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FIRST REPORT OF *SPARASSIS LATIFOLIA* (*BASIDIOMYCOTA*) FROM PAKISTAN: EVIDENCE FROM MORPHO-ANATOMICAL AND nrDNA DATA

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Abstract

Sparassis latifolia was found growing near *Pinus wallichiana*. It was characterized by having loosely arranged flabellae, pale to creamy on hymenial and ab-hymenial surfaces, turning to dark brown, undulating margins, amonomitichyphal system with frequent clamps in all parts of basidioma. Molecular phylogenetic analysis of nrDNA data also confirmed its relatedness with *S. latifolia*. It is a new record for Pakistan.

Key words: Cauliflower, Conifers, Genetic divergence, Himalayan, Saprobic.

Introduction

Cauliflower fungus, *Sparassis*, is a genus composed of brown rotting fungi usually associated with conifers and *Fagales*. This mode of decaying is derived from dominating white-rot fungi in *Polyporales* (Hibbett & Donoghue, 2001). Recently, its taxonomy and systematics were much focused (Blanco-Dios *et al*., 2006; Desjardin *et al*., 2004; Wang *et al*., 2004; Dai *et al*., 2006; Zhao *et al*., 2013). Species in the genus *Sparassis* can be differentiated on the basis of clamp connections, among other featurestheir presence in a given species among different tissues is constant. For example, *S. brevipes* Krombh., *S. spathulata* (Schwein.) Fr. and *S. subalpina* Q. Zhao *et al*. lack clamp connections in their cortical tissues and present in sub-hymenium and at the base of basidia, while *S.crispa*, *S. cystidiosa*, *S. latifolia* and *S. radicata*) have clamp connections both in their cortical tissues and in sub-hymenium as well as at the base of basidia (Desjardin *et al*., 2004; Wang *et al*., 2004; Zhao *et al*., 2013).

Dai *et al*., (2006) re-evaluated the Asian isolates of *Sparassis* and classified them into three groups; one group of *S. crispa* (from Europe and eastern North America), second group of *S. radicata* (from western North America) and third group of *S. latifolia* (from Asia) based on phylogenetic relationship. The holotype of *S. latifolia* was collected from Jilin, China and was found distributed throughout eastern Asia (Dai *et al*., 2006). From the present report, its distribution in Southeast Asia has also been confirmed (Fig. 4).

Material and Methods

During the exploration of macrofungi of Himalayan moist temperate forests of Pakistan, a member of the *Polyporales* belonging to *Sparassis* was collected from Helipad, Khanspur-Ayubia, Khayber Pukhtunkhwa, Pakistan, among conifers. Field notes were recorded and the specimens were photographed. Macroscopic characters were described using fresh material. Small portions of the hymenium (~1 cm) were placed in 2% CTAB buffer in 1.5 mL Eppendorf vials and kept frozen at –20°C for molecular characterization. The remaining specimen was dried, placed in air-tight plastic bags, sealed, labelled and deposited in the Fungal Biology and Systematics Laboratory, Department of Botany, GC University, Lahore.

Macro- and micro morphological features; Colour, shape of Basidiocarp, hymenial and ab-hymenial surfaces, basidiospores, basidia, basidioles, fambrillae hyphae were described and measured following Hanif *et al*., (2014).

DNA extraction and polymerase chain reaction: CTAB method followed by Gardes & Bruns (1993) was used for DNA extraction. The method was modified as followed by Khalid & Hanif (2017). Polymerase chain reactions were performed as described by Hanif *et al*. (2019). The amplified PCR product was sequenced from Macrogen, Korea. The DNA sequence of *Sparassis latifolia* was deposited in GenBank (accession KF866226).

The phylogenetic placement of *S. latifolia* was confirmed by Maximum Parsimony. The analysis involved 34 nucleotide sequences including a sequence of *S. latifolia* from Pakistan. These sequences were retrieved from GenBank following Zhao *et al*., (2013). *Grifola frondosa* (Dicks.) Gray (AY218415) and *Oligoporus rennyi* (Berk. & Broome) Donk (AY218416) were used as outgroup. Sequence alignment and editing were performed as described by Hanif *et al*., (2022). Percentage identities (PID) were calculated following Hanif *et al*., (2012). Data sheet of aligned and trimmed sequences was used for the construction of the phylogenetic tree (Fig. 3).

Molecular and phylogenetic analysis: ITS region of nrDNA was amplified using ITS primers and sequenced to produce 661 bp sequence. BLAST analysis indicated that the sequence from current specimen of *Sparassis latifolia* was 99% similar (100% query coverage) with *S. crispa* (AF308851). However, the morpho-anatomical details of our specimen did not match with *S. crispa*. When we compared their shared genetic characters using Jalview software (Waterhouse *et al*., 2009) which indicated the two shared 95% of their genetic characters with *S. crispa*.

The sequences obtained were aligned with related sequences from *Sparassis* from GenBank and UNITE. Of the total 690 characters in the ITS dataset, all characters were of type 'unord' and had equal weight, 306 characters were constant, 359 were variable parsimony-uninformative, and 267 were parsimony-informative characters, gaps were treated as "missing". Molecular phylogenetic analysis was inferred as described by Hanif *et al*. (2014). Evolutionary analyses were conducted described by Hanif *et al*., (2022).

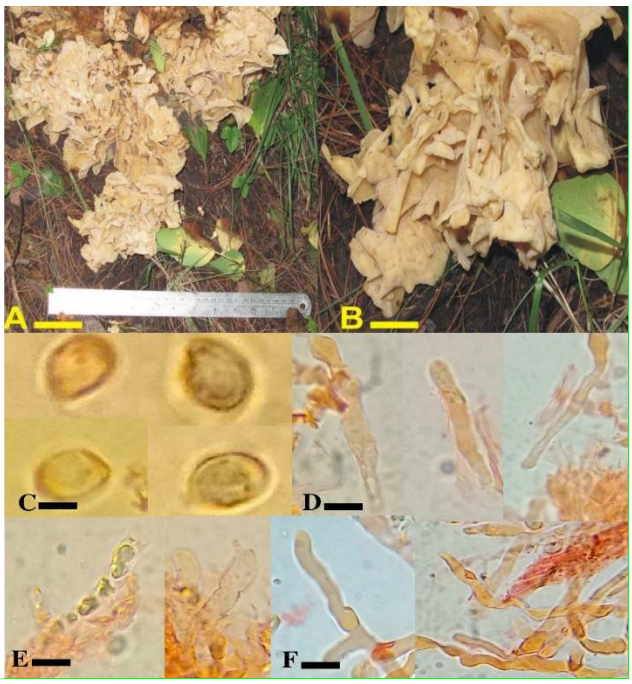

Fig. 1. A-F. ***Sparassis latifolia.*** (A-B). Basidiomata (C). Basidiospores (D). Cystidia (E). Basidia (F). Skeletal hyphae. **Scale bars (1cm): A (1.4cm)** = 6cm, **B (1.4cm)** = 3cm, **C** = 3µm, **D** = 8.5µm, **E** = 5.4µm, **F** = 10µm.

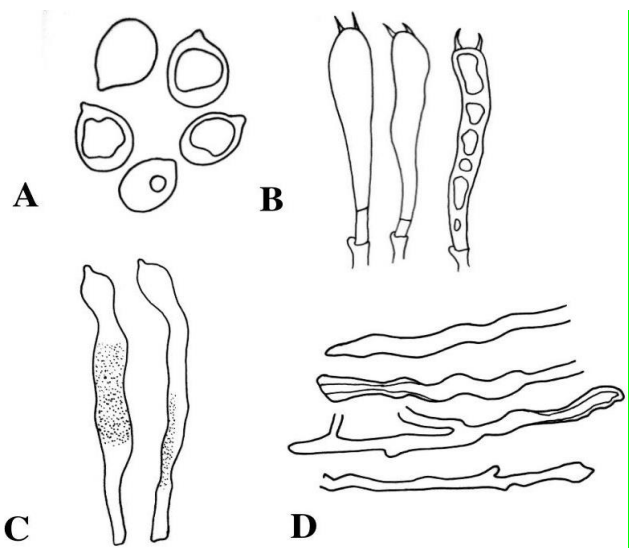

Fig. 2. A-D: *Sparassis latifolia.* (A). Basidiospores (B). Basidia (C). Cystidia (D). Skeletal hyphae.

Results

The ITS dataset demonstrates three clades (Fig. 3). The collection of *S. latifolia* from Pakistan clustered within *S. latifolia* with 88% bootstrap support. It shared a clade with *S. radicata* and *S. crispa* with 100% bootstrap support. *Sparassis latifolia* from Pakistan had 99 PID and 0.3% genetic divergence with *S. latifolia*, 93 PID and 2.7% genetic divergence with *S. radicata* and 96 PID and 3.0% genetic divergence with *S. crispa*. The shared genetic characters of *S. latifolia*, *S. radicata* and *S. crispa* were also calculated. *S. latifolia* shared 99, 97 and 95% of analyzed genetic characters with *S. latifolia, S. radicata* and *S. crispa*, respectively.

Taxonomic enumeration

Basidiocarps: 35 cm wide, up to 20 cm high, with numerous loosely arranged flabellae, solitary, stipitate, pale to creamy on hymenial and ab-hymenial surfaces, turning to dark brown on the lower surface, several broad flattened layers of tissues, mostly originate from a central mass, much dissected and contorted, with wavy/undulating margins. Stipe up to 12 cm long, thick at the base, covered under severe undulations of layered flabellae.

Hyphal structure: Hyphal system monomitic; septate generative hyphae; no reaction with KOH.

Context: White inside, contextual hyphae hyaline, monomitic, branched, interwoven, 5-12 µm diam.

Flabellae: Three layered; thin-walled hyaline tramal hyphae, frequently branched and interwoven, 4–9 µm diam. A large number of basidia and basidioles in hymenia; Basidia with four sterigmata and clavate, 24–28 × 4.5–7 µm; Basidioles similar in shape to basidia, sparsely branched, 26–30 × 5–7 µm, cystidia long, hyaline 33–51 × 4–6 µm.

Basidiospores: 5–6 × 4.5–5 µm, smooth, hyaline, broadly ellipsoidal to ovoid, flattened adaxially, large central guttule.

Material examined: Pakistan, Khyber Pakhtunkhwa province. Hazara division, Abbottabad district, Ayubia-Khanspur, Nathiagali, and Dungagali, 1972 m a.s.l. associated with *Pinus wallichiana*. *Hanif* # MHS22810.1 (Accession KF866226). August 22, 2010

Habitat and distribution: Solitary in mass, found in Himalayan moist temperate forests of Pakistan.

Discussion

The investigations regarding *Sparassis* inventories are scanty from Pakistan. According to the recently available database, there are only two species of *Sparassis* described from Pakistan viz; *S. laminosa* from Kalam, Swat (Ahmad, 1956, 1972; Hattori and Murakami, 1993) and *S. crispa* from Kaghan Valley and Sharan (Ahmad, 1969, 1972). In the present work another species of *Sparassis*, *S. latifolia* has been presented as a new addition in the fungi of Pakistan. It was collected from Himalayan moist temperate forests among conifers *Pinus wallichiana*. Initially, this species was tentatively given a name based on some morphological features as *S. crispa*. But morpho-anatomical and molecular data confirmed it as *S. latifolia* (Figs. 1-3). There are several reports about the misidentification of *S. latifolia* as *S. crispa* because of their similar morphological features (Imazeki *et al*., 1988; Mao, 1998, 2000; Ying & Zang, 1994; Yuan & Sun, 2007; Zang *et al*., 1996). Recently, Ghafoor & Niazi (2023) conducted a study on culturability, cultivation potential, and element analysis on *S. latifolia*. They collected the specimen from Ayubia, KP, Pakistan growing under *Abies pindrow* Royle. Regarding taxonomic identification of the species, only a photograph of basidioma, and GenBank accession no.s for its ITS-nrDNA sequences are given. However, morphological description, molecular phylogenetic analysis and comparison with the allied taxa, necessary for species identification is missing in that article. Moreover, both morphological and molecular data of specimens described in

two studies (Ghafoor & Niazi, 2003 vs. current study) is compared. As a result, it is found that the fruiting body of *S. latifolia* described in Ghafoor & Niazi (2023) is very small (7 cm x 8 cm) when compared with the specimen in current study (35 cm x to 20 cm), and with Type specimen (30 cm x 25 cm; Dai *et al.*, 2006). ITS-nrDNA sequences (OM417066, OM417067) of *S. latifolia* (Ghafoor & Niazi, 2023) also exhibit differences from the ITS-nrDNA sequences of *S. latifolia* generated during current study, although both collections were made from the same locality. Since focus of the study (Ghafoor & Niazi, 2023) was not taxonomy, therefore it is assumed that the authors had tentatively identified their taxon. However, it can be suggested to Ghaffor & Niazi (2023) to re-confirm the identity of their taxon (LAH28820) by performing detailed morphological and molecular-phylogenetic analysis. *Sparassis latifolia* was previously reported from northeastern China by Dai *et al*., (2006). In the current investigation, *S. latifolia* clustered with strong bootstrap support with *S. crispa* and *S. radicata* due to the presence of clamp connections in the tissues (Fig. 3). These findings are in agreement with Zhao *et al*. (2013). In their analysis, these species clustered within the same clade with strong statistical support. *Sparassis crispa* and *S. latifolia* can be differentiated based on macro and micro-morphological features as well as their habitat. One of the most distinguishing features is the length of stipe. *S. latifolia* has long stipe compared with *S. crispa*. Zhao *et al*., (2013) detected intraspecific divergences of *S. latifolia* within the Chinese populations and divided them into two subclades, namely, the Northeast Asia subclade and the southwestern China subclade. Interestingly, our sample was clustered with the southwestern China subclade (subclade I Fig. 3).

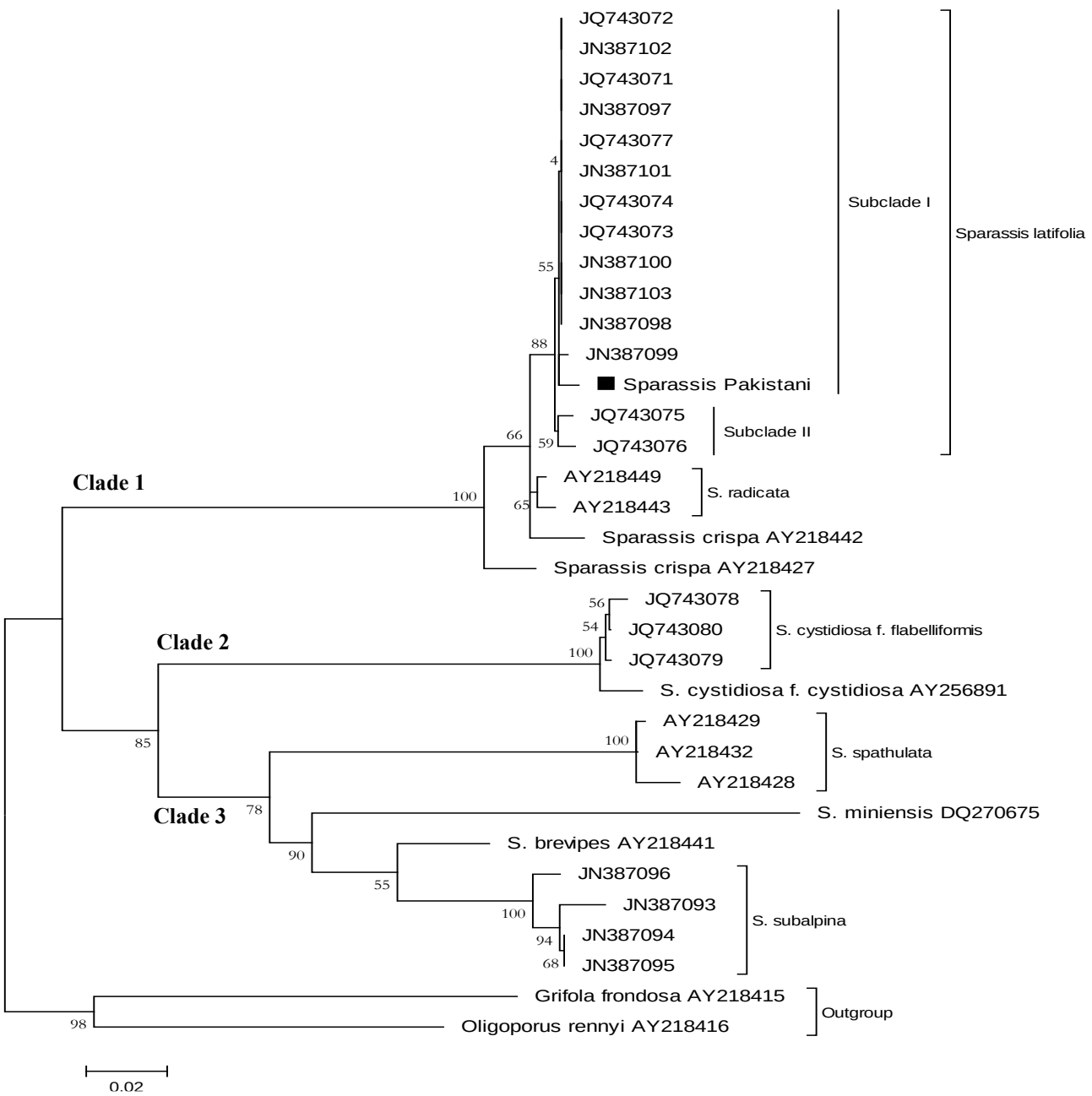

Fig. 3. Phylogenetic analysis of *Sparassis latifolia*
Phylogenetic relationship of *Sparassis latifolia* with other members of *Sparassis* inferred from nrITS sequences using Maximum Likelihood from 34 sequences.

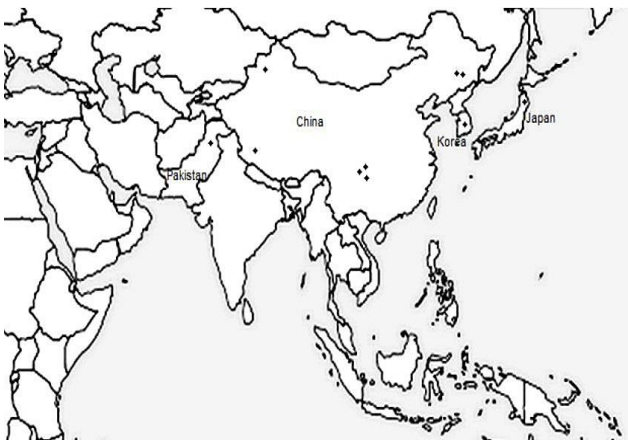

Fig. 4. Geographical distribution of *Sparassis latifolia* in Asia. Cubical dots (♦) representing the regions/countries who reported *S. latifolia*.

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