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Morphological and Molecular characterization of *Pleurotus flabellatus* from Paechiparai forest of Tamil Nadu, India

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ABSTRACT

An isolate of *Pleurotus flabellatus* was collected from Paechiparai forest, Tamil Nadu, India. In this manuscript macroscopic, microscopic and molecular systematic details of this mushroom have been documented. The rDNA sequencing analysis was carried out to determine the inter species and intra species relation using phylogenetic tree construction. The rDNA sequencing data revealed 98% match to represent *P. flabellatus*.

KEYWORDS: *Pleurotus flabellatus*, rDNA sequence, phylogenetic construction

INTRODUCTION

According to forest report in the year of 2013, in India sixty nine million hectares are reported to be under forest cover which represents 20.64% of the geographic area (Gudikandula *et al*., 2015). In mushrooms approximately 23,000 species are reported to be under *Basidiomycota* which represent the second largest phylum of Kingdom Fungi (Hawksworth *et al*., 1995). The genus *Pleurotus* is one such mushroom with a wide range of geographical distribution in temperate and tropical regions (Delmas, 1989 ; Yang, 1986; Upadhyay *et al*., 2017). Only a fraction of fungal wealth has been studied by mycologists and scientific scrutiny continue to unravel the unexplored and hidden wealth. One-third of the fungi of the world are reported to be present in India of which only 50% are characterized (Manoharachary *et al*., 2005). From India, approximately 1989 taxa of Agaricomycetous mushrooms have been recorded (Upadhyay *et al*., 2017) in comparison to more than 21000 taxa of these fungi documented the world over in the Dictionary of Fungi (Kirk *et al.,*2008). Wild mushrooms have variety of applications including biological, commercial, economical, and environmental (Cohen *et al*., 2002). Some of the wild edible mushrooms have been reported from different parts of India including South West India (Sathe and Kulkarni, 1987; Atri *et al*.,2010). So far from various parts of India as many as 26 species of *Pleurotus* are reported (Upadhyay *et al*., 2017) all of which are edible and medicinally and nutraceutically important. *Pleurotus* species are reported to utilize lignolytic and hydrolytic enzymes and produce their fruit bodies for growth and adaptation (Mikiashvili *et al*., 2006). Some of its cultivated species include *Pleurotus sajor-caju*, *Pleurotus pulmonarius*, *Pleurotus flabellatus*, *Pleurotus cystidiosus*, *Pleurotus sapidus*, *Pleurotus ostreatus*, *Pleurotus abalones*, *Pleurotus citrinopileatus*, *Pleurotus salmoneostramineus*, *Pleurotus floridanus*, etc. (Wang *et al*., 2001; Maziero, 1990). Many taxonomic studies have been carried out on species of *Pleurotus* (Vilgalys *et al*., 1996) but the main problem of taxonomic nomenclature and phylogenetic relationships remain unresolved. The taxonomic disagreements are primarily due to the initial misidentification, absence of type specimens, limited reports on physiological characteristics, instability of morphological characters due to environmental changes and the lack of mating compatibility studies (Zervakis and Balis, 1996). To date, many studies on the mating compatibility of species have identified intersterility groups among species of *Pleurotus*. Molecular and biochemical criteria, including isozyme electrophoresis with isoeletric focusing analysis have been used to detect the intraspecific and interspecific relationships among the species of *Pleurotus* (Zervakis and Labarere, 1992 ; Zervakis *et al*., 1994). Molecular identification based on RFLP of total DNA (Sagawa *et al*., 1992), ribosomal DNA sequence (Iracabal *et al*., 1995 ; Bunyard *et al*., 1996), mitochondrial DNA (Toyomasu *et al*., 1992), structure analysis of mitochondrial rRNA sequence (Gonzalez and Labarere, 2000), polymorphism of the 5' portion of 26S ribosomal DNA (26S rDNA) sequence (Dapeng *et al*., 2004) and ribosomal DNA (Neda and Nakai, 1995) sequence analysis have also been used for understanding phylogenetic relationships as well as the taxonomic identification of *Pleurotus* species. The nucleotide substitution rate in the mtSSU rDNA (mitochondrial small subunit ribosomal) gene is 16 times greater than in the homologous regions of ribosomal rDNA for ten members of the order *Boletales* (Bruns and Szaro, 1992). Mitochondrial SSU rDNA has also been used to investigate the interspecies relationships in the genera *Polyporus* (Ko and Jung, 2002) and *Amylostereum* (Slippers *et al*., 2000). The phylogenetic relationships among the closely related biological species of genus *Pleurotus* have been described by using the partial mitochondrial small subunit ribosomal rDNA sequences (Dapeng *et al*., 2004). In the present study, the morphological and molecular characterization of *Pleurotus flabellatus* (Berk. & Broome) Sacc. has been carried out.

The rDNA sequencing analysis was carried out to determine the inter species and intra species relation using phylogenetic tree construction.

MATERIAL AND METHODS

a) Fruit bodies collection and preservation: The fruit bodies of *Pleurotus flabellatus* (Berk. & Broome) Sacc. were collected from the fallen dead tree in the forest of Dam area, Paechiparai, Tamil Nadu, India during rainy season. Photographs of the fresh specimens were taken both in the collection place as well as in the laboratory (**Fig.1A**). Spore print was obtained by removing one mature pileus from the stipe at the point of attachment, placed over a white cardboard paper in such a way that the lamellae facing downwards, then

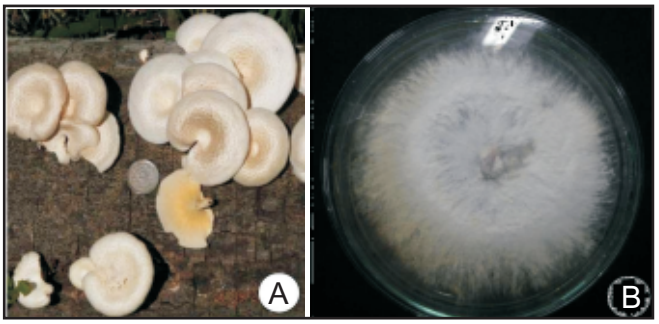

Fig. 1. Habitat features of *Pleurotus flabellatus* (A) Basidiocarps in natural habitat (B) Mother culture plate.

it was covered with petri dish. After an hour the pileus was removed and spore print was air dried. After recording the spore print color, it was properly tagged and preserved in polythene cover containing naphthalene, which prevent these from pests attack for further studies. The color of the spore print is a most valuable character for the identification The specimens were tagged with collection number and air dried in an electric dryer. The fungal specimens were preserved in sealed polythene bags with naphthalene balls which protect these from insect and pest attack The preserved specimen was deposited in the Herbarium of Madras University Botany Laboratory (MUBL) for future microscopic observation

b) Study of macroscopic and microscopic characters: Macroscopic features were studied following Largent *et al.* (1986). Kornerup *et al*. (1978) color chart was followed to determine the color of the fresh specimen. Microscopic features were studied with a compound microscope with camera attached Monitor.

A Carl Zeiss Axiotar microscope equipped with bright field and phase contrast were used for observing the microscopical characters of the fungi. Photographs were taken by the camera attached to the microscope. The line diagrams of the microscopic character were illustrated with the aid of a POM prism type camera lucida fixed to a Labomed Trinocular Microscope (model-CXL plus).

c) Pure culture: The collected basidiomata were cultured for their mycelial growth. The fruiting body was cut equally and washed with 0.01 % $HgCl_2$ to remove contamination in the surface region. After surface sterilization, it was washed with distilled water for three times to remove $HgCl_2$ and transferred to the slant of PDA medium. The mother culture slants of *P. flabellatus* were incubated at 21°C for 5 -7 days on the PDA agar medium. The pure culture was maintained and stored for further investigation **(Fig. 1B)**.

d) Extraction of DNA: *P.flabellatus* was grown using Potato Dextrose Broth (PDB). After fourteen days, the mycelia were harvested and washed with sterile double distilled water. The mycelia were blended with pestle and mortar and the mixture was used for isolation of DNA. A 1.5 mL centrifuge tube was filled 1/3rd with freeze dried mycelial mixture. Extraction buffer (0.5M NaCl, 10Mm Tris HCl (pH7.5), 10Mm Na EDTA, 1% SDS) 0.5 mL was added to each tube and mixed well and then allowed to stand for 15- 20 min at 65°C and centrifuged at 3000g for 10 min. Supernatant was mixed with 0.5 mL of phenol and incubated for 15 min at room temperature. After incubation, 0.5 mL of Sevag reagent (Chloroform Isoamyl alcohol (24:1 (v/v)) was added, mixed and incubated for 15 min and again centrifuged at 13,000g for 20 min.

Supernatant with 400°C of Sevag reagent was added and centrifuged at 11,000g for 10 min. The supernatant collected with 0.6 volumes of ice-cold isopropanol was added to precipitate the DNA and samples were left for 1 hour on -20˚C and again centrifuged at 13,000g for 20 min. The pellet was washed with 100 µl 70 % (v/v) ethanol. The DNA pellet was resuspended in 300 µL of 0.2 M ammonium acetate and left overnight at 4°C. The pellet was precipitated by adding 600 µL of ethanol. The pellet was dissolved in 50 µL in sterile double distilled water. The isolated DNA was quantified using UV Spectrophotometer.

e) PCR amplification and rDNA sequencing: The internal transcribed spacer region (ITS I, 5.8S and ITS 2) of the rDNA was amplified with oligonucleotide primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3' and 5'-TCCTCCGCTTATTGATATGC-3'). The PCR cocktail with a total reaction volume of 25 µL contained the following ; MgCl2 (25 mM) - 1.0 µL, PCR Buffer (10x) - 2.5 µL, Forward primer (10 µm) - 1.0 µL, Reverse primer (10 µm) - 1.0 µl, Taq polymerase (5 µg/µL) - 0.15 µl, dNTP mix (2mM) - 1.0 µL, DNA - 1.0 µl and Glass distilled water - 17.35 µL. The reaction mixture was overlaid with equal volume of mineral oil to prevent dehydration. A control was included along with the sample with 10 µL of glass distilled water instead of DNA. The samples were centrifuged at 4000g for 30 seconds. Two µL of amplified DNA fragments with 3 µL of gel loading dye were separated on a 1 % agarose gel pre stained with standard molecular marker using TAE buffer. The gels were run at 50V for 40 to 60 min and visualized under UV light. The size of the amplified fragments was determined by using gene ruler 100bp ladder, MBI fermentas as a stand MW marker. The amplified PCR product carrying ITS rDNA gene was purified using Qiagene minicolumn. One gram of agrose with 100 mL of TAE buffer (1x) mixed together and melted in micro oven until the agarose was completely dissolved. Five micro liter of ethidium bromide (10 g/1 mL) was added before casting the gel into the tray. Two µL of genomic DNA was mixed with 3 µL of gel loading dye (Bromophenol blue 0.25 %, Sucrose 40 %, 10 Mm Na_2 EDTA mixed together with 100 mL D.H_2O) loaded to all the wells. Ten µL of Gene Ruler 1000 bp ladder was loaded into one well as a standard molecular weight marker. Electrophoresis was carried out at 60 V for 60 min. The purified PCR product of the strains was partially sequenced using Applied Biosystem Instrument (ABI) Prism version 3. A 400 ng of rDNA typical reaction with 3.2 pmol of ITSI and ITS4 Primer. The PCR product was sequenced in the forward and reverse direction. Basic Local Alignment Search Tool (BLAST) (http;//www.ncbi.nlm.nih.gov/blast) provides a method for rapid searching of nucleotide database. Multiple sequence alignments (MSA) are an extension of pairwise alignment to more than two sequences from incurring part at a time (Corpet 1988). MSA methods align all the sequences in a

given query set. Multiple alignments are often used in identifying conserved sequence regions across a group of sequences hypothesized to be evolutionary related. Such alignments are also used to aid in establishing evolutionary relationships by constructing phylogenetic trees. Multiple sequence alignment was done using the programme multalin (http;//bioinfo.Genetoul.Frlmultaline/multialigm /html).

f) Construction of a phylogenetic tree: Sequence data of ITS region were aligned using multalign online programme. Final alignments were visually examined and adjusted manually, phylogenetic analysis was conducted using the programme MEGA version 4 (Kumar *et al*., 1993). The combined ITS I and ITS II data sets were analysed by maximum parsimony (MP) analyses. Missing data / gaps were treated as pairwise deletions. A heuristic search was performed using close neighborhood interchange (CNI) branch swapping on starting trees generated with twenty random addition sequences. A strict consensus tree was derived from the results. The robustness of the internal branches were evaluated by 100 bootstrap replications using a heuristic.

TAXONOMY

Figs. 1(A-B);2(A-B);3(a-e);4 and 5

Macroscopic features: Basidiocarps in troops. Pileus 3.4 7.6 cm dia, centralized with depressed base; convex surface pure white (4A1) when young, later becomes ivory (4A2), smooth, moist; appearing squamose when moist, margin entire, decurved. Lamellae decurrent, yellowish to white, closely arranged, up to 4.8 mm wide with lamellulae of four to five lengths; edge crenate. Stipe lateral, 0.2- 0.4× 0.3-0.5 cm, cylindrical, solid, surface white. Partial veil absent. Volva

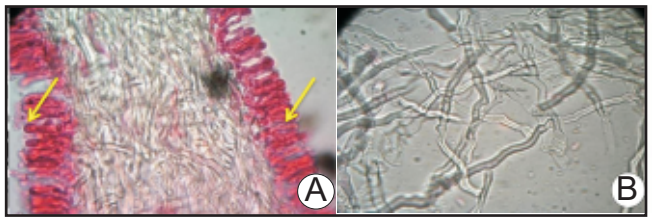

Fig. 2 Microscopic features of *Pleurotus flabellatus* (A) Cheilocystidia and Pleurocystidia, (B) Hyphal system and Clamp connection.

absent. The nature of fruit body was pleurotoid.

The external morphological details of this mushroom are in conformity with the description given by Largent *et al.,* (1986) for similar strain of *P. flabellatus*.

Microscopic features (Figs. 3a-e)

Generative hyphae up to 4.31 µm dia., hyaline, thin walled with clamp connection,. Spores 7.89 -10.8 × 3.5- 4.67 µm (Q = 3.24) cylindrical to bacilli form, hyaline, thin walled, smooth, Basidia 27.69 - 36 × 6.3-7.8 µm, narrowly clavate, bearing stigmata. Cheilocystidia 23.49 -31.39 × 3.35 - 4.58 µm, clavate. Pleurocystidia 29.4 - 38.8 × 6.7 - 7.9 µm, thin-walled. The length and width of the spores was determined by measuring the spores in profile view and for rhomboidal

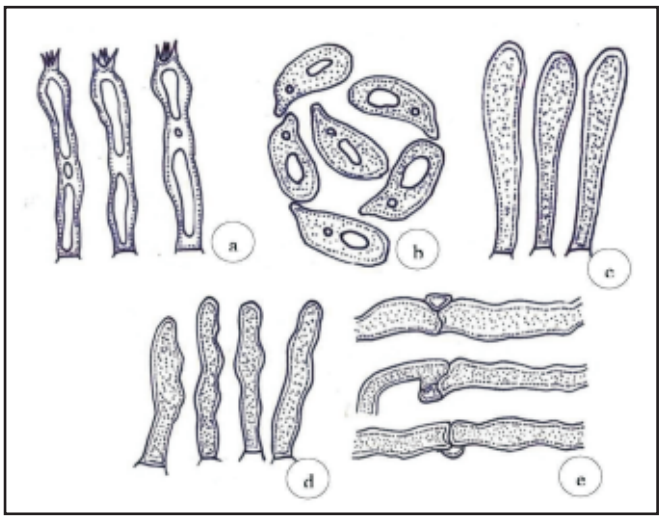

Fig. 3. ***Pleurotus flabellatus* (10 µm)**. (a) Basidia, (b) Basidiospores, (c) Cheilocystidia, (d) Pleurocystidia, (e) Generative hyphae with Clamp Connection.

spores the measurement was taken by both profile and face view. The hyphal nature with clamp connection and spore ornamentation were similar to that of Largent *et al.* (1986).

PCR amplification and rDNA sequencing: The genomic DNA was isolated from the *P. flabellatus* mycelia mat. The isolated DNA was subjected to the gel electrophoresis on 1% of agarose gel and good yield of genomic DNA was obtained (250µg/ µL) with the size of 1500bp. PCR analysis was carried out on rDNA of *P. flabellatus* using the ITS1 and ITS 4 primer pairs for amplified internal transcribed spacer (ITS I and ITS II) regions. The final amplified PCR product showed approximately 650 bp rDNA in 1% agarose gel **(Fig. 4)**. The purified PCR products were used for the sequencing using an automated Applied Biosystem Instrument (IABI Prism). The DNA sequence was analyzed according to the sequencer manufacturer's instructions. The sequence data of *P. flabellatus* were deposited in the GenBank database under the accession number **KT970056**. The purified PCR product was analyzed for sequence arrangements with the primers ITS 1 and ITS 4 at forward direction. The sequence results showed that the purified PCR product was 650 bp. The sequence data from ITS regions of *P. flabellatus* was aligned against available sequence data from NCBI GenBank and analysed using the data analysis programs Multalign online software. Natural habitat and taxonomy of the genus *Pleurotus,* morphological features of *P. flabellatus* were also considered. rDNA and the sequence data have been determined as valuable insights for various basidiomycete groups having evolutionary relationships with biogeography. The rDNA locus of the ITS regions has been considered the most useful part for relationship analysis of closely linked species of genus *Pleurotus*, including cultivated mushroom species (Isikhuemhen *et al*., 2000). The PCR product of isolated rDNA was 650bp as observed by earlier investigators (Martin *et al.,* 2004).

Construction of evolutionary tree: The aligned sequences was analyzed again for evolutionary relationships using the software MEGA version 4. The analyses performed with

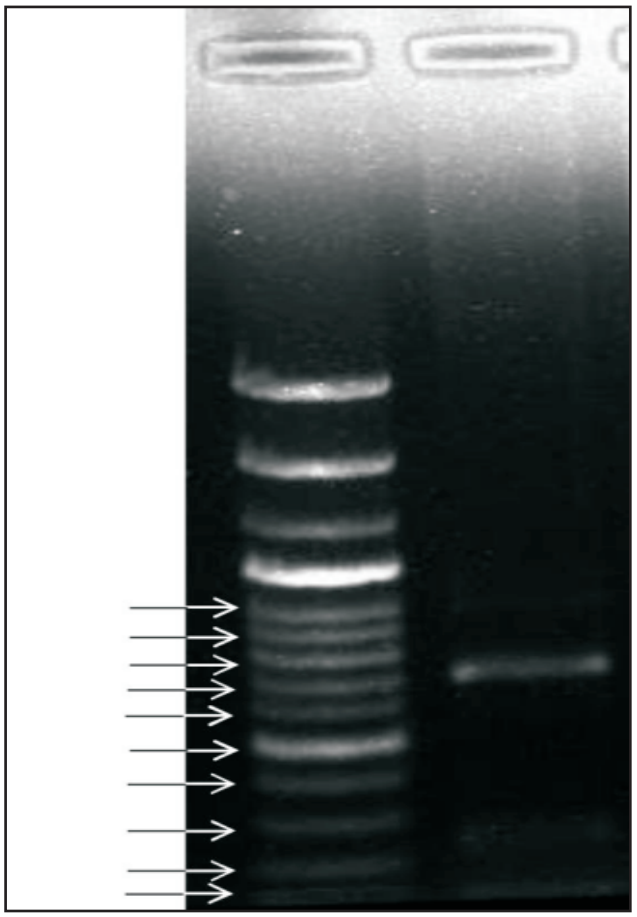

Fig. 4. **Genomic DNA profile of *Pleurotus flabellatus*.**
Lane 1. DNA ladder basepair (bp).
Lane 2. PCR amplified product.

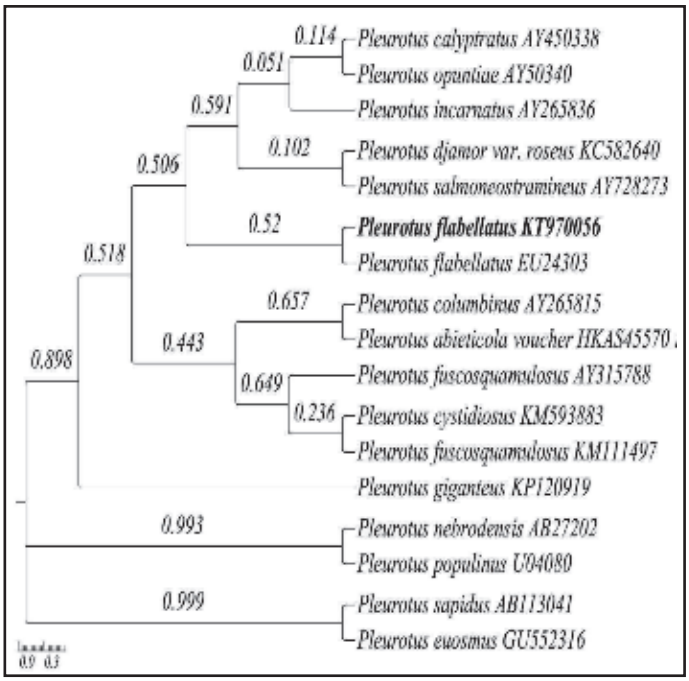

Fig. 5. Phylogenetic analyses of relationships among species of *Pleurotus flabellatus*

available closely related *Pleurotus* species from GenBank database. Aligned sequences were used to estimate evolutionary relationships between *P. flabellatus* and a wide range of species of *Pleurotus*. The evolutionary tree data were analyzed using Maximum Parimony method and tree building method. Complete and pair wise deletion were investigated by close neighbourhood interchange (CNI) method. The consensus tree produced by both models had the same topology and only differed in the statistical support of internal branches. The evolutionary tree produced from the combined data set of ITS regions is shown in **Fig. 5.** Interestingly, *P. flabellatus* showed clustering with different species of *P. flabellatus* with 98 % of the bootstrap value in phylogram. The rDNA, RAPD and other molecular identification were strongly supported and clearly identified (Ravash *et al*., 2009). The results of the present investigation genetically support the isolated strain as *P. flabellatus*. *P. ostreatus*, *P. eryngii, P. columbinus, P. calyptratus, P. opuntiae, P. incarnates, P. djamor* var. *roseus* and *P. salmoneostramineus* were in the same subgroup of cluster. These species were positively correlated and morphological behavior were similar or probably in the ancient period from the same ancestors after some evolutionary change in relationship of different species (Stajic *et al*., 2005). According to Chandra et al. (1990), the *Pleurotus* species were intermediate as is apparent from the present study. Khan *et al*. (2011) found the maximum similarity of *P. flabellatus* of 80% compared with other species and highest relationship from the cluster.

CONCLUSION

In conclusion, strong patterns of *P. flabellatus* strain application of integrated approaches, usually employing one or more molecular techniques, have contributed to the elucidation of such ambiguous issues in Basidiomycota. The amplified nuclear rDNA sequence was used for assessment of population dynamics of *P. flabellatus*. The genetic variation and relationships were identified using genetically supported evolutionary trees. On the other hand, ribosomal DNA internal transcribed spacers sequencing provided valuable data for supporting species delimitations in Basidiomycota.

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