Bioprospection of Basidiomycetes and molecular phylogenetic analysis using internal transcribed spacer (ITS) and 5.8S rRNA gene sequence

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Macrofungi belonging to the phylum Basidiomycota are mostly used as medicinal mushrooms in many countries. In the present study, hundred basidiocarp of macrofungi were collected from Tamilnadu during rainy season. The basidiocarp was found in association with root/trunk of living trees, wood log and decayed matter. Among the hundred basidiocarp, 49 were grown into axenic cultures. Notable variations in the macroscopic characteristics of the basidiome and culture morphology were observed. To study the genetic diversity, the molecular taxonomy of the isolates was carried out using internal transcribed spacer (ITS) and 5.8S rRNA gene sequence marker. Thirty-two strains belonging to the order Polyporales, Hymenochaetales and Russuales under the division Basidiomycota were classified based on phylogeny analysis. This study provides first evidence for the occurrence of species *Fulvifomes fastuosus* (LDCMY39 and LDCMY43) and *Ganoderma wiirense* (LDCMY02, LDCMY08, LDCMY11, LDCMY17 and LDCMY19) from southern India. Molecular evidence for the existence of *Phellinus badius* was given for the first time as well. These data enhance our understanding on the diversity of macrofungi in India, which could be further exploited for biomedical applications.

The kingdom fungi are a distinct group of eukaryotic organisms encompassing about 1.5 M species1,2, where 77,000 fungal species are identified by ITS sequence and been reported in GenBank repository3. They are identified by filamentous mycelium, absence of motile cells and chlorophyll, presence of chitin-rich cell walls and secretion of external digestive enzymes to degrade the food. Their mode of reproduction is via asexual and sexual spores4. These are considered to be the key decomposers of terrestrial ecosystems and known to play crucial ecological role5–7. Wild mushrooms from the natural habitat have profound biological and economic impact due to their major role in ecosystem maintenance8–10. Destruction of environment is the major threat for fungal diversity; exploration of diversity of macrofungi and their taxonomy are acquired importance for reforestation programmes11.

The phylum Basidiomycota includes largely of fleshy fungi (e.g., mushrooms, toadstools, rusts) and ranked second with approximately 23,000 species4. Abundant growth of Basidiomycetes are prevalent in the rainy seasons where the environmental conditions such as temperature, relative humidity and sunshine are favourable, which aids them in the breakdown of dead organic tissue12. These are the potential indicators of environmental quality13. Many fleshy fungi are edible and harmless, but few are poisonous14. However, approximately 700 species of Basidiomycetes were reported to exhibit notable pharmacological activities15,16. These mainly aids in immune system enhancement, regulation of biorhythm, maintenance of homeostasis and are considered to be the biofactor of effective compounds to cure various diseases as anti-fungal, anti-inflammatory, anti-tumor, anti-viral, anti-bacterial, hepatoprotective, anti-diabetic, hypolipidemic, anti-thrombotic and hypotensive activities17,18.

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Though countless number of macrofungi demonstrates an array of medicinal values only a small fraction has been subjected to scientific examination.

India is rich in fungal biodiversity and consists of one-third of global fungal diversity in which only 50% is characterized and explored. Until 1975, study on mushrooms was neglected in states such as Tamil Nadu, Kerala, Karnataka, and Andhra Pradesh in South India. Natarajan and colleagues worked on the prospecting of mushrooms from southern and south-western region excluding Kerala and, listed 230 agaric and bolete species belonged to 67 genera.

The diversity of Basidiomycetes is studied by classical and molecular methods. It involved collection of basidiome, in vitro culture, molecular identification, and preservation of the macrofungi. Classical taxonomy of macrofungi involves description of macro- and micro-morphological characters such as attachment of basidiocarp, types of basidiocarp, pileus surface, margin, pore surface, hyphal system, setae, basidia, basidiospore and reaction to KOH, Meltzer's reagent etc.21–23. Traditional survey alone cannot detect many species of fungi, as they do not produce visible basidicarp or species-specific characteristics. Those can be studied using molecular methods.24–26

The focus of the present study was to explore the diversity of ethnomycologically important Basidiomycetes in Southern Tamil Nadu, India and we have employed molecular methods for the identification of macrofungi.

Many methods have been used in molecular systematics of macrofungi namely DNA-DNA hybridization; restriction enzyme analysis - RFLP (restriction fragment length polymorphism), rDNA (nuclear ribosomal DNA), mtDNA (mitochondrial DNA); and sequencing analysis – spacers (ITS-internal transcribed spacer), SS nuclear rRNA, mitochondrial rRNA.37 The universal primer for fungal phylogenetics comprised of fungal ribosomal operon: large subunit (26S or 28S), small subunit (18S) and the ITS comprising of ITS1 and ITS2 containing the conserved 5.8S38–40. The ITS1 and ITS4 primers amplify the highly variable ITS1 and ITS2 sequences surrounding coding sequence of 5.8S and it's exclusively specific for basidiomycetes.31,32 This study focussed on sequencing the entire ITS1, 5.8S RNA and ITS2 for identification of isolated macrofungi. Based on phylogenetic analysis, thirty-two strains belonging to the division Basidiomycota were classified. This study provided additional information to the present knowledge on the data of diversity of fungi in Tamilnadu and also to understand their bioprospects.

Results

This study is the first report on the occurrence of species Fulviformes fastuosus and Ganoderma wiiroense from India. In addition, molecular evidence for the existence of Plellinus badius in southern Tamilnadu is also provided. In the present study, hundred basidiomata were collected from different locations: Lady Doak College Campus (Fig. 1), Nagamalai (Fig. 2), Pudhupatti (Fig. 3), Ayyanar falls and Kovai Kutralam (Fig. 4), and Tirunelveli (Fig. 5). The collection details such as habitat, host, attachment pattern and position of basidiome on the tree are mentioned in Table 1. The species richness was found in the following order: Lady Doak College Campus (22%), Pudhupatti (21%), Nagamalai (19%), Ayyanar falls (23%), Tirunelveli (13%), Kovai Kutramal (1%), and Thenkasi (1%). The host of the isolates are as follows: Albizzia sp, Azadirachta sp, Canthium dicoccum, Cocos nucifera, Nerium sp, Tamarindus sp, Tabernanthe iboga, and stipitate (33%).

Among the hundred basidiome collected only forty-nine isolates (49%) could be grown in axenic cultures. The mycelial growth significantly varied from 7 days to 30 days. The colour of the mycelia varies for each strain: white, orange white, yellowish white, pale yellow, greyish orange, light yellow, pale orange and brownish orange (Fig. 6, Table 2). The pure cultures of all isolates were stored in mineral oil till further use.

Genomic DNA was obtained and 5.8S ribosomal RNA gene segment was amplified using sequence specific primers. Thirty-two isolates were successfully sequenced and the size of the amplicon ranged from 599 bp to 902 bp. The sequences were deposited in GenBank and accession numbers were obtained (Table 3). Variation in genetic makeup was observed among the isolates from the same environment. Molecular phylogenetic analysis was carried out using 52 ITS sequences in which 20 reference sequences were retrieved from GenBank, NCBI to clarify the variation among the sequences. The phylogenetic tree constructed using maximum likelihood (ML) method (Fig. 7). The basidiomycete species were clustered into three clades: Clade 1 - Polyporales, Clade 2 - Hymenochaetales and Clade 3 - Russulales. The three clades are detailed below:

Clade 1: Polyporales - Found in all study sites except Ayyanar falls. Eighteen strains were grouped under this clade and fifteen sequences were further categorised under the family Ganodermataceae, two under Polyporaceae and one in Fomitopsidaceae. The isolated strains belong to the Polyporales were Coriolopsis capitata, Fomitopsis ostreiformis, Ganoderma resinaceum, Ganoderma sp., Ganoderma wiiroense and Trametes elegans. Coriolopsis capitata LDCMY42 collected from Nagamalai showed 99% similarity with the strain Coriolopsis capitata DK01 (AM237457). Monophyletic origin of Fomitopsis ostreiformis was determined with 100% bootstrap support. Four strains were identified as Ganoderma wiiroense (LDCMY19, LDCMY08, LDCMY11, LDCMY17 and LDCMY02) and showed highest similarity with the strains reported from United States of America (KT952361 and KT952363). Variations in the genetic makeup as well in the morphology of the Ganoderma wiiroense strains were observed. Majority of the Ganoderma strains were found to be stipitate. Based on molecular analysis, this is the first evidence for the occurrence of Ganoderma wiiroense from India.

The Clade 1 was supported by 99% bootstrap value and it was further categorized into 6 groups (1.1–1.6). Three groups (1.1–1.3) in this clade consisted of strains from Ganoderma sp. Five strains of Ganoderma wiiroense were grouped in 1.1 and supported by 95% bootstrap value. The mean difference between the sequences in this group was very low (0.000878851). The group 1.2 included Ganoderma sp., which is supported by 90% bootstrap with the mean difference of 0.019876893. The group 1.3 included Ganoderma sp. from different places, which was supported by 95% bootstrap value with the mean difference of 0.049142826. The group 1.4 included...
Trametes elegans LDCMY37, Thenkasi showed similarity with two strains reported from Nepal and India, and supported by 99% bootstrap value with the mean difference of 0.004707472. The group 1.5 included Fomitopsis ostreiformis LDCMY21 isolated from Nagamalai supported by 100% bootstrap value with the mean difference of 0.001759814. The group 1.6 included Coriolopsis caperata LDCMY42 from LDC Campus and it was supported by 99% bootstrap with the mean difference of 0.003519628.

Clade 2: Hymenochaetales - the isolates categorized in this clade were found in all study sites except Thenkasi. Twelve isolates belonging to the genus Fulvifomes, Phellinus and Inonotus were categorized in this clade. They are Fulvifomes fastuosus (LDCMY39 and LDCMY43), Inonotus rickii (LDCMY52), Phellinus badius (LDCMY36) and Phellinus sp. (LDCMY36). Molecular phylogeny analysis confirmed that two strains (LDCMY39 and LDCMY43) obtained from Lady Doak College campus as Fulvifomes fastuosus. The isolates showed highest similarity with the strains reported from Sri Lanka (KR867653) and South Korea (AY558615) and supported with 95% bootstrapping. The host for both the strains were Albizzia sp. We further provided the first significant report on more precise identification of Fulvifomes fastuosus on the basis of the genetic information. A strain collected from Ayyanar falls was identified as Inonotus rickii (LDCMY52) that shared 100% similarity with the strains previously reported from India. The genus Phellinus was found to be present in all study sites. Phellinus badius LDCMY36 shared 93% relatedness with the strain CBS 449.76 from South Korea. This was the first molecular evidence of the species Phellinus badius from India.

This Clade 2 was supported by 100% bootstrap value and consisted of 4 groups (2.1–2.4). The Group 2.1 includes Fulvifomes fastuosus (95% bootstrap) with the mean difference of 0.082737938; Group 2.2 was supported by 94% bootstrap and includes Phellinus sp. (0.100297219); Group 2.3 has only Inonotus rickii and supported by 100% bootstrap value and the mean difference was 0.27677544. Phellinus badius (99% bootstrap) along with few strains of Phellinus sp. were categorised in Group 2.4. The mean difference within the group was 0.096520676.

Clade 3: Russales - This group consisted of samples collected only from Tirunelveli and supported by 100% bootstrap value and consisted of 2 groups (3.1 & 3.2). Two strains (LDCMY57 and LDCMY58) supported with
93% bootstrap value and identified as *Amylosporus* sp. belonging to the family Bondarzewiaceae and grouped in 3.2. The mean difference among the isolates in this group was 0.134112602. These isolates showed similarity with the strains reported from India (BAB-5055 and BAB-5255), China (Dai 7803) and USA (JV080620).

The morphological and culture characteristics of first time reported strains from India *Ganoderma wiiroense* and *Fulviformes fastuosus* along with *Phellinus badius* are given below.

**Ganoderma wiiroense.** Annual, pileate, basidiocarp, sessile, woody hard, white to creamy yellow when dry. Size of the pileus 10.5 cm × 7.5 cm; Hymenophore poroid, Hyphal system trimitic, generative hyphae with clamp connections, hyaline, thin-walled, branched, 2–4 μm in diameter; skeletal hyphae occasionally branched, 2.5–7.5 μm thick; binding and skeleton-binding hyphae hyaline. Spores ellipsoid (Fig. 8). Colonies of *G. wiiroense* on PDA was fast growing, 22–37 mm diameter after 3 days and took 7 days to completely colonize 80 mm diameter plates.

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**Figure 2.** Field photographs of Basidiomata collected from Nagamalai, Madurai District. The macrofungi grown on the host species: *Albizia* sp., - LDCBIF32, LDCBIF33, LDCBIF35, LDCBIF72, LDCBIF76; *Azadirachta* sp., - LDCBIF30; *Cocos* sp., - LDCBIF24 and *Tamarindus* sp., - LDCBIF15, LDCBIF36. Few isolates were collected from the decayed matter (LDCBIF23, LDCBIF25, LDCBIF26, LDCBIF28, LDCBIF29, LDCBIF31) and wood log (LDCBIF14, LDCBIF27, LDCBIF34).
**Fulvifomes fastuosus.** Perennial, pileate, basidiocarp, sessile, woody hard and without odour or taste when dry. Size of the pileus 4.5 cm × 2 cm; Hymenophore poroid, hyphal system Dimitic; generative hyphae without clamp connections, hyaline, thin-walled, simple septate, occasionally branched, 2–3 µm in diameter; skeletal hyphae thick-walled with broad lumen, unbranched, 3–5 µm in diameter. Tissue darkening in KOH. Hymenial setae absent. Spores: subglobose, yellowish, thick-walled, smooth 3.4–5.7 × 3.1–4.2 µm. Yellowish brown, dark reddish brown in KOH (Fig. 9). Colonies of Fulvifomes fastuosus on PDA plate was slow compared to Ganoderma strains, 25–28 mm diameter after 7 days and took 20 days to completely colonize 80 mm diameter plates.

**Phellinus badius.** Perennial, pileate, basidiocarp, sessile, woody hard, easily detachable from the host. Hymenophore poroid, hyphal system dimitic; generative hyphae thin walled, simple septate, clampless, moderately branched, hyaline to pale yellow, 3.47 µm; skeletal hyphae thick walled (4.35 µm); Hymenial setae absent. Spores: ellipsoid, moderately thick walled, 4.21–5.54 × 2.83–4.13 µm. Yellowish brown, dark reddish brown in KOH (Fig. 10). The growth of Phellinus badius on PDA was slow, 23–24 mm diameter after 7 days and took 15 days to completely colonize 80 mm diameter plates.

**Discussion**

Fungi are ubiquitous in nature and distributed in all ecosystem. It can survive in diversified habitats such as air, water, soil, litter etc. It contains 1.5 million species, of which 74,000 species are named\(^4\). The phylum basidiomycota consist of 37% of all described fungal species\(^33\). Threats to fungi due to habitat destruction are a global concern as they play an important role in human welfare\(^19\). To understand the distribution and diversity of macrofungi in South India, the basidiomata were collected from living trees, wood log and leaf litters during the rainy season (November to January).

The Basidiomycetes were usually classified based on phenotypic traits; however, classification based on morphological characteristic features alone will be flawed and misleading and the use of molecular classification was found to be more reliable\(^34,35\). So far, only 5% of fungal strains were isolated as pure cultures and several described species were acknowledged only as herbarium specimens\(^19\). In the present study, pure culture (Fig. 6) was raised...
from 49% of the isolates and the molecular data were obtained for 65% of the isolates. These molecular data helped in identification of the isolates and was used for construction of genetic diversity among the macrofungal isolates.
Molecular phylogeny of the macrofungal isolates. The molecular systematics of macrofungi has been studied by various methods using DNA-DNA hybridization, restriction enzyme analysis - RFLP, rDNA, mtDNA and sequencing analysis of ITS\(^27\). Pectinase isoenzyme\(^36\), manganese superoxide dismutase\(^37,38\), ITS and 25S ribosomal sequences\(^34,35,39\) were used to construct molecular phylogeny in macrofungal species. Later, ITS was used as a DNA barcode for fungal identification\(^32,40,41\). In this study, amplification of nuclear ribosomal ITS was used to identify the isolates. The identified isolates belong to three families namely Polyporales, Hymenochaetales and Russuiales. The representative strains of the Polyporales from this study were *Coriolopsis caperata*, *Fomitopsis ostreiformis*, *Ganoderma resinaceum*, *Ganoderma sp.*, *Ganoderma wiiroense* and *Trametes elegans*. The isolated strains belonging to Hymenochaetales were *Fulvifomes fastuosus*, *Inonotus rickii*, *Phellinus sp.* and *Phellinus badius*. *Amylosporus* sp. was the only strain found in our study from the family Russuiales. We are the first to report the occurrence of *Ganoderma wiiroense* and *Fulvifomes fastuosus* with morphological and molecular evidence; and also provided the molecular evidence for *Phellinus badius* from India.

Figure 5. Field photographs of Basidiomata collected from Tirunelveli District. The macrofungi grown on the host species: *Nerium* sp., - LDCBIF88; *Canthium* sp., - LDCBIF89; *Albizzia* sp., - LDCBIF90 - LDCBIF98 and *Tamarindus* sp., - LDCBIF99 & LDCBIF100.
| S. No. | Basidiome Id | Host | Attachment to the Host | Position of basidiome on the tree | Size L * W (in cm) | Xanthochroic |
|--------|--------------|------|------------------------|-----------------------------------|------------------|-------------|
| 1.     | LDCBF01*     | Albizzia sp | Stipitate | Root | 15, 10.5 | — |
| 2.     | LDCBF02*     | Decayed material | Stipitate | — | 10.5, 7.5 | — |
| 3.     | LDCBF03*     | Wood Log | Sessile | Root | 11, 9 | — |
| 4.     | LDCBF04*     | Wood Log | Sessile | Root | 7.5, 4.5 | — |
| 5.     | LDCBF05*     | Wood Log | Sessile | Root | NA | — |
| 6.     | LDCBF06*     | Wood Log | Sessile | Root | NA | — |
| 7.     | LDCBF07*     | Wood Log | Sessile | Root | 6, 5 | — |
| 8.     | LDCBF08*     | Tamarindus sp | Stipitate | Root | NA | — |
| 9.     | LDCBF09*     | Azadirachta sp | Stipitate | Root | 16, 13.5 | — |
| 10.    | LDCBF10*     | Decayed material | Sessile | — | 4, 5 | — |
| 11.    | LDCBF11*     | Wood Log | Stipitate | Root | 12, 5 | — |
| 12.    | LDCBF12*     | Wood Log | Stipitate | Root | 15, 7 | — |
| 13.    | LDCBF13*     | Wood Log | Stipitate | Root | 9.5, 7 | — |
| 14.    | LDCBF14*     | Wood Log | Sessile | Root | 15.2, 8 | — |
| 15.    | LDCBF15*     | Tamarindus sp | Sessile | Root | 19, 10.5 | — |
| 16.    | LDCBF16*     | Albizzia sp | Stipitate | Root | 4, 2 | — |
| 17.    | LDCBF17*     | Albizzia sp | Stipitate | Root | 9, 5 | — |
| 18.    | LDCBF18*     | Albizzia sp | Stipitate | Root | 7, 4.5 | — |
| 19.    | LDCBF19*     | Albizzia sp | Stipitate | Root | 9, 7 | — |
| 20.    | LDCBF20*     | Albizzia sp | Stipitate | Root | 5.5, 3 | — |
| 21.    | LDCBF21*     | Albizzia sp | Stipitate | Root | 5, 3 | — |
| 22.    | LDCBF22*     | Albizzia sp | Sessile | Root | 12,7,5 | — |
| 23.    | LDCBF23*     | Decayed material | Stipitate | Root | 7,5, 6 | — |
| 24.    | LDCBF24*     | Cocos sp | Sessile | Root | 39, 20 | — |
| 25.    | LDCBF25*     | Decayed material | Stipitate | Root | 6, 3 | — |
| 26.    | LDCBF26*     | Decayed material | Stipitate | Root | 7.8, 6 | — |
| 27.    | LDCBF27*     | Wood Log | Sessile | Root | NA | — |
| 28.    | LDCBF28*     | Decayed material | Stipitate | Root | 8,5, 7 | — |
| 29.    | LDCBF29*     | Decayed material | Stipitate | Root | 7,6 | — |
| 30.    | LDCBF30*     | Azadirachta sp | Stipitate | Root | 5,8, 3.5 | — |
| 31.    | LDCBF31*     | Decayed material | Sessile | Root | 5, 3 | — |
| 32.    | LDCBF32*     | Albizzia sp | Sessile | Root | 25, 16,5 | — |
| 33.    | LDCBF33*     | Albizzia sp | sessile | Root | 11.5, 7 | — |
| 34.    | LDCBF34*     | Wood Log | Sessile | Root | 4,5, 2.5 | — |
| 35.    | LDCBF35*     | Albizzia sp | Sessile | Root | 14, 6 | — |
| 36.    | LDCBF36*     | Tamarindus sp | Sessile | Trunk | 15,5, 10 | — |
| 37.    | LDCBF37*     | Wood Log | Sessile | Root | 25, 18 | — |
| 38.    | LDCBF38*     | Wood Log | Sessile | Root | 12,10.5 | — |
| 39.    | LDCBF39*     | Albizzia sp | Sessile | Trunk | 4.5, 3 | + |
| 40.    | LDCBF40*     | Albizzia sp | Sessile | Trunk | 4.8, 2.8 | + |
| 41.    | LDCBF41*     | Albizzia sp | Sessile | Trunk | 4.5, 3.5 | + |
| 42.    | LDCBF42*     | Albizzia sp | Sessile | Trunk | 5.5, 4 | + |
| 43.    | LDCBF43*     | Albizzia sp | Sessile | Trunk | 5, 3.5 | + |
| 44.    | LDCBF44*     | Albizzia sp | Sessile | Trunk | 10, 6 | + |
| 45.    | LDCBF45*     | Albizzia sp | Sessile | Trunk | 7.5, 3 | + |
| 46.    | LDCBF46*     | Albizzia sp | Sessile | Trunk | 6, 4.5 | + |
| 47.    | LDCBF47*     | Albizzia sp | Sessile | Trunk | 12, 5.6 | + |
| 48.    | LDCBF48*     | Albizzia sp | Sessile | Trunk | 15, 6.5 | + |
| 49.    | LDCBF49*     | Albizzia sp | Sessile | Trunk | 19,5, 9 | + |
| 50.    | LDCBF50*     | Albizzia sp | Sessile | Trunk | 10.5, 6 | + |
| 51.    | LDCBF51*     | Albizzia sp | Sessile | Trunk | 12, 9.5 | + |
| 52.    | LDCBF52*     | Albizzia sp | Sessile | Trunk | 10, 5.5 | + |
| 53.    | LDCBF53*     | Albizzia sp | Sessile | Trunk | 14, 8 | + |
| 54.    | LDCBF54*     | Albizzia sp | Sessile | Trunk | 11,5,7 | + |
| 55.    | LDCBF55*     | Albizzia sp | Sessile | Trunk | 5.5, 4.5 | + |

Continued
| S. No. | Basidiome Id | Host | Attachment to the Host | Position of basidiome on the tree | Size L * W (in cm) | Xanthochroic |
|--------|--------------|------|------------------------|-----------------------------------|-------------------|-------------|
| 56.    | LDCBF56*     | Albizia sp. | Sessile | Trunk                  | 7,4               | +           |
| 57.    | LDCBF57*     | Albizia sp. | Sessile | Trunk                  | 8,5, 6            | +           |
| 58.    | LDCBF58*     | Albizia sp. | Sessile | Trunk                  | 13, 5, 5          | +           |
| 59.    | LDCBF59*     | Albizia sp. | Sessile | Trunk                  | 9, 6              | +           |
| 60.    | LDCBF60*     | Albizia sp. | Sessile | Trunk                  | 6, 4, 5           | +           |
| 61.    | LDCBF61*     | Albizia sp. | Sessile | Trunk                  | 4, 2              | +           |
| 62.    | LDCBF62*     | Albizia sp. | Sessile | Trunk                  | 7, 5, 4           | +           |
| 63.    | LDCBF63*     | Albizia sp. | Sessile | Trunk                  | 5, 2, 5           | +           |
| 64.    | LDCBF64*     | Albizia sp. | Sessile | Trunk                  | 6, 5, 3           | +           |
| 65.    | LDCBF65*     | Albizia sp. | Sessile | Trunk                  | 7, 5              | +           |
| 66.    | LDCBF66*     | Albizia sp. | Sessile | Trunk                  | 5, 5              | +           |
| 67.    | LDCBF67*     | Albizia sp. | Sessile | Trunk                  | 11, 7             | +           |
| 68.    | LDCBF68*     | Albizia sp. | Sessile | Trunk                  | 11, 4, 8          | +           |
| 69.    | LDCBF71*     | Albizia sp. | Sessile | Trunk                  | 8, 5, 5           | +           |
| 70.    | LDCBF72*     | Albizia sp. | Sessile | Trunk                  | 5, 5, 3, 5        | +           |
| 71.    | LDCBF73*     | Albizia sp. | Sessile | Root                   | 6, 5              | +           |
| 72.    | LDCBF74*     | Albizia sp. | Sessile | Trunk                  | 3, 2              | +           |
| 73.    | LDCBF75*     | Albizia sp. | Sessile | Root                   | 5, 5, 3           | +           |
| 74.    | LDCBF76*     | Albizia sp. | Sessile | Trunk                  | 6, 4, 5           | +           |
| 75.    | LDCBF77*     | Albizia sp. | Sessile | Trunk                  | NA                | +           |
| 76.    | LDCBF78*     | Wood Log    | Sessile | —                      | 11, 5, 7          | —           |
| 77.    | LDCBF79*     | Decayed material | Stipitate | —                     | 3, 3              | —           |
| 78.    | LDCBF80*     | Decayed material | Stipitate | —                     | 7, 5              | —           |
| 79.    | LDCBF81*     | Decayed material | Stipitate | —                     | 6, 5, 8           | —           |
| 80.    | LDCBF82*     | Albizia sp. | Sessile | Root                   | 4, 2, 5           | +           |
| 81.    | LDCBF83*     | Albizia sp. | Sessile | Root                   | NA                | +           |
| 82.    | LDCBF84*     | Albizia sp. | Sessile | Root                   | NA                | +           |
| 83.    | LDCBF85*     | Wood Log    | Sessile | —                      | NA                | —           |
| 84.    | LDCBF86*     | Wood Log    | Stipitate | Root                  | NA                | —           |
| 85.    | LDCBF87*     | Wood Log    | Sessile | Trunk                  | NA                | —           |
| 86.    | LDCBF88*     | Nerium sp. | Sessile | Root                   | 16, 8, 4          | —           |
| 87.    | LDCBF89*     | Caithium sp. | Sessile | Root                   | 11, 8             | —           |
| 88.    | LDCBF90*     | cocos sp.   | Stipitate | Root                  | 9, 1, 8           | —           |
| 89.    | LDCBF91*     | cocos sp.   | Stipitate | Root                  | 3, 3, 5           | —           |
| 90.    | LDCBF92*     | Albizia sp. | Stipitate | Root                  | 8, 5              | —           |
| 91.    | LDCBF93*     | Albizia sp. | Stipitate | Root                  | 5, 4, 3           | —           |
| 92.    | LDCBF94*     | Albizia sp. | Stipitate | Root                  | 7, 2, 5, 1        | —           |
| 93.    | LDCBF95*     | Albizia sp. | Sessile | Root                   | 3, 2, 8           | —           |
| 94.    | LDCBF96*     | Albizia sp. | Stipitate | Root                  | 4, 3, 8           | —           |
| 95.    | LDCBF97*     | Albizia sp. | Stipitate | Root                  | 7, 5, 2           | —           |
| 96.    | LDCBF98*     | Albizia sp. | Stipitate | Root                  | 4, 8, 3, 4        | —           |
| 97.    | LDCBF99*     | Tamarindus sp. | Sessile | Root                  | 10, 8, 6          | —           |
| 98.    | LDCBF100*    | Tamarindus sp. | Stipitate | Root                  | 4, 4              | —           |
| 99.    | LDCBF101*    | Anacardiaceae sp. | Sessile | Root                  | 7, 8, 6, 8        | —           |
| 100.   | LDCBF104*    | Decayed Material | Stipitate | —                     | NA                | —           |

Table 1. Basidiomata collected. $, €, ®, ≠, *, used to denote the strains collected from different places. $Ayyanar falls; €Coimbatore; ®Lady Doak College Campus; *Nagamalai; ≠Thenkasi; ®Pudhupatti; ®Tirunelveli.

G. wiiroense belonging to the Family Polyporales was first reported from Upper Western region of Ghana. There were only 8 strains available in the GenBank for G. wiiroense, where two from Ghana and the rest from this study. Crous et al. reported that G. lucidum (TVK1, India; GenBank FJ982798) was closer to G. wiiroense. In our study, we also found that the G. lucidum FJ982798 was closer to G. wiiroense than any other Ganoderma strains reported in this study.

The genus Phellinus belonging to the Family Hymenochaetaceae were important owing to their medicinal values. Phellinus species have been reported from Kerala, where two from Ghana and the rest from India. Eighteen Phellinus species have been reported from Kerala, including P. nilghieriensis (Mont.) Cunn., P. shafteri from Gujarat, and P. badius was described morphologically from Punjab. This study provides the first report on molecular evidence for P. badius from India.
Axenic cultures from collected basidiomata

Figure 6. Axenic culture of collected basidiomata. The mycelium culture on PDA plates. Variations in growth and the color of the mycelium was observed (See Table 2). The identified strains by sequencing; *Amylosporous* sp. - LDCMY57 & LDCMY58; *Coriolopsis caperata* - LDCMY42; *Fomitopsis ostreiformis* - LDCMY21; *Fulvifomes fastuosus* - LDCMY39, LDCMY43; *Ganoderma resinaceum* - LDCMY01; *Ganoderma* sp. - LDCMY04, LDCMY05, LDCMY06; LDCMY12, LDCMY14, LDCMY16, LDCMY18, LDCMY22, LDCMY41. *Ganoderma wiarioense* - LDCMY19, LDCMY08, LDCMY11, LDCMY17 and LDCMY02; *Inonotus rickii* - LDCMY52; *Phellinus badius* - LDCMY36; *Phellinus* sp. - LDCMY23, LDCMY24, LDCMY27, LDCMY28, LDCMY29, LDCMY31, LDCMY34, LDCMY45; *Trametes elegans* - LDCMY37.
The radial expansion was measured on the 3rd day (shown in bold) and 7th day. The measurements are given in mean ± SD. The total number of days taken for complete colonization (80 mm) in initial radial expansion (in mm) and complete colonization (in days) are given in Table 2. The radial expansion was measured on the 3rd day (shown in bold) and 7th day. The measurements are given in mean ± SD. The total number of days taken for complete colonization (80 mm) in PDA medium varied among the isolates and ranged from 5–30 days for different strains.

Table 2. Growth and characteristics of mycelium culture. *S.c.*, *P.c.* and *G*. Used to denote the strains collected from different places. *Ayyanar falls; ‡Coimbatore; §Lady Doak College Campus; *Nagamalai; *-Thenkasi; *Pudhupatti; ‡Tirunelveli.* The radial expansion was measured on the 3rd day (shown in bold) and 7th day. The measurements are given in mean ± SD. The total number of days taken for complete colonization (80 mm) in PDA medium varied among the isolates and ranged from 5–30 days for different strains.
| S.No | Organism Name | Strain/Isolate Name | Source of DNA | Geographical Origin | Sequence Length (ITS1/ITS4) | Accession No |
|------|---------------|---------------------|---------------|--------------------|----------------------------|--------------|
| 1.   | Amylosporous sp. | LDCMY58® | Mycelium | Tirunelveli, South India | 741 | KY491656 |
| 2.   | Amylosporous sp. | LDCMY57® | Mycelium | Tirunelveli, South India | 774 | KY491657 |
| 3.   | Amylosporous sp. | BAB-5055 | — | India | 897 | KR155100 |
| 4.   | Amylosporous sp. | BAB-5255 | — | India | 775 | KT186196 |
| 5.   | Amylosporous sp. | Dai 7803 | — | China | 748 | KM213668 |
| 6.   | Amylosporous campbellii | JVO80620j | — | Southern Florida | 807 | JF692201 |
| 7.   | Amylosporous campbellii | JVO80620j | — | Southern Florida | 810 | JF692200 |
| 8.   | Coriolopsis caperata | LDCMY42* | Mycelium | Lady Doak College Campus, Madurai, South India | 614 | KY111254 |
| 9.   | Coriolopsis caperata | DK01 | — | New Delhi | 585 | AM237457 |
| 10.  | Fomitopsis ostreiformis | LDCMY214* | Mycelium | Nagamalai, Madurai, South India | 599 | KY111252 |
| 11.  | Fomitopsis ostreiformis | X1412 | — | Indonesia | 1600 | KC599920 |
| 12.  | Fomitopsis ostreiformis | Xoe62 | — | Karnataka- India | 636 | KJ174431 |
| 13.  | Fomitopsis ostreiformis | X1393 | — | Finland | 1600 | KC599921 |
| 14.  | Fulvifomes fastuosus | LDCMY39* | Mycelium | Lady Doak College Campus, Madurai, South India | 756 | KX957798 |
| 15.  | Fulvifomes fastuosus | LDCMY43* | Mycelium | Lady Doak College Campus, Madurai, South India | 738 | KY491659 |
| 16.  | Fulvifomes fastuosus | CBS 213.36 | — | South Korea | 768 | AY586165 |
| 17.  | Ganoderma destructans | CMW43670 | — | South Africa | 640 | KR183856 |
| 18.  | Ganoderma lucidum | TVK1 | — | India | 603 | FJ982798 |
| 19.  | Ganoderma multiplexus | BSN020 | — | Japan | 832 | LC149613 |
| 20.  | Ganoderma resinaecon | LDCMY01* | Mycelium | Lady Doak College Campus, Madurai, South India | 614 | KX557799 |
| 21.  | Ganoderma sp. | LDCMY04* | Mycelium | Lady Doak College Campus, Madurai, South India | 610 | KY009866 |
| 22.  | Ganoderma sp. | LDCMY05* | Mycelium | Lady Doak College Campus, Madurai, South India | 620 | KX557800 |
| 23.  | Ganoderma sp. | LDCMY06* | Mycelium | Lady Doak College Campus, Madurai, South India | 608 | KY009865 |
| 24.  | Ganoderma sp. | LDCMY124 | Mycelium | Pudhupatti, South India | 606 | KY471289 |
| 25.  | Ganoderma sp. | LDCMY16* | Mycelium | Nagamalai, Madurai, South India | 607 | KY111251 |
| 26.  | Ganoderma sp. | LDCMY184 | Mycelium | Nagamalai, Madurai, South India | 722 | KY009870 |
| 27.  | Ganoderma sp. | LDCMY224 | Mycelium | Nagamalai, Madurai, South India | 619 | KY009871 |
| 28.  | Ganoderma sp. | LDCMY144 | Mycelium | Nagamalai, Madurai, South India | 614 | KY009872 |
| 29.  | Ganoderma sp. | LDCMY411 | Mycelium | Lady Doak College Campus, Madurai, South India | 642 | KY111250 |
| 30.  | Ganoderma wiiroense | LDCMY024 | Mycelium | Lady Doak College Campus, Madurai, South India | 608 | KY009864 |
| 31.  | Ganoderma wiiroense | LDCMY084 | Mycelium | Lady Doak College Campus, Madurai, South India | 618 | KY009867 |
| 32.  | Ganoderma wiiroense | LDCMY114 | Mycelium | Pudhupatti, South India | 611 | KY111253 |
| 33.  | Ganoderma wiiroense | LDCMY174 | Mycelium | Nagamalai, Madurai, South India | 612 | KY009869 |
| 34.  | Ganoderma wiiroense | LDCMY194 | Mycelium | Nagamalai, Madurai, South India | 647 | KY009873 |
| 35.  | Ganoderma wiiroense | UMN-20-GHA | — | USA | 769 | KT952361 |
| 36.  | Ganoderma wiiroense | UMN-21-GHA | — | USA | 722 | KT952363 |
| 37.  | Inonotus rickii | LDCMY52® | Basidiome | Ayyanar falls, Dindigul, South India | 902 | KJ471287 |
| 38.  | Inonotus rickii | CAA-32 | — | Rajasthan- India | 747 | HQ882221 |
| 39.  | Inonotus rickii | CAA-28 | — | Rajasthan - India | 750 | HQ882217 |
| 40.  | Phellinus badius | LDCMY364 | Mycelium | Pudhupatti, South India | 688 | KY111249 |
| 41.  | Phellinus badius | CBS 449.76 | — | South Korea | 714 | AY586609 |
| 42.  | Phellinus sp. | LDCMY234 | Mycelium | Pudhupatti, South India | 709 | KY491658 |
| 43.  | Phellinus sp. | LDCMY244 | Mycelium | Pudhupatti, South India | 668 | KY471286 |
| 44.  | Phellinus sp. | LDCMY274 | Mycelium | Ayyanar falls, Dindigul, South India | 662 | KX557801 |
| 45.  | Phellinus sp. | LDCMY284 | Mycelium | Ayyanar falls, Dindigul, South India | 693 | KX557802 |
| 46.  | Phellinus sp. | LDCMY294 | Mycelium | Ayyanar falls, Dindigul, South India | 683 | KX557803 |
| 47.  | Phellinus sp. | LDCMY314 | Mycelium | Ayyanar falls, Dindigul, South India | 685 | KX557805 |
| 48.  | Phellinus sp. | LDCMY344 | Mycelium | Nagamalai, Madurai, South India | 681 | KX557804 |
| 49.  | Phellinus sp. | LDCMY454 | Basidiome | Ayyanar falls, Dindigul, South India | 677 | KJ471288 |
| 50.  | Trametes elegans | LDCMY374 | Mycelium | Thenkasi, South India | 606 | KX009868 |
| 51.  | Trametes elegans | UOC SIGWI S25 | — | Nepal | 655 | KP780433 |
| 52.  | Trametes elegans | BAB-4765 | — | India | 637 | KJ549949 |

Table 3. Species and their GenBank accession number used for constructing molecular phylogeny.

| Species and their GenBank accession number used for constructing molecular phylogeny. |
|---------------------------------------------------------------|
| Used to denote the sequence data generated from the strains collected from different places. |
| Ayyanar falls; Kovai kutralam; Lady Doak College Campus; Nagamalai; Thenkasi; Pudhupatti; Tirunelveli. |
The genus Fulvifomes Murrill was segregated from Phellinus Quel., Murrill and typified with F. robiniae (Murrill). It was not accepted as a separate genus and treated as a subspecies of Phellinus till 1999. Later, comprehensive evidences based on molecular phylogenetic analyses proved that it as an independent genus closely associated with Auricularia Reid and Phylloporia Murrill. The key characteristics of Fulvifomes are pileate basidiocarps, a dimitic hyphal system, coloured basidiospores and absence of setae. Species with resupinate basidiocarps were included into Fulvifomes based on morphological studies. Recently, species with monomitic hyphal system were included in Fulvifomes by Zhou.

Fulvifomes fastuosus was described by Bondartseva and Herrera. There are 162 reports available in GenBank on the genus Fulvifomes based on molecular data and among them only 18 sequences were on F. fastuosus. The species F. fastuosus was described from China, Thailand and Sri Lanka. In this study based on molecular phylogeny, two strains collected from Lady Doak College, Tamilnadu, India were identified as Fulvifomes fastuosus.

Macro and micromorphological characteristic features of G. wiiroense, P. badius and F. fastuosus.

The identification based on molecular means has been checked with the macro- and micro-morphological characteristic features and were found to be similar with the reported strains. However, the observation on basidiospores was different from the other reports for P. badius and F. fastuosus. The basidiospores of P. badius are ovoid to subglobose to globose and 4–6 × 4–5.5 μm. Singh and colleagues reported that basidiospores were broadly ellipsoid to subglobose. Our observation shows the P. badius basidiospores were ellipsoid and 4.21–5.54 × 2.83–4.13 μm. The basidiospores of F. fastuosus were subglobose, thick-walled, smooth 4.49 × 4.01 μm. According to Dai, the basidiospores were 5.61 × 4.2–5.6 μm. Our observations shows the basidiospores were 3.4–5.7 × 3.1–4.2 μm, which was smaller than Dai, but similar to Edirweera et al. However, the variation in the ratio (Q) was the same as previously reported of F. fastuosus strains. The variation in the size of basidiospores might be due to their geographical niche as well as depending on their nutrients from the host species.

Host preference by the macrofungal isolates. There are several factors that influence the distribution of fungi namely ecological niche, climatic conditions, host/substrate type, distribution of fauna and flora. To study host preference, basidiomata were collected from the living trees, wood log, and leaf litter. Later, the basidiomata was identified by molecular classification.

In India, the information on Ganoderma was first published in the early 1900s. Nearly 144 hosts were recorded in India. Among them coconut, betelnut, Casuarina, Areca catechu, Dalbergia sissoo and Toona cil., was observed as obvious host of Ganoderma sp. In India and Sri Lanka, Cocus nucifera showed high incidence as a host for Ganoderma species. From this study, it was observed that Ganoderma sp. grown on the following host species: Albizia sp., Tamarindus sp., Azadirachta sp. and Coccus nucifera. Fomitopsis ostreiformis belonging to Ganodermataceae has the host species Albizia sp., and Copiorolips caperata from wood log. The newly reported Ganoderma wiiroense has been collected from the trees of Albizia sp. (Table 1).

The species Fulvifomes fastuosus belongs to the family Hymenochaetaceae and reported to have medicinal properties. The F. fastuosus was observed in the trees of Xylocarpus granatum. In this study, F. fastuosus were found in the host trees of Albizia sp. The genera Phellinus have wide host range. Globally Quercus sp. is the more susceptible host and, in India Mangifera sp. followed by Acacia, Arctocarpus and Albizia are the predominant host of Phellinus. The genera Amylosporus was first reported in India among the Asian countries with bamboo as their host. In this study, the Amylosporus sp. was found in the host Neurium sp. and Albizia sp. Interestingly from this study, Albizia sp. is found to be the host preferred by most of the macrofungal isolates. This might be due to the abundance of this species in the vicinity of the collected macrofungi.

To conclude, we have identified and report two new macrofungal species G. wiiroense and F. fulvifomes and molecular evidence for P. badius from India. It was observed that Albizia sp., as the host preferred by most of the macrofungal isolates. Our data provide the existence of G. wiiroense in India; however, we were unable to trace out the origin of how G. wiiroense might have cross boundaries. We can only speculate G. wiiroense already exists in India; because of the lack of intense mycological study prior, this is the first report on it. These data gains us insight on macrofungal diversity in India, which can be used for the prospection of macrofungi in biomedical and industrial applications.

Methodology

Sample Collection and culture of isolates. Fresh basidiomata of the wild mushrooms belonging to the division basidiomyctera were collected from different locations in Dindigul (Ayyanar falls), Madurai (Lady Doak College Campus, Nagamalai, Pudupatti), Coimbatore (Kovai Kutralam), Thenkasi and Tirunelveli, Tamilnadu (India) during 2013–2017 on rainy seasons i.e., November to January. The basidiomata were cleaned and aseptically transferred to the lab. After surface sterilization with 70% ethanol, small pieces from the contextual layer of basidiomata were transferred to sterile potato dextrose agar (PDA) medium supplemented with streptomycin. The plates were incubated at 37°C for 5–7 days. The pure culture was obtained by continuous sub culturing and used for further analysis. The isolates were stored in PDA plates and slants. The basidiomata were then dehydrated with naphthalene balls for future studies.

The radial growth of the mycelium of all the isolates on the PDA medium was measured using a ruler. Five-millimetre mycelial plugs were removed from the growing edge of the 7-day-old pure culture and inoculated on to the centre of the 80 mm petriplates containing PDA. According to Tomkin and our observation, the growth is not constant in the early stage. The lag phase was shorter (1 day) in some strains and longer (5 days) in some strains. The radial/lateral expansion was measured after three days (i.e., 3rd day for strains with shorter...
lag phase and 7th day for strains with longer lag phase) in diameter (in mm), and the number of days taken to completely colonize 80 mm petridish was recorded. All the measurements were made in triplicates. The representative voucher specimens were deposited in the Department of Biotechnology, Lady Doak College, Madurai, Tamilnadu, India. Taxonomical identification of the isolates was carried out based on molecular identification methods.

After identification, the macromorphological characteristic features such as shape, color, hymenial surface of the basidiomata were studied according to published description. Microscopical observations (hyphal system, figure 7. The evolutionary relationship was inferred using the maximum Likelihood method in MEGA6. The analysis involved 52 nucleotide sequences; thirty two sequences generated in this study are highlighted. The initial trees were obtained with the random addition of sequences. All positions containing gaps and missing data were eliminated. Numerical values above the internodes are the percentage of 1000 bootstrap replications. Bootstrap values higher than 60% are indicated. Scale bar 0.05 represents nucleotide substitutions per position. Three clades were predicted Clade 1: Polyporales; Clade 2: Hymenochaetales; Clade 3: Russules. The abbreviated letters next to accession number indicates the localities from which the sample is collected: IN - India, GH - Ghana, CH - China, ID - Indonesia, FL - Finland, NE - Nepal, SA - South Africa, SF - South Florida, SK - South Korea, SL - Sri Lanka. The diversity within subpopulation was predicted as 0.1, the diversity within entire population - 0.3 with a Mean inter population Diversity - 0.3 and Coefficient of differentiation - 0.8.
presence/absence of setae and basidiospores) were carried out using brightfield microscope (Olympus system microscope model CX41). Slides were prepared using 5% KOH and cotton blue.

Molecular characterization of the isolates. Genomic DNA Isolation, PCR amplification and sequencing. Genomic DNA of all the isolates were extracted as described by Moncalvo et al. 10 mg of mycelial biomass was homogenized with 3% SDS extraction buffer (3 g SDS, 50 mM Tris, 150 mM NaCl and 80 mM Na₂EDTA) and
incubated at 60 °C for 20–30 min. The 5.8S nuclear ribosomal RNA gene was amplified using ITS1 (CTTGGTCAT TTAGGGAAGTAA) and ITS4 (CAGGAGACTTGTACACGGTGTCAG) primers. PCR amplification was carried out using the following condition: initial denaturation (95 °C, 2 min), denaturation (94 °C, 45 sec), annealing (50 °C, 45 sec), extension (72 °C, 1.30 min), final extension (72 °C, 5 min). The PCR products were purified and sequenced (Chromous Biotech Pvt. Ltd, Bangalore). The sequences were read bidirectionally for both strands of the entire ITS1, 5.8S rDNA and ITS2 region. The DNA sequence obtained from both the strands was edited and contig assembly was carried out using DNA Baser sequence assembly software (V.4.36.0). The assembled sequences were submitted to GenBank Database.

**Phylogenetic analysis.** Additional ITS sequences of Basidiomycetes were downloaded from GenBank to clarify the interspecies relationship. The phylogenetic tree was constructed by maximum likelihood (ML) analysis in MEGA 6 software. The tree inference options were set as follows: Heuristic Method Nearest-Neighbor-Interchange (NNI) with the very strong branch swap filter with 1000 bootstrap replicates, gaps were treated as missing.

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Author Contributions

T.M. conceived the study, designed and executed the wet lab experiment, designed the evolutionary study, produced figures, analyzed the data and prepared the manuscript. PJ. helped with the experiments. A.A.P.A. produced figures, analyzed the data, reviewed and helped with the manuscript. A.A.P.A. and R.S. made critical revisions and approved final version. All authors reviewed and approved of the final manuscript.

Additional Information

Competing Interests: The authors declare no competing interests.

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