SKUAST Journal of Research 25(2): 199-206; (2023)
(*10.5958/2349-297X.2023.00027.2*); NASS rating: 4.17

Research Paper

Morpho-molecular characterization of *Flammulina filiformis* of Northeastern Himalayan Jammu and Kashmir: a new record from the Indian subcontinent

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(Received November 20, 2022; accepted December 30, 2022)

ABSTRACT

***Flammmulina* species are among the most cultivated as well as naturally occurring edible mushrooms occurring worldwide including Asia especially China, Japan and some parts of India. Morphological characterization based on macroscopic and microscopic characters like basicdiaocarp, basidiospores, basidia, pileus, stipe, different cystidial elements (pleurocystidia, cheilocystidia, pileocystidia) etc. of genus *Flammulina* was carried out. Molecular characterization based on sequencing of internal transcribed spacer (ITS) region using PCR based ITS1 and ITS4 primers, and phylogeny reconstructed, *Flammulina filiformis* was identified and is proposed as new record from India. The sequence was submitted to GenBank, NCBI under accession number ON844192.**

Key words: *Agaricales*, distribution ecology, edible species, *Flammulina filiformis,* internal transcribed spacer, new report, *Physalacriaceae*, taxonomy

Flammulina mushrooms occupies an important place among the Basidiomycetes in addition to being useful in the production of medicines. These mushrooms (trivial mime: enoki-take) have been traditionally cultivated since ancient times in China, Japan, and other oriental countries because of their nutritional and therapeutic value and is renowned for its anti-cancer properties (Stamets., 1993; Yamanaka., 1997; Hughes *et al.,* 1999; Methven *et al.,* 2000; Psurtseva., 2005). This genus is represented by 15 species worldwide viz. *Flamullina callistosporioides* (Singer) Singer, *F. cephalariae* Pérez-Butrón & Fern. -Vic., *F. elastica* (Sacc.) Redhead & R.H. Petersen, *F. fennae* Bas, *F. ferrugineolutea* (Beeli) Singer, *F. glutinosa* G. Stev., *F. lupinicola* (Redhead & R.H. Petersen) C. Hahn, *F. mexicana* Redhead et al., *F. ononidis* Arnolds, *F. populicola* Redhead & R.H. Petersen, *F. rossica* Redhead & R.H. Petersen, *F. similis* E. Horak, *F. stratosa* Redhead et al., *F. velutipes* (Curtis) Singer, and *F. yunnanensis* Z.W. Ge & Zhu L. Yang (Redhead *et al.,* 1998; Redhead and Petersen., 1999; Redhead *et al.,* 2000; Methven *et al.,* 2000; Ge *et al.,* 2008; Petersen *et al.,* 2012; Vizzini *et al.,* 2012; Hahn., 2016; Wang *et al.,* 2018) both from the Northern and Southern hemisphere. This genus was represented by only one species i.e. *Flammulina velutipes* (W.Curt.: Fr.) Singer before 1980's despite the fact that several authors pointed out varieties and forms of the species. The first taxonomical record of *Flammulina* from Western Europe was given by Bas in 1983. In nature, *Flammulina* is a cosmopolite inhabiting deciduous forests during cold seasons. It can survive even mildly cold temperatures and hence, has been given the name "winter mushroom". *Flammulina* species are known to occur naturally on different deciduous tree species *viz*., willows (*Salix* spp.), maple (*Acer* spp.) poplar (*Populus* spp.), elms (*Ulmus* spp.) plum (*Prunus* spp.), and birch (*Betula* spp.) as a parasite and later as a saprophyte growing on the trunks or stumps of these broad-leaved trees from late autumn to early spring.

Flammulina filiformis previously known as *F. velutipes* is an important commercially cultivated mushroom with high medicinal and dietary value. Its production contributes to more than 10% of the world's cultivated mushrooms (Royse *et al.,* 2017; Wang *et al.,* 2018; Yan *et al.,* 2019).

MATERIALS AND METHODS

Survey and sample collection: The macrofungi specimen for the present investigation was collected from Dachigam National Park (DNP) in the Zanskar mountain range of Kashmir, India through periodic surveys in different seasons. The collected specimen was brought to the Mushroom Research and Training Centre Laboratory of the Division of Plant Pathology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-Kashmir), Shalimar, Srinagar for detailed investigations. The Kashmir valley lies between the coordinates 32º 17' and 36º 58'N latitude and 73º 26' and 80º 30'E longitude and is situated between Pir Panjal range and the Zanskar range covering an area of 15,948 k sq.km (Qazi., 2005). Dachigam National Park, which has been presently selected as the area of investigation is a part of the state of Jammu and Kashmir well known as the biomass state of India. Dachigam National Park placed across the Zanskar mountain range of the Northwest Himalayan biogeographic region (2A), is located 21 km Northeast of Srinagar city of the Jammu and Kashmir state (Rodgers and Panwar., 1988; Rodgers *et al.,* 2000). DNP has more or less native vegetation and is of great ecological and aesthetic importance for exhibiting strong vegetation as opposed to areas just outside the park as they are exposed to various types of biotic interferences (Singh and Kachroo., 1978). DNP lies between the coordinates 34° 05″ 00′ N and 34° 10″ 32′ N and 74° 53″ 50′ E and 75° 09″ 16′ E covering an area of 141 sq km ranging from 1700 to 4700 m (Rodgers and Panwar., 1988; Rodgers *et al.,* 2000). Dachigam National Park is bordered to the Northeast by the Sindh Valley, in the far east by Tarsar, Lidderwath, Kolhai from Lidder Valley and Overa Aru Wildlife Sanctuary, in the Southeast by Tral range; and in the West and Southwest by Harwan, Brain and Nishat (Holloway and Wani., 1970; Kurt., 1978). The climate of the study area with higher degrees of variation in dryness and precipitation can be described as typically temperate sub-Mediterranean, with the bixeric regime (Singh and Kachroo., 1978; Mani., 1981). Dachigham has an annual minimum and maximum rainfall ranging between 32 and 546 mm (Bhat., 1985). The data with respect to the collected specimen from DNK along with their location (Latitude & Longitude), altitudinal range and forest type surveyed is summarized in Table 1 and Fig 1.

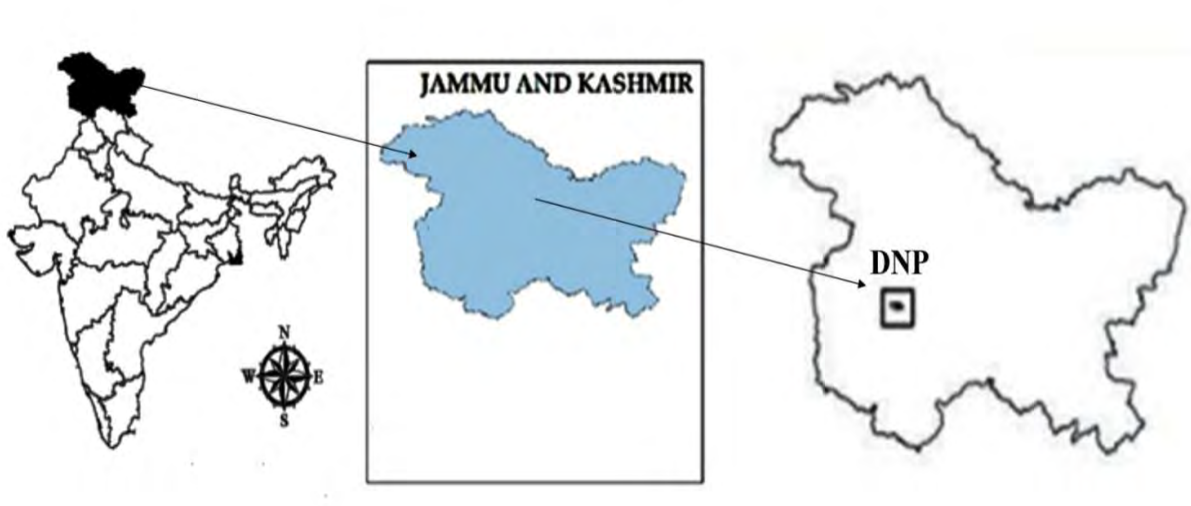

Fig. 1: Magnified Map depicting Dachigam National Park (DNP), India (Ahmad and Farooq, 2018)

Table 1. Distribution, habitat, seasonal availability and putative ECM association of investigated taxa

Name of family, genus and species	Locality (Altitude)	Date, Month & Year of collection	Growing habit	Habitat	Edibility
Family *Physalacriaceae*					
Genus *Flammulina* P. Karst					
Flammulina filiformis	Dachigam National Park (1650 m)	30th December, 2021	Caespitose clusters	Mixed coniferous forest on living stalk of *Populus indica*	Edible

Sampling, Identification and Characterization

Collection of material: Sporocarps were collected from Dachigam National Park in the Zanskar mountain range, during the winter season of 2021. Basidiomes were collected with care using a sharp knife, waste newspapers, hand lens, camera, paper and pen to take field notes regarding locality, GPS position, altitude, date of collection, collection number, habit, habitat, substrate and their association with the surrounding forest vegetation. While collecting the specimens, care was taken to collect the fresh and healthy carpophores and to avoid old decaying fruit bodies or those infected with insects etc. Precautions were taken to avoid mixing of all the collections. For this purpose, each collection was wrapped individually. Every effort was made to collect the sociobiological information by interacting with the local people in the field regarding the particular agarics during collections. The collected specimen was taken to the temporary laboratory set up where they were further analyzed, dried and packed. Sporocarps were air dried at 40–45^0 C in a drier specially designed for drying mushroom specimens (Atri *et al.,* 2005) which were finally packed in a cellophane paper packet for permanent preservation.

Morphological characteristics: The macro-morphological characters were carried out in the field from fresh collection with respect to the shape, colour and size of carpophore, striated or non-striated cap margin, presence or absence of universal veil, volva, annulus, attachment of gills, colour changes of the carpophore parts, etc. as per the standard protocol given by (Atri *et al.,* 2005, 2017) and the colour names and codes were followed as given by Kornerup and Wanscher., 1978.

Spore print: In order to note down the colour of the spore print white coloured reference cards measuring 13.5 × 5.0 cm were used. Fresh and mature fruit bodies were selected to obtain spore prints. A triangular piece of the cap of the carpophore was cut towards the attachment of the stipe and placed with blades facing downwards on a post card sized white paper marked with the appropriate collection number, along with a wet cotton plug in order to provide humidity for the pileus to remain turgid and this arrangement was covered with a petriplate and left undisturbed for one hour. Although sincere efforts were made to get the spore print for all the collections but in order to get a thick spore print the time required for smaller agarics with thin texture is more as compared to the larger fleshy basidiocarps.

Preservation: After recording the morphological characters on the field key (Atri *et al.,* 2005), some pieces of sporocarps from cap and stipe were preserved in liquid preservative (25 ml Rectified alcohol (95%) + 5 ml Formalin (37%) + 70 ml distilled H_2O, Hawksworth *et al.,* 1983) for studying the microscopic characters.

Microscopic observations: The microscopic details were studied by cutting free hand sections of revived part of the dried specimen and staining them in Congo Red and the internal details of the pileus cuticle, stipe cuticle, hymenophore trama and various cystidial elements were recorded for further use in taxonomic categorizaton. The microscopic details were worked out as per standard methodology (Singer., 1986; Atri *et al.,* 2000, 2017).

Molecular characterization

DNA extraction, PCR and sequencing: Total genomic fungal DNA from the fungus was isolated using modified CTAB (Cetyl trimethyl ammonium bromide) extraction method (Murray & Thompson 1980). The fungal fruiting bodies were ground into fine powder by constant crushing using autoclaved and pre-chilled mortar and pestle in liquid nitrogen, to make the fungal fruiting bodies brittle and reduce DNase activity. About 40-50 mg of fine powdered fruiting bodies powder was transferred into 1.5 ml polypropylene centrifuge tube containing about 700 µl pre-heated (65°C) 2X CTAB extraction buffer [100 mM Tris HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% CTAB, 1% PVP and 0.5% -mercaptoethanol (added just before use)]. The powder was suspended in the buffer by inverting and rotating the tubes properly. The tubes were incubated at 65°C for 60 minutes in thermomixer (Eppendorf). After incubation, 700 µl of chloroform isoamyl alcohol (24:1) was added and the tubes were swirled, till it made an emulsion. The tubes were centrifuged at 12,000 rpm for 12 minutes at room temperature. The supernatant was transferred to a clean sterile 1.5 ml polypropylene centrifuge tube using large bore tip and then again 700 µl mixture of chloroform isoamyl alcohol in the same ratio was added and that step was repeated. After transferring the supernatant to a new tube, equal volume of pre-chilled isopropyl alcohol (approximately 600-700 µl) was added and the tubes were inverted gently several times and kept at-20ºC overnight. The microfuge tubes were then centrifuged at 12,000 rpm for 12 minutes at 4ºC and supernatant decanted. The DNA pellet was rinsed twice with 70 per cent ethanol for five minutes to remove any residual salts followed by re-centrifugation. Pellet was collected and leftover ethanol was dried up completely

by turning down microfuge tubes on a blotting paper and allowed to air dry (at room temperature). The pellet was dissolved in 200 µl of 1X TE (Tris EDTA buffer-10mM Tris HCl, 1mM EDTA, pH 8.0), and left for few hours at room temperature to dissolve the DNA. Heat-treated RNase (Fermentas) was then added to a final concentration of 10µg/ml and subsequently incubated at 37ºC for 30 minutes. DNA of the fungal fruiting body was obtained in a similar way and stored at-80ºC for further use.

Assessment of quality and quantity of DNA: The quantity of DNA was assessed by Agarose gel electrophoresis and Nano-drop (Eppendorf). Agarose (0.8 g) was dissolved in 100 ml of 0.5X Tris acetate EDTA (TAE) electrophoresis buffer. The mixture was heated till the agarose was dissolved completely and formed a transparent and clear solution. It was cooled down to 60°C with constant stirring, ethidium bromide added to the final concentration of 0.5 µg/ml of buffer. Then the agarose solution was poured into an already prepared gel mould with combs and was left for 20-30 min for solidification. DNA samples for loading were prepared by adding 2 µl loading dye (6X) (0.25% w/v bromophenol blue, 50% glycerol in sterile water) to 8 µl DNA such that the final concentration of loading dye was 1X. The DNA samples were loaded into wells with the help of micropipette. Along with the DNA samples, marker of known concentration was also loaded. The gel was run for about 1-2 hours and visualized under UV transilluminator using photo gel documentation system (Alfa Imager EC, Protein Simple, USA), and the DNA samples photographed. The intensity of fluorescence of the sample was compared with that of a standard marker and then DNA concentration of the sample ascertained. Similarly, DNA quantification was done on Nano-drop (Eppendorf) by placing 1 µl DNA stock on cuvette and absorbance recorded. The final concentration of DNA was maintained to 20-25 ng/µl. The quality of DNA samples was judged based on whether DNA formed a single high molecular weight band (good quality) or a smear (degraded/poor quality band). The DNA was then diluted to 25 ng/l by adding double distilled sterile water and used for PCR.

Selection of Internal Transcribed Spacer (ITS) primers for PCR amplification: The consensus primers ITS1 and ITS4 were selected for PCR amplification of DNA from representative fungal fruiting body and confirmation of identity of fungal fruiting body (White *et al.,* 1990).

Polymerase Chain Reaction (PCR) amplification: PCR amplification of DNA samples of selected fungal fruiting body was carried out in 0.2 ml PCR tubes using ITS primers (Saiki & al., 1988). PCR reaction was carried out in 0.2 ml PCR tubes with 25 µl reaction volume containing 1X buffer (10 mM Tris-HCl pH 8.0; 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 U of *Taq* DNA polymerase (Fermentas, Thermo Fisher Scientific, Waltham, MA, USA), 40-50 ng of DNA template, 0.4 µM of primer and 15.8 µl of sterilized distilled water. PCR amplifications were performed in thermal cycler programmed for initial denaturation at 94ºC for 5 min followed by 35 cycles at 94ºC for 1 minute, annealing temperature of 55ºC for 1 minute, extension at 72ºC for 2 minutes and a final extension of 10 minutes.

Visualization of PCR products: To 25 µl of the amplified product, 4 µl of 6X loading dye was added to make the final concentration of loading buffer 1X in the reaction samples. PCR products were resolved on 1.2 per cent agarose gel. The gel was prepared in 0.5X TAE buffer. Ethidium bromide was added at concentration of 0.5µg/µl. The gel was run at 5 V/cm, visualized under UV light and photographed using Gel Documentation System (Alfa Imager EC Protein Simple, USA).

DNA Sequencing and phylogenetic analyses: After PCR amplification using ITS primers, 5 µl of PCR product of the selected fungal fruiting body was electrophoresed to ensure successful amplification and remaining 20 µl PCR product was sent for custom sequencing (Biokart Lab., Bangalore India). The retrieved sequences were used for analysis using various software for confirmation of identity of fungal fruiting body at molecular level. The sequences for each forward and reverse primer were retrieved from chromatograms received. The sequence alignment was carried out for both forward and reverse sequences in BioEdit version 7.0 to obtain consensus sequences for each isolate (Hall, 1999). Similarly, the ITS sequence obtained was analyzed through BLASTn programme of NCBI (http://www.ncbi.nlm.nih.gov/BLAST) to compare it with the available database of National Centre for Biotechnological Information (NCBI) (http://www.nlm.nih.gov/ HYPERLINK "http://www.nlm.nih.gov/nuccore"nuccore).The consensus sequence of ITS gene for fungal isolate obtained was compared with the other sequences along with an out group sequence retrieved from NCBI database using molecular evolutionary genetic analysis(MEGA XI) software version-11 for phylogenetic analysis (Tamura *et al.,* 2011). An optimal tree was generated by using Kimura-2-parameter substitution model (Kimura, 1980). The sequence was submitted to GenBank National Centre for Biotechnological Information (NCBI) for allotment of Accession number.

RESULTS AND DISCUSSION

Morphological characterization of *flammulina* species:

Taxonomic observations: *Flammulina filiformis* (Z.W. Ge, X.B. Liu & Zhu L. Yang) P.M. Wang, Y.C. Dai, E. Horak & Zhu L. Yang, *Mycological Progress* 17 (9): 1021 (2018) Fig. 2 (A-H). MycoBank No.: MB 819132

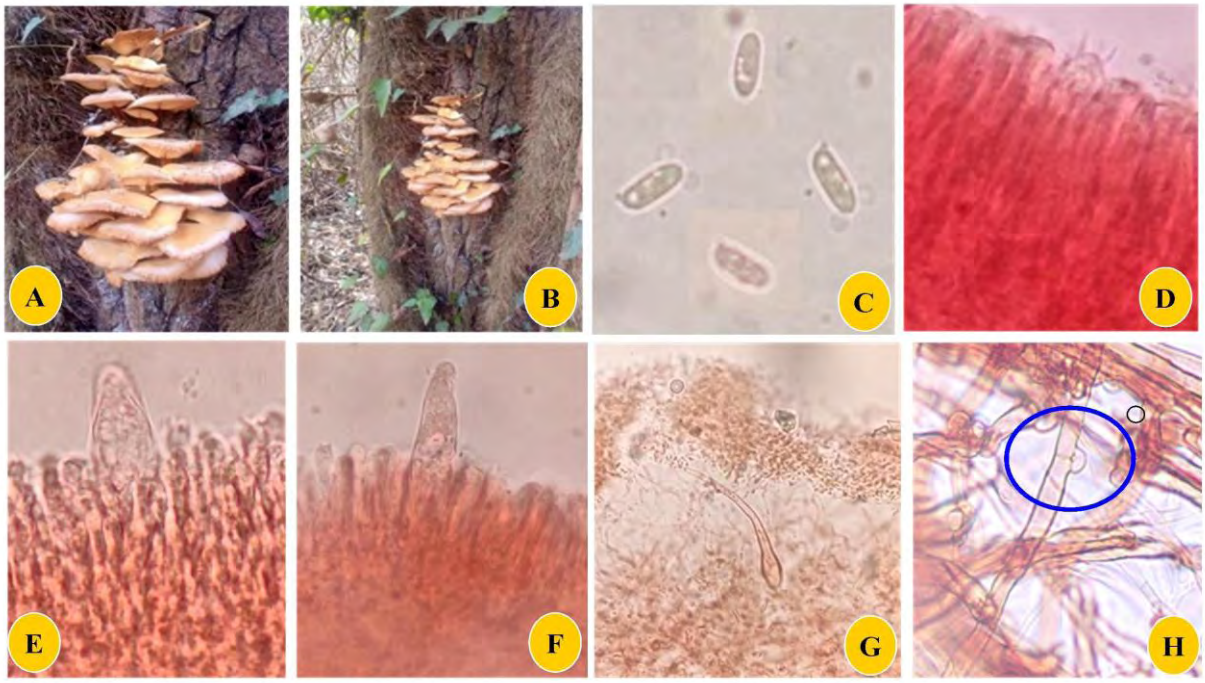

Fig. 2: Morphological and microscopic features of *Flammulina filiformis* (A. Basidiocarp growing in caespitose clusters. B. Basidiocarp growing in their natural habitat on living stalk of *Populus indica*. C. Microphotograph of inamyloid basidiospores. D C.S through hymenophore bearing tetrasterigmate basidia. E. C.S. through lamellae showing Pleurocystidia. F. C.S. through gill edge showing utriform Cheilocystidia. G. C.S. through pileus showing cuticular details and contex with prominent pileocystidia. H. C.S. of Stipe showing clamp connections on stipe cuticle)

Basionym: *Fammulina velutipes* var. *filiformis* Z. W. Ge *et al., Mycosystema* 34: 598 (2015).
Synonym: *Flammulina velutipes* var. *himalayana* Z. W. Ge *et al., Mycosystema* 34: 600 (2015).

Description: - Basidiocarps 5.5–18.5 cm in height. Pileus 2.0–7.5 cm broad, convex to appplanate with uplifted margins at maturity; surface, creamy to nearly white (4A1, 1A1) with a orange tinge in the centre in young basidiocarps, golden yellow (2A6–2A8) or ochraceous yellow (3B8), to ochraceous brown (4B8) in mature basidiocarps; scales absent; margin irregular, splitting at maturity; sub viscid, dry at maturity; cuticle half peeling; flesh up to 0.1-0.4 cm thick, creamy white, unchanging; taste and odour mild. Pileal veil absent. Lamellae up to 0.6 cm broad, adnate, unequal, crowded to moderately broad, creamy white (4A1) to yellowish white (2A2); brownish at maturity, lamellulae present; gill edges smooth. Stipe central to excentric, 6.0–16.0 cm in length, up to 0.8 cm broad near the apex, up to 1.2 cm broad near the base, somewhat subcylindrical; creamy white (4A1) to pale yellow (4A4) in young basidiocarps, yellowish brown (4B6) to light to dark brown (5E8) at maturity; unchanging, surface velvety to pruinose; exannulate.

Basidiospores (4.8) 5.6–8.0 (8.8) × 3.32–4.98 µm (excluding apiculus), Q = 1.4, elongate, sometimes ellipsoid, granular, single walled, thin walled, inamyloid; apiculate, apiculus up to 0.8 µm long. Basidia (18.2) 24.0–33.6 × 4.0–5.6 µm, clavate, narrow, granular, tetrasterigmate; sterigmata 3.2–4.0 µm long. Gill edge sterile. Pleurocystidia 30.4–62.4× 9.6–14.4 µm, utriform with broad to capitate apex, granular in upper half, much protruding from basidial layer, not deeply seated; cheilocystidia 28.8–49.6 × 7.2–14.4 µm, utriform to fusiform, granular. Hymenophoral trama regular. Gill edges heteromorphous. Pileus cuticle an ixotrichoderm with pileocystidia, made up horizontally entangled septate, hyphae, pileocystidia 65.12–207.57 × 8.14–18.3 µm broad, abundant to scarce, narrowly lageniform, to fusiform, thick-walled; context hyphal, made up of 3.2–7.2 µm broad, septate, irregularly placed, hyaline, septate, abundantly clamped hyphae. Stipe cuticle hyphal, made up of 2.4–7.2 µm broad, longitudinally tangled, septate, hyaline to granular hyphae, giving rise to a regular turf

of 4.8–14.4 µm broad, septate, clamped, branched, hyaline to granular, projecting hyphae; context hyphal, made up of 6.4–24.0 µm broad, longitudinally placed, granular, inflated hyphae. Clamp connections present in all parts of basidioma. Based this characterization, and compared with the available literature, the fungus was identified as *Flammulina filiformis.*

Distribution and Ecology: *Flammulina filiformis* was reported to be growing on decaying corticated wood of *Broussonetia papyrifera*, *Salix* spp., *Betula platyphylla*, *Dipentodon sinicus*, *Neolitsea* spp., and other broad-leaved trees from East Asia (Wang *et al.,* 2018). Presently examined collection was found growing in caespitose clusters on living stalk of *Populus indica* in mixed coniferous forest in the month of December at an altitude of 1650 mfrom Dachigam National Park,Srinagar, Jammu and Kashmir, A North Eastern Himalayan region of India.

Collection Examined: Jammu and Kashmir, Srinagar, Dachigam National Park (1650 m) 34.1372° (N– 75.0377° E), growing in caespitose clusters, on living stalk of *Populus indica*, in mixed forest. Collected by Tariq Ahmad Sofi and Naseema Aqbar Wani, 30th, December 2021 (Holotype).

Edibility: *Flammulina filiformis* is placed in the category of edible mushrooms for production and consumption (Yan *et al.* 2019).

Comments: Present collection matches well with the description of *Flammulina filiformis* as given by Wang *et al.,* 2018. For confirmation at molecular level by sequencing the ITS region using PCR based ITS 1 and ITS 4 markers. The sequence results confirmed the identity of this mushroom as *Flammulina filiformis*. This collection was also compared with an allied species, *F. velutipes* but it differs in the size of basidiospores which were slightly larger in *F. velutipes* compared to *Flammulina filiformis* with slightly smaller basidiospores.

Molecular characterization: After the custom sequencing of PCR amplified product, the sequences were retrieved from chromatogram and consensus sequence obtained was blasted against the available database of NCBI, which showed the highest level of identity with the *Flammulina filiformis,* thus confirming the results of morphological characterization and identity of the fungus. The sequence was submitted to GenBank (NCBI) under accession number ON844192. For phylogenetic analysis, three sequences each of *Flammulina velutipes* and *F. filiformis* and an out-group sequence of *Morchella esculenta* retrieved from NCBI, grouped the present sequence with the *Flammulina filiformis*, again confirming its identity and phylogenetic relationship of this fungus (Fig. 3).

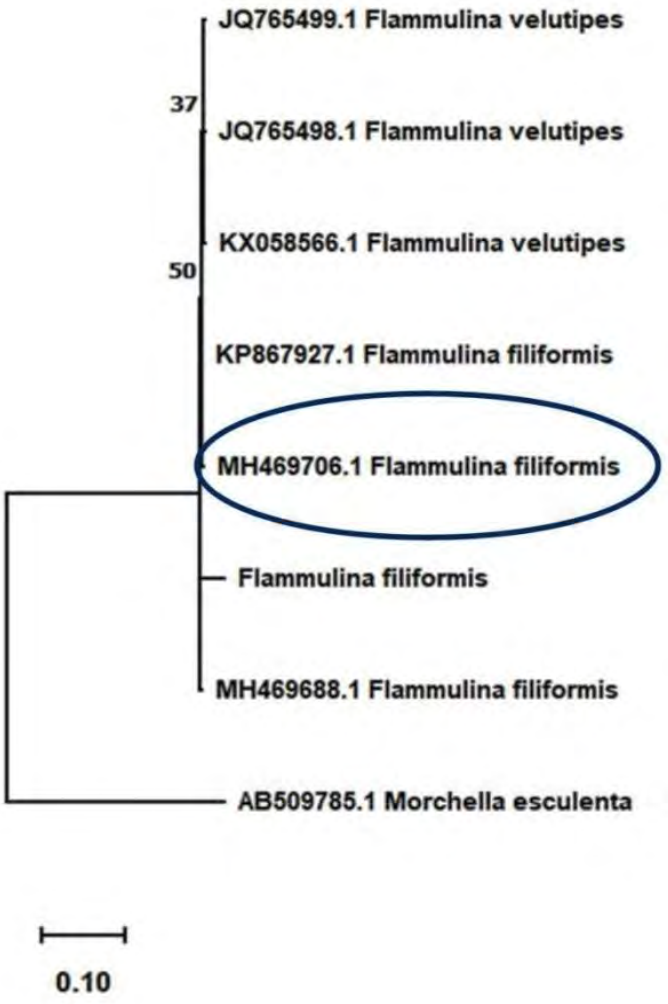

Fig. 3: Phylogenetic relationships among *Flammulina filiformis* based on sequences of ITS region (sequences of present study and those obtained from NCBI)

The morphological as well as sequence (molecular) results confirmed the identity of this edible mushroom naturally growing in DNP forests as *Flammulina filiformis* and as per the available literature, it forms the first report from India.

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