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MORPHOMOLECULAR APPROACH TO IDENTIFY *FLAVODON FLAVUS* (KL.) RYV.: A POTENTIAL MACROFUNGI OF BANGLADESH

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Abstract

This study emulated the morphomolecular identification of *Flavodon flavus* (Kl.) Ryv. from the Chittagong University campus, Bangladesh. The morphological features of the fruit body of the fungus, such as the shape, size and texture of the basidiocarp, basidiome structure, surface characteristics of fruit body, spore-bearing surface of fruit body, nature of the hymenophore, and dimitic hyphal system; absence of stipe and volva; nature of colony and microscopic features such as hyphal diameter, branching pattern, shape and size of spores corroborated the identity with *Flavodon flavus*. The identity of the fungus was further confirmed through sequencing and analysis of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA). A BLASTn search of the ITS region sequences of this macrofungus revealed that the sequence was 97% similar to that of *F. flavus*.

Introduction

Flavodon flavus (Kl.) Ryv., a monotypic genus derived from the basionym *Irpex flavus* Klotzsch (1833) was introduced in mycology by Ryvarden in 1973. It is a basidiomycetous wood decay fungus lives on decayed, coarse wood under moist condition. It is under the family Meruliaceae (Hattori 2000). *Flavodon flavus*, the most abundant wood-decaying fungi in nature can degrade all the cell wall components of wood, mostly lignin and cellulose by utilizing some wood-decomposing enzymes to simplest form, carbon dioxide and water, and subsequent use for their metabolism (Boominathan and Reddy 1992). This process contributes immensely to a forest ecosystem by regenerating and accelerating soil health and nutrients.

In Bangladesh, the study of macro-fungi and their application in medicinal, agricultural, biotechnological, and even environmental fields is scanty. A few studies on morphological identification were carried out which is insufficient to contribute effectively to the present situation (Shayesta and Rahman 1992). Moreover, the traditional morphological approaches are not often dependable due to the presence of similar reproductive structures of macro-fungi and even coincidental paraphyletic taxonomy (Hossain 2022). Therefore, the aim of this study was morpho-molecular identification of *Flavodon flavus*.

Materials and Methods

The study was conducted in Chittagong University campus, Bangladesh. Fresh sample of the fruit body was collected from the hilly forest of Chittagong University campus during the early Autumn (October and November) and the rainy season (May to June) of 2022-23. Field notes on habits and habitats as well as clear photographs were taken for documentation. Proper sanitation was maintained during the collection followed by preserving the sample under dry (oven dry at 40°C) and wet (in 70% alcohol or formaldehyde solution) conditions. Morphological features *i.e.* size, shape, color, structure, texture, pore type, and zonation of the samples were studied and

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then cultured on PDA medium to observe the characters such as colony growth and pattern, hyphal structure, basidia, and basidiospore in the laboratory. The morphological characters of the present fungus were justified by studying scientific papers (Simmons *et al*. 2016, Das *et al*. 2017, Saha *et al*. 2018, Ador *et al*. 2023), books (Ying *et al*. 1987), websites (https://champignouf.com/, https://www.mushroomexpert.com/, https://firstnature.com/, https://www.indiabiodiversity.org/) and personal communication with mushroom experts.

About 1 mg mycelial mat was taken from the freshly grown fungal colony to extract total genomic DNA using Maxwell® FSC DNA IQ™ Casework Kit (Promega Corporation, USA). The PCR amplification of internal transcribed spacer of the nuclear ribosomal DNA (ITS nrDNA) region was conducted using the forward and reverse primers set of ITS1 and ITS4 (White *et al*. 1990) following the protocols: initiative denaturation step at 95°C for 3 min, 35 cycles of denaturation, annealing and extension at 95, 49 and 72°C, respectively for 30 seconds, final extension at 72°C for 5 min and ended with 4°C. The PCR products were purified using Wizard® Genomic DNA Purification Kit (PCR Clean-up System, Promega Corporation, USA) and sequencing was done at Apical Scientific Laboratory, Seri Kembangan 43300, Selangor, Malaysia. The raw data obtained through sequencing were edited and arranged using the Molecular Evolutionary Genetics Analysis (MEGA) software version 11 (Tamura *et al*. 2021).

The obtained data of nucleotide sequences was deposited in the GenBank for accession number and subjected to the Basic Local Alignment Search Tool (BLAST). A dataset was prepared by comparing the newly identified species with similar species from the National Centre for Biotechnology Information (NCBI) Databases. Several distantly related sequences were also taken as outgroups to elucidate the differences between the species. The multiple sequences of the data set were aligned altogether using the ClustalW alignment of MEGA 11 (Tamura *et al*. 2021). A phylogenetic tree was constructed using the same software's Maximum Likelihood method, with 1000 bootstrap replications of relative branches.

Results and Discussion

Flavodon flavus is basidiomycetous wood decay fungus belonging to the class- Agaricomycetes, order- Polyporales, and family- Meruliaceae. It is a common white-rot wood-decaying fungus, capable of degrading all the cell wall components mainly lignin and cellulose. It grows gregariously on deadwood or rotten timbers of angiosperms as a crust fungus, largely in moist condition.

Basidiocarp annual, firmly attached with gregarious growth, resupinate to pileate, dimidiate, semicircular, adjacently imbricate, mostly attached to a hard surface, pileus up to 90 × 50 × 10 mm, flat to umbellate, hairy on upper surface layers with slight concentric rays, margin entire, wavy and lobed, dark-greyish color at the center, whitish to yellowish towards the margin, hymenophore poroid at a young stage that turn to irpicoid or hydnoid with up to 2.5 mm long toothed outgrowths undersurface after ages, round to angular up to 3 per mm and 5 mm deep, basidiome greenish to yellowish color, soft to hard and rough texture, dry to spongy in monsoon weather (Fig. 1a, b).

On the PDA surface the fungus showed a filamentous to rhizoidal growth pattern, flat and filiform towards the margin, white-colored hairy appearance of mycelia, cream-white to yellowish color spores first appear on the mature hyphae of the center of the colony (Fig. 1c).

Dimitic hyphal system *i.e*. generative hyphae and skeletal hyphae present. Generative hyphae were hyaline, simple, moderately branched, interwoven, simple septate, thin to slightly thick-walled, up to 2-5 µm in diameter, clamp connection absent (Fig. 1d, e). Skeletal hyphae were hyaline, thick-walled, rarely branched and tangled, encrusted, up to 7 µm in width, dominating in

the setting, apical encrusted skeletal hyphae may project into the hymenium; basidia present at the tip of a hyphal branch, clustered or single, clavate, thin and slender, thicker sterigmata, size up to 25-30 × 4-5 µm in diameter, basidiospore hyaline, circular to semi-circular or ellipsoidal, thin to thick-walled, size 4.5- 5.0 × 2.5-3.0 µm in diameter (Fig. 1f, g). The morphological features *i.e.* resupinate to pileate basidiocarp, shape, size, and color of pileus and basidiospores, nature of hymenophore and basidiome, and dimitic hyphal system corroborate the identity of the fungi with *F. flavus* as reported by Nnagadesi and Arya (2015), Saha *et al*. (2018), Kezo *et al*. (2019) and Gore and Mali (2021).

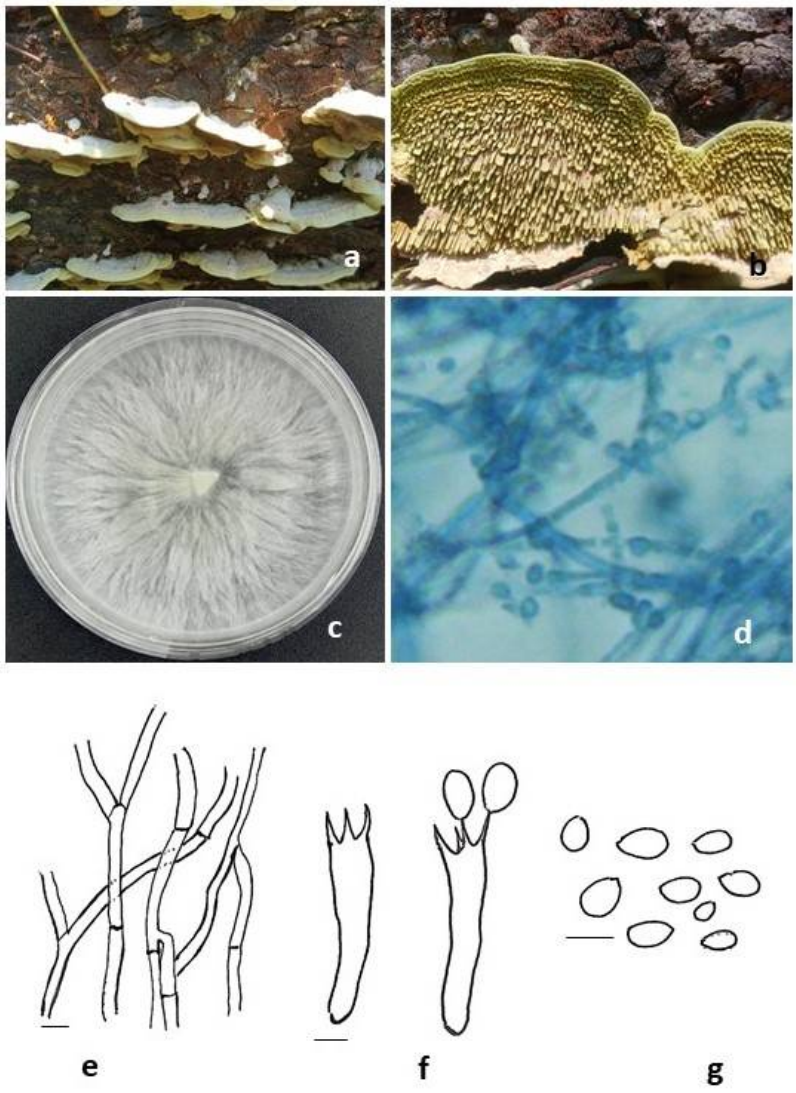

Fig. 1. Characteristics of *Flavodon flavus*. a: Basidiocarp grown on wood, b: Lower surface of basidiocarp, c: Mature colony, d: Generative hyphae with basidia and basidiospores, e: Generative hyphae, f: Basidium with basidiospore, and g: Basidiospores. [Scale bars: 5 µm]

A phylogenetic tree was constructed by clustering consensus sequence with eight other nucleotide sequences of similar species and two distantly related species from GenBank databases to categorize their highest similarity or differences (Fig. 2). The BLASTn search of ITS region sequences of this macro-fungus exhibited maximum similarity (97%) with *F. flavus*. The evolutionary history was inferred using the Maximum Likelihood method and the Tamura-Nei model (Tamura and Nei 1993) with the highest log likelihood (*InL*= -2305.55). Initial trees were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model (Tamura and Nei 1993) and then selecting the topology with a superior log likelihood value.

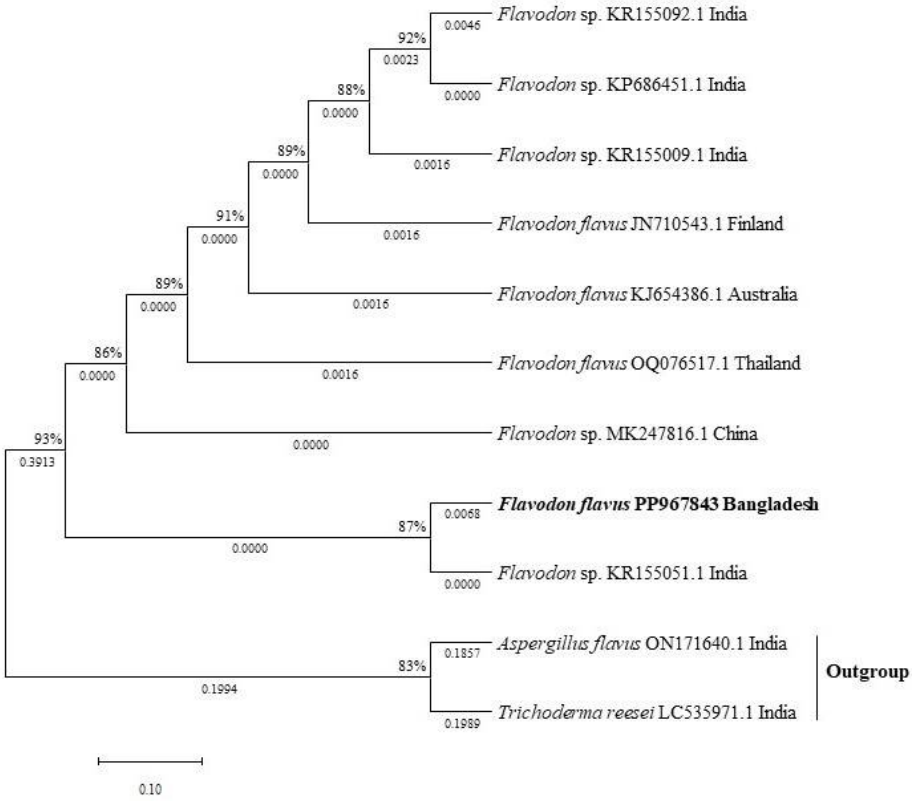

Fig. 2. Phylogenetic tree showing the taxonomic position of *Flavodon flavus* with its related taxa from varied regions. Maximum likelihood bootstrap (BS) values >50% and PP values >0.50 are shown.

The analysis involved 11 nucleotide sequences and 707 positions in the dataset. In the phylogenetic tree, 9 nucleotide sequences of *F. flavus* of diverse regions were clustered showing a statistical frequency of 93% bootstrap (BS). The newly obtained sequence of *F. flavus* from Bangladesh (GenBank accession No. PP967843) was clustered in a clade with other sequences of *Flavodon* sp. generated from different parts of the world with a statistical frequency of 87% BS. Two other sequences of distant taxa as out group were clustered in a clade with statistical support of 92% BS. The distance between the nucleotide sequences of *F. flavus* and the outgroup (*Trichoderma reesei* and *Aspergillus flavus*) was demonstrated by showing their clusters in two different branches (Fig. 2).

The molecular analyses contingent from ITS sequences of nrDNA shared phylogenetic affinities with *F. flavus*. The phylogenetic tree was generated by using some sequence data of the genus *Flavodon* and other Polypores species from the GenBank through Neighbor-joining and Maximum Likelihood method that showed a distinct evolutionary branch within the subsect. DNA-based phylogenetic approaches have positioned wood decay fungi taxonomy in a situation where it can give clear answers to these queries raised in morphological identification (Kamei *et al*. 2005, Miettinen *et al*. 2012, Simmons *et al*. 2016). Among the different genetic markers used in molecular identification, the ITS regions of nrDNA seems to be the most efficient, reliable, widely used, and powerful genetic marker for the precise identification of wood decay fungi (Schoch *et al*. 2012, Khosrow 2016). The ITS regions are established as the formal DNA barcode in fungi as it validates a clear barcoding gap for a wide range of ancestries and is frequently in good agreement with biological/morphological species concepts and could, therefore, be exploited for identification resolutions (Schoch *et al*. 2012, Badotti *et al*. 2017, Fryssouli *et al*. 2020).

The ITS-based molecular identification methods might be an important complement to conventional fungal identification methods. This study provides detailed morpho-molecular information on *F. flavus* and the techniques adapted for this fungus will facilitate isolating and accurately identifying other wood decay fungi.

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