.

Polymerase Chain Reaction (PCR) cocktails were achieved with the following concentrations (25µL): 16.3µL of molecular bio. H20, 2.5µL of 10 x buffer, 1µL MgCl2 (25mM), 1.0µL dNTP mix, (10 mM), 1.0µL of each primer (8 µM), 0.2 units of Taq polymerase (Accutaq) and 1µL Genomic DNA. PCR reactions were carried out on

BIOMETRA Thermal Cycler. Cycling parameters were as follows: starting denaturation, at 94^0C for 5 minutes, followed by 94^0C for 30 seconds, 50^0C for 45 seconds in annealing of primers, 72^0C for 1 minute in primer extension and the final extension at 72^0C for 10 minutes and completed with 30 cycles. PCR products were subjected to agarose gel electrophoresis and purification utilizing Qiaquick PCR Purification Kit (Qiagen, Germany).PCR products of *ITS* and *LSU* regions were purified using Qia- Quick Purification Kit (Qiagen). Purified PCR products were sent to Macrogen, Korea for sequencing.

2.3 Data Analysis

The PCR success rate was assessed thru the percentage of successfully amplified DNA. Codon Code Aligner v 4.2.5 was utilized in assembling and editing DNA sequences. Basic Local Alignment Search Tool and GenBank nucleotide sequences were utilized in analyzing sequence similarity and identity. The MEGA 6.0 package was used in the alignment of sequences on Clustal W, in measuring the *inter* and *intra specific* divergence in p-distance with pairwise deletion options and in the construction of Maximum Likelihood Phylogenetic tree in Kimura 2- parameter (K2P) distances in which resolution of species was analyzed. Wilcoxon Mann-Whitney test was performed to support statistical significance between the mean inter- and intraspecific genetic divergences of the two barcoding loci.

3. RESULTS AND DISCUSSION

3.1 PCR and Sequencing Success Rates

The effectiveness in PCR amplification and sequencing is an important factor to consider an efficient barcoding loci. A total of 28 samples were collected at five study sites. *ITS* yielded 78.57% PCR success and 81.82 % sequencing. On the other hand, *LSU* amplified 64.29% and 88.89% sequencing success rate. The sequence quality was assessed after it has been edited and assembled into contigs in codon code aligner software. The contigs of ITS sequence quality was accounted to 79.85 % with sequence coverage ranging from 471bp (*Dictyophora indusiata* collected in Site 4) to 815bp (*Volvariella volvacea* collected in Site 3). While LSU was higher than ITS at 90.85% and sequence coverage of 638bp (*Ganoderma applanatum* collected in Site 1 to 1351bp (*Ganoderma lucidum* collected in Site 5). Thus, the mean sequence coverage of bidirectional reads was higher in *LSU* with 91% and only 80% in *ITS*. In the evaluation of universality, *ITS* performed better than *LSU* in PCR success rate. Whereas, *LSU*

showed better in sequencing success rate and sequence quality. The nucleotide sequences generated were all subjected to National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) matched search to confirm the identity of collected samples. Tables 8 and 9 contain the taxa collected from different sites, sequence length, BLAST homology of all nucleotide sequences, percent identity/similarity of generated nucleotide sequences to its respective BLAST homologous sequence and GenBank accession number. All samples had matched identities in GenBank as shown in table 2 and 3.

Table 2: *ITS* NCBI BLAST homology of collected samples

Taxa collected from different Sites (1-5)	Sequence Length	NCBI Taxa Homology	% Identity / Similarity (accession no.)
Auricularia auricula_3	653	*Auricularia aff. Auricula-judae* MEL 2382634	96 % (KP012996.1)
Auricularia mesenterica_2	709	*Auricularia mesenterica* (Dicks)Pers.	92 % (KM396800.1)
Auricularia polytricha_1	774	*Auricularia polytricha* (Mont.) Sacc.	85% (KJ2617301)
Auricularia polytricha_2	583	*Auricularia polytricha* (Mont.) Sacc.	99% (KJ627785.1)
Dictyophora indusiata_4	471	*Dictyophora indusiata* (Vent.) Desv. (1809)	87% (AF324161.2)
Ganoderma applanatum_1	655	*Ganoderma applanatum* (Pers.) Pat.	99 % (JN008873.1)
Ganoderma applanatum_2	634	*Ganoderma applanatum* (Pers.) Pat.	99% (GU213473.1)
Ganoderma applanatum_3	729	*Ganoderma applanatum* (Pers.) Pat.	99% (KF494999.1)
Ganoderma applanatum_4	726	*Ganoderma applanatum* (Pers.) Pat.	95% (AJ608709.1)
Ganoderma applanatum_5	630	*Ganoderma applanatum* (Pers.) Pat.	99 % (GU213472.1)
Ganoderma lucidum_1	658	*Ganoderma lucidum* (Leys.)Karst.	88 % (EU021459.1)
Ganoderma lucidum_4	647	*Ganoderma lucidum* (Leys.)Karst.	88% (GU726927.1)
Ganoderma lucidum_5	658	*Ganoderma lucidum* (Leys.)Karst.	95 % (HM053462.1)
Schizophyllum commune_1	630	*Schizophyllym commune* Fr.	99% (AB369909.1)
Schizophyllum commune_2	656	*Schizophyllym commune* Fr.	100 % (KP012945.1)
Schizophyllum commune_3	730	*Schizophyllym commune* Fr.	100 % (KP0689214.1)
Schizophyllum commune_5	559	*Schizophyllym commune* Fr.	100 % (LN808976.1)
Volvariella volvacea_3	815	*Volvariella volavacea* (Bull.)Sing.	90 % (JN086668.1)

Table 3: *LSU* NCBI BLAST homology of collected samples

Taxa collected from different Sites (1-5)	Sequence Length	NCBI Taxa Homology	% Identity / Similarity (Accession no.)
Auricularia auricula_2	859	*Auricularia aff. Auricula-judae* (Mont.) Sacc.	98 % (KM396826.1)
Auricularia auricula_3	864	*Auricularia aff. Auricula-judae* (Mont.) Sacc.	98 % (KM396825.1)
Ganoderma applanatum_1	638	*Ganoderma applanatum* (Pers.) Pat.	99 % (KF495009.1)
Ganoderma applanatum_2	853	*Ganoderma applanatum* (Pers.) Pat.	99 % (KF495010.1)
Ganoderma applanatum_3	1351	*Ganoderma applanatum* (Pers.) Pat.	98 % (KF495011.1)
Ganoderma applanatum_4	866	*Ganoderma applanatum* (Pers.) Pat.	99 % (EU232274.1)
Ganoderma applanatum_5	897	*Ganoderma applanatum* (Pers.) Pat.	99 % (EU2322274.1)
Ganoderma lucidum_1	1007	*Ganoderma lucidum* (Leys.) Karst.	97 % (DQ208411.1)
Ganoderma lucidum_2	818	*Ganoderma lucidum* (Leys.) Karst.	99 % (DQ208413.1)
Ganoderma lucidum_3	998	*Ganoderma lucidum* (Leys.) Karst.	98 % (DQ208410.1)
Ganoderma lucidum_4	763	*Ganoderma lucidum* (Leys.) Karst.	98 % (DQ208413.1)
Schizophyllum commune_1	893	*Schizophyllym commune* Fr.	100 % (HM595605.1)
Schizophyllum commune_2	856	*Schizophyllym commune* Fr.	99% (FJ372711.1)
Schizophyllum commune_3	824	*Schizophyllym commune* Fr.	100 % (DQ071725.2)
Schizophyllum commune_4	763	*Schizophyllym commune* Fr.	99% (FJ372712.1)
Schizophyllum commune_5	849	*Schizophyllym commune* Fr.	99 % (HM595605.1)

3.2 Mean Inter – and Intraspecific Divergence

The divergence was analyzed between inter- and intra-species using Kimura 2-Parameter Analyses (Table 4). The LSU with 90% (0.09) showed high mean interspecific sequence divergence that was significantly higher than ITS which had 43%. (0.43). Whereas, in intraspecific divergence ITS had 16% (0.16) while LSU had 0% (0.00). The mean intraspecific divergence of ITS showed a high discrimination within species and very minimal variations with 16%. While LSU showed an extremely high discrimination within species, which resulted in no variation within species and showed that nucleotide sequences from different sites were of the same species. Table 5 and 6 data showed that Wilcoxon Mann – Whitney test is significant (2-tailed) for both fungal barcode scores on the ITS and LSU. Both fungal barcodes loci showed a comparable level of i*nter* and *intra specific* divergence, results are highly significant ($p < 0.001$). The value of mean ranking indicates that the LSU fungal barcode significantly worked better in inter and intra specific divergence than the ITS.

Table 4: Mean Interspecific and Intraspecific divergence of *ITS* and DNA Barcodes.

Sequence Divergence	DNA Barcodes	
	ITS	*LSU*
Mean Inter-specific divergence (%)	0.43 ± 0.16	0.90 ± 0.05
Mean Intra-specific divergence (%)	0.16 ± 0.17	0.00 ± 0.00

Table 5: Test Statistics on Wilcoxon Mann –Whitney Test of Interspecific and intraspecific divergence of *ITS* and *LSU*.

	Interspecific Divergence	Intraspecific divergence
Mann-Whitney U	730.000	15.000
Wilcoxon W	5195.000	366.000
Z	-12.938	-5.649
Asymp. Sig. (2-tailed)	.000	.000

Table 6: Ranks on Wilcoxon Mann –Whitney Test of Interspecific and intraspecific divergence of *ITS* and *LSU*

Fungal Barcode	N	Mean Rank	Sum of Rank
Inter specific divergence *LSU*	94	55.27	5195.00
ITS	212	197.06	41777.00
Total	306		
Intra specific divergence *LSU*	26	14.08	366.00
ITS	18	36.67	624.00
Total	44		

3.3 RESOLUTION OF SPECIES

The maximum likelihood (ML) Phylogenetic topology tree of the partial nucleotide sequence of *ITS* region revealed notable clades of 18 taxa and was identified and confirmed under eight species and five genera (Figure 4). The clades were supported by high bootstrap support values of 100% resolution of species. Likewise, ML Phylogenetic tree of partial nucleotide sequences of LSU displayed notable clades of 16 taxa under four species and three genera (Figure 5). All species showed monophyletic group for both DNA barcodes.

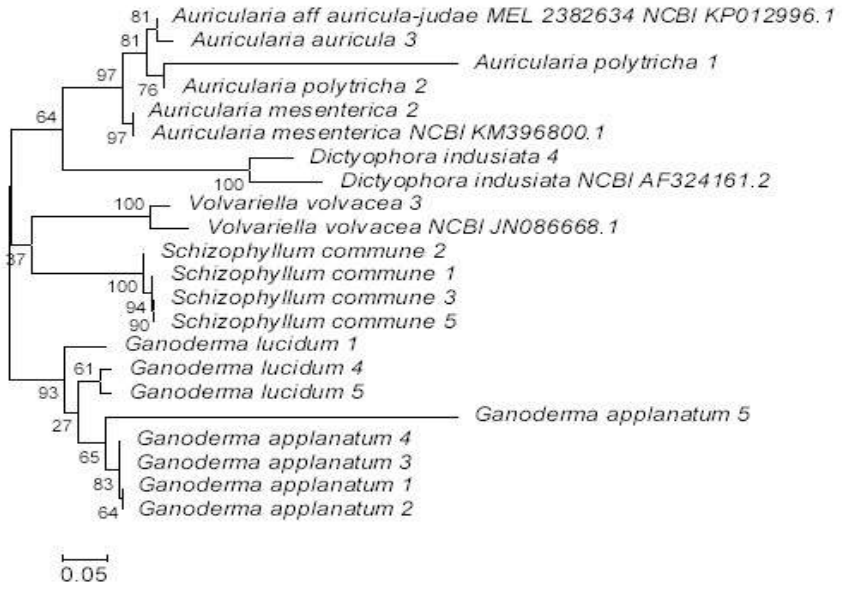

***Fig.*4: *ITS* - Resolution of Species using Maximum Likelihood Phylogenetic Tree. ML Bootstrap consensus trees showed 100% resolution of species for the *ITS* barcode region using K2P Model.**

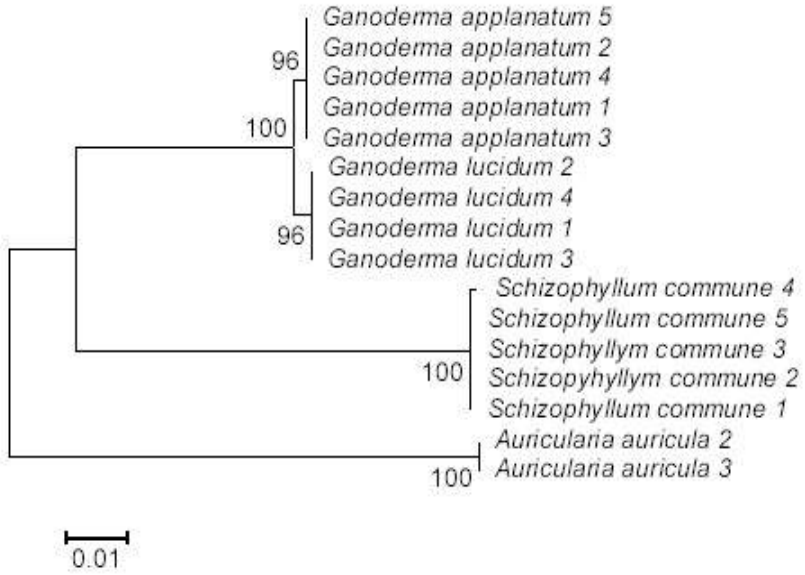

***Fig. 5*: *LSU* - Resolution of Species using Maximum Likelihood Phylogenetic Tree. ML Bootstrap consensus trees showed 100% resolution of species for the *ITS* barcode region using K2P Model.**

4. DISCUSSION

The nuclear ribosomal internal transcribed spacer (*ITS*) is the proposed universal DNA barcode for fungi by Fungal Barcoding Consortium, due to its utmost possibility in species identification for large number of fungi. While the nuclear ribosomal a large subunit (*LSU*) with higher species resolution in other fungal group is found to be secondary DNA barcode for fungi [14]. The results in PCR success rates were *ITS* with 78.57% rate and 64.29% for *LSU*. These data confirm that *ITS,* based on [14]. works better in PCR amplification than other fungal barcode they tested including *LSU.* In the sequence success rate, LSU was higher at 88.89% to *ITS* at 81.82%. The *ITS*-generated 22 nucleotide sequences and four of which were contaminated. On the other hand, *LSU* generated 18 sequences and two of which were contaminated. A total of six sequences from *ITS* and *LSU* were contaminated with microscopic fungi: *Aspergillus spp.* and *Candida spp.* The study of [15]. supports this contamination factors that could possibly cause unsuccessful identification of nucleotide sequences from this study. Fungal contamination of airborne spores (conidia of *Aspergillus*), during DNA extraction process, may lead to false identification. These contaminants can be *Aspergillus fumigatus, Saccharomyces cerevisiae, and Acremonium spp*. The *LSU* has higher sequence quality with 90.85% as compared to 79.90% of *ITS*. In terms of mean sequence divergence, the ability to discriminate between species or interspecific divergence was higher in *LSU* with 90% or 0.09 while *ITS* had 43% or 0.43. Wherein, mean *intraspecific* divergence resulted from 0.00% in *LSU* and 16% or 0.16 in *ITS.* The standard sequence divergence for *intraspecific* variations ranged from 0 to 72% and 0 to 58% for interspecific variations [14].

Based on the result of this study, the species discriminatory ability of *LSU* is higher than *ITS*. In terms of intra species discriminatory ability *LSU* was able to show that samples are of the same species. But, the results for *ITS* intra and inter specific discriminatory ability still lies within the standard average range. *ITS* can also discriminate species to other species and within species. The Wilcoxon Mann – Whitney test revealed that the two barcoding loci are significantly different from each other in terms of inter and intra specifics divergence with .000 which is less than .001. The standard statistical test results state that if the result is $p < 0.001$ it is considered as highly significant. Species resolution using

Maximum likelihood tree formed a monophyletic clade. Thus, both *ITS* and *LSU* identified and resolved all the samples respectively, giving a 100% resolution of species.

5. CONCLUSION

All eight medicinal macrofungi collected in five sites used in this study were initially identified using morphology and successfully confirmed by their molecular identity using DNA barcoding. Nuclear ribosomal DNA internal transcribed spacer (*ITS*) and Large Sub unit (*LSU*) facilitated the molecular identification of all eight medicinal macroscopic fungi. In this study, ITS is further supported to have higher PCR rate success [14] than LSU and both barcode loci yielded 100% species resolution but in other parameters (nucleotide sequencing rates, sequence quality, sequence coverage and mean sequence divergence) LSU worked better than ITS. Findings revealed that in the DNA barcoding of eight medicinal macrofungi utilized in this study, LSU performed better as macrofungal barcode than ITS.

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