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Original Article

Addition of new flammulina species via DNA, molecular characterization and phylogenetic investigation

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Abstract

Flammulina was found frequently distributed in the District Mansehra during the present research work. The genus was ignored and not studied for prevalence previously, as F. velutipes was the exclusively reported species from the research vicinity. During the present research work, three new species were explored i-e F. hazariansis (N. J201177, N. J201178), F. solatium (N. J201188, N. J201167) and F. dwarftype (N. J201187, NJ201139) were amassed from the surrounding areas of Hazara University Mansehra. Our findings revealed that the reported species are novel, the molecular, morphological, and phylogenetic characterizations proved it. The specimens were collected from various habitats, vegetation, damp places, and forest areas with rich organic soil favor the mushroom's growth from May to November. The species were studied for morphological characteristics like size, shape and color of the pileus, stripe, and spore size were also recorded. The species were preserved by sun and oven drying strategies. The Kit methods for molecular characterizations were used for the extraction of DNA, and for PCR, ITS4 primer was designed from conserved regions described in previous studies. The amplified PCR products were sequenced from Microgen Korea. Phylogenetic analysis of the obtained sequences was done based on the maximum likelihood method using Mega version 6.0. Our findings based on morphological and phylogenetic analysis confirmed the existence of three new species in the already described genera from the region. The area of District Mansehra is enriched with natural vegetation and can be explored for brand-spanking new species.

Keywords: Agaricomycetes, Flammulina, Morphology, Molecular characterization, Phylogenetic analysis, New records

1. Introduction

The genus *Flammulina*, encompassing a diverse group of fungi for fascinating functions and ecological momentous, has been a matter of top-notch concern in mycological studies and researches. *Flammulina* is assessed in the family Physalacriaceae and inside the order of gilled mushrooms, *Agaricales* [1] The species inside the genus are extensively allotted on morphological diversification within the international, main to the taxonomic studies to delineate relationships [2]. *Flammulina sp*ecies usually characteristic convex to flat caps with flat clean surfaces, usually mellowness in yellowish brown colorations, to the stem gills are attached [3,4].

Gilled mushrooms with distinguished pleurocystidia, and definite sticky caps growing on wood, are common in wintry weather. The main producer of iciness mushrooms is Japan [5]. *Enokitake* (*Flammulina sp*ecies) has nutritious and medicinal residences and considered as fourth biggest fit to be eaten mushroom in China [6]. *F. velutipes* carries extraordinary vitamins, like carbohydrates, proteins, mineral elements, vitamins, and crude fibers, commonly known as the enoki mushroom, is one of the exclusive species of the genus and is prized for its culinary uses, cultivated commercially, and consumed in numerous traditions all over the international [7, 8, 9]. A few dominant micro-organisms that cause infection in *F. velutipes* fruiting bodies, for instance, *Lactococcus lactis* were remoted and identified [10]. Presently, the genus consists of 15 species, added by mycologists. *Flammulina* is worldwide in distribution inclusive of subtropical and temperate regions across North America, Europe, Asia, and different continents [11]. More than ten species were reported from Europe and North the USA [12]. The maximum common species of *Flammulina* in China are *F. Velutipes*, *F. Rossica*, *F. Mexicana*, *F. Finlandia*, and *F. Stratosa* are constrained to unique geographical regions within the globe [13,

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14, 15, and 16]. A few of the species are confined to the host, for instance, *F. Ononidis* and *F. Populicola* [17, 18].

Most of the species are determined clearly on diverse soils and trees [11]. *Flammulina* is a famous gilled mushroom and may be discovered in Australia and South the USA [12]. *Flammulina sp*ecies confer distinguished role to surrounding procedures by breaking down woody substrates into organic natural matter and taking part in complex ecological interactions [18]. The molecular characterization of fungal species is a vital factor for identity because the morphological aspects of the examination are insufficient to explain in the right way. In the present research work the location of Mansehra was explored for the myco-flora diversity and stated 3 new species within the genus *Flammulina.*

2. Materials and Methods

The research work on collection, accumulation and identification of macro-fungi species was conducted during 2017-2020. Approximately a hundred and eighty specimens were collected from the surrounding areas of district Mansehra and among those 06 specimens were identified from the genus *Flammulina* and 3 novel species were remoted.

2.1. Morphological observations

Species were collected from the grassy ground of Hazara University and its surrounding areas and studied for their macro and micro-morphological characteristics; ecological facts were recorded at the time of collection. The species was photographed by Canon Power Shot A 460 5.0 MP Digital Camera (Digital Japan Camera) at the substrate and determined for morphological records like size, shape, and color of the sporocarp as well as the stem. Altitude and Latitude were taken with the aid of Garmin e trex 10 – GPS (China). Samples have been gathered in a paper bag, added to the agriculture lab, and washed with distilled water to put off the soil particles. Sun and oven drying strategies have been used for preservation, maintaining the species for 5-6 hours in the solar for two days. Microscopy was performed to observe the spores of the fungus under the binocular microscope using a lens scale. The Protocol followed from "collecting and describing macro-fungi" [19,20] and from mushroomexpert.com

2.2. DNA extraction protocol

All the samples were solar-dried and crushed the sporocarp and stripe to powder form, EZ- 10Spin column Genomic DNA Mini-preps kit, turned into used for fungi DNA extraction (according to the manufacturer's protocol).

a. PCL (Plant C ll Lysis) solution was utilized by adding RNase A (100μg/ ml) helpful for analyzing DNA contents of tissue or callus chemically combining with vortex (mechanical) chopping [21]. The entire content of RNase A was added into the PCL solution and stored at 4ºC. The precipitates were formed when the PCL solution was stored.

The precipitate was dissolved by warming up to room temperature 2mg, and 10mg of proteinase lactase K, 6678 respectively. For ones' samples, a long duration of garage time was required, and the proteinase K solution was kept at-20ºC.

b. The solution was prepared and added 48ml of 100% ethanol 212 ml wash solution and 120 ml of 100% ethanol 230 ml wash solution (volume of added ethanol: volume of wash solution = 4:1).

c. Elution buffer is 2.0mm tris-HCl pH 8.0~8.5 (TE buffer pH 8.0).

2.3 Procedures for isolation of genomic DNA and sample preparation

The dried sample was grounded under liquid nitrogen to a fine powder using a mortar and pestle. After the evaporation of liquid nitrogen, the powder was transferred immediately to a 1.5 ml Eppendorf tube. Without wasting time, step 2 was followed. Due to the processing of the samples and time-taking procedures, other prepared samples were kept with frozen nitrogen at 20 ºC. However, the concentration gradients of tissue samples may vary depending on researcher own needs and choice [22].

Approximately 60-80 mg of grounded material was used and added to an equal volume of approximately 150 µl) of PCL solution (mushroom cell lyses solution). Vortexes and shake the tube several times. Incubated at 65 ºC for 20 minutes, vortex or pipette up and down to further remove any clumps. For better results, the clumped tissues were removed. At 25µl PP solution, mix well and keep the solution on ice for 15 minutes. Centrifuged at 4ºC, 8,000 multiplied by g (10,000 rpm) for 5 minutes. Applied the clear lysate to an EZ-10 Spin Column. Added 300 µl PB buffer to the EZ-10 Spin Column. Mixed gently by inverting the tube. Incubated the mixture for 3 minutes at room temperature. During incubation, mix occasionally by inverting the tube. Centrifuged at 4ºC, 8,000 x g (10,000 rmp) for 2 minutes.

For amplification the rDNA ITS region (Ribosomal Internal Transcribed Spacer region of the DNA), fungal specific forward primer, ITS1F CTTGGTCATTTAGAAGTAA 5′to3′ in combination with the reverse primer, ITS4 TCCTCCGCTTATTGATATGC were used [23]. Both ITS1F and ITS4 primers are highly specified for fungi, especially for basidiomycetes [24]. PCR was performed in 20µl PCR tubes containing 10µl 2X Ready mix (Sigma-Aldrich), 8.3µl water, and 0.1µl of each primer with 1.5µl of DNA extract. PCR reactions were performed with 3.00 minutes of initial denaturation and 30s of final denaturation at 95ºC followed by 35 cycles at 53ºC for 35s, initial extension for 1.35+5s at 72°C and final extension for 2 minutes at 72°C [23].

2.4. Phylogenetic analysis

The PCR products were sequenced from Microgen, Korea. The DNA sequences have been edited using Sequence Scanner (v1.0) and Bio-edit (v 7.0) and BLAST-searched for sequence comparison and identification by submitting the query in the Gen-Bank database.

Sequences have been aligned using Mega 6.0 [25]. The divergence in rDNA-ITS was measured by comparing sequence pairs reconstructed by using Meg Align (DNA STAR). For Phylogenetic analysis, MEGA 6.0 was used. Employing the Tamura-Nai model, Maximum Likelihood (ML) trees were constructed, and bootstrap consensus trees were generated. The bootstrap consensus trees were inferred from 1000 replicates and corresponding bootstrap values were cited in the tree.

Accession No: The sequences of all the species were submitted to the Gene Bank and NCBI and were given Accession No. as shown in Table 1.

Table 1. Species accession No. deposited in Gene Bank and NCBI.

S.No	Novel spp	NCBI Accession No.	Gene Bank Accession No.
1.	*F. hazariansis N. Jabeen*201177	MZ044825	MK079641
2.	*F. hazariansis N. Jabeen*201178	MZ044826	----------
3.	*F. solatium N. Jabeen201*188	MZ044828	MK079647
4.	*F. solatium N. Jabeen*201167	MZ044824	MK079635
5.	*F. dwarftype N. Jabeen201*187	MZ044827	MK079646
6.	*F. dwarftype N. Jabeen*201139	MZ044823	----------

3. Results

3.1 *Flammulina hazariansis* (N. Jabeen201177& N. Jabeen201178) spp. nov.

a. Synonymy: *F. velutipes* (Curtis) [33], Lilloa 22: 307 (1947)

F. velutipes F. longispora, F. vinaceoroseolus, F. velutipes var. campolameirana, F. velutipes var. cytiseicola, F. velutipes var. cytisicola, F. velutipes var. filiformis, F. velutipes var. himalayana, F. velutipes var. lactea, F. velutipes var. lupinicola, F. velutipes var. pratensis, F. velutipes var. radicans, F. velutipes var. velutipes

b. Holotype: Pakistan Khyber Pukhtun Khawah, (KPK) Mansehra, Chakia, and Baffa (N. Jabeen201177& N. Jabeen201178) (November 2018).

c. Etymology: The species name is given from the origin of the collection, the Hazara region, village Chakia, which is surrounded through stunning woodland mountainous, vicinity, and luxurious green grassland. The species was collected from logs and buried logs as shown in Fig.1.

d. The description on the morphological basis: Pileus was observed rounded yellow to brown, gilled, convex, moist, and sticky, grown in clusters, and measured 5.4cm (average). The stripe was yellowish brown, long, 5-5.5cm x 3.5mm in thickness (average). The spore was smooth, elliptical, hyaline, in-amyloid, pleurocystidia not clearly visible cheilocystidia present in scattered form; the basidiospore print was white and measured as 7.6 x 3.6 µm. Basidia. Basdidiocarp and sketches can be seen in Figures 1 and 2. The hyphae were branched, cylindrical, and clamped. The comparative study reveals that the species *F. hazariansis N. Jabeen* shows similarities and differences in morphological characteristics with *F. himalyana* in certain aspects has showed in Table 2 and is also found close in the Phylogenic tree constructed.

e. Habitat: Deciduous trees and logs. (Saprophyte)

f. Specimen Region: The most suitable time for hunting is winter, found abundantly in the forest [26]. The species was found in the forest area of villages Chakia and Baffa 8km and 16km respectively from Mansehra, collected during November.

g. Location Coordinates: N= 34º 20’0” E=73 º 12’ 0” Alt= 1063m (Chakia)
N= 34º 26’0” E=73 º 13’0” Alt= 922m (Baffa)

F. Scale bars: 10µm.

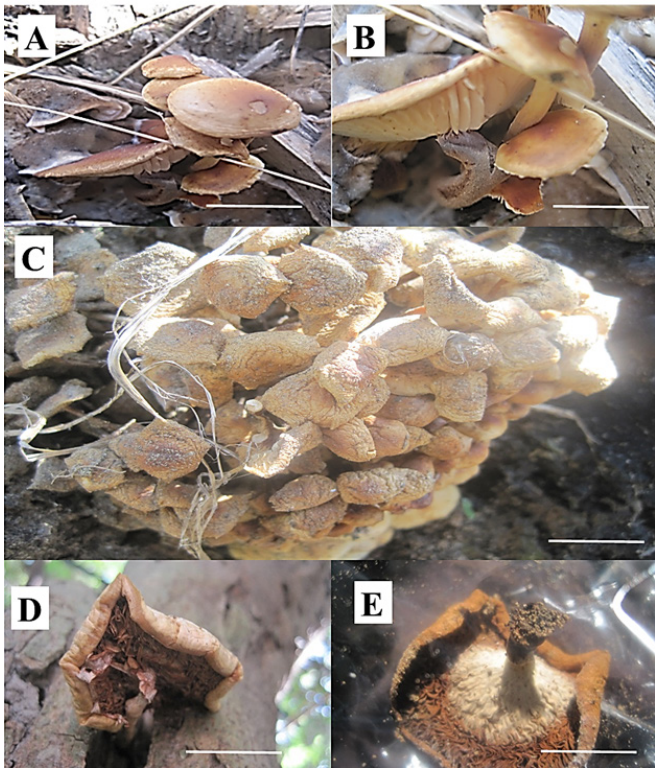

Fig. 1. ***F. hazariansis*** **(N. J201177, N. J201178)**, A: Basidiomata of holo type (F. hazariansis N. J) grown on substrate, B, D&E: Gills, C. Aggregation of Basidiocarps, D: Mature gills with basidiospores. Scale bars: A -E 3-5.5 cm.

3.2 *F. solatium N. Jabeen201*88, and *N. Jabeen201167* sp.nov.

a. Holotype: Pakistan Khyber Pakhtunkhwa, (KPK) Mansehra, from Jaba (N. J201188) and from Anayatabad (N. Jabeen201167) (Nov 2018)

b. Etymology: The same species N. Jabeen201188 *and* N. Jabeen201167 were collected from different regions of Hazara Valley and grown solitary on the ground, so the name of the species is strongly referred to as *F. solatium.* As we observe in Fig. 3 the species was grown single in

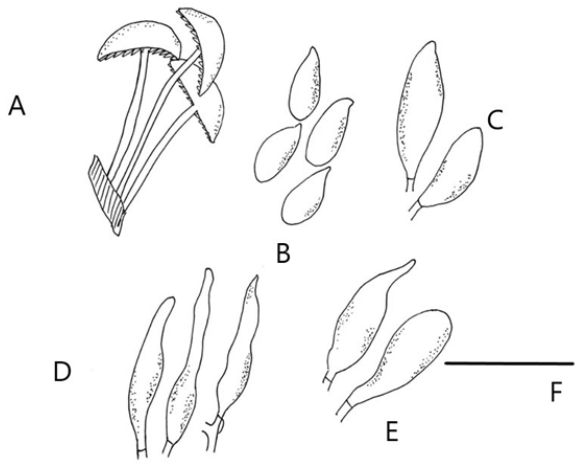

Fig. 2. Sketches of Microscopy A. Basidiomata; B. Basidiospores; C.D.E. Basidium. F. Scale bars: 10µm.

Table 2. Morphological comparison of *F. hazariansis* and *F. himalyana.*

Character	*F. hazariansis N. Jabeen*	*F. himalyana*
Habitat/ Substrate	Deciduous trees and logs. (Saprophyte)	Clustered on the stump of Betulaceae in a mixed forest dominated by Picea and Abies.
Stripe	The stripe was yellowish brown, long, 5-5.5cm x 3.5mm in thickness (average).	Cylindrical, brown, short, size 0.5-1mm in length
Basidia	The hyphae branched, cylindrical, and clamped.	Powdery-cream texture, gilled, dark on top surface
Basidiospore	The spore was smooth, elliptical, hyaline, inamyloid, pleurocystidia not clearly visible cheilocystidia present in scattered form.	Elongate ellipsoid, smooth, hyaline, thin-walled, non-amyloid.
Spore print	The basidiospore print was white and measured as 7.6 x 3.6 µm.	Basidiospores print was hyaline and 6–7 × 3–4µm.
Pileus	Pileus was observed rounded yellow to brown, gilled, convex, moist and sticky, grown in clusters, and measured 5.4cm (average).	Pileus 2–4cm in diam., Plano-convex; surface ochraceous, paler toward the margin, viscid when wet; context white.
Species characterized and reported Nomenclature	*F. hazariansis N. Jabeen* *N. Jabeen et al.* (2021)	*F. himalyana* [35]

the forest area.

c. The description on the morphological basis: Pileus was observed thickened, convex, smooth, grown solitary on logs, light brown, gilled (attached), grown, with no discoloration towards the center, and cap size ranges from 2.5-6.5cm on average. The stipe was found light brown to yellowish brown, cylindrical, fusiform, hollow, and measured as 2-7.6cm long (average). The basidiospores were broadly ellipsoid smooth hyaline and the average measurement was 9.2 x 3.7 µm. The basidium 4 spore, clavate, and 25-35µm (av). Pleurocystidia were thick-walled, clavate, ellipsoid-pedunculate, and 30-55.5 x 10-20 µm in average measurement. Cheilocystidia are present abundantly. The spores and basidia can be observed in Fig. 4. The species was observed close to *F. filiformis* in the Phylogenetic tree and showed similarities but differences in a few morphological characteristics given in Table.3. The species was found to be solitary growing on the ground whereas F. filiformis was observed in clusters [27].

d. Habitat: The species was observed in the dense forest area.

e. Specimen Region: The species N. Jabeen201167 was collected from Anayatabad 7km and N. Jabeen201188 from Jaba situated about 20.6km from Mansehra during November.

f. Location Coordinates: N= 34º 30’21” E=73º 16’14” Alt= 1070m (Anayatabad)
N= 34º 26’20” E=73 º 15’30” Alt= 3221m (Jaba)

3.3. *F. dwarftype N. Jabeen201187* and *N. Jabeen201139* sp.nov.

a. Holotype: Pakistan Khyber Pukhtun Khawa, (KPK) Mansehra, from Hazara University Pheasant area (N. J201187) and from Attar Sheesha (N. J201139) (Nov 2018). Fig. 5 shows the species growing on rich organic matter soil and among deciduous trees.

b. Etymology: The species *F. dwarftype N. Jabeen201187* and *F. dwarftype* N. Jabeen201139 *were* the same, collected from different regions of Hazara Valley and grown on the ground as well as buried logs, smaller in stripe size as compared to our new specie *F. solatium* and *F. velutypes* so the name of the specie strongly referred to as *F. dwarftype N. Jabeen.*

c. The description on the morphological basis: Pileus

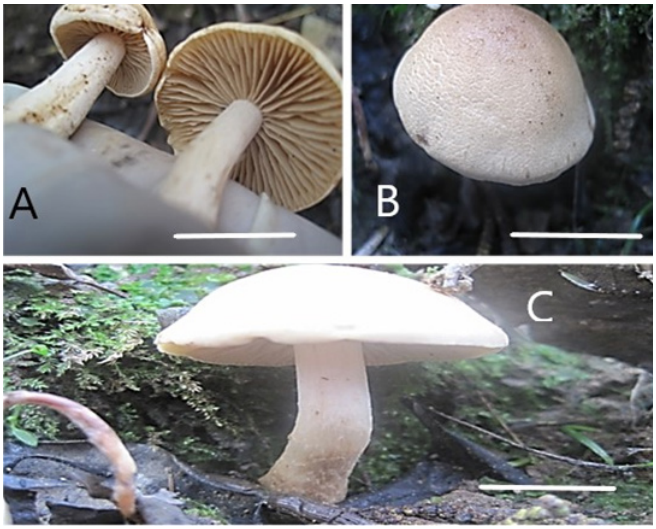

Fig. 3. ***F. solatium N. Jabeen*201188 and *N. Jabeen*201167spp.nov** A: Morphological features of mushroom (Hymenium) B: Basidiocarp C: Habitat and Substrate of specie D: Scale bars: A -C 3-5.5 cm.

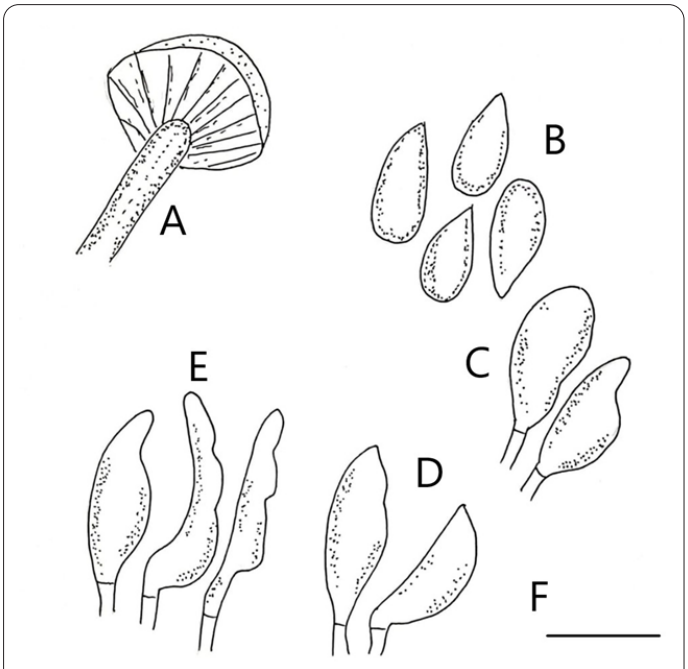

Fig. 4. Sketches of Microscopy A: Basidiomata B: Spores C: Basidium D: Pleurocystidia; E Cheilocystidia F; Scale bar=10µm.

Table 3. Morphological comparison of *F. solatium N. Jabeen* and *F. filiformis.*

Character	*F. solatium N. Jabeen*	*F. filiformis*
Habitat/ Substrate	Observed in dense forest area	Found in clusters on substrate i-e dead logs
Stipe	Stipe was found light brown to yellowish brown, cylindrical, fusiform, hollow, and measured as 2-7.6cm long (average)	Central, sub cylindrical, densely covered with hairs (brown velvety) and measured as 1.5-8x 0.2-0.6cm (average)
Basidia	The basidium 4 spore, clavate, and 25-35µm (av).	Narrowly clavate, 22-27 x 4-5.5 µm Hyaline and thin-walled.
Basidiospore	The basidiospores were broadly ellipsoid smooth.	Ellipsoid to oblong ellipsoid, smooth hyaline.
Spore print	hyaline and the average measurement was 9.2 x 3.7 µm.	Hyaline and measured as5.5-7.5(8) x 2.5-3.5 µm
Pileus	Pileus was observed thickened, convex, smooth, grown solitary on logs, light brown, gilled (attached), grown, with no discoloration towards the center, and cap size ranges from 2.5-6.5cm on average	Found in clusters, convex to applanate, surface observed as smooth, shiny, greasy, center brown to yellowish brown
Species characterized and reported Nomenclature	*F. solatium N. Jabeen* N. Jabeen *et al*. (2021)	***F.* velutipes *var. filiformis*** *Z.W.* Ge, X.B. Liu (2015). http://journals-myco.im.ac.cn & Zhu L. Yang, var. nov.

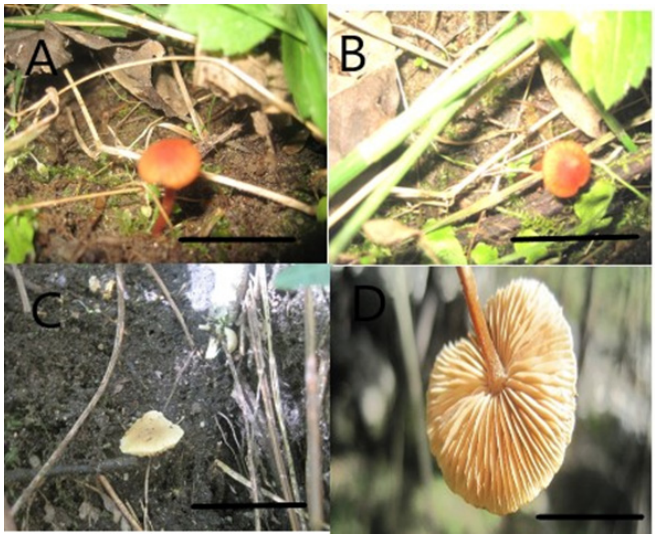

Fig. 5. ***F. dwarftype*** **N. Jabeen sp.nov** A&C: Basidiocarp B: Growing with substrate D: Morphological features of mushroom (Hymenium) Scale bars: A -D 2.5-5.4cm.

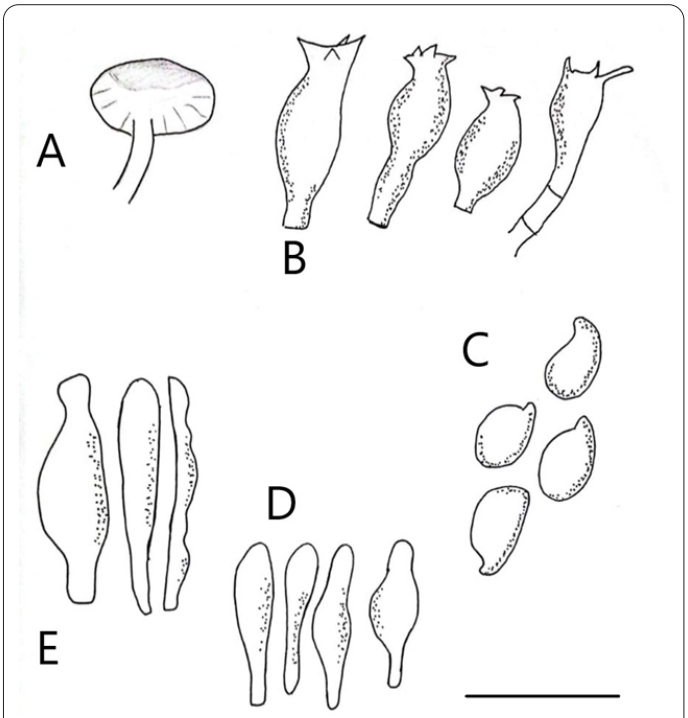

Fig. 6. Sketches of Microscopy. A: Basidiomata, B: Pro basidium, C: Spores, D: Pleurocystidia, E: Cheilocystidia, F: Scale bar. Scale Bar= 10µm.

was smooth, semi-convex, and rounded, observed thickened, grown on soil and trees, gilled (attached), light brown with dark discoloration towards the center and cap size ranges from 2.5-5.8cm in average. The stipe was found brown to reddish brown, cylindrical, fusiform hollow, and measured as 2-5.4cm long (average). The basidiospores were ellipsoid smooth hyaline; the average measurement was 7.5-8.9 x 3-6.7 µm. The basidium 4 spore, clavate, and 25-35µm (av). The sketches can be observed in Fig. 6. Pleurocystidia were clavated, ellipsoid-pedunculate, and 30-55.5 x 10-20µm in average measurement. Cheilocystidia present scattered. Few similitaries and differences of species *F. dwarftype N. Jabeen* and *F.velutype* were observed and shown in Table.4.

d. Habitat: Deciduous trees and rich organic soil.

e. Specimen Region: The species *F. dwarftype* N. J201187 has collected from the pheasant area near Hazara University 14km and *F. dwarftype* NJ201139 from Attar Sheesha, situated about 14.6km from Mansehra, in November.

f. Location Coordinates: N= 34º 26'0" E=73º 15'0" Alt= 977m (Hazara University)

N= 34º 24'0" E=73º 19'0" Alt= 1103m (Attar Sheesha)

3.4. Evolutionary analysis by Maximum Likelihood method

Evolutionary history was inferred by using the Maximum Likelihood method and the Hasegawa-Kishino-Yano model [34, 27]. The tree with the highest log likelihood (-2624.64) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio N. Jabeen algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood

Table 4. Morphological comparison of *F. dwarf type N. Jabeen* and *F. velutypes.*

Character	*F. dwarftype N. Jabeen*	*F. velutype*
Habitat/ Substrate	Collected from the forest area as well as from logs in the Pheasant area of Hazara University.	Found in clusters on tree trunks and buried woods.
Stipe	Stipe was brown to reddish brown, cylindrical, fusiform, hollow, and measured as 2-5.4cm long (average).	Equal or larger near the base, hard and tough, yellowish-brown or orange-brown in the young stage with a coating of dark rusty brown to blackish velvety covering towards maturity, measured 2-11cm long.
Basidia	The basidium 4 spore, clavate, and 25-35µm (av).	Sub clavate to clavate, cylindrical, thin-walled; 40-65 µm.
Basidiospore	The basidiospores were ellipsoid smooth, and hyaline; the average measurement was 7.5-8.9x3-6.7 µm	Smooth, more or less elliptical; inamyloid and measured as 6-9.5x3- µm.
Spore print	hyaline and the average measurement was 2.5-4.5cm.	Hyaline and measured as5.5-7.5(8) x 2.5-3.5 µm
Pileus	Pileus was smooth, semi-convex, and rounded, observed thickened, grown on soil and trees, gilled (attached), light brown with dark discoloration towards the center, and cap size ranges from 2.5-5.8cm in average.	Found in clusters, convex to applanate, surface observed as smooth, shiny, greasy, center brown to yellowish brown
Species characterized and reported Nomenclature	***F. dwarftype N. Jabeen*** N. Jabeen *et al.* (2021)	***F.* velutipes** Kuo. M, 2013 *F. populicola.* Retrieved from the *MushroomExpert. Com* Website: http://www.mushroomexpert.com/flammulina_populicola.html

(MCL) approach and selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1592)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 30 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. There was a total of 819 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [28, 32].

3.5. Phylogenetic analysis and molecular characterization of *Flammulina* species:

Three sequences amplified using ITS1F and ITS4 primers pair were identified on the basis of BLAST analysis to find out the closest match to the sequences found in Gen-Bank. BLAST result showed the maximum identity of amplified products to *Flammulina species*. A total of 30 sequences were aligned in Clusta-W. The phylogenetic tree was constructed with these sequences the results showed that all the collected species are in the second clade. Two sequences NJabeen201167and NJabeen201188 have maximum similarities and are close to *F. filiformis*, blast results indicated that the species NJabeen201167 and NJabeen201188 are the same, showing 99.17% and 99.20% similarities with *F. filiformis* matches with 93% identity and 100% query value. (Table.5) shows the similarities and dissimilarities in distances of all the species and their comparison. Besides three amplified and sequenced rDNA-ITS sequences, twenty-two similar sequences from NCBI Gen-Bank data were used to construct a phylogenetic tree with maximum likelihood as an optimality criterion. After sequence alignment using Mega 6.0, unaligned regions were trimmed from both the 5′ and 3′ ends of the alignment data sheet prior to analysis. *F. stratosa* (MH8627351, AF0478721) was used as an outgroup.

Phylogenetically, the six *Flammulina spp* isolates from the Mansehra region clustered in a separate clade, and the reported species showed the results that occupying new positions on the tree with three novel species, *F. hazarian-sis (*NJ201177, NJ201178), *F. solatium* NJ*201*188, and NJ201167 and *F. dwarf type* NJ*201*187, and NJ201139 as in Fig.8. All the species were showing the 100% bootstrap value.

4. Discussion

Flammulina was found diversely distributed in the Mansehra region of KPK Pakistan during our research work. The Fig. 7 (map of Mansehra) shows the diversity of *Flammulina* collected from the surroundings of Mansehra. Almost all the locations are covered with dense vegetation. Found and described three novel species of the genus on morphological and molecular basis and phylogenic

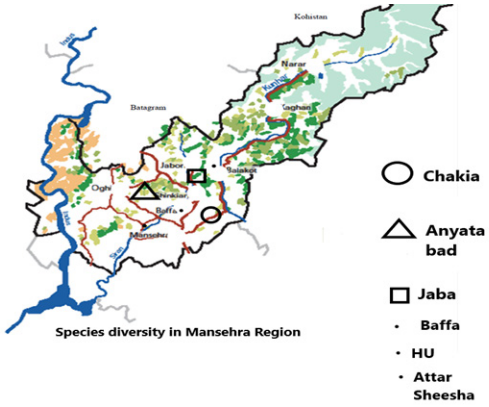

Fig. 7. ***F. dwarftype*** **N. Jabeen sp.nov** A&C: Basidiocarp B: Growing with substrate D: Morphological features of mushroom (Hymenium) Scale bars: A -D 2.5-5.4cm.

Table 5. Comparison of species with others for similarity and dissimilarities.

S. No	Species	Species	Dist	Similarities	Dissimilarities
1	*F. hazarianis* N. J201177	*F. velutipes var himalayana* KP8679211 HKAS80829	0.0191122598	98.09	1.91
	F. hazarianis N. J201177	*F.stratosa MH8627351_{outgroup}*	0.1227333460	87.73	12.27
2	*F. hazarianis* N. J2011778	*F. yunnanensis* KP8679231	0.0638657596	93.61	6.39
3	*F. hazarianis* N. J2011778	*F. velutipes var filiformis* MH4697071	0.0258630802	97.41	2.59
	F. hazarianis N. J2011778	*F. velutipes var himalayana* NR 1539981 HKAS 80829	0.0258038953	97.42	2.58
	Flammulina sp N. J201188	*F. velutipes var filiformis* KP8679241 voucher HKAS83890	0.0174903288	1.75	1.75
4	*Flammulina sp* N. J201188	*F. velutipes var filiformis* MH4697071	0.0093819296	99.06	0.94
5	*Flammulina sp* N. J201167	*Flammulina sp* N. J201188	0.0000000000	100.00	0.00
6	*Flammulina sp* N. J201167	*F. velutipes var filiformis* MH4697071	0.0096937577	99.03	0.97
	Flammulina sp N. J201167	*F. velutipes var filiformis* KP8679241 voucher HKAS83890	0.0180739538	98.19	1.81
7	*Flammulina sp* N. J201187	*F. velutipes var filiformis* MH4697071	0.0240414448	97.60	2.40
	Flammulina sp N. J201187	*F. populicola* KY2002191	0.0500917169	94.99	5.01
	Flammulina sp N. J201187	*F. velutipes var himalayana* NR 1539981 HKAS 80829	0.0258038953	97.42	2.58
8	*Flammulina sp* N. J201139	*F. velutipes var filiformis* MH4697071	0.0216972761	97.83	2.17
9	*Flammulina sp* N. J201139	*Flammulina sp* N. J201187	0.0000000000	100.00	0.00
10	*Flammulina sp* N. J201139	*F. velutipes var filiformis* KP8679241 voucher HKAS83890	0.0272285736	97.28	2.72

analysis Table 1 shows the comparison of all the collected species with their similar and non-similar characteristics.

Our species *F.hazariansis N. Jabeen* is newly added to the genus *Flammulina* as a few characteristics regarding morphology and its position in the phylogenetic tree are different, constructed by the likelihood method. *F.hazariansisN. Jabeen* is near *F himalayan* in phylogenetic (tree) analysis. The comparative studies reveal that the Pileus 2-4cm, paler towards the margins, and the stripe 3-4 x 0.5-0.7cm, was densely covered by hairs in *F. himalyana. So F.hazariansisN. Jabeen* is bigger comparatively and with no hairs on the stripe. The collection sites were enriched with plantations and high humidity as well. Pileus 2–4cm in diam., Plano-convex; surface ochraceous, paler toward the margin, viscid when wet; context white. Whereas Pileus was observed rounded yellow to brown, gilled, convex, moist and sticky, grown in clusters, and measured 5.4cm (average) in *F. hazariansis N. Jabeen* [27].

The ecology and habitat reveal that *F. solatium N. Jabeen* was grown solitary on the ground enriched with organic matter as compared to the *F. filiformis; grow in clusters and aggregations as* observed close to *F. filiformis* in the Phylogenetic tree (Fig.8) and showed similarities but differences in a few morphological characteristics [28].

F. velutipes, with few differences in morphological and molecular, exist all around the world and are treated as a separate species, namely *F. filiformis*. *F. dwarftype N. Jabeen* is phylogenetically near to *F. velutipes* as shown in Fig.8. but different in a few morphological characteristics as Pileus was smooth, semi-convex, and rounded, observed thickened, grown on soil and trees, gilled (attached), light brown with dark discoloration towards the center and cap size ranges from 2.5-5.8cm in average and *F. velutypes* was convex, becoming broadly convex to flat; moist and sticky when fresh; bald; color fairly variable—

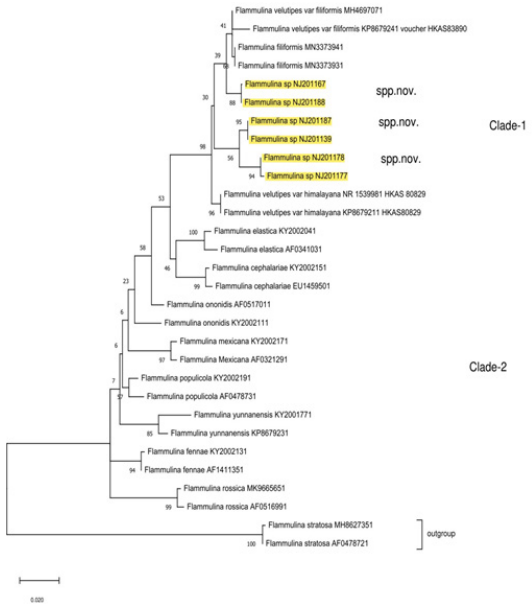

Fig. 8. Phylogenetic tree of *Flammulina spp*. Generated with 100 bootstrap values based on sequences of different species and *F. stratosa* as an outgroup.

dark orange-brown to yellowish brown, often fading with maturity measured as1-7 cm. Our reported species was dwarf, size of spore was different as well as color and texture also varied comparatively. Two species with few differences can be explained by their macro- and micro-morphological characteristics but the findings were strongly supported by molecular studies i-e Phylogenetic studies [16]. Our knowledge of fungal evolution has been revolutionized by molecular techniques in recent years [36]. Previous studies revealed that *F. mexicana* belonged to the same species complex as *F. populicola* [29, 16, 28, 27]. In

our phylogenetic tree, the three species *F. hazariansis N. Jabeen* Fig. 8. (shown in clade-1) were closely related to *F. himalyana. F. solatium* N. Jabeen was found near *F. filiformis* Fig. 8. (shown in clade 3), *F. dwarftype N. Jabeen* was near and *Flammulina velutypes* Fig. 8 (shown in clade -3) strongly supported as novel according to phylogenetic analysis. [29, 39, 31, 28, 27]. The two different species can be explained by their macroscopic and microscopic morphological characteristics, but these findings are strongly supported by molecular studies (i.e. phylogenetic studies) [36].

The northern areas of Pakistan and especially the surrounding areas of Mansehra were found enriched with macro-fungi diversity. Specially encountered for the *Flammulina* biodiversity center for *Flammulina.* In addition to *F. hazariansis N. Jabeen, F. solatium N. Jabeen*, and *F. dwarftype N. Jabeen* the region can be explored for valuable edible species of this genus.

5. Conclusions

In the present research work, different areas including Chakia, Baffa, Hazara University, Attar Sheesha, and Jaba were explored for mushroom collection and many areas enriched with mushroom diversity. The collected species were identified on a morphological and molecular basis. In this way, we identify the mushroom up to the species level. The Phylogenetic tree constructed based on similarities shows three new explorations in the genus *Flammulina*. Much research can be conducted further to observe nature and fungal ecology.

1. List of abbreviations (used in this paper)

1. **DNA** Deoxyribose nucleic acid
2. **PCR** Polymerase chain reaction
3. **ITS** Internal Transcribed Spacer
4. **NCBI** National Center for Biotechnology Information
5. **BLAST.** Basic Local Alignment Search Tool
6. **MEGA6.** Molecular Evolutionary Genetics Analysis 6
7. **F.** *Flammulina*
8. **A.** *Agaricus*
9. **Av.** Average
10. **K.** Potassium
11. **EZ.**Easy
12. **EDTA.** Ethylenediamine tetra-acetic acid
13. **TE.** Tris-EDTA
14. **N.** Nadia

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Novelty statement

The current work was conducted to explore the species of Basidiomycota in the Mansehra Region and identify different reported and unreported species from the area. The described species were found novel due to their morphological as well as molecular characterization. Compared with the species in NCBI and Gene Bank.

Conflict of interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate.

The whole work based on collection and identification of macro-fungi so approved ethically.

Informed consent

The authors declare that no such material other than fungi was used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Nadia Jabeen; A. Mubeen Lodhi: Conduction the research, exploration, and collection of specimens, and drafted the manuscript; Alia Gul; Abdul Basit: Contribution of molecular work and conducted the conceptualization and validation of study; Sanaa Almowallad3, Adel I. Alalawy, Amnah A. Alharbi: reviewed and edited the article; Mohamed Sakran; Sezai Ercisli; Mohamed El-Sharnouby; Ayman El Sabagh interpretation of manuscript.

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