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# PHYLOGENETIC STUDY OF TWO UNREPORTED EDIBLE POLYPORES FROM PAKISTAN: *SPARASSIS LATIFOLIA* AND *GRIFOLA FRONDOSA*

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**Abstract**

Grifola Gray and Sparassis Fr. are among the most well-known Polyporales genera due to their immense mycogastronomic significance. Despite their economic importance and widespread distribution, they have been largely overlooked in taxonomic studies across different regions. Our analysis of morphological features and nuclear rDNA regions (ITS and nrLSU) from the voucher specimens, has led to the identification of two previously unreported species in Pakistan i.e., *G. frondosa* and *S. latifolia*. To reconstruct phylogenetic relationships of the species, the current study used three methods: maximum parsimony, maximum likelihood, and Bayesian analyses on the concatenated dataset (ITS + nrLSU). Phylogenetically, our specimen sequence from *Grifola* grouped together with *G. frondosa*, showing a robust support (100% MP, 100% ML and 1.00 BPP). However, it differed from *G. frondosa* in many distinct characteristics such a thin, non-fleshy pileoli, shallow, radially elongated pores, distinctly largest generative hyphae, lack of skeletal hyphae (monomitic hyphal system) and highest Q value, short sterigma, and spore size. Similarly, the morpho-anatomical characteristics of the *Sparassis* specimens closely resembled those of *S. latifolia*, *S. crispa*, and *S. radicata*, and were hardly distinguishable. The result of the phylogenetic analysis showed a strong association with *S. latifolia*, forming a single clade with high support (96% MP, 93% ML, and 0.98 BPP). This taxonomic study is a vital contribution to the list of polypores in the country and provides useful data about the biogeographic distribution worldwide.

**Key words:** Grifola, Sparassis, Mushroom, Phylogeny, Morphology

## Introduction

Polyporales is one of the largest order in the fungal kingdom, consisting of 18 clades and 37 families (Justo *et al*., 2017). The current study examines two polyporoid species, one of which belongs to *Sparassis* genus that falls under the "antrodia clade" - the largest group of brown rot polypores (Hibbett & Donoghue, 2001; Ortiz-Santana *et al*., 2013; Justo *et al*., 2017; Cui *et al*., 2019; Yuan *et al*., 2022). Another species is the member of *Grifola* - which was initially classified under the "antrodia clade" (Wu *et al*., 2004; Binder *et al*., 2005; Yu *et al*., 2010; Garcia-Sandoval *et al*., 2011). However, the genus was later transferred to the "core polyporoid clade" by Justo & Hibbett, (2011). It has been shown by the Binder *et al*., (2013) that the taxonomic position of the genus still remains uncertain and does not belong to either the "antrodia clade" or to the "core polyporoid clade". Nonetheless, it shows sister relationship with the latter (Justo & Hibbett, 2011; Binder *et al*., 2013).

The *Sparassis* Fr. is a type genus of edible mushrooms that belongs to Sparassidaceae Herter (Jülich, 1981). The genus is morphologically characterized by highly branched hymenophore which grows from thick stipe, forms flabellate (fan-shaped) branches with initially amphigenous hymenial surface and conspicuously creamy to white or yellowish color, possessing a monomitic hyphal structure composed of septate generative and gloeoplerous hyphae. Cystidia are rarely present and basidiospores are typically hyaline and ellipsoid to subglobose shaped. These characteristic have been documented in numerous studies (Wang *et al*., 2004; Desjardin *et al*., 2004; Zhao *et al*., 2013; Liu *et al*., 2023; Binder *et al*., 2005; Kirk, 2008).

The genus has been the subject of classification and taxonomic refinement since the late nineteen century. Martin & Gilbertson (1976), and Burdsall & Miller (1988) identified three common species, namely *S. brevipes* Krombh, *S. crispa* (Wulf.) Fr., and *S. spathulata.* Schw., Fr. However, based on phylogenetic data, Desjardin *et al*., (2004) and Wang *et al*., (2004) recorded seven species. Dai *et al*., (2006) and Ryoo *et al*., (2013) further refined the phylogenetic relationships of *Sparassis* species by studying geographical distribution of isolates using both ITS and LSU sequence data. They classified the genus into three species, namely *S. crispa* from Europe and eastern North America, *S. radicata* from western North America, and *S. latifolia* from Asia. Subsequently, Hughes *et al*., (2014) and Petersen *et al*., (2015) re-examined these findings and discovered that *Sparassis* can be divided into two complexes: the *Sparassis crispa* complex and the *Sparassis spathulata – S. brevipes* complex. The former complex includes species with curled or crisped branches such as *S. crispa*, *S. radicata*, *S. latifolia*, and *S. americana*, while the latter complex comprises species with erect or stiff, blade-like margins such as *S. spathulata*, *S. brevipes*, *S. laminosa*, *S. nemecii*, *S. subalpina*, *S. miniensis*, and *S. cystidiosa*. Hughes *et al*., (2014) also proposed the two new taxa, namely, *S. americana* and *S. americana* f. *arizonica*, and identified several monophyletic clades within the *Sparassis crispa* complex. Recently, the mitochondrial genome was used to differentiate between the Asian and European specimens and the American collections in *S. crispa* complex, leading to the identification of eight clades (Bashir *et al*., 2020).

*Grifola* Gray was originally described and classified in the family Hymenotheceae by Gray (1821) and later transferred to Grifolaceae Jülich (Hibbett *et al*., 2000; Justo *et al*., 2017). Name of the genus was typified on the basis of cosmopolitan species of *Grifola frondosa*. According to

Justo *et al*., (2017), Grifolaceae is a separate family within Polyporales. These fungi produce a white rot in their substrate and are clearly distinct from brown rot fungal lineages in the antrodia clade. The genus is characterized, with eccentric simple or branched stipe and imbricate semicircular compound, annual basidiomes which consist of numerous petaloid pilei or multipileate or flabellate, with angular pores, 2-4 per mm, decurrent on stipe, possessing monomitic or dimitic hyphal system and usually clamp connections in generative hyphae and having ovoid to ellipsoid and inamyloid basidiospores (Gilbertson & Ryvarden 1986; Zhao & Zhang 1992; Shen *et al*., 2002). *Grifola* spp. are distributed throughout the globe from temperate forest to subtropics and are commonly associated with broad leaved trees (Dai, 2012; Acharya *et al*., 2015; Gargano *et al*., 2020; Wu *et al*., 2022).

Gray (1821) initially attempted to morphologically classify the genus into five species, which was later refined by Murrill (1904). However, subsequent taxonomic investigations by Gilbertson & Ryvarden, (1986), and Zhao & Zhang, (1992) have revealed that the genus is likely monotypic. This taxonomic investigation revealed that North American and Asian isolates were morphologically similar. The molecular data analyses of Lee *et al*., (2012) showed that species from the Japan exhibit little variation but may require revision due to difference in nomenclature. Recent analyses showed that *G. umbellata* (Pers.) Pilat is the widely distributed species and constitute phylogenetically distinct lineage (Sotome *et al*., 2008; Xing *et al*., 2013). Similarly, *G. sordulenta* (Mont.) Singer is associated with *Nothofagus* species from southern Argentina and south-central Chile also exhibit distinct relationship in the phylogenetic studies of Rajchenberg & Greslebin, (1995), and Shen (2001). Recent phylogenetic analyses of Rugolo *et al*., (2023) found that *Grifola* spp., in Southern Hemisphere are represented by four taxa including *G. sordulenta* and *G. colensoi*, *G. gargal* and a newly reported species *G. odorata* from New Zealand. The study highlights the complex evolutionary history of *Grifola* species evolving from Gondwanan ancestors.

These studies highlight the importance of molecular, morphological and geographical distribution data for accurate species identification and determining phylogenetic relationship among the species of both *Grifola* and *Sparassis*. The literature review further shows a lack of complete taxonomic information about these economic fungi from many countries, hindering a comprehensive understanding of their origins and biogeographic distribution on a global scale. A broader range of collection and diversity is required from all over the world to address questions and determine the phylogenetic relationships of these fungal groups. Many Asian countries including Pakistan, although possess a significant richness in mycoflora have received inadequate attention in term of taxonomic research. Observation further suggests that there is a significant amount of diversity yet to be described and resolved. Therefore, the current study aims to examine some representative vouchers materials and infer the phylogenetic relationship using both molecular and morphological data to expand the understanding of polypore diversity in the country.

## Materials and Methods

**Morphological characterization:** The study analyzed voucher specimens collected in temperate forest of district Swat KP, Pakistan, which is located between 35º22' 42" N and 72º10'47" E in the Hindu-Kush mountain range. The ephemeral macro-morphological features of basidiocarps were recorded in the field notebook. For color terms Petersen (1996) was adopted, and the voucher specimens were deposited at the University of Malakand (UM) herbarium.

A thorough microanatomical examination of the basidiocarps were carried out using microscopic techniques and routine notations, in accordance with Ji (2022). Freehand anatomical sections were analyzed microscopically for the hyphal system, septal features, and hymenial elements, including basidial and sterile elements, and spore characteristics. Reagents such as 5% KOH, 2% congo red, lactophenol cotton blue, and Melzer's reagent (IKI) were used to mount and stain the samples. The reactions were classified into amyloid or Melzer's-positive (IKI+), and inamyloid or Melzer's-negative (IKI-) according to Banik *et al*., (2012). The mean spore length (L), width (W) were calculated as the arithmetic average of all spores, and the variation in the L/W ratios between the specimens studied was denoted as Q. The number of spores measured from a given number of specimens was recorded as n. The structure measurements was performed with Image J software (Tsujikawa *et al*., 2003).

**Cultural characterization:** The isolation was done from piece of the context of freshly collected basidiocarps. The tissue was inoculated onto 90 mm PDA and MEA plates following Lindner & Banik (2011) and Virginia & Catalin (2013) and observed under a stereomicroscope for macro-morphological features (Stalpers, 1978).

**DNA extraction:** DNA extraction was done using standardized CTAB method, as described by Murray & Thompson (1980) and Stirling (2003), with modifications. Approximately 25 mg of piece from the pore surface was homogenized in 400 µl of 2% CTAB buffer with a multi-beads shocker at 3000 rpm/1min, and then incubated at 65°C for 50 minutes. To purify the homogenate, 350 µl of chloroform: isoamyl alcohol (24:1) was added and vortexed for one minute, followed by centrifugation at 13200 rpm at 4ºC for 20 minutes. The aqueous phase was transferred to a new autoclaved microtube. Precipitation was performed with 133.33 µl of ice-cold iso-propanol, followed by centrifugation at 13200 rpm at 4ºC for 20 minutes, and the supernatant was discarded. The resulting pellet was washed twice by adding 500 µl of ice-cold ethanol, followed by brief vortexing and centrifugation for 3 minutes. After drying the pellet was re-suspended in distilled water, and stored at -20ºC until further use.

**PCR and Sanger sequencing:** PCR amplification of the ITS1, 5.8S, and ITS2 gene regions of rDNA, as well as the nrLSU region was carried out using primers ITS-1-ITS4 and LR0R-LR5 (Gardes & Bruns, 1993; White *et al*., 1990; Vilgalys & Hester, 1990). PCR reactions were performed on a Veriti thermal cycler (Applied Biosystems). In 20 µl reaction

mixtures containing 2 µl of genomic DNA (~100 ng), 0.5 µl of each primer, 14.17 µl of sterile deionized water (Fisher Scientific), 26 µl of 2×Taq PCR Mastermix, 0.5 µl Taq DNA polymerase (Takara BIO INC.), 2.0 µl MgCl2, and 0.5 µl dNTPs (Promega). The following temperature regime was used: an initial denaturation step at 95°C for 3 min, followed by 35 cycles of 95°C for 30 s, an annealing phase at 55°C for 30 s, and an extension step at 72°C for 30 s. The final extension was carried out at 72°C for 8 min. PCR products were confirmed using gel electrophoresis on 1% (wt/vol) agarose gels (Fisher Scientific) prepared in 0.5x TAE buffer and stained with ethidium bromide (Stefanova *et al*., 2022). A 1Kb ladder (Promega) was used as a standard marker in the first well, and gel documentation was carried out for visualization. Amplicons were purified using the total fragment DNA purification Kit (MEGA quick-spinTM PLUS, Thermo Fisher Scientific) according to the manufacturer's instructions. The Purified samples were sequenced using automated Sanger sequencing at Macrogen Inc. Seoul (https://dna.macrogen.com) sequencing facility in South Korea.

**Phylogenetic analyses:** To confirm the identity of query nucleotide sequences, Basic Local Alignment Search Tool (BLASTn) was used against the nucleotide database in GenBank. To ensure correct identification and retrieval of right reference sequences, protocols recommended by Nilsson *et al*., (2012) and Schoch *et al*., (2014) were followed. Sequence alignment was carried out using the MAFFT 7 online multiple sequence alignment program (https//mafft.cbrc.jp/ alignment/server/; Katoh *et al*., 2019). We reviewed the alignment and manually adjusted misaligned sites, excluding ambiguous sites at both ends, and removed most gaps, treating remaining gaps as missing data in all analyses using BioEdit 7.2.5 (Shen *et al*., 2002; Hall *et al*., 2011). *Wolfiporia dilatohypha* (FP72162) served as the outgroup. We performed three different phylogenetic analyses on the combined ITS + nLSU data set. Using jModelTest2 (Darriba *et al*., 2012), we determined the best-fit substitution model based on AIC criterion.

The parsimony analyses (MP) were conducted using PAUP v.4.0.b10 (Swofford, 2002). The analysis involved 1000 heuristic search replicates, utilizing random taxon addition searches and tree-bisection-reconnection (TBR) branch swapping. A 50% majority-rule consensus tree was generated, and the tree's topology was evaluated by calculating tree length, consistency index (CI), homoplasy index (HI), and retention index (RI) (Justo & Hibbett, 2011). Maximum Likelihood (ML) estimates were computed on the combined data sets (ITS+ nLSU) using IQ-TREE version 1.6.12 (Nguyen *et al*., 2015). Substitution models of TIM2ef+G, TrN+I, and TrN+I were selected for the respective partitions (Chernomor *et al*., 2016). Branch support was determined through bootstrapping with 1000 replicates (Hoang *et al*., 2018). Bayesian Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) inference was performed on the data set using MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003). The substitution models for each partition were specified as nst=6, rates=gamma (ITS), and nst=6, rates=propinv (nLSU and rpb2). The base frequencies, substitution rates, gamma shape, and p.inv. parameters were set based on the best-fit models obtained through the Akaike Information Criterion (AIC) as determined by JModelTest2. The analysis was run for 2 million generations with four chains, and trees were sampled every 100 generations. The first 5000 trees (25% of the total) were excluded as burn-in and were not used in constructing the consensus tree. The stop rule was set at stopval=0.01 (Lindner and Banik 2008). The trees were analyzed using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and later edited. The optimal topologies from maximum likelihood (ML) analyses were displayed and validated based on the ML-BS score (≥75), MP-BS score (≥50), and Bayesian posterior probabilities (BPPs) (≥0.95) (Ji, 2022).

## Results

**Phylogenetic analyses:** Three methods - MP, ML, and Bayesian analyses - were employed to reconstruct the phylogenetic relationships. The dataset used for the analysis consisted of 65 sequence variants, including 39 ITS and 26 nrLSU sequences. The representative voucher samples, MUS27 and MUBS92, were included in the analysis (Table 1). Figure 1 illustrates the topology of the ML analysis, indicating the bootstrap proportion (MP), bootstrap maximum likelihood (ML), and Bayesian posterior probability (BPP) values. The analysis included a total of 14 species, including, *S. radicata*, *S. crispa*, *S. spathulata*, *S. subalpine*, *S. brevipes*, *S. miniensis*, *S. cystidiosa*, *S. latifolia*, *S. americana*, *G. frondosa*, *G. sordulenta*, *G. colensoi*, *G. umbellatus*, and outgroup species such as *P. schweinitzii* (DA-38) and *Wolfiporia dilatohypha* (FP72162). Among the 1309 nucleotide characters in the aligned data matrix, including gaps, 845 (64.6%) were constant sites, 90 (6.88%) were variable but uninformative for parsimony analysis, and 374 (28.57%) were parsimony-informative characters.

A heuristic search was conducted using maximum parsimony analysis, which produced 100 trees with equal parsimony scores on a single island, using tree-bisection-reconnection (TBR) branch swapping. These trees were used to create a 50% majority rule consensus parsimonious tree, which had a tree length of 1072. The consensus tree had a consistency index of 0.647, a retention index of 0.872, a rescaled consistency index of 0.564, and a homoplasy index of 0.353, indicating the proportion of homoplastic characters in the dataset. A maximum likelihood (ML) analysis was performed on a combined dataset with the initial log-likelihood of the dataset was -6775.564. The optimal evolutionary model was found to be the GTR+I+G, with an estimated proportion of invariant sites (p-invariance) of 0.2450 and a shape parameter for the gamma distribution of 0.4810. A Bayesian analysis was conducted using the same dataset with the "lset nst = 6" option. The analysis was run until convergence was achieved after 20,000,000 generations, with a standard deviation of split frequencies of 0.009968. The results of both Bayesian and likelihood analyses revealed similar tree topologies and clade distributions, with most lineages supporting species-level clades.

Our sample sequence MUS27 grouped together with *G. frondosa*, showing strong support (100% MP, 100% ML and 1.00 BPP), whereas the MUBS92 showed relationship with *S. latifolia* (96% MP, 93% ML and 0.98 BPP) (Fig. 1).

**Table 1. List of species specimens and accession of GenBank used in the phylogenetic analyses.**

| S. No. | Species | Voucher | Country | ITS | nrLSU |
|---|---|---|---|---|---|
| 1. | *Sparassis radicata* | TENN50232/ss32 | USA | AY218449 | AY218410 |
| 2. | *Sparassis radicata* | TENN52558 | USA | KC987592 | KF053405 |
| 3. | *Sparassis radicata* | FP-133458 | USA | KC987572 | KF053408 |
| 4. | *Sparassis radicata* | UBC-F12464/ss | USA | AY218443 | - |
| 5. | *Sparassis crispa* | MBUH-PIRJO&ILKKA94-1587/ss5 | USA | AY218427 | AY218389 |
| 6. | *Sparassis crispa* | MBUH-DORISLABER/ss25 | USA | AY218442 | AY218404 |
| 7. | *Sparassis crispa* | CBS120826 | USA | KC987552 | KF053374 |
| 8. | *Sparassis crispa* | CBS423c2 | USA | KC987554 | KF053375 |
| 9. | *Sparassis spathulata* | zw-clarku001/ss7 | USA | AY218428 | AY218391 |
| 10. | *Sparassis spathulata* | zw-clarku004/ss11 | USA | AY218432 | AY218395 |
| 11. | *Sparassis subalpina* | HKAS57488 | China | JN387093 | JN387104 |
| 12. | *Sparassis subalpina* | HKAS57511 | China | JN387094 | JN387105 |
| 13. | *S. brevipes* | MBUH-ILKKA96-1044/ss24 | USA | AY218441 | AY218403 |
| 14. | *S. brevipes* | WU 30050 | USA | KP100501 | KP100475 |
| 15. | *Sparassis miniensis* | LOU-fungi 18390 | USA | DQ270675 | DQ270676 |
| 16. | *Sparassis cystidiosa* | HKAS59856 | China | JQ743079 | JQ743090 |
| 17. | *Sparassis cystidiosa* | HKAS59855 | China | JQ743078 | JQ743088 |
| 18. | *Sparassis cystidiosa* | Desjardin 7410 | USA | AY256891 | - |
| 19. | *Sparassis latifolia* | HMJAU2007 | China | JQ743072 | JQ743082 |
| 20. | *Sparassis latifolia* | HKAS59854 | China | JQ743071 | JQ743081 |
| 21. | *Sparassis latifolia* | HMJAU2955 | China | JQ743073 | JQ743083 |
| 22. | *Sparassis americana* f. *americana* | CMFR: OKM7058 | USA | KC987581 | KF053389 |
| 23. | *Sparassis americana* f. *americana* | TENN66366 | USA | KC987594 | KF053388 |
| 24. | *Grifola frondosa* | zw-clarku005 | USA | AY218415 | AY218413 |
| 25. | *Grifola frondosa* | M036 | USA | AY049119 | - |
| 26. | *Grifola frondosa* | WC834 | USA | AY049139 | - |
| 27. | *Grifola frondosa* | CIRM-BRFM 1162 | France | GU731562 | - |
| 28. | *Grifola frondosa* | strain-10 | Germany | FR686557 | - |
| 29. | *Grifola frondosa* | Dai 19172 | China | ON417161 | ON417211 |
| 30. | *Grifola sordulenta* | G01 | USA | AY049142 | - |
| 31. | *Grifola sordulenta* | AFTOL-ID 562 | USA | AY854085 | AY645050 |
| 32. | *Grifola sordulenta* | PDD:86931 | New Zealand | GU222266 | - |
| 33. | *Grifola colensoi* | CBS 326.49 | Australia | MH856542 | - |
| 34. | *Grifola umbellatus* | WA0000052306 | Poland | KX756417 | - |
| 35. | *Grifola umbellatus* | Pen13513 | China | KU189772 | - |
| 36. | *Wolfiporia dilatohypha* | FP72162 | USA | EU402556 | EU402517 |
| 37. | *Phaeolus schweinitzii* | DA-38 | USA | EU402585 | EU402514 |
| 38. | *Sparassis latifolia* | MUS27 | Pakistan | OQ453180 | OQ453213 |
| 39. | *Grifola frondosa* | MUBS92 | Pakistan | OQ453162 | OQ453163 |

***Grifola frondose* (Dicks. Fr.) S. F. Gray**

Vouchers specimen: Pakistan, KP Province: (i) *Grifola frondose* voucher no. MUBS92, Sailand, district Swat, (34º59'23" N and 72º10'56" E, 2751 m asl), mixed coniferous forest on dead tree roots of *Quercus semicarpifolia*, September, 2020. Depository info: Mycology section of University of Malakand (UOM); (ii) *Grifola frondosa* voucher no. MUBSg21, Miandam, district Swat, (35º04'28" N and 72º35'42" E, 2820 m asl), in mixed coniferous forest on ground near *Quercus* sp., September, 2021.

**Basidiocarps:** Basidiocarps annual, multipileate, substipitate, imbricate, non-bruising, and large sized approx. 20-36 cm across, 12-24 cm high, rosette of multiple overlapping tongue shaped caps (Flabellae/Pileoli**)** fused to a common short stipe forming irregular whorl. Individual cap is deltoid, spathulate or dimidiate, about 8-12 cm long, 3-5 cm wide, and 0.8-1.2 mm thick, non-fleshy and non-juicy with mild odour, pleasant taste, and leathery texured which become crumbly and lightweight when dry; upper surface pale grayish and dark grayish towards the margin become dark gray or dark brown to blackish when dry, faintly zonate, 3-4 concentric zones at the margin, glabrous, showing radial vaguely streaked or striation. Margin wavy, whitish or paler, blunt and almost flat. Stipe stout, short, sub cylindrical and fleshy and massive and up to 1 cm in length. Pore surface whitish or cream in appearance when fresh become light yellowish when dry, nearly decurrent on stipe. Pores are usually shallow, minute nearly rounded to slit like, number of pores 5-7 or usually 5/mm; Dissepiment is non-uniform less than 0.1 mm in thickness, and non-lacerate. Tube layer closely affixed to the context, pale whitish, about 0.1 mm in thickness, single layered both context and tube layers are concolorous. Context: indistinctly paler covered by pileal black coat or cuticle, and about 0.8- 0.9 mm at the widest point. Context to tube layer ratio is 9:1 (Fig. 6).

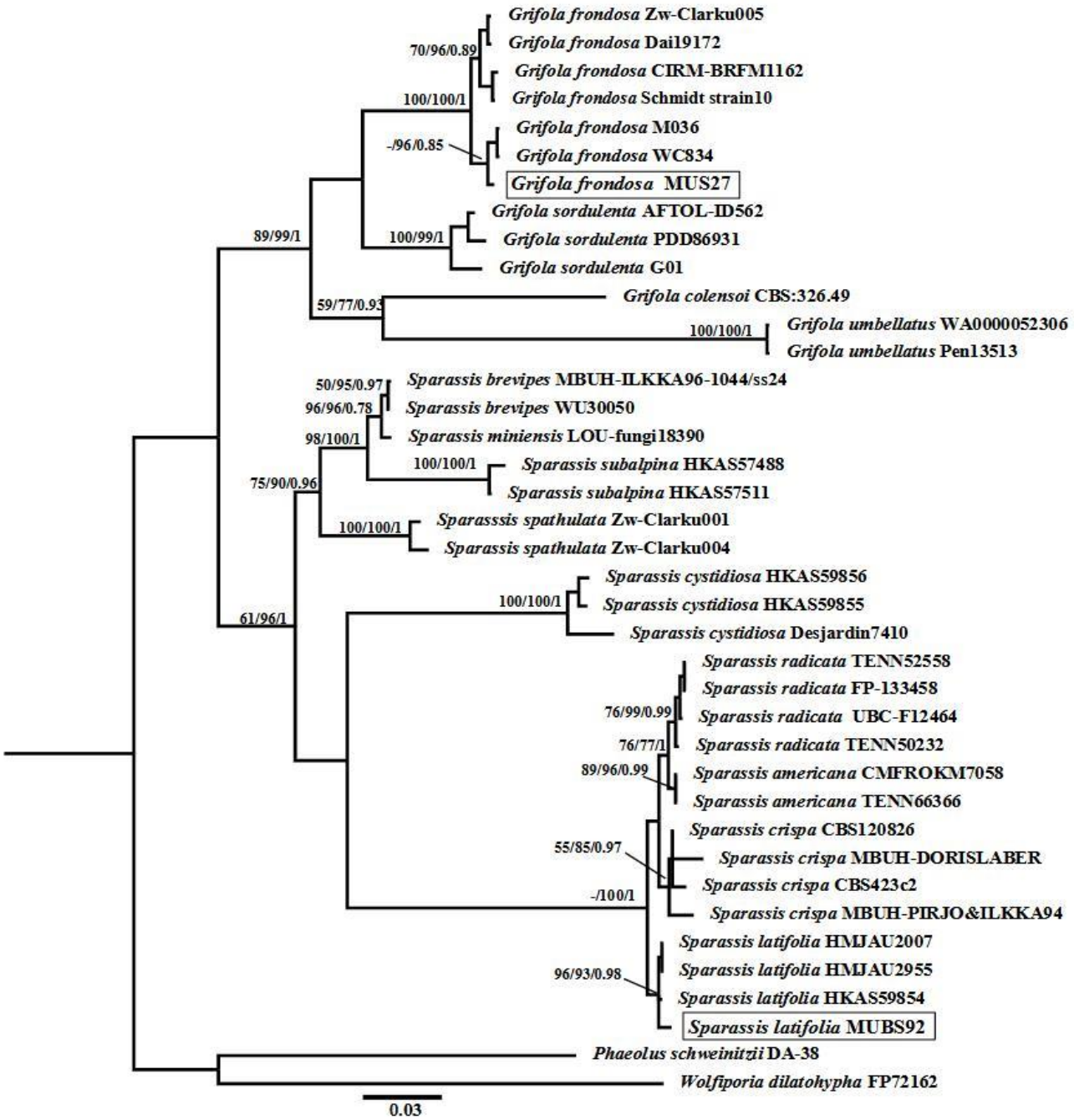


Fig. 1. The strict consensus tree determined through maximum likelihood depicts the phylogenetic relationships among *Grifola* and *Sparassis* spp., inferred from ITS + nrLSU sequences. Branch support values indicated before the corresponding node bootstrap MP/ bootstrap ML/ posterior probabilities BPP. Tip labels after taxa name designate voucher specimen number.

Hyphal system is monomitic. The contextual hyphae are wider, pale to hyaline, 11-23.5 (12.0) µm, and 0.77 to 1 µm wall thickness, frequently septate, branched or anastomosed, regular, more or less parallel, and unchanged with KOH. Tramal hyphae consist of wider generative hyphae in the subhymenial layer somewhat similar to the contextual hyphae. Whereas the generative hyphae from the hymenial layer are narrower, range 2.5-4.0 (6.6) µm in diam., thin walled, yellowish in color, interwoven, branched, rarely septate, possess abundant clamps, with lateral protuberances and swollen hyphal termii. The skeletal hyphae were not seen in the tramal layer. Basidia: clavate, with short sterigmata and a simple septum at the base, 15–27 (15.7) × 5.5–8 (6.3) µm. Basidioles and cystidia are absent. Basidiospores ellipsoidal or ovoid, hyaline, uniguttate, thin and smooth walled, non-dextrinoid or inamyloid, acyanophilous, 6.0–8.6 × 3.4–4.7µm, L = 7.1 µm, W = 3.8 µm, Q = 1.65-2.27, (n = 35/2) (Fig. 2).

**Cultural characteristics (Figs. 3, 4)**

Form and texture: Circular or irregular, homogeneous, silky to fleecy. The mat was flat, showing mostly aerial and sparsely grown, usually radial, azonate growth; Advancing edge: Outline was uneven, usually silky or fibrillar and raised; Colony odour: Not detected, Exudates: Not found

Front color: Whitish to lightly brown, The brown to blackish color of the mycelium can be visualized when hold against the light; Obverse color: Pale to yellowish In vitro teleomorph formations: Denser lumps were observed on the margin in the old culture while the center is left uncovered KOH test: Light brown; Growth rate: The average GR was measured as 5.1 (± 0.5) mm day-1 . The colony achieved the total radial growth of 40.95 mm on day 9 at 25°C.; Hyphal characteristics (Fig. 3): The hyphae were usually long, branched, septate, rarely clamped, hyphal diameter was ranging from 2-4.5µm. Hyphal loops and swelling were rarely examined in the culture. Short lateral branches and protuberances were appeared on the hyphae. No chlamydospores were found. Comments: Inoculum was obtained internally from the context of basidiocarp collected from the base of Oak trees. Colony initiation and establishment was slightly slow. Culture viability was lost after few sub-culturing.

**Morphological similarity:** The morphological character-matrix revealed that the species within the *Grifola* genus exhibit a little variation among the species. All of the described species were found to produce annual, imbricate, and multipileat, flabellate, stipitate, rosette-like basidiocarps. The pileoli were observed to be irregularly lobed to petaloid or spatulate and radially rugose, and finely tomentose surface. The size of the fruiting bodies remains relatively consistent, typically ranging from 20-35 cm in width, although minor variations have been observed across the species (Table 2). Additionally, slight differences in coloration on the pileal and pore surfaces have been recorded. Our specimen exhibits several distinctive features that distinguish it from other species in the genus. In *G. colensoi*, *G. gargal*, and *G. odorata*, the pores were irregular, labyrinthine, and elongated, while *G. frondosa* possessed somewhat irregular to rounded or elongated pores. When compared pore size, hyphal structure, basidial and basidiospores our specimen exhibits relationship with *G. frondosa*. In our specimens, the pore size was smaller, ranging from 5-7, which is notably smaller than the pores found in *G. colensoi*, *G. gargal*, *G. odorata*, and *G. sordulenta*, which typically range from 1-2/mm. Notably, distinct characteristics were recorded in our specimen including monomitic hyphal structure, shallow pores (0.1 mm), largest generative hyphae (11-23.5), highest Q value (1.86), short sterigma does not match with the previously described species in the genus. Further, few of the species *G. gargal*, *G. odorata*, *G. sordulenta* monomitic hyphal system was recorded for the species rest of species were dimitic hyphal structure. The recorded distribution indicated that *G. amazonica*, *G. americana*, (North American) *G. umbellate* (rare European) *G. frondosa* (cosmopolitan species) were showing the norther hemispheric distribution while the *G. colensoi*, *G. gargal G. odorata*, *G. sordulenta* were found in the southern hemisphere.

***Sparassis latifolia* Y.C. Dai & Zheng Wang.** Vouchers Specimen: Pakistan, KP province: (i) *S. latifolia* voucher no. MUS27, Sailand, district Swat, (35º05'53" N and 72º35'14" E, 2587 m asl), mixed coniferous forest on roots of dead stumps of *Abies pindrow*, July, 2018. ii) *S. latifolia* voucher no. MUCS11, Chail, district Swat, (35º07'15" N and 72º34'12" E, 2327 m asl), in mixed coniferous forest on ground near *Abies pindrow*, August, 2019. Depository info: Mycology section of University of Malakand (UOM).

Spore print: white; Rot type: white rotting

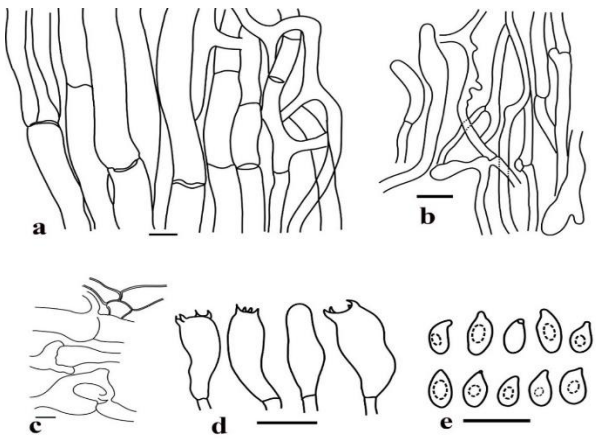


Fig. 2. Microscopic structure drawn from voucher specimen of *G. frondosa* a. Generative hyphae of context showing branches; b. Generative hyphae of hymenial layer; c. Clamps and branching points of the hyphal system and hymenium structure; d. basidia e. basidiospor Scale bars: a,b,c = 10 µm.

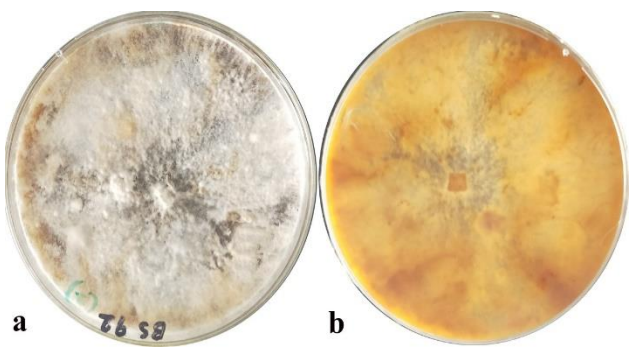


Fig. 3. Cultural characteristics; MEA grown culture of *Grifola frondosa* a. front view; b. reverse view (90 mm plate).

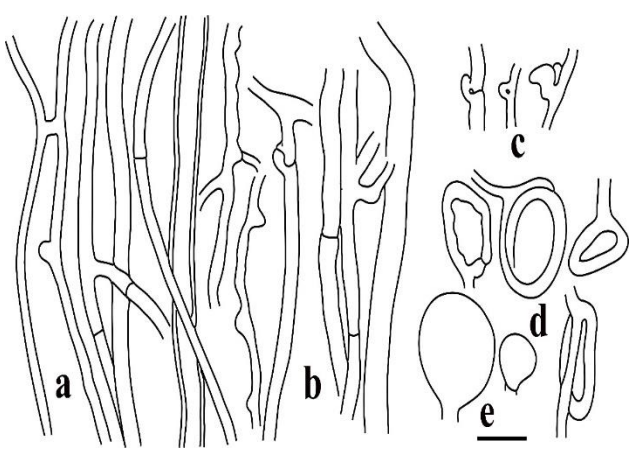


Fig. 4. *In vitro* hyphal characteristics of *Grifola frondosa*; a. Surface thick and thin hyphae showing H-shaped anastomosis; b. Submerged hyphae with short lateral protuberances and branches; c. Hyphal clamps and knots; d., e. Hyphal swelling and loops, (100x, Bar = 10 µm).

**Basidiocarps:** Basidiocarp large sized, solitary, annual, stipitate, imbricate, about 15-30 cm across, 10-12 cm high, consisting of multiple, dissected, highly contorted flabellae, forming irregular rosette arise from stout subcylindrical rooted stipe. Flabellae: white to cream colored, azonate, non-bruising, approximately 1.5-3 cm broad, 2.5-3.5 cm long, and 0.5 mm thick. Basidiocarps having pungent odor, pleasant walnut taste, leathery-textured when fresh and become lightweight and crumbly when dry. Upper surface deeply cream or yellowish colored become light cream or pale when dry. Surface glabrous or glistening, isomorphic and concolorous hymenial and abhymenial surfaces. Flabellar margin is wavy or contorted (crisped) and entire, blunt become dark brown or burnt black when dry. Stipe is short, stout, and branched, up to 6 cm long and 3-5 cm in diam., sub cylindrical to flattened and white colored (Fig. 6).

Hymenial layer closely affixed to the context, both layers are indistinguishable. Hyphal system is monomitic, hyphae are septate, unchanged with KOH. Context is white colored. Contextual hyphae are wider pale 4.4-9.5 (7.0) µm, frequently septate and rarely branched, reticulate or interwoven arrangement, agglutinated, showing large sized medallion clamps. Encrusted gloeoplerous hyphae are rarely found 6.3-9.6 (7.5) µm in the context and stipe mycelia. Tramal hyphae long, thin walled, pale yellowish, regular and more or less parallel showing fairly abundant clamps and septation, 3.5-10.5 (5.6) µm, frequently vesiculose or irregularly inflated and agglutinated. Stipe hyphae are 3.8-11.6 (6.6) µm, septate, branched, and have abundant clamps. Dark brown to black colored spherical microcrystals were found in the context and stipe layers. Basidia: sub-clavate, 4-sterigmate, short sterigmata, with a simple septum and clamp at the base, 25–30 (28.5) × 6.5–7.5 (7.3) µm. Basidioles are abundant, narrowly elongated, 16–32 (23) × 3.5–5 (4.5) µm and cystidia are absent. Basidiospores ellipsoidal or subglobose, hyaline, uniguttate, thin walled, smooth walled, non-dextrinoid, and non-amyloid, 4.0–7.3 × 3.8–5µm, L = 5.7 µm, W = 4.4 µm, Q = 1.0-1.6, (n = 30/2) (Fig. 5). Rot type: brown rotting.

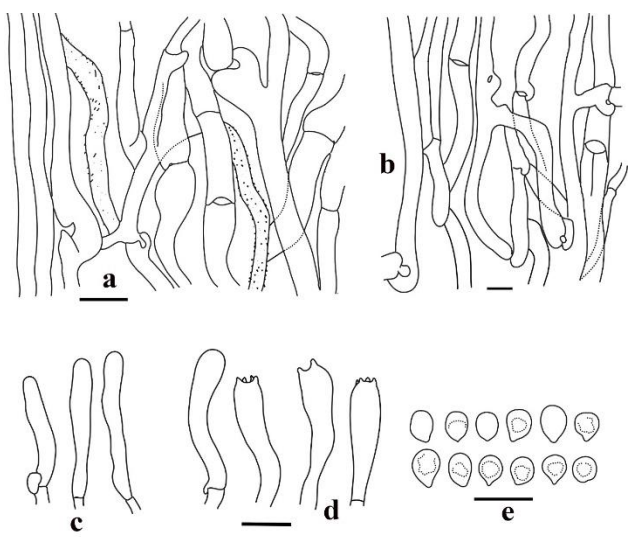


Fig. 5. Microscopic structure drawn from voucher specimen of *S. latifolia* a. hyphae of context showing gloeoplerous hyphae; b. Generative hyphae from trama; c. basidioles d. basidia e. basidiospores Scale bars: a, b, d, e = 10 µm.

**Table 2. Morphological characters of *Grifola* species obtained from the original description.**

| Species | *G. colensoi* | *G. gargal* | *G. odorata* | *G. sordulenta* | *G. amazonica* | *G. frondosa* | *G. frondosa* | *G. umbellata* |
|---|---|---|---|---|---|---|---|---|
| Flabellar shape | divided lobes | foliose or lamellate | flabellate lobes | flabellate | flabellate | flabelliform | flabelliform | Orbicular caps branches |
| Flabellar size (cm) | NA | 8 × 7 | –x11 | NA | NA | –x4 | 12x 5 | NA |
| Basidiocarp (cm) | 32 × 27x25 | 30w | 35 × 22 × 24 | 35 × 15 ×30 | NA | NA | 12-24 x20-36 | 50w |
| Pileal surface color | dark brown or purplish black | cream yellow, light beige, light brown or grey | grey-brown, brown or white | cream-color, light cinnamon or grey | brown | pale olive-buff | pale grayish, darker on the margin | grayish brown |
| Pore surface color | pale cream-color | white | white | pale cream-color | white, pale greyish | cream to ivory white | pale whitish or cream | pale cream |
| No. Pores/mm | NA | 1–2 | 1–2 | 1–2 | 3–4 | 2–4 | 5–7 | 1–2 |
| Pores depth(mm) | 4 | 5 | 2 | 5 | 2 | 4 | 0.1 | 0.5-1 |
| Hyphal system | dimitic | monomitic | monomitic | monomitic | dimitic | dimitic | monomitic | dimitic |
| Gen. Hyphae (µ) | 3–6 | NA | 2.5–9.5 | NA | NA | 5 | 11-23.5 | NA |
| Basidiospores (L) | 5.20–7.10x3.90–5.10 | 7–8x5–6 | 5.8– 8.5x5.0–7.0 | 6–7x4–5 | 4–4.5 x3–3.5 | 5.5–6.5/7 x3.5–4.5/5 | 6.0–8.6x3.4–4.7 | 7.5-10 x 3-3.5 |
| Q range | 1.35 | 1.46 | 1.24 | 1.35 | NA | NA | 1.86 | NA |
| Geo. Distribution | Australia, Tasmania New Zealand | Argentina | New Zealand | Argentina | NA | NA | Pakistan | Europe |
| Host | Fuscospora Eucalyptus | Lophozonia Amomyrtus, Eucryphia, Populus. | Metrosideros Fuscospora, Salix | Nothofagus | NA | Quercus, Castanea, Fagus, Carpinus | Quercus sp | hardwood |

**Morphological similarity:** Analysis of the morphological character-matrix indicated that all species documented within the genus *Sparassis* share a range of morphological features, making the intraspecific delimitation difficult. These include a solitary, annual, compound fruiting body that generates a complex of branched and contorted lamellate basidiocarps, which typically exhibit an anastomosing branching and monomitic hyphal system. In addition, these species possess a 4-sterigmate clavate basidia, with basal clamps and septa, as well as ellipsoidal basidiospores with slight variation in dimension of basidial and basidiospores size (Table 3a, 3b). Based on the morphological examination of our specimens demonstrated a close relationship with *S. crispa*, *S. latifolia*, and *S. radicata*, making the differentiation challenging. Nevertheless, our specimen and S. latifolia share only a few characteristics, including the size of the basidiome, the presence of stout branched stipe, azonate flabellae, short basidia, thin generative hyphae, and longer basidiospores (5-9 µm). The *S. latifolia* is distinct from several other species, including *S. crispa*, *S. subalpine*, *S. cystidiosa*, *S. spathulata* herbstii, *S. crispa*, and *S. miniensis*, in that it has well-developed stipes. The flabellar margin of several species, such as *S. spathulata*, *S. brevipes* f. nemecii, *S. americana*, *S. radicata*, and *S. miniensis*, is characterized by lobate, leciniate, or of frilled type. On the other hand, the margin of *S. americana* and *S. radicata*, *S. crispa* is crisped, while slightly contorted in the *S. latifolia*. Among the species analyzed, a slightly zonate flabellar surface was recorded for *S. spathulata*, *S. spathulata* herbstii, *S. brevipes*, *S. brevipes* f. nemecii, and *S. crispa*. On the other hand, the flabellar surface of *S. subalpine*, *S. spathulata* herbstii, *S. americana* f. americana, *S. radicata*, *S. miniensis*, and some other species were identified as matte and pruinose, whereas other species had a glabrous surface. Similarly, the presence or absence of clamp connections in the tramal hyphae was found to vary among the species. Specifically, *S. subalpine*, *S. spathulata*, *S. spathulata* herbstii, and *S. brevipes* were identified as clampless tramal hyphae, while other species had clamp connections in their trama.

## Discussion

The two genera, *Grifola* and *Sparassis*, are among the most well-known species in the Polyporales order which having an immense mycogastronomic significance. Despite these importance (Wu *et al*., 2019), they have been largely neglected in biogeographic, ecological, and taxonomic studies. By examining the morphological features of the collected specimens and further looking at two nuclear rDNA regions (ITS and nrLSU), we were able to identify two species which were not previously reported in Pakistan. The observed *Grifola* specimen was markedly differ from other species in the genus in many important features including thin non-fleshy pileoli, shallow, radially elongated pores, distinctly largest generative hyphae, highest Q value, short sterigma, and spore size. The hyphal construct in the previously recorded *G. frondola* species was recorded dimitic, our study noticed the monomitic system consisting of two different types of generative hyphae i.e., broader generative hyphae in the context while narrower in the trama and lack of the skeletal hyphae. Similar record was found in the past taxonomic study of Pouzar (1966). The gross morphological variation of the species confirmed that the *G. frondosa* clade may further be divisible into several morphological or cryptic species, as previously reported in several studies (Rajchenberg & Greslebin, 1995; Shen, 2001; Shen *et al*., 2002; Sotome *et al*., 2008; Lee *et al*., 2012; Xing *et al*., 2013). However, based on combined data of ITS, LSU, and multiple phylogenetic analyses, has been observed that the specimen belongs to a larger clade consisting of isolates of *G. frondosa*, from USA and China. So far, four clearly defined intraspecific clades were identified in the *Grifola* genus.

In the genus *Sparassis*, the morphological comparison from original descriptions showed several unrecorded or even overlapping features of taxonomic interest to cause problem in species delimitation. However, the phylogenetic analyses based on sequence data derived from rDNA (ITS + nrLSU) revealed that high degree of morphological resemblance among the *Sparassis* species was found to be merely superficial. The morpho-molecular characteristics of our *Sparassis* specimen, collected were consistent with the previously published descriptions of the species in question (Dai *et al*., 2006). In a study conducted by Dai *et al*., (2006), most of the Asian collection of *Sparassis* was identified and named *S. latifolia*. while, Park *et al*., (2005), Ryu *et al*., (2009) and Ryoo *et al*., (2013) from South Korea has shown the potential misidentification of *S. latifolia* as *S. crispa*. Based on morphological examination and phylogenetic analyses of our specimens demonstrated a close relationship with *S. crispa*, *S. latifolia*, and *S. radicata*, and *S. americana*, thus allied under same clade. Similar conclusion was derived by Wang *et al*., (2004), Dai *et al*., (2006) who revealed that the phylogenetic relationship between *S. radicata* or *S. crispa* found in western North America and *S. crispa* found in Europe belong to the same clade. Our study suggests that even geographically distant species are more closely related to each other than they are to species in closer geographic proximity. This fungal distribution could possibly be attributed to long-distance dispersal. Similar finding were deeply addressed in the previous study of Ryoo *et al*., (2013).

In conclusion, the current study provides insights into the diversity and relationships among species within the *Grifola* and *Sparassis*. Analyses of molecular data within different clads revealed a strong correspondence with morphological characteristics and biogeographical distribution. Some phylogenetic clads still require re-evaluation and taxonomic clarification to refine our understanding of the biogeography and evolutionary diversity. Further work is needed to confirm edibility of these species and their potential value in food and drugs biotechnology.


Fig. 6. Basidiomes of *Grifola frondosa* (a), and *Sparassis latifolia* (b) from natural habitat; (c) pores surface (fresh) of *G. frondosa* (d) Basidiocarp of *S. latifolia* showing stipe portion (e) Pores of *G. frondosa*, (f) Flabellar arrangement and margin of *S. latifolia*; Bars: a, b = 3 cm, d, f = 2 cm, c, e = 1division = 1 mm.

**Table 3a. Morphological characters of *Sparassis* species obtained from the original description.**

| S. No. | Species | Basidiocarp (L x W) cm | Stipe | Flabellar shape | Flabellar margin | Zonation | Abhymenial surface | Hymenial surface | Clamps in trama |
|---|---|---|---|---|---|---|---|---|---|
| 1. | *S. cystidiosa* f. *flabelliformis* | 25 x 30 | Poor | Broadly flabelliform | Entire or dissected, slightly contorted | Azonate | Grayish brown to yellowish brown | NA | Present |
| 2. | *S. latifolia* | 30 x 35 | Robust | Broadly laminar | Dissected, contorted | Azonate | White to cream-shallow brown | NA | Present |
| 3. | *S. subalpina* | 16 x 15 | Poor | Broadly laminar | Entire or leciniate, contorted | NA | Grayish or brownish | NA | NA |
| 4. | *S. latifolia* | 30 x 25 | Robust | Laminar | Dissected, slightly contorted | Azonate | White cream | NA | Present |
| 5. | *S. cystidiosa* | - x 25 | Poor | Flabelliform | Entire or dissected, slightly contorted | Azonate | Yellowish brown to brown | Bicolorous | NA |
| 6. | *S. spathulata* | NA | Robust | Spathulate or fanshaped | Entire or lobate, contorted | Zonate | Off-white or pale ochraceous buff | Unilateral | NA |
| 7. | *S. spathulata herbstii* | NA | Poor | Spathulate or fanshaped | Entire, contorted, labyrinthine | Zonate | Off-white | Unilateral | NA |
| 8. | *S. brevipes* | NA | Robust | Laminar | Labyrinthine, non-crisped | Zonate | Off-white | Unilateral | NA |
| 9. | *S. brevipes* f. *nemecii* | 21 x 30 | Robust | Lobate or gyrose | Lobate, non-crisped | Zonate | Paler, non-waxy | | NA |
| 10. | *S. crispa* | NA | Poor | Laminar rarely lobate | Entire, crisped | Zonate | Off-white, pale ochraceous buff | Unilateral | Present |
| 11. | *S. americana* f. *americana* | 20 x 35 | Robust | Petaloid | Entire or lobate, crisped | NA | Paler, light ochraceous buff, off-white | NA | NA |
| 12. | *S. americana* f. *arizonica* | 20 x 30 | NA | Petaloid or frilled | Lobate, crisped | NA | Creamy yellow to yellowish brown | Unilateral | Present |
| 13. | *S. radicata* | 30 x 25 | NA | Thin lacunose | Entire or dissected, crisped | NA | Off-white or pale pinkish cinnamon | Unilateral | Present |
| 14. | *S. miniensis* | 21 x 18 | Poor | Broad | Leciniate, contorted | Azonate | Whitish | NA | Present |
| 15. | *S. latifolia* | - x 40 | Robust | Broadly laminar | Dissected, slightly contorted | Azonate | White to yellowish or cream | NA | Present |
| 16. | *S. latifolia* | 12 x 30 | Robust | Laminar | Entire, contorted | Azonate | White to cream colored | NA | Present |

**Table 3b. Morphological characters of *Sparassis* species obtained from the original description.**

| S. No. | Species | Gloeoplerous hyphae (µm) | Tramal gen. hyphae (µm) | Cystidia | Basidia (LxW) µm | Basidiospores (LxW) µm | Geo. Distribution | Host plants | References |
|---|---|---|---|---|---|---|---|---|---|
| 1. | *S. cystidiosa* f. *flabelliformis* | 2– 11 | | Absent | 55–70x7–8.5 | 7–9x6–7 | China | Quercus spp. | Zhao *et al*., 2013 |
| 2. | *S. latifolia* | 7–12 | 5–15 | NA | 55–68x5–7 | 4.5–5.5x3.5–4 | Russia, Japan, China | Conifers and Fagales spp. | Zhao *et al*., 2013 |
| 3. | *S. subalpina* | 4–10 | 4– 10 | NA | 73–85x5.5–7.5 | 5.5–6.5x4–5 | China | *Picea likiangensis*, Rhododendron sp., *Quercus* sp. | Zhao *et al*., 2013 |
| 4. | *S. latifolia* | 7–12.5 | 4.5–9.5 | NA | 25–29x2.6–8 | 4.5–5.5x3.5–4 | China, Europe, North America | conifer forest | Dai *et al*., 2006 |
| 5. | *S. cystidiosa* | 2– 11 | 3–8 | Present | 65–74 x3 8–9.5 | 7–9x3–7 | Thailand | *Quercus eumorpha* | Desjardin *et al*., 2004 |
| 6. | *S. spathulata* | NA | NA | NA | 50–65x6.5–7 | 6–7x4.5–5 | North Carolina, Mississippi, Maryland, Louisiana, Tennessee | Quercus, Tsuga, Fagus, Betula, Acer and Magnolia, Pinus | Petersen *et al*., 2015 |
| 7. | *S. spathulata herbstii* | NA | 5–12 | NA | 30– 42x6–8 | 6–7.5x4–5 | Alabama, Columbia, New Jersey, Pennsylvania, South Carolina, Tennessee | NA | Petersen *et al*., 2015 |
| 8. | *S. brevipes* | NA | 5.5–11 | NA | 28–35x5–7 | 6–6.5x4–4.5 | Czech Republic, Ukrain, Germany Europe | *Abies alba*, *Picea excelsa*, *Fagus silvatica* or *Quercus* spp. | Petersen *et al*., 2015 |
| 9. | *S. brevipes* f. *nemecii* | | 4–8.5 | NA | 40–43x5.5–7 | 5.5–6.5x4–4.5 | Czech Republic, Germany, Europe | *Fagus* sp. | Petersen *et al*., 2015 |
| 10. | *S. crispa* | 5–10 | 5–16 | NA | 37–48x7–8.5 | 4.5–5x3.5–4 | Germany, Austria, Europe | *Pinus sylvestris* | Hughes *et al*., 2014 |
| 11. | *S. americana* f. *americana* | 9.5 –14 | 4–15 | NA | 50–66x6–7 | 4.5–6x3.5–4 | Tennessee, North America | *Pinus* spp. | Hughes *et al*., 2014 |
| 12. | *S. americana* f. *arizonica* | NA | NA | NA | 40 –60x4 – 8 | 5–6x3.5 –4 | United States, Arizona | *Pseudotsuga menziesii*, *Pinus* spp. | Hughes *et al*., 2014 |
| 13. | *S. radicata* | NA | NA | NA | 38–60x6.5–8 | 5–6.53.5–4 | North America | *Pseudotsuga* sp. | Hughes *et al*., 2014 |
| 14. | *S. miniensis* | NA | 2.5–7 | Absent | 30.5–56 x4.5–9.5 | 6.5–7.5x4.5–5 | Galicia, Spain | *Pinus pinaster* | Blanco-Dios *et al*., 2006 |
| 15. | *S. latifolia* | NA | 3.0–7.0 | NA | 39–51x5.5–8.0 | 5.0–5.2x4.0–4.1 | Korea | *Pinus koraiensis* | Ryoo *et al*., 2013 |
| 16. | *S. latifolia* | 6-9.6 | 3.5-10.5 | Absent | 25–30 x6.5–7.5 | 4.0–7.3x3.8–5 | Pakistan | *Abides pindrow* | This study |

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