GAU Research Journal 46(4) : 15-21 (October 2021)

Molecular Characterisation and Phylogenetic Analysis of Two ildmushroom Species from Pathanapuram in Kerala, India

Dr. Sona A[1]*, Arya S Vijayan[2]
[1]Assistant Professor, SAS SNDP Yogam College, Konni
[2]Student, SAS SNDP Yogam College, Konni

(Period of research: March-June 2020)

ABSTRACT

The aim of the study to determine the evolutionary relationship of two wild mushrooms based on 18s rRNA sequences. Genomic DNA was isolated by CTAB method, and 18S ribosomal RNA amplified by PCR using forward ITS4 and reverse ITS5 primers. Sequencing was done and similarity identified with BLAST. The analysis revealed 98.89% similarity with *Earliella scabrosa* 99.20% with *Fomitopsismeliae.*Phylogenetic tree was constructed using Neighbor-Joining tree, Minimum evolution tree methods using MEGAXI software.

Key words: phylogenetic tree, BLAST, evolutionary relationship, molecular markers, fungi

INTRODUCTION

Fungi are eukaryotes with complex cellular organization being heterotrophic in nature is responsible for decomposition and nutrient cycle thereby maintain an ecological balance. Mushrooms are fleshy fruiting bodies that comprise 80-90% water and are excellent source of vitamins and minerals except iron and calcium (Manikandan, 2011). Mushrooms are used as functional foods; their low-calorie protein rich diet are of importance in diet of rural and urban dwellers. Mushrooms are priced vegetable owing to their increasing cultivation and consumption. Classification of fungi and basidiomycetes is based on standard barcoding markers of which the internal transcribed spacers (ITS) region is widely used. ITS markers enable successful molecular characterization and species identification of wild edible mushrooms and are used in the construction of phylogenetic tree (Khaund and Joshi, 2014; Schoch et al., 2012). The polymorphic nature of rDNA (Gardes and Bruns, 1993) enable speciation (Martin et al., 2004), morphological, ecological adaptation. *Earliella scabrosa* is a bracket and puffball type fungus proposed in the global fungal red list is a well distributed species throughout the world. This species of fungus is mainly found on newly fallen trees (Adarsh et al., 2015). This species is known to possess antioxidant activity and anticancer activities and hence used in therapy (Zmitrovich et al., 2017). *Fomitopsis meliae*is a brown rot fungus found on living or dead conifers and hardwoods known to produce endoglucanase. Though known as a saprophytic fungus, the aim of the experiment is to extract the rDNA-ITS fragments of two wild mushrooms species and amplify using ITS4 and ITS5 primers and determination of nucleotide sequence. Sequence was analyzed for similarity using BLAST of NCBI database to identify the species. The FASTA format was used to create Phylogenetic tree. Mushrooms find wide applications in marketing as a source of income, apart from medicinal use, in ceremonies, dyeing wood and natural fibres, as fire starters and gun powder.

MATERIALS AND METHODS

Sample Collection: Both the wild mushrooms found on tree bark were collected from Pathanapuram (9.079944/76.821610) on 16th March 2020. Mushroom with its base intact was removed by hand and stored in a polythene bag under refrigeration until processing.



Figure 1: SAMPLE A



Figure 2: SAMPLE B

ISOLATION OF GENOMIC DNA (Healey *et al.*, 2014)

Material required: Mushroom fruiting bodies

Preparation of CTAB buffer:2 g CTAB (Cetyl trimethyl ammonium bromide); 10 ml 1 M Tris pH 8; 4ml 0.5 M EDTA pH 8 (Ethylenediaminetetraacetic acid di-sodium salt); 28 ml 5M NaCl; 40.0ml H_2O; 1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) MW 40,000). Adjust all to pH 5.0 with HCl and make up to100ml with H_2O.

Liquid Nitrogen, Absolute Ethanol (ice cold), 70% Ethanol (ice cold), 7.5M Ammonium Acetate, Chloroform:Iso Amyl Alcohol(24:1), Water(sterile), RNase (10μg/ml) 200mg of plant tissue was ground to a fine powder with liquid nitrogen and added approximately 500µl of CTAB buffer. Transferred CTAB/plant extract mixture to a microfugetube and incubated the CTAB/plant extract mixture for about 15 min at 55°C in a water bath. After incubation, the CTAB/ plant extract mixture was centrifuged at 12000 rpm for 5 min and the supernatant transferred to clean microfuge tubes.

To each of the tubes added 250µl of chloroform: isoamyl alcohol (24:1) and mixed the solution byinversion. The tubes were centrifuged at 13000 rpm for 10minutes. The upper aqueous phase (containing the DNA) was transferred to a clean microfuge tube. To the tubes added half volume of chloroform: isoamyl alcohol and mixed well by inversion. After mixing, the tubes were centrifuged at 13000rpm for 10minutes. Then, added 50µl of 7.5 M ammonium acetate followed by 500µl of ice-cold absolute ethanol. The tubes were inverted slowly several times to precipitate the DNA. The tubes were placed at -20˚C for 1hrtoprecipitatethe DNA. The tubes were centrifugedat13000rpmfor 5min and the supernatant decanted. 500µlofice cold 70% ethanol was added and slowly the tube was inverted. The tubes were centrifuged at 13000rpm for 1 min to pellet the DNA. The process was repeated twice. Removed the supernatant and allowed the DNA pellet to air dry (approximately 15 min). Resuspended the DNA in sterile DNase free water(10µlRNaseA can be added to the10ml water prior to dissolving the DNA to remove any RNA in the preparation). After resuspension, the DNA was incubated at 65˚C for 20 min to destroy any DNases that may be present and stored at 4˚C. Further the DNA was stored at–14˚C. Quantitation of DNA was done using spectrophotometer and the quality analysed by gel electrophoresis.

POLYMERASE CHAIN REACTION: Taken PCR buffer (10X), dNTP (100mM), Taq polymerase (5U/µl), deionised water, Primer (Forward ITS4 and reverse ITS510 pmol/µl), template DNA 11ng/µl|), Ethidium bromide (10mg/ml). Materials Required: PCR machine, microcentrifuge, crushed ice, adjustable micropipettes, plastic tips, PCR eppendorf (0.5ml).

FOR REACTION VOLUME: Mixed 2xPCRMasterMix- 12.5 µl;10µM ITS4 - 2.0µl; 10µM; ITS5- 2.0µl; DNA10ng/µl -2.0µl; Deionised water - 6.5µl

PCR CONDITIONS: Initial denaturation for 95˚C 1.00 min followed by Denaturation at 95˚C 0.30 min; Annealing at 53˚C 1.00min; Extension at 68˚C 1.40min; repeat for 30 cycles; Final extension at 68˚C 5min; Finally stored at 4˚C.

PHYLOGENETIC ANALYSIS

Sequence analysis was performed using online tool BLAST of NCBI database. Forward and reverse sequences were assembled, and coding was generated after trimming the low-quality bases. The trimmed genetic sequences were then compared to different 18S rRNA gene of different mushrooms in there reference RNA sequence (18SrRNA) databases of NCBI nucleotide BLAST website to identify the genus of the selected mushroom. The query sequences were constructed to FASTA format and then used to create a Phylogenetic tree using Neighbor Joining and Minimum evolution tree construction methods.

RESULTS AND DISCUSSION

DNAISOLATIONBYCTABMETHOD

Genomic DNA was extracted with the established CTAB method. Briefly cell walls of fungal mycelia were broken down by grinding with glass rods or in the presence of liquid nitrogen. The CTAB extraction buffer was then added, and after incubation, purified with phenol chloroform isoamylalcohol and precipitation with isopropanol were conducted. Finally, the DNA was isolated and performed quantification by spectrophotometry and analyzed quality by agarose gel electrophoresis.

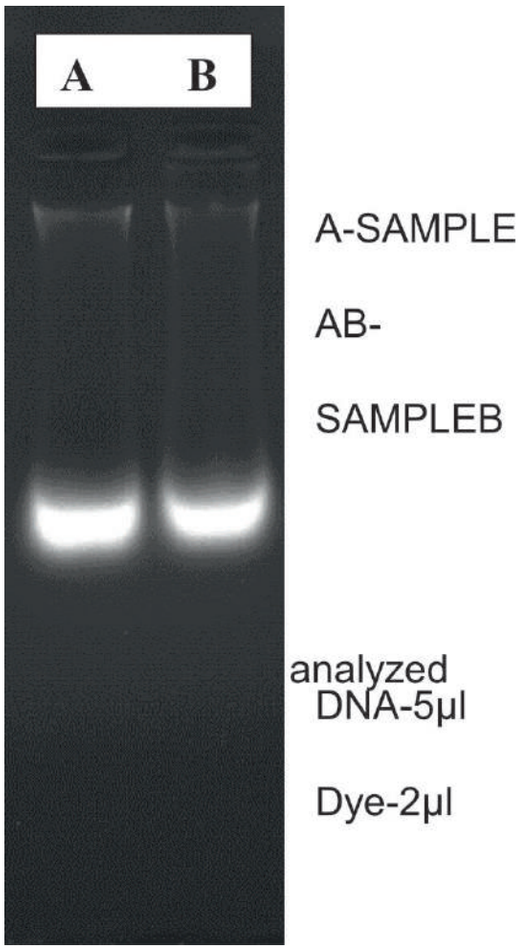

Figure 3: Gel Electrophoretic pattern of DNA of mushroom samples A and B

POLYMERASECHAINREACTION

The genomic DNA of the mushroom was extracted, the rDNA-ITS fragment of the genomic DNA was amplified using ITS4 and ITS5 primer and subjected to nucleotide sequence AA, Ganesh, P.N. (2015). Decomposition of wood by Polypore Fungi

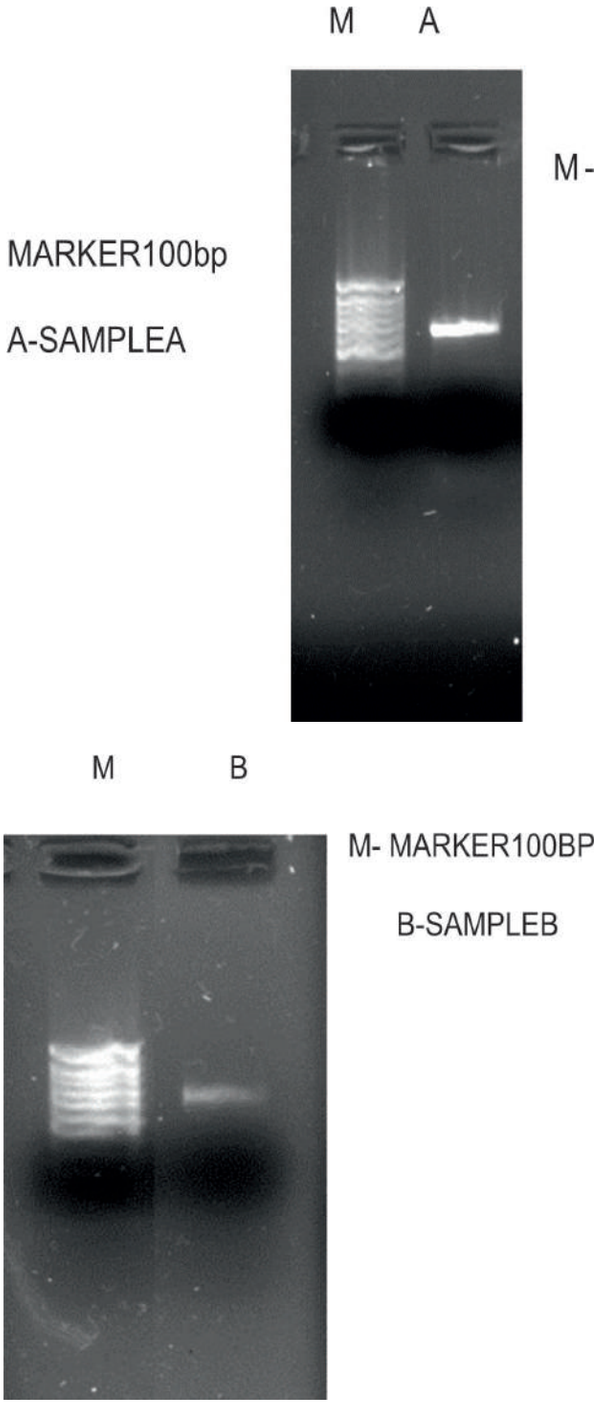

Fig:4: Gel Electrophoretic pattern of PCR amplified 18S rRNA of mushroom samples A and B

CODING

SAMPLE A

GGGACTGGAGTCTACCTGATTTGAGGTCAGAGGTCATAAGAAGCTGTCTCTTGAG
CGAGACGATTAGAAGCCAGCCAAACGCTTCACGGTCGCGGCGCAGATAATTATC
ACACCGAGAGCCGATCCGCAAGGAAACAGGCTAATGCATTTAAGAGGAGCCGAC
TTGCGAGAAGCCAACAAAAACCTCCAAGTCCAAGCCTACAAACCCGCAAAGATTT
GTAGGTTGAGAATTTCATGACACTCAAACAGGCATGCTCCTCGGAATACPSCAAG
GAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCACATT
ACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCGTTGC
TGAAAGTTGTATATAGATGCGTTACATCGCAATACACATTCTGATACTTTGTAGTT
GGTAGTAAAACGTAGACCCAGTAACGTCACGGCGTTGAAAGCCGCGGCGCGCC
TGAAGCCTACAGTAAGTGCACAGGGGTAAGAGTGGATGAGCAGGGCGTGCACA
TGCCTCGGAAGGCCAGCTACAACCCGTTTCAAAACTCGTTAATGATCCTTCCGCA
GGTTCACCTACAAAGAGACATTGTTACGACTTTTAATTCCCA

The BLAST annotation shows 98.89% identity with Earliella scabrosa.

SAMPLE B

AGGACGGTACGTACTGATTTGAGGTCAAGGTCAAAGTGATTGTCCTCGAGTAAAA
GGACGATTAGAAGCCGATCCCATTAACATGCTTCACAGCAACGGCGTAGACAAT
TATCACACCGATAGCTGATCCACAAAGGTTCGAGCTAATGCATTCAAGAGGAGC
CGATCACAGACATACCGGCAATAAACCTCCAAGTCCAAGCTCTATTCACAAAAGC
AAATAGAGTTGAGAATTCCATGACACTCAAACAGGCATGCTCCTCGGAATACCAA
GGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTCA
CATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCG
TTGCTGAAAGTTGTATTTAAATGCGTTAGACGCAAGAGTGACATTCTTTAAACTGA
AGTAGTTTGTAATAAAACATAGGAAGGCCTACAACCAAAGCAGACACACAGAGTG
CCCACTCCAGCCACAAACCGACCTACAGTGTGTGCACAGGTGTGTGAGGTGGAT
AATGATCAGGGCGTGCACATGCCGCTGACGGCCAGCAACAACCCCTTTCAGAAT
TCATTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTTGTTACGACTTTTAC
TTCCCAA

The BLAST annotation shows 99.20% identity with Fomitopsis meliae.

The PCR products of ITS regions of Earliella scabrosa and Formitopsis meliae are 640 and 660 base pairs respectively in consistent with that of Hussein et al., (2014), Gardes and Bruns (1993) and Maeta et al. (2008) as the fragment size of the DNA target region is 600-800 bp. However according to Blaalid *et al.* (2013) fungal ITS region varies roughly between 450 and 750 base pairs (bp) in length.

PHYLOGENETICTREE

Phylogenetic tree based on a comparison of the 18S rRNA sequences of two mushroom samples and some of their close phylogenetic relatives, the tree was created by the Neighbor Joining, and Minimum Evolution methods.

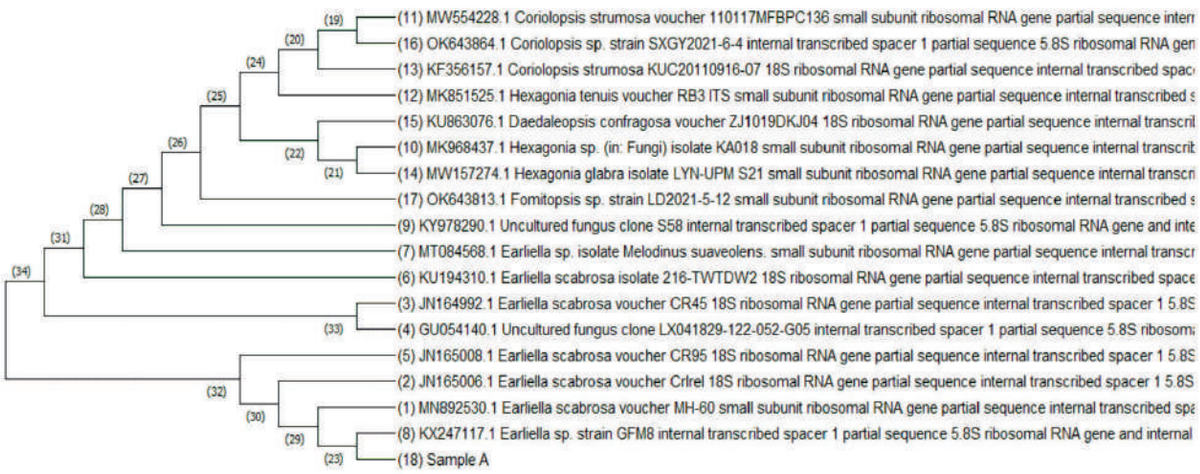

Fig5: Phylogenetic tree of mushroom sample A using Neighbor Joining Method



Fig6: Phylogenetic tree of mushroom sample A using Minimum Evolution Method

PHYLOGENETICTREEOFSAMPLEA

Earliella scabrosa is a fungus of family Polyporaceae, found in the tropical region associated with trees of Acacia, Ficus and dead dying trees. Though known as a saprophytic fungus, in-vitro cultivated Earliella scabrosa showed antifungal activity against certain wood-degrading fungi of rubberwood (Teoh and Don 2013). It is found in rain forests of both eastern and western hemispheres. Some reports have described its antioxidant properties and free radical scavenging ability. Earliella scabrosa contains isocoumarin used in cancer therapy (Zmitrovich et al., 2017).



Fig7: Phylogenetic tree of mushroom sample B using Neighbor Joining Method.



Fig8: Phylogenetic tree of mushroom sampleB using Minimum Evolution tree Method.

PHYLOGENETICTREEOFSAMPLEB

Species of *Fomitopsis* cause brown roton living or dead conifers and hardwoods. The genusis characterized by its sessile to effuse-reflexed, white to pinkish or brown to dark-brown basidiomata, poroid, white to pinkish pores surface with round, angular to irregular pores, dimitic or trimitic hyphal system, presence or absence of cystidia, and sub-globose to cylindrical, hyaline, smooth basidiospores. *Formitopsis meliae* CFA2 brown rot fungi is a potent produce of multi-enzyme cocktail in particular endoglucanase that can be used for enzyme saccharification (Patel et al., 2021). This fungi *Formitopsis meliae* could be grown to check for the production of multi-enzyme cocktail, paves way for further studies. Xylanase and ligno-cellulosic materials can use in saccharification (Lin et al., 2018) and for the production of biofuels (Wu and Shin, 2016).

CONCLUSION

Variation in morphological features might often mislead identification of wild mushrooms. In combination with molecular data, it enables better species identification and characterization. In this study, the ITS markers helped in exact identification of the two species of wild mushrooms. Further study is needed to check for the nutritional, medicinal and industrial use of these two species.

REFERENCES

Adarsh, C. K., Vikas, K., Vidyasagaran, K., Ganesh, P.N. (2015). Decomposition of wood by Polypore Fungi in tropics- Biological, Ecological and Environmental factors – A Case Study. *Research Journal of Agriculture and Forestry Sciences*; 3(8), 15-37.

Blaalid, R., Kumar, S., Nilsson, R., Abarenkov, K., Kirk, P., Kauserud, H. (2013). ITS1versus ITS2 as DNA metabarcodes for fungi. *Molecular Ecology Resources*; 13, 218–224.

Gardes, M., and Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. *Molecular Ecology;*2: 113–118.

Healey, A., Furtado A., Coopper T., Henry R.J. (2014). Protocol: A simple method for extracting next generation sequencing quality genomic DNA from recalcitrant plant species. *Plant methods;*10(1) : 21.

Hussein, J. M., Tibuhwa D. D., Mshandete, A. M., Kivaisi AK. (2014). Molecular phylogeny of saprophytic wild edible mushroom species from Tanzania based on ITS and nLSU rDNA sequences. *Current Research in Environmental and Applied Mycology;* 4(2): 250-260.

Khaund, P., and Joshi, S. R. (2014). DNA barcoding of wild edible mushrooms consumed

by the ethnic tribes of India. *Gene*; 550(1): 123-130.

Lin, H., Sun, M., Li, J., Xu, Q., Yang, B., Wang, Q., Xie, W., Sun, S., Hu, K., and Zhang, L. (2018). Purification and characterization of xylanase from spent mushroom compost and its application in saccharification of biomass wastes, *BioRes;* 13(1), 220-230.

Maeta, K., Ochi, T., Tokimoto, K., Shimomura, N., Maekawa, N., Kawaguchi, N. (2008). Rapid species identification of cooked poisonous mushrooms by using real-time PCR. *Applied and Environmental Microbiology*; 74: 3306–3309.

Manikandan, K. (2011). Nutritional and Medicinal values of Mushrooms. In book "Mushrooms Cultivation, Marketing and Consumption", Directorate of Mushroom Research, Solan; Editors: Singh, M., Vijai, B., Kamal, S., Wakchaurae, G. C.

Martin, P., Muruke, M., Hosea, K., Kivaisi, A. K., Zerwas, N., Bauerle C (2004). A rapid PCR-RFLP method for monitoring gene variation among commercial mushroom species. Biochem Mol Biol Educ. 32, 390-394.

Patel, A., Divecha, J., and Shah, A. (2021) *Fomitopsis meliae* CFA 2, a novel brown rot for endoglucanase: emphasis towards enhanced endoglucanase production by statistical pproach. *Mycology;*DOI: 10.1080/21501203.2021.1918277

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J. L., Levesque, C. A. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences;*109: 6241-6246.

Teoh, Y. P., Don, M. M.(2013). Antifungal activity of In-vitro grown Earliella scabrosa, a Malaysian Fungus on selected wood-degrading fungi of Rubberwood. *Journal of Physical Therapy Science*; 24(2): 21-33.

Wu Y., Shin H. (2016). Cellulase from the fruiting bodies and mycelia of edible mushrooms: A review. *Journal of Mushrooms;* 14(4):127-135.

Zmitrovich, I. V., Ezhov, O. N., Ranadive, K. R., Wasser, S. P.(2017). Profiles of little known medicinal polypores: *Earliella scabrosa* (Agaricomycetes).*International Journal of Medicinal Mushrooms;*19(11): 1023-1027.