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Unlocking the Ecological and Molecular Characterization of *Agaricales* from Temperate Forests of Pakistan

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MUSHROOMS are a valuable group of fungi essential for their consumption and play a vital role in the ecosystem. Their fruiting bodies possess a variety in their shape, color, and ornamentation;these features are the reason for their identification. This study was carried out in Kumrat Valley, Upper Dir District of Khyber Pakhtunkhwa, Pakistan, to explore the ignored native flora ofPakistan and examine their detailed characterization. Fifteen species of order Agaricales were collected and characterized during the survey and sample collection. The highest percentage frequency (100%) was calculated in the case of *Inocybe rimosa* and *Hygrocybe conica,* while the lowest frequency (25%) was for Amanita species and developed a detailed Arc GIS map of the sampling site. DNA was isolated and amplified on PCR by using ITS primers. PCR products were sequenced and submitted to NCBI for accession numbers. A phylogenetic relationship between the species of the same order was made using Mega X. Similar studies are not common in Pakistan. Therefore, examining biodiversity and the different varieties of native mushrooms in forested areas of Pakistan is recommended.

Keywords: Basidiomycetes, Biodiversity, Distribution, Frequency, GIS, Macro-fungi, Mushrooms, Molecular characterization.

Introduction

Fungi are an essential part of an ecosystem for their bio-geochemistry, recycling of significant environmental chemicals, and biologically active compounds. They support the growth of forest trees through mycorrhizal associations with roots and plant pathogens. Their absence can decline the population of trees to the brink of extinction (Wojciechowska, 2017; Jones et al., 2018). However, only 6.7% of 1.5 million fungal species have been identified and characterized, and a larger portion of these species are in temperate regions. Tropical regions have the highest fungal diversity but are not yet fully explored (Hawksworth, 2001). These fruiting bodies are also known for their medicinal and edible values. Since ancient times, people have consumed these mushrooms for food, medicine, and culinary purposes and cultivated them to earn money. Besides its cultivation, mushroom hunting is still a most adventurous hobby, and people love to collect edible and medicinal mushrooms from the wild (Miles & Chang, 2004). The common understanding about agaricales is a fruiting body with stipe, pileus, lamella, volva, and ring.

On the contrary, aphyllophorales have minute pores underneath the pileus, and the fruiting body appears to be woodier and harder. Mushrooms represent the fleshy delicate, and woody fruiting bodies of different morphology belonging to various taxonomical classes of

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Ascomycetes and Basidiomycetes (Rashid et al., 2016). Naturally, mushroom growth can be seen only in a particular season and most likely during the rainy season. These mushrooms occupy a specific niche determining their morphology (Srivastava & Bano, 2010). They grow on various substrates, for example, fallen forest leaves, dead and decaying woods, organic matter, and mycorrhizal association with the roots of higher plants (Arora, 2008; Karwa & Rai, 2010). Each mushroom produces its basidiospores or ascospores, with their color, shape, and other identification features (Svrcek, 2000). These mushrooms, commonly called macrofungi, are abundant in Pakistan, especially in dense forests and undisturbed pastures of the Himalayan range. Still, the residents of these areas are not much aware of the significance of these mushrooms (Jabeen et al., 2014). Human activities have been threatening the biodiversity of the mushrooms and their natural niche for the last decade at a rapid rate. Usually, these fruiting bodies grow saprophytically, but most of them play a vital role in forest decomposition. In addition, some mushrooms make a symbiotic relationship with the roots of higher plants and live symbiotically (Dwivedi et al., 2012). Mushrooms are identified and characterized through conventional approaches, such as reactions of fungal mycelium to different chemicals, morphology, biochemistry, and physiology (Hyde et al., 2020a). These traditional approaches have significantly helped other associated disciplines, such as fungal ecology, biochemistry, plant pathology, and phytoremediation (De Souza & Declerck, 2003; Duong et al., 2008; Evidente et al., 2008; Hyde & Soytong, 2008). However, later on, Fungal taxonomists realized that the applications of the recent development in DNA sequence, its analysis, and phylogenetic study provide more comprehensive information about the evolutionary relationship among fungi (Manamgoda et al., 2011; Schoch et al., 2012; Jeewon et al., 2013). Thus, a recent trend toward fungal identification has moved towards applying morphological characterization combined with phylogenetic analysis, molecular biology, ecology, and chemotaxonomy (Senanayake et al., 2017, 2018; Manawasinghe et al., 2019; Hyde et al., 2020a; Phukhamsakda et al., 2020; Samarakoon et al., 2020; Wibberg et al., 2020). Applying these advanced approaches has provided practical insight into studying the diversity, ecology, taxonomy, and phylogenetic relationships between fungal species (Hongsanan et al., 2017; Hyde et al., 2020b). These approaches have strengthened the conventional techniques and made the identification process easier (Ekanayaka et al., 2017). Studying the diversity of *Agaricales* is a significant part of global biodiversity and an integral portion of fungal ecology and diversity (Li et al., 2012). Extensive research on mushrooms has been carried out in the USA, European countries, Canada, Australia, and other western countries, while tropical countries like Pakistan are less explored and studied. Therefore, there is a strong need to adopt some advanced, scientific, and rational procedures to identify mushrooms, which was the focus of this study. Therefore, this study aims to bridge the gap in examining the variety and diversity of mushrooms in different forested areas of Pakistan.

Material and Methods

Research work was conducted at Kumrat Valley, District Upper Dir, Khyber Pakhtunkhwa, and Sustainable Development Study Centre, Government College University, Lahore.

Sample collection

Mushroom samples were collected from different sites in the forested Kumrat valley (35°32'N & 72°13'E), as shown in Fig. 1. The area under study has an annual rainfall of about 80%, with a mean temperature range between 25-31°C. During the season of monsoon, the relative humidity remains over 80%. The pH of the soil was between 6 and 6.5. Most mushroom vegetation grew on natural organic matter, leaf litter, or dung. Sampling was carried out with a sharp knife and studied following a systematic sampling procedure. Their morphological features include color, texture, structure, natural substrate, ecology, host plants, length of the pileus, type of lamella and their attachment with the pileus, size of the stipe, and the presence or absence of volva were recorded at the sampling time. Forests at Kumrat valley were deciduous and mixed. Soil pH was calculated by a pH meter and temperature with a thermometer. The frequency of fungal species and density of the collected mushrooms were calculated by following formulas (Rashid et al., 2016). A detailed GIS map of the study area was also developed using Arc GIS, Arc Map version 10.7.

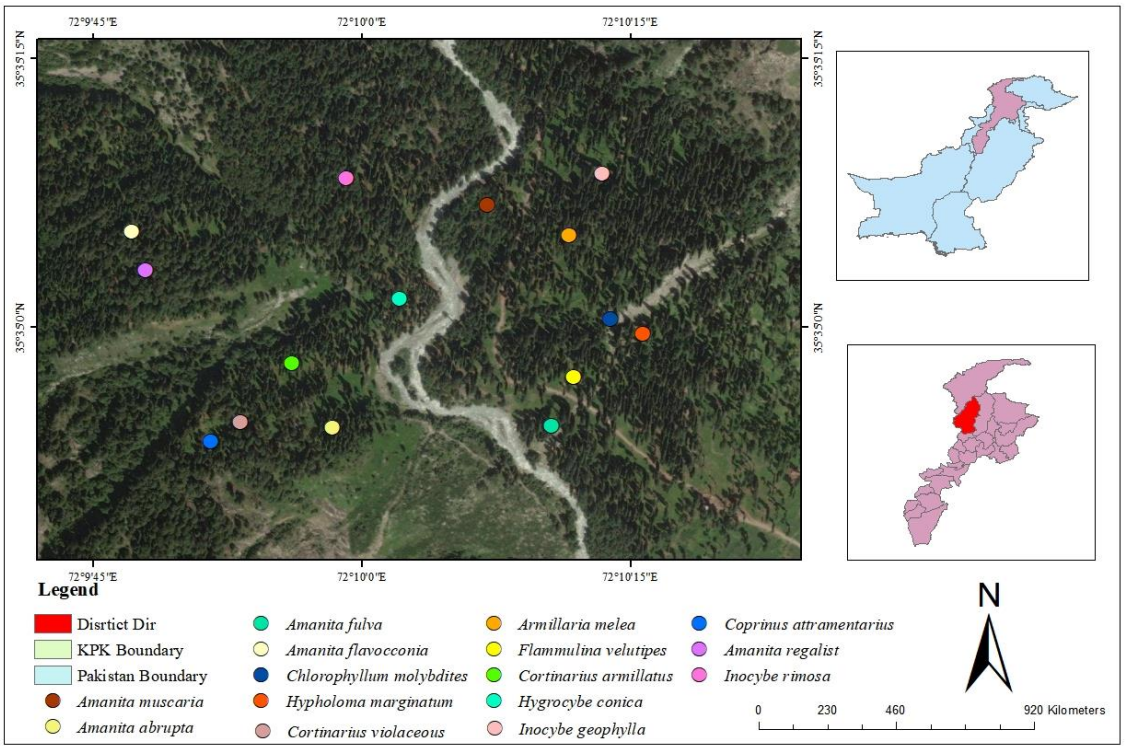


Fig. 1. Arc GIS satellite map of the sampling sites in Kumrat Valley highlighting the sampling site for each of the mushroom

% Frequency= (No,of sites in which species is present)/(Total No.of sites) X 100

% Density= (Total No.of indvidual species)/ (Total No.of species) X 100

Preparation of mushroom samples

Most Agaricales have a perishable fruiting body that can quickly change shape and other features after collection. So, for the sort, term preservation mushroom samples were collected in a paper box and dried with the help of a heater fan. After drying, samples were preserved in polythene bags for further study.

Morphological and taxonomic characterization of mushrooms

For morphological characterization, variation in pileus shapes (spherical, convex, depressed, umbonate, umbilicate, infundibuliform), edges of the pileus (grooved, plicate, split) (Badalyan et al., 2004), spore color, shape, form, and ornamentation were studied. The shape of gills, the spacing pattern between the gills, gills color, stipe length, width, color, presence or absence of annulus ring, and presence or absence of volva were also recorded at the time of sample collection (Srivastava & Bano, 2010). Freehand sections of hymenium and stipe were cut with a sterilized sharp blade and studied under a trinocular microscope to study the spores' color, shape, and ornamentation. Spore sizes were also measured by an ocular micrometer (Svrcek, 2000). Taxonomic and morphological characterization was verified from the literature and identification keys available in Alexopoulos et al. (1996), Huffman et al. (2008), Russell (2017), and Rubina et al. (2017).

DNA extraction

Dried fruiting bodies of the mushroom samples (oven-dried at 30ºC) were processed for DNA extraction through maceration in a mortar and pestle with liquid nitrogen. Two milliliters of nucleon reagent B [400 mM Tris, pH 8.0; 120mM EDTA, pH 8.0; 150mM NaCl and 1% (w/v) sodium dodecyl sulphate (SDS). were added to ground material to suspend it. 10mg mL^{-1} RNaseA (0.5µL) was mixed with cell suspension and kept for thirty minutes at 37°C. Two mL of sodium perchlorate (5M) and 2mL chilled chloroform was added and mixed briskly to the cell mixture. After centrifugation (at 4000rpm) for 10min, the supernatant shifted to a new sterile tube, and 2mL of chilled ethanol (95%) was added. Collected DNA

(deoxyribonucleic acid) was centrifuged for two minutes at 10,000rpm. Ethanol C_2H_5OH (70%) was used to rinse the DNA pellet that was then dried and finally assorted in TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0). Isolated DNA was stored at -20 °C after maintaining at 65°C for ten minutes (to denature DNAase enzyme). One percent agarose gel was prepared to evaluate the quality and concentration of DNA. A UV transilluminator was used to analyze and visualize DNA bands and gel's approximate size and quality (Gašparcová , 2017).

PCR Amplification of the ITS region

The amplification was done by using isolated genomic DNA as a template. Two universal primers, ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3'), forward primer, and ITS-4 (5'-TCC TCC GCT TAT TGATAT GC3'), reverse primer, were used for the amplification of the Internal transcribed spacer regions (ITS) of the mushrooms (Siddiquee et al., 2010). The reaction mixture (25µL) was prepared by adding 5µL (10-50ng) genomic DNA, 12.5µL Master Mix (GeneAll Biotechnology Co, LTD), and 1µL (10pmol μL^{-1}) each of forward and reverse primers primer. Double distilled water was used to raise the volume up to 25µL. PCR (Thermocycling polymerase chain reaction) involved initial denaturation at 94°C for 5min, 40 cycles of 95 °C for 45s, 60 °C for 45s, and 72°C for the 20s for denaturing annealing, and extension, respectively, followed by 72°C for 10min (on PLT-06 Machine). One percent agarose gel was prepared to visualize PCR products.

Nucleotide sequencing

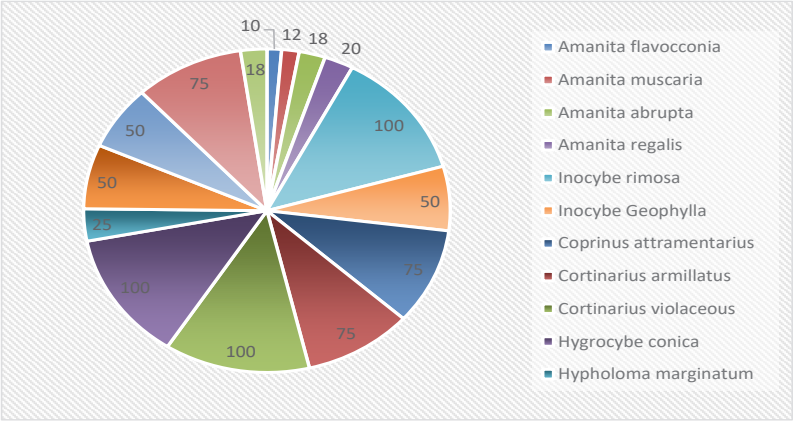
Amplified PCR products were submitted to 1st Base Sequencing Singapore Co., Ltd. for bidirectional sequencing. The purified, sequenced 18S rDNA sequencing results were submitted to NCBI (National Center for Biotechnology Information) database for BLAST alignment search. The sequence data were assembled and analyzed using Mega 6 version 6 and aligned with ClustalW. Nucleotide sequence comparisons were performed using Basic Local Alignment Search Tool (BLAST) network services against the National Center for Biotechnology Information (NCBI) database. The phylogenetic tree was constructed using the Neighbor-Joining method MEGA 6 version 6.0 (Tamura et al., 2011).

Results

Percentage frequency and density of species

Mushroom samples were collected from the forested areas (Panjkora, Jahaz Banda, Dugayi, and kumrat) of Kumrat valley (Fig. 2). The ecological niche and morphological characterization of each species were studiedDuring the survey and sample collection, 15 species of mushrooms belonging to 9 different genera of *Agaricales* were collected and characterized morphologically (Table 1). Among the collected mushroom species, the highest frequency was recorded forthe *Inocybe rimosa* and *Hygrocybe conica* (100%), followed by *Coprinus attramentarius* and *Cortinarius armillatus* (75%). The lowest frequency (10%) was observed for three species of the *Amanita flavoconia* (Fig. 2 A). Maximum density was recorded in the case of *Inocybe geophylla* (62.3%), followed by *Inocybe rimosa* (60.5%) and *Cortinarius armillatus* (58.8%). A minimum value of species density in the case of *Amanita muscaria* (21.8%) (Fig. 2 B). Morphological characterization of all the agaricales was also recorded (Fig. 3), substrate details , usage and NCBI accession numbers are also recorded (Table. 2).

(2A)



(2B)

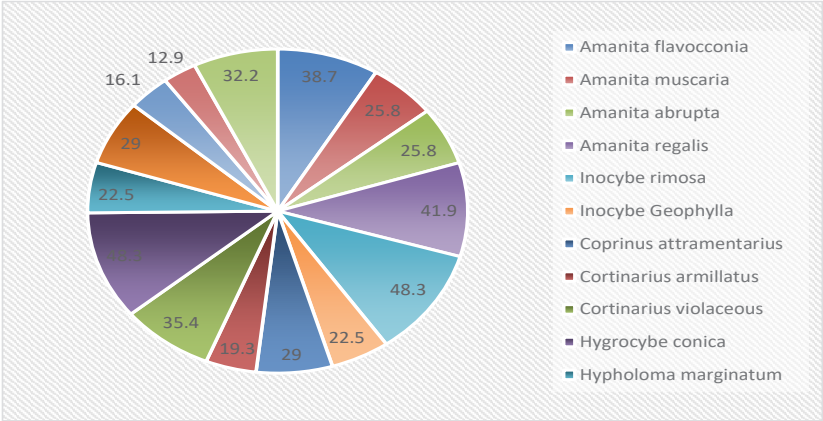


Fig. 2. A: Percentage frequency of the mushroom species at sampling sites of Kumrat Valley; B: Percentage species density of the collected mushroom samples from Kumrat Valley

Molecular characterization

The PCR amplification products showed *A. abrupta*, *A. flavoconia*, *A. fulva*, *A. muscaria*, *A. regalis,* and *A. mellea* gave about 738bp, 733bp, 817bp, 696bp, 663bp and 690bp amplified band each, respectively. Whereas *C. molybdites*, *C. atramentarius*, *C. armillatus*, *C. violaceus*, *F. velutipes*, *H. conica*, *H. marginatum*, *I. geophylla*, *I. rimosa* showed 838bp, 647bp, 606bp, 696bp, 763bp, 609bp, 650bp, 709bp, and 731bp amplification bands, respectively (Fig. 4). The analysis involved 15 nucleotide sequences. The phylogenetic tree revealed three main clads from which further branches emerged. Being placed in the same clad, a close relationship was observed among *A. fulva, A. mellea* and *C. molybdites, F. velutipes*. Similarly, *A. muscaria*, *C. violaceus*, and *A. abrupta* shared the same clad and showed close affinity. All other mushroom species including *A. flavoconia*, *I. rimosa*, *I. geophylla*, *C. atramentarius*, *C. armillatus, A. regalis*, *H. conica* and *H. marginatum*, being placed in one clad showed close affiliation among each other while they show a distant association with the species of the other two Clads (Fig. 5). The BLAST results were obtained by searching with partial nucleotide sequences in the GenBank database. The mushrooms were identified as *A. abrupta* [% identity 98 (AB015685) Japan], *A. flavoconia* [% identity 98 (EU819463) USA], *A. fulva* [% identity 98 (MT229861) USA], *A. muscaria* [% identity 98 (ABO15700) Japan], *A. regalis* [% identity 99 (JF907764) USA] *A. mellea* [% identity 98 (KT822250) China], *C. molybdites* [% identity 99 (MK541990) Sudan], *C. atramentarius* [% identity 99 (AF345814) Korea], *C. armillatus* [% identity 99 (HQ845116) Finland], *C. violaceus* [% identity 99 (KM253741) Finland], *F. velutipes* [% identity 99 (JN047545) China], *H. conica* [% identity 99 (JF908057) USA], *H. marginatum* [% identity 99 (MH612933) USA], *I. geophylla* [% identity 99 (MT451922) China] and *I. rimosa* [% identity 99 (JQ408784) USA] (Fig. 5).

Discussion

The present study was conducted during the summer of 2019, right after monsoon rains, because the percentage of precipitation on these days is favorable for spore germinations. It was observed that dead and decaying woods in the forest are the best hosts for these mushrooms. Moreover, morpho-ecology of mushrooms was also observed and recorded (Table 1). Figure 1 explains the enriched areas of kumrat valley regarding such mycoflora. Similarly, Lakhanpal (2014) also collected the species of *Agaricales* from North-western Himalayans in India during the summer season. Malik et al. (2017) also concluded that most of the mushrooms understudy grew on soil with a high level of organic matter and dead and decaying woods. Likewise, Sharma et al. (2009) found about 300 species of mushrooms from 15 different families of Agaricales during a survey in the northwest Himalayas in India. In this research, the highest frequency of the species was calculated for *Hygrocybe conica,* while the lowest value was observed for the species of *Amanita.* The same pattern was also observed while studying species density in concerned areas. A similar study was also done by Rubina et al. (2017) and found *Russula nobilis* with a density of 5% in the National Botanical Garden, Dhaka. In another study, Das et al. (2016) found and morphologically identified six species of the genus *Agaricus* from the mangrove forest of Bangladesh.

TABLE 1. Morphological and ecological descriptions of collected mushrooms

Sr. #	Name	Ecology	Pileus	Stipe	Lamellae
1	*Amanita flavocconia*	Grows in mycorrhizal association with hardwoods of oaks	Pileus was convex to flat, orange-yellow; the surface was sticky and ornamented with scattered warts, 2-8cm in diameter	Stipe was thick, tapering toward the base, 5-12cm long, rough, yellow, and annulated with volva at the bottom	Lamellae were free, highly crowded, and white-yellow colored
2	*Amanita muscaria*	Grows in mycorhizal association with conifers, crowded and forms fairy rings.	Pileus is 5-27 cm in diameter, bright red, oval, or flat-convex, smooth surface with white warts	Stipe is 5- 19cm long, white, equal, and annulated with volva at the base	Lamellae are white, crowded, and free.
3	*Amanita abrupta*	Grows saprobically in coniferous forests and in groups	Pileus was 5-10cm in diameter, white-colored, convex, with a central depression and angular warts on the surface	Stipe was 12cm long, smooth, slender with a bulbous base and hairy surface	Lamellae were white, moderately crowded, and free
4	*Amanita regalis*	Grows scattered in mycorrhizal association with hardwood of conifers	Pileus was convex to flat,brown fragments of veils on the surface and 10-20cm in diameter	Stipe was smooth, thick, annulate, 12-25cm long with volva at the base	Lamellae were free, highly crowded, and pale yellow
5	*Amanita fulva*	Grows in mycorrhizal association with hardwoods of conifers	Pileus was a convex, brown, sticky surface, lined at margins and 5-10cm in diameter	Stipe was thick, tapered towards base, hairy, pale brown with volva at the base and 8-16cm long	Lamellae were free, white and highly crowded
6	*Inocybe rimosa*	Grows scattered in mycorrhizal association with hardwoods of conifers	Pileus was conical with a central bump, dry, hairy, yellowish-brown, 3-8cm in diameter with split margins	Stipe was thick,equal, silky surface, pale yellowish, and 4-9cm long	Lamellae were adnexed, closely crowded, and brown
7	*Inocybe geophylla*	Grows in mycorrhizal association and in small clusters	Pileus was 1-3cm, conical, white, with a smooth surface	Stipe was 1-4cm long with a dry and silky texture	Lamellae covered by Cortina, greyish brown, slightly crowded, and free.

TABLE 1. Cont.

Sr. #	Name	Ecology	Pileus	Stipe	Lamellae
8	*Coprinus attramentarius*	Saprobically grows on decaying woods and stumps	Pileus was oval to conical, greyish brown, smooth, and 2-6cm in diameter	Stipe was smooth, hollow, white, and 6-14cm in length	Lamellae were free, slightly crowded, and black at maturity
9	*Cortinarius armillatus*	Saprobically grows scattered on dead and decaying matter	Pileus was convex, flat, and dry with a slight ornamented surface, brown, 4-9cm in diameter	Stipe was pale yellow, silky, swollen at , 8-15cm long	Lamellae were slightly crowded, attached, and rusty brown
10	*Cortinarius violaceous*	Grows saprobically scattered on fallen pine leaves	Pileus was purple, scaly, convex, flat, and 5-11cm in diameter	Stipe was purple, hairy, 5-15cm long, thick with club-shaped base	Lamellae were adnate, moderately crowded, and greyish black
11	*Hygrocybe conica*	Saprobcally grown in clusters on hardwoods and conifers	Pileus was conical, slimy, orange red, and 2-7cm in diameter	Stipe was orange-red, white base, and 6-10cm long	Lamellae were adnate, slightly crowded, and white
12	*Hypholoma marginatum*	Saprobically grew on wood, leaf litter, and organic matter	Pileus was convex, smooth, 3-6cm in diameter, olive-brown with a smooth surface	Stipe was central, cortinoid, annulate, 9-12cm long, scaly and narrow at the base	Lamellae were adnate, highly crowded, and cinnamon-colored
13	*Armillaria melea*	Parasitically grows on forest trees and causes white rot infection	Pileus was flat, honey-yellow, smooth, and 4-15cm diameter	Stipe was thick, tapering at the base, with rhizomorph, smooth surface, annulate and yellow	Lamellae were adnate, moderately spaced, and reddish-brown
14	*Chlorophyllum molybdites*	Grown saprobically in grassy lawns in the shape of fairy ring	Pileus was convex to flat, scaly, white, and 10-40cm diameter	Stipe was light brown, equal, thick with a smooth surface, and 4- 24cm long	Lamellae were free, slightly crowded, and dark greyish
15	*Flammulina velutipes*	Saprobically grows on stumps, logs, roots and other hardwoods	Pileus was flat, moist surface, yellowish brown and 1-6cm in diameter	Stipe was 2-11cm long, orange brown and enlarged at the base	Lamellae were adnexed, highly crowded and pale yellow in color

Fig. 3. Pictorial Presentation of collected mushrooms in their natural niche; A: *Amanita flavocconia*; B: *Amanita muscaria*; C: *Amanita abrupta*; D: *Amanita regalis*; E: *Amanita fulva*: F: *Coprinus attramentarius*; G: *Inocybe rimosa*; H: *Inocybe geophylla*; I: *Cortinarius violaceous*; J: *Cortinarius armillatus*; K: *Flammulina velutipes*; L: *Hygrocybe conica*; M: *Cholorophyllum molybdites*; N: *Armillaria melea*; O: *Hypholoma marginatum*

TABLE 2. Details of collected mushrooms samples from Kumrat Valley

S. No	Scientific Name	Substrate	NCBI accession numbers	Families	Use
1.	*Amanita flavocconia*	Mycorhizal with conifers	MW553142	*Amanitaceae*	M/P
2.	*Amanita muscaria*	Mycorhizal with spruce	MW553144	*Amanitaceae*	P
3.	*Amanita abrupta*	Mycorhizal with conifers	MW553141	*Amanitaceae*	M/P
4.	*Amanita regalis*	Mycorhizal with conifers	MW553145	*Amanitaceae*	P
5.	*Amanita fulva*	Mycorhizal with conifers	MW553143	*Amanitaceae*	P
6.	*Inocybe rimosa*	Beeches	MW553157	*Inocybaceae*	P
7.	*Inocybe Geophylla*	Mycorhizal with Pines	MW553156	*Inocybaceae*	P
8.	*Coprinus attramentarius*	Dead wood	MW553148	*Agaricaceae*	M
9.	*Cortinarius armillatus*	Mycorhizal with conifers	MW553151	*Cortinariaceae*	P
10.	*Cortinarius violaceous*	Mycorhizal with birches	MW553152	*Cortinariaceae*	P
11.	*Hygrocybe conica*	Grassy fields	MW553154	*Hygrophoraceae*	U
12.	*Hypholoma marginatum*	Hardwoods	MW553155	*Strophariaceae*	I
13.	*Armillaria melea*	Dead roots and stumps	MW553146	*Physalacriaceae*	E
14.	*Chlorophyllum molybdites*	Conifer needles	MW553147	*Strophariaceae*	I
15.	*Flammulina velutipes*	Dead hardwoods	MW553153	*Physalacriaceae*	I

M/P= Medicinal/Poisonous, U= Unknown, I= Inedible, E= Edible

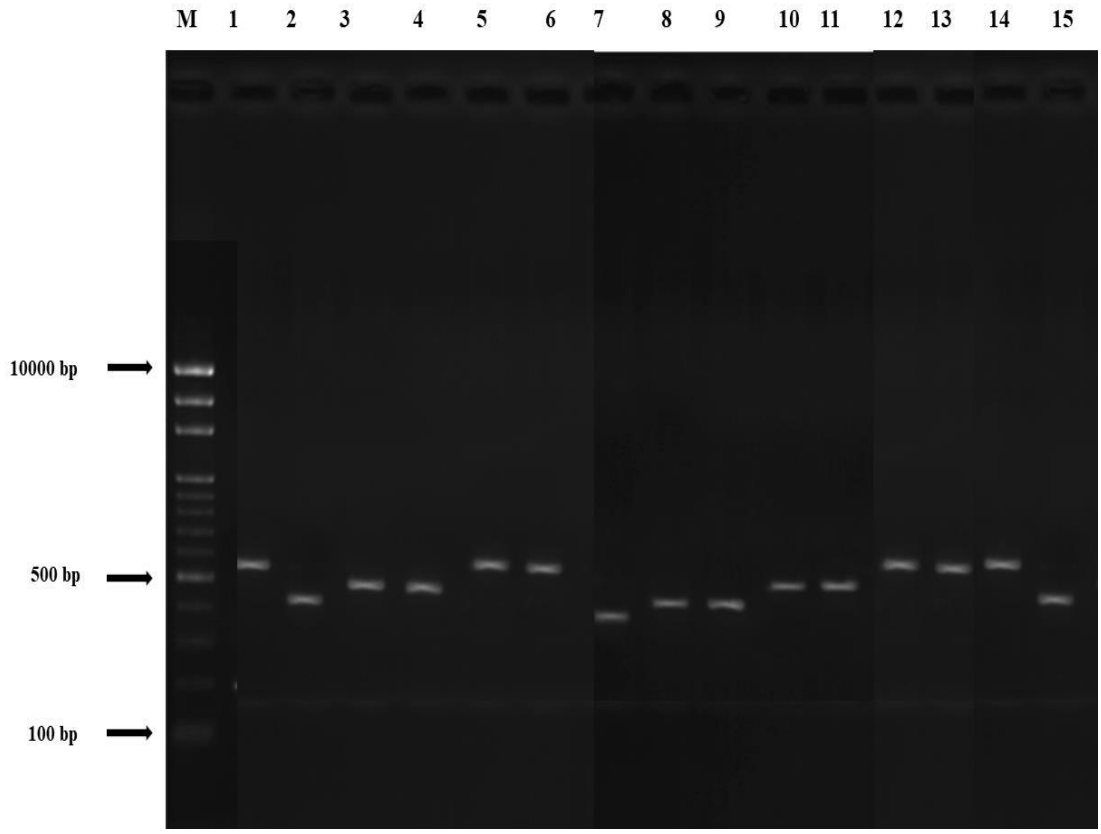


Fig. 4. Agarose gel electrophoresis of PCR amplified products of ITS regions of *A. abrupta*, *A. flavoconia*, *A. fulva*, *A. muscaria*, *A. regalis*, *A. mellea, C. molybdites*, *C. atramentarius, C. armillatus, C. violaceus, F. velutipes, H. conica, H. marginatum, I. geophylla* and *I. rimosa* [M=DNA marker (100bp)]

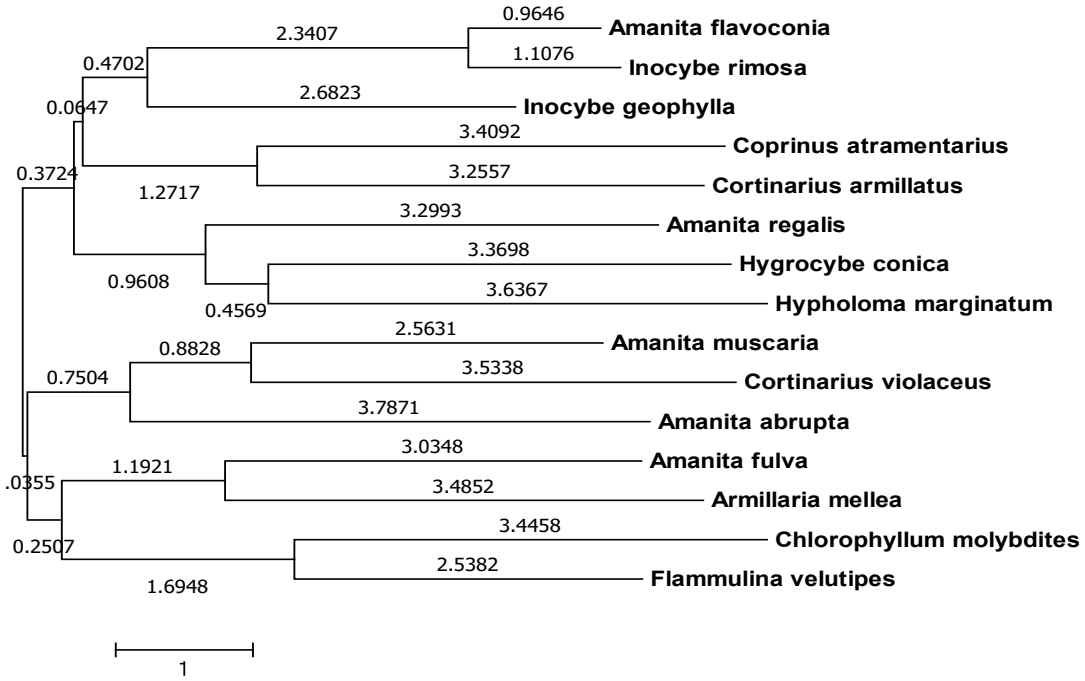


Fig. 5. Phylogenetic tree depicting relationship among mushroom samples. Evolutionary analyses were conducted in MEGA6

On the other hand, Lee et al. (2007) calculate percentage frequency and density for Amanita species as 12.4% and 6.8%, respectively, isolated from Coconut in northern Thailand. Such low count of frequency and density for *Amanita* can be due to its angiocarpic type of growth. Another reason can be its eye-catching colors, making it visible from a distance to mushroom hunters and other predators. The species count for a higher percentage of frequency and density because of their cluster growth. This growth in the forest makes them dominant and competitive with other mycoflora.

Similarly, Kumar et al. (2013) have investigated some mushrooms' diversity and nutritional significance in Nagaland. Their investigations recorded a new variety of *Rhodocybe popinalis* belonging to the *Entolomataceae* family of order *Agaricales* from a coprophilous environment. Mycologists have recently given priority to molecular characterization of mycoflora to develop and study phylogenetic relationships between species (Kodsueb et al., 2007; Swe et al., 2008; Maharachchikumbura et al., 2016). The following research showed that rDNA sequences of the collected mycoflora proved to be more effective in identifying and studying their phylogenetic relationships. Hyde et al. (2016) discussed that DNA sequences from ribosomal genes or other genes had become common in fungal identification. The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). In this research work, DNA was extracted directly from the parts of basidiocarps due to a lack of mycelial growth in culture plates. It was observed that fungal species have a specific growth pattern with a specific nutritional medium, and the unavailability of the particular media is the main hurdle in mycelial growth. Likewise, Izumitsu et al. (2012) have also extracted DNA and performed PCR amplifications direct from basidiocarps. They have discussed that although direct DNA extraction from basidiocarps was challenging, it yielded satisfactory results. Kuhnert et al. (2015) emphasized that rather than targeting multi-locus genes, other advanced approaches, such as chemotaxonomic procedures, must also be adapted to understand species relationships better. Stielow et al. (2015) discussed more advanced molecular techniques to strengthen morphological characterizations of phylogenetic relationships between the fungal species.

Conclusions

This research work concluded that the temperate forests of Pakistan are enriched with native mycoflora. Such an ecological niche favors the

mushroom diversity in these regions. As the study of mushrooms is much neglected in Pakistan, so it is a need of the hour to study this subject in all possible dimensions. This study provides an accurate and authentic characterization of *Agaricales,* which can be helpful in their usage as diet or medicine and for research purposes. In addition to identifying valuable native mycflora this research has also found the genetic similarity and variability among the studied mushrooms.

Competing interests: The authors report no conflicts of interest regarding this work.

Authors' contributions: Muhammad Ali and Faiza Sharif conceptualized the research work, Shinawar Waseem Ali, Muhammad Shahzad and Muhammad Ali conducted the experimental work, Sidra Javed and Ahmad Ali Shahid helped in DNA isolation, PCR amplification and gene sequencing. All the authors jointly written, read and approve this final manuscript file.

Ethics approval: Not applicable.

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