MORPHOLOGICAL CHARACTERIZATION AND DNA BAR CODING OF *MORCHELLA* SPECIES GROWING IN DIFFERENT REGIONS

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Abstract. The molecular diversity of 15 different *Morchella* species collected from three different ecological zones (Chinar, Miandam, and Kalam) of District Swat, Khyber Pakhtunkhwa, Pakistan in 2018-2019. Considerable morphological variations exist concerning some species of *Morchella*, however, classical taxonomy is helpful in the identification of morel species. The focus of this study was morphological, anatomical, and molecular characterization of morel species. Phylogenetic analyses by maximum parsimony, neighbor joining, and maximum likelihood method revealed that majority of species are morphologically like *M. esculenta* and *M. vulgaris*. DNA sequence-based phylogenetic analysis may be useful in identifying the morel taxonomy. The DNA consensus sequences were finally aligned and analyzed using FASTA and BLAST sequence format. Morpho-anatomical traits were used to identify four species (M. esculenta (Linn.) Pers., M. vulgaris (Pers.) Boud, M. angusticeps Peck., and M. brunnea M. Kuo, sp. nov) among the collected *Morchella* specimens. While the two morphologically distinct taxa, such as the *Morchella* (unidentified) and the M. esculenta clade, were discovered using ITS-1 and ITS-2 sequence data in an internal transcribed spacer (ITS) based molecular approach. These two species have demonstrated phylogenetically shared similarities with M. Brunnea and M. angusticeps. An elaborate study is required to determine the exact limit of genetics and species diversity among all ecotypes on a national scale, which is genotypically adapted to specific ecological environments. here has been a limited effort in Pakistan thus far to identify species using both conventional taxonomy and the most recent DNA techniques. Thus, the goal of the current study is to identify some morel species that grow locally based on their morpho-anthropometric traits, with a focus on their ecological and soil traits.

Keywords: *morels, morphology, phylogeny, DNA sequencing, DNA barcoding, ITS sequence, soil parameters*

Introduction

Unlike plants and animals, fungi are eukaryotic organisms that are heterotrophic in nature and have a global distribution (Chauhan et al., 2014). Significantly, *Morchella* is a type of mushroom that belongs to the Ascomycetes family (Okan et al., 2013). According to Okan et al. (2013), morel mushrooms are a type of edible wild fungus that is part of the Morchellaceae family. Morel plants are found in temperate regions all over

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the world. According to Goldway et al. (2000), it was commonly found in America, Europe, and the Asian Himalayan region, including the northern regions of Pakistan, such as Chitral, Dir, Kohistan, and Kalam. The elevation range of 1800-3000 m above sea level is where black morels can be found. According to Keefer et al. (2010), morel fruiting is one of the most highly valued edible fungi and is shipped all over the world in both fresh and dried forms, along with pine mushrooms and chanterelles. Humankind has utilized wild edible mushrooms (*Morchella*) as a food source from the beginning of time. The world's highest native source of protein foods is edible mushrooms. They are prized for their potent nutritional qualities, unique flavor, and scent. Protein, vitamin, and mineral ranges are high, but other content is poor. According to Bhatt et al. (2016), mushrooms are regarded as a suitable diet that provides easily digestible, high-quality protein. Compared to other vegetables, edible mushrooms (*Morchella*) are a rich source of nutrients and contain minerals, vitamins B, C, and D. It is made up of 2% fat and 32.5% protein. 190 mg/g of iron and 0.18 mg/g of potassium are present, along with other chemical constituents (Ajmal et al., 2015).

One of the most dangerous infections that people confront worldwide is infectious diseases. Many antibacterial chemicals that had a positive therapeutic effect and were employed in traditional folk medicine were identified from various mushrooms. The *Morchella* species is highly valued and has been used to treat a variety of illnesses in China, Japan, and Malaysia in the last 2000 years ago. According to a reported study, people gathered and ate wild edible mushrooms for their nutritional value and therapeutic properties (Okan et al., 2013). Its immunomodulatory, anti-neoplastic, and fat-reducing properties are used to treat infections. Taşkın et al. (2016) describe *Morchella* as edible macrofungi that are part of the *Morchella*ceae family. Because of their unique flavor and scent, *Morchella* species, or true morels, are consumed all over the world and are therefore very marketable and profitable. The demand for it is growing daily.

It is essential to assess the genetic diversity of the current germplasm for human consumption due to the growing demand. Therefore, molecular, and morphological characterizations are widely used in species variations estimation and classification for the estimation of genetic diversity; however, because morphological characterizations are highly impacted by environmental changes (Ihsan et al., 2021, 2024), DNA-Barcoding is widely used to characterize morels (Pildain et al., 2014). Morels can be described in a variety of ways, but internal transcribed spacers, or ITS, are the most effective molecular methodology for species identification (Gessner, 1995). The ITS rDNA region has been the only locus used in numerous research to evaluate *Morchella* genetic diversity in comparison to other (Du et al., 2012). The DNA barcoding approach is also used to identify species based on segments that are identical, or a combination of segments created by changes in DNA sequences. An increasingly popular technique for obtaining taxonomic data about unknown organisms is DNA barcoding. This is the research area's first attempt at identifying morel species at the molecular level. Given the enormous diversity of fungi, investigations pertaining to their identification are crucial given the problems pertaining to their protection and exploration. The present exploratory effort could serve as a foundation for more research.

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Material and methods

The current study was carried out from 2018 to 2020 at the University of Malakand's Department of Botany. The plant parts were collected from different morels that were growing in Pakistan's Khyber Pakhtunkhwa province's Swat district. In Khyber Pakhtunkhwa, Pakistan, various exploration trips were planned to Chinar, Miandam, and Kalam valley District Swat. Five sites from each region were studied, and 5 plants from each site were collected and data was recorded. While conducting the exploration, pictures of the morel growth region were taken. *Figure 1* shows the various geographical zones within the District Swat, Khyber Pakhtunkhwa, Pakistan study region.

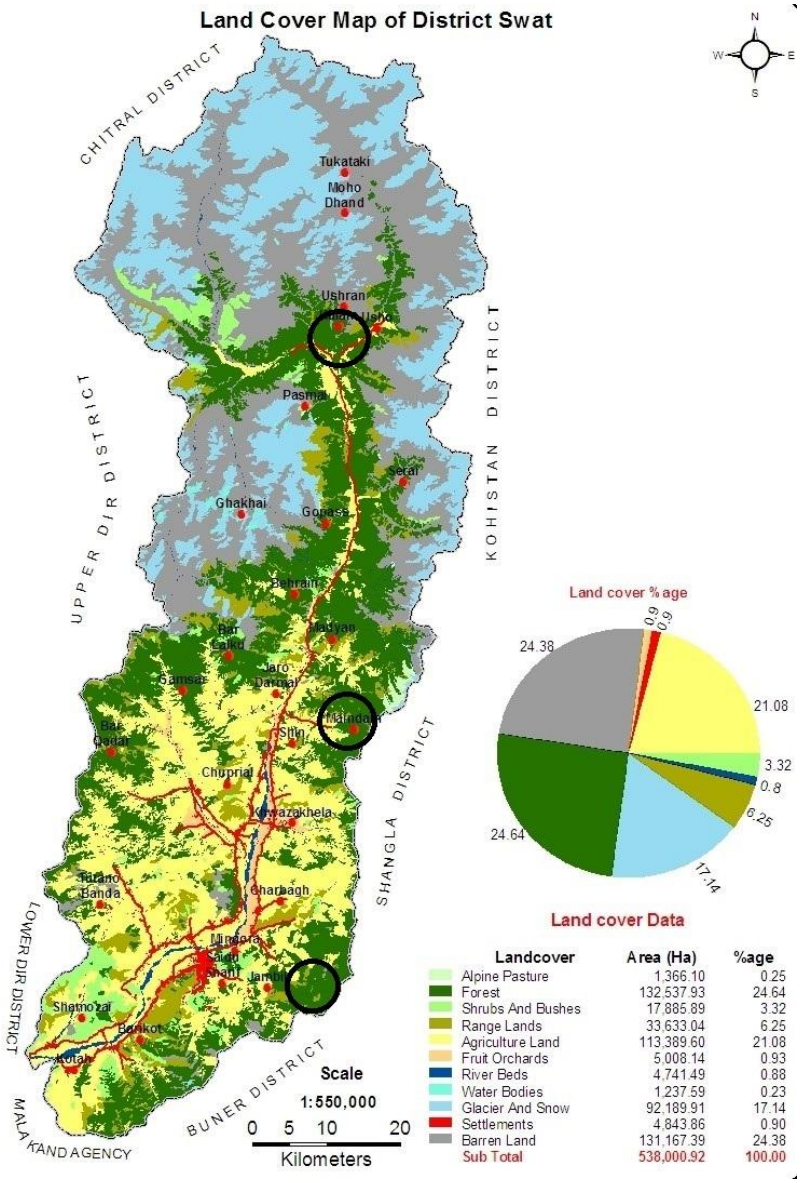

Figure 1. *Geographical zones of the studied area of District Swat, Khyber Pakhtunkhwa, Pakistan*

Field visit

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Various field visits were planned to the area under investigation. Geographical coordinates, such as height, longitude, and latitude, were recorded during the field visit. Every specimen's photo was taken there and then. Each location's soil was taken for analysis, including measurements of the pH, organic matter, nitrogen, phosphorus, and potassium contents. Only morel mushrooms (*Morchella*) were sampled, dried methodically, and kept in accordance with the *Morchella* species.

Morphological characterizations

Four distinct morphological traits—color, shape, cape, and stalk—were noted. The specimens were gathered, appropriately photographed on the spot, and their appearance, pit, and rib orientation, were noted as well.

Anatomical character

Under a microscope, the specimen's internal characteristics, including spore size, shape, color, asci length, asci width, spore arrangement, and sterile paraphysis, were noted. The described procedures for staining and microscopic analysis were followed (Loizides et al., 2020). Twenty randomly chosen ascospores were extracted from each specimen and placed in a 3% KOH solution for the spore's dimension. The equation of Borges et al. (2009) was used to measure the "Q" value.

Molecular characterization

DNA isolation was used for the molecular characterization of *Morchella* species, and ITS-1 and ITS-2 markers were used in PCR analysis (Borges et al., 2009).

DNA-bar coding

ITS region PCR amplification was performed using isolated DNA and primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'TCCTCCGCTTATTGATATGC– 3'). Starting at 95°C for 5 min, the polymerization process (PCR) was run through 35 cycles at 94°C, 54°C, and 72°C (for 45, 30, and 45 s each cycle), ending at 72°C for 10 min. Primers ITS4 were used to sequence the PCR products, which were examined in a 1% agarose gel (Clowez et al., 2014).

DNA sequencing

Base Clear (the Netherlands) successfully performed Sanger sequencing on the purified DNA samples. According to the reported method (Loizides et al., 2016), 20 µl (6 ng/µl DNA; 25 pmol primer LR0R or primer LR5) volumes were needed for DNA sequencing and the sample concentration was 15 ng/µl per 100 bp.

Alignment and phylogenetic analysis

The consensus sequences were aligned and examined using the FASTA and BLAST sequence formats after the raw trace files were modified. The three techniques used for phylogenetic analysis were maximum parsimony, neighbor joining, and maximum like hood. The greatest likelihood evolutionary model, which was built using aligned data, was the most effective in determining the genetic links. The morphological characteristics matched the molecular information as well.

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Results

Morpho-anatomical characteristics of Morchella esculenta (Linn.) Pers.

This species was found in fruit tree orchards and deciduous plantations and gathered near crop fields. Late March through April marked the beginning of the sprouting season. exhibited gregarious behavior of fructification and appeared singly. *Table 1* shows the flora connected with M. esculenta and the soil's composition. The base was lacunose and enlarged, while the stipe had a dull, off-white, or pale yellow color. The stipe's length varied between 4 and 5.5 cm, while its breadth varied between 2 and 3.5 cm. Hymenophore in Pileus: oval to sub cylindric in shape; specimens showed no sinus; pale yellow with numerous reddish dots on ridges; apex obtuse, like a cone; edge was

non-looped. The Pileus measured between 35 and 47 mm in length and 32 and 90 mm in breadth. The pits were longer, deeper, and ranged in color from yellow to pale white. At first, ridges had a color similar to pits, but as they grew older, they turned yellowish. Eventually, reddish to orange patches appeared, most of which were delicate and velvety. Many vertical ridges and a concave appearance for the horizontal ridges (*Fig. 2*). The ascospore print measured 16.25–26.2 μm × 10–18 μm and was creamy to white in color. It was oval to elongated in shape, with two walls. Transparent, granular components were investigated in the ooplast of the spores. The asci were cylindrical in shape and were 200.15–250.20 μm × 18.70–20 μm. They contained eight spores organized end to end (*Fig. 2*).

Figure 2. *Morphological and Anatomical characters of M. esculenta growing in Chinar Tehsil Babuzai District Swat, Khyber Pakhtunkhwa, Pakistan*

Morpho-anatomical characteristics Morchella vulgaris (Pers.) Boud.

M. vulgaris is typically found on sloping areas with damp, humus-rich soil beneath deciduous trees in the lower plain. appeared in early April and persisted through May. M. vulgaris grows either alone or in clusters. *Table 1* displays the soil composition, geographic locations, and flora that are related with M. vulgaris. The stem was clavate-

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shaped, tubular, dull, somewhat mealy, or light pink in color, ranging from light yellow to off-white, with a massively inflated base measuring 3 to 5 cm in length and 1-2 to 5 cm in width.

The Pileus had a round to sub-oval shape, measuring 2.5–5 cm in length and 2–5 cm in width at its widest point. It was dirty at first and turned tan or yellowish in color as it matured. The apex was dome- or oval-shaped, without a sinus and without a looping edge. The deep, labyrinthine pits, which measured up to 35 mm in size, had an uneven appearance, were elongated and polygonal, and were darker than the ridges. ageing while dying. The color of the ridges ranged from creamy yellow to pale brown. The main ridges have powerful curves that are sinuate or completely irregular, bony, appear incomplete or erupted, and contain orange to reddish spots that abrade with phases. The color of the pits, ridges, minor ridges, edge, concave ridges, and stipe appears to be scarcely littered (*Fig. 3*).

The ascospores are narrowly ovoid to elliptical, dual smooth encased, hyaline, and have comparable content. They measure 30.14–43.2 μm × 20.6–23 μm. The asci were cylinder-shaped, eight-spored, and attached ascospores from side to side or end to end in each ascus, measuring 143.7–187.76 μm × 6.8–8.19 μm. The size of the paraphysis was 161–165.57 μm × 8.11–14.29 μm; it was transparent, septated, and tubular in shape (*Fig. 3*).

Figure 3. *Morphological and anatomical characters of M. vulgaris growing in Mindam Tehsil Khwazakhela District Swat, Khyber Pakhtunkhwa, Pakistan during 2018-2019*

Morpho-anatomical characteristics of Morchella brunnea M. Kuo

Morchella brunnea grows in high moisture, humus-rich soil found in deciduous plantations at lower altitudes. Fruiting occurred in April and May and was solitary or dispersed. *Table 1* displays the soil composition, geographic locations, and flora that are related with M. vulgaris. The lower portion of the stem fruiting body is erect, rod-

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shaped, and clavate in shape. Its color is dull, off white, and pale yellow, and its dimensions range from 1 to 1.7 cm in width to 6 to 8 cm in length.

The upper portion of the fruiting body can be oval or elongated in shape; it is greyish at first and turns reddish brown as it ages. It has a non-looping edge, no sinus, and a length of 3 to 4 cm and a width of 2 to 3 cm. Within the pileus are pits that can range in size from 30 mm to enormous, extended polygonal, somewhat uneven, and enlarged pits. There are 11 to 19 vertical ridges that run from base to top, unfinished, concave horizontal ridges, ridges that are dark brown to tan brown in color, up to 2 mm wide, irregular margins, and fractures that turn dull brownish to tan brown as they mature.

Pits and stipe appear to have the same color, yet ridges have various colors (*Fig. 4*). The ascospores have a creamy to white print, with an elliptical shape, two smooth walls, a hyaline wall, and a homogenous content. Their length and width range from 25.7 to 27.7 µm. The asci were tubular, eight-spored, and placed inside each ascus either end-to-end or side-wise, measuring 293.6 to 588 µm × 18-37 µm (*Fig. 4*).

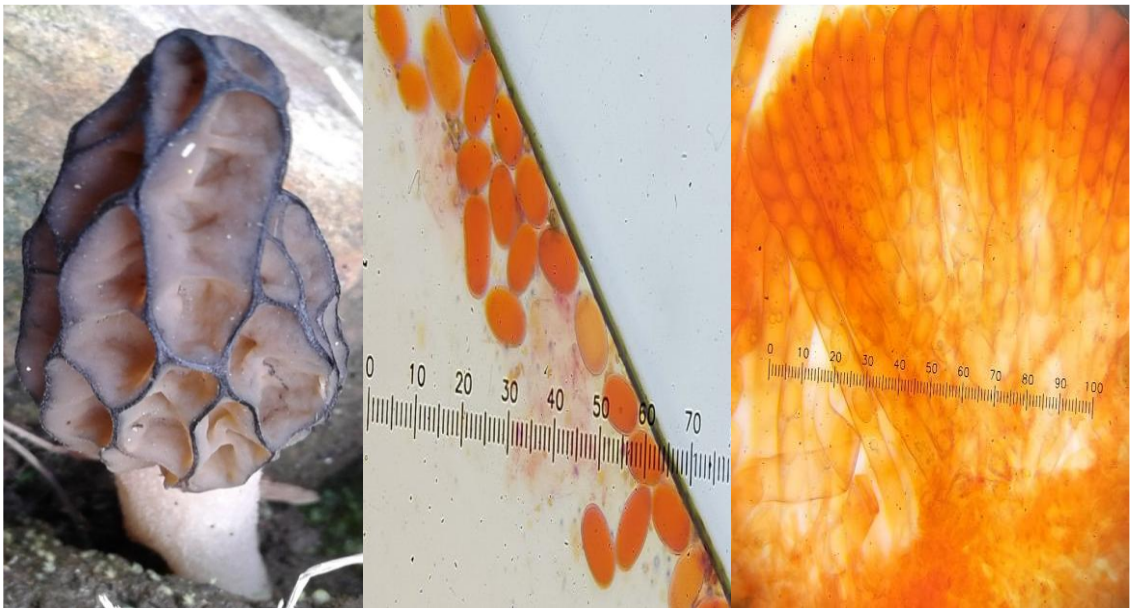

Figure 4. *Morphological and anatomical characters of M. brunnea collected from Chinar, District Swat, Khyber Pakhtunkhwa, Pakistan during 2018-2019*

Morpho-anatomical characteristics of Morchella angusticeps Peck.

M. angusticeps is a higher-growing plant that is primarily found in coniferous woods. It is typically connected with higher plants. Dark brown soil with a high moisture content and humus-rich composition. The growth season lasted from April until May. *Table 1* displays the soil parameters, geographical circumstances, and associated flora of M. angusticeps. The stalk measured 4-6 cm in length and 1.5–2 cm in width. It was upright, tube-shaped, clavate-shaped, dull, off-white, and pale yellow in color, with a significantly enlarged base.

Pileus were measured to be 0.5 to 1 cm in diameter, round or sub-conical in shape, 3 to 4.5 cm long and 2 to 4 cm wide, initially greyish and turning brownish light to black in maturity, with a dome-shaped apex, no sinus, and a non-looping edge. The pits are deep, up to 25 mm in size, uneven, elongated, and occasionally spherical, with an

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extended pit liner. There are 17–24 vertical ridges that range in color from brown to dark smoky, with a smooth edge and a thickness of 1–2 mm. When they mature, they rupture and turn dull brown to dark brownish. Many horizontal ridges run from the base to the top of the vertical ridges, giving the appearance of being incomplete (*Fig. 5*).

Ascospores are egg-shaped, double smooth walled, hyaline, and have a comparable composition. Their print ranges from cream to white and they measure 26.65–27.68 µm × 16.2–19.3 µm. Asci are cylindrical, eight-spored, 199.14–248.18 µm long, and 19.63–21 µm broad. They have ascospores grouped sidewise within each ascus (*Fig. 5*).

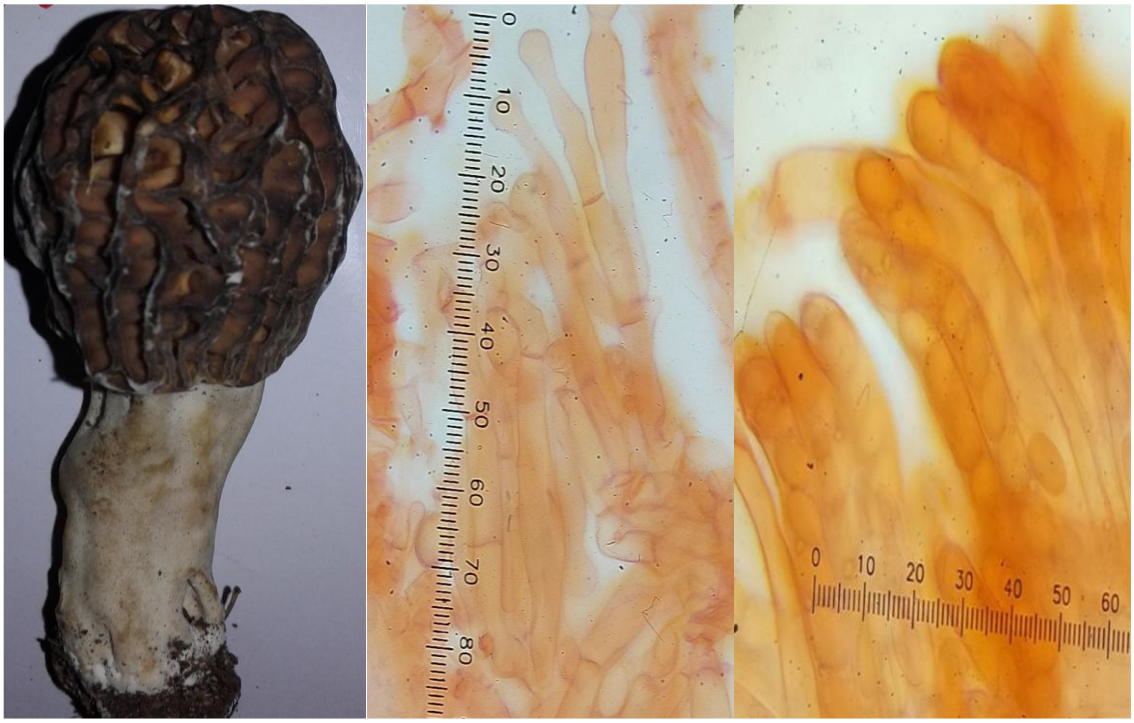

Figure 5. *Morphological anatomical characters of M. angusticeps growing collected from Miandam, District Swat, Khyber Pakhtunkhwa, Pakistan during 2018-2019*

Morpho-anatomical characteristics of Morchella esculenta clade

The *Morchella* esculenta clade is found predominantly in evergreen forests at high elevations beneath pine needle forests. The humus-containing soil has a dark brown color and is wet. During April and May, they can be seen growing alone or in a cluster. *Table 1* displays the soil characteristics and geographic circumstances of the unnamed M. esculenta clade along with the related flora.

At maturity, the stems are hollow, clavate, erect, or occasionally twisted. They are dull, off white, and pale yellow in color, measuring 4 to 5.5 cm in length and 2 to 3.5 cm in width. At maturity, pileus have an elongated or ovoid shape, are columnar, 7 cm long and 3 to 5.5 cm wide, and are initially greyish to blackish before turning pale yellow with a rounded apex dome, absent sinus, and non-looping edge. At first, pits are big, deep, and elongated brownish, but as they mature, they become pale yellowish, irregular, and occasionally form ladder-shaped pits with extended pit linings. When ridges reach maturity, they change from being brown to becoming yellowish in color, frail and delicate, and wrinkled in 18 to 28 vertical ridges, Numerous horizontal ridges

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emerge as a ladder inside the vertical ridges, extending from base to top and having an unfinished, concave appearance (*Fig. 6*). Asci are ranged from 232.6 to 318.2 µm long and 21.53 to 25.57 µm broad; rod-shaped, eight spores, ascospores side wise organized within each ascus; ascospores are leaving cream to white print; 14.25 to 27.2 µm long and 9.41 to 18 µm wide; oval shaped, double smooth walled, transparent, similar content (*Fig. 6*).

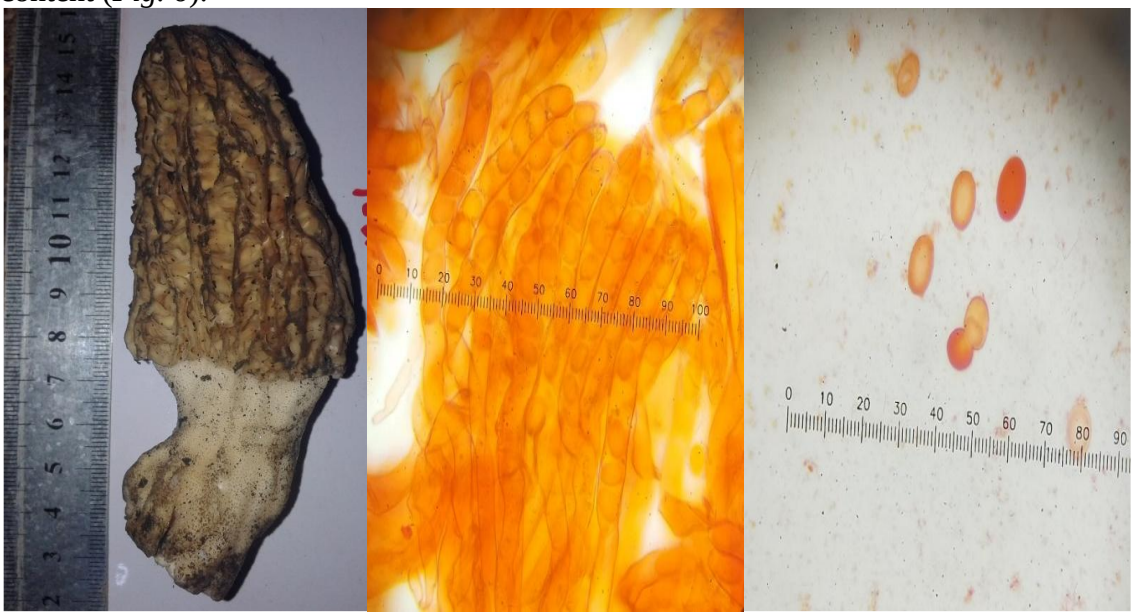

Figure 6. *Morphlogical and anatomical characters of M. esculanta clade (unidentified) collected from Chinar, District Swat, Khyber Pkhtunkhwa, Pakistan during 2018-2019*

Morpho-anatomical characteristics of Morchella (unidentified)

At high altitudes, primarily evergreen forests with pine needles are home to *Morchella*. The soil has a dark brown hue and is wet and rich in humus. emerge in April and May and can grow alone or in a cluster. *Table 1* displays the soil characteristics, geographic location, and related vegetation of *Morchella* (unidentified).

Table 1. *Soil parameters and geographical conditions of studied genotypes with associated flora*

S. No	Botanical name	Geographical coordinates	N kg/mg	P Kg/mg	K Kg/mg	$CaCO_3$ %	Organic matter %	PH	Associated flora
01	*Morchella esculenta* (L.) Pers.	34° 41N 72° 26E	0.48	1.58	244	7.5	5.76	5.96	*Quercus dilatata, Diospyrous kaki, Berberis lyceum, Rumex dentatus, Viola* sp.
02	*Morchella vulgaris* (Pers.) Boud.	35° 02N 72° 35E	0.04	10.2	56	6.25	0.96	7.6	*Abies pindrow, Taxus baccata, Viburnum grandiflorum, Fragaria nubicola.*
03	*Morchella brunnea* M. Kuo	35° 02N 72° 35E	0.09	2.312	374	6.75	0.2	6.62	*Quercus dilatata, Diospyrous kaki, Berberis lyceum, Rumex dentatus, Viola* sp.
04	*Morchella angusticeps* Peck.	35° 02N 72° 35E	0.03	7.1	60	7.5	0.75	8.75	*Abies pindrow, Taxus baccata, Viburnum grandiflorum, Fragaria nubicola*
05	*Morchella esculenta clade* (unidentified)	35° 27N 72° 37E	0.03	34	140	8.75	0.69	7.5	*Cedrus deodara, Picea smithiana, Viburnum grandiflorum, Fragaria nubicola*

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06	*Morchella* (unidentified)	35° 27N 72° 37E	0.04	88	52	7.5	0.89	7.3	*Cedrus deodara, Picea smithiana, Viburnum grandiflorum, Fragaria nubicola*

Unknown *Morchella* stems are upright, deeply cleft, and clavate in shape. When fully grown, their color ranges from off white to creamy, measuring 2 to 3 cm in length and 2 to 4.5 cm in width. When mature, pileus are oblique and convolant to spherical in shape; when they are off white, they seem greyish, and at the apex, they are suppressed and oblique like, No sinus, non-looping margin, measuring 2.5–3.7 cm in length and 4-5.5 cm in width. Pits are big, deep, uneven, and occasionally form ladder-shaped pits with extended pit linings. At maturity, they become elongated and greyish. Ridges: when they reach maturity, they take on a dark greyish hue, become frail and delicate, wrinkle 18–28 vertical ridges, and have numerous horizontal ridges inside the vertical ridges that resemble a ladder and run from base to top, seeming incomplete and concave (*Fig. 7*). The ascospores, which are oval-shaped, double smooth walled, transparent, and have identical content, are leaving a cream to white print. The asci, on the other hand, are rod-shaped, 106.32 to 116.55 in length, and 6 to 7.8 µm in diameter. The spores are expelled from each ascus. Paraphysis are translucent, septate cylindrical, measuring 85.9 to 118.8 × 4.1 - 5.5 µm (*Fig. 7*).

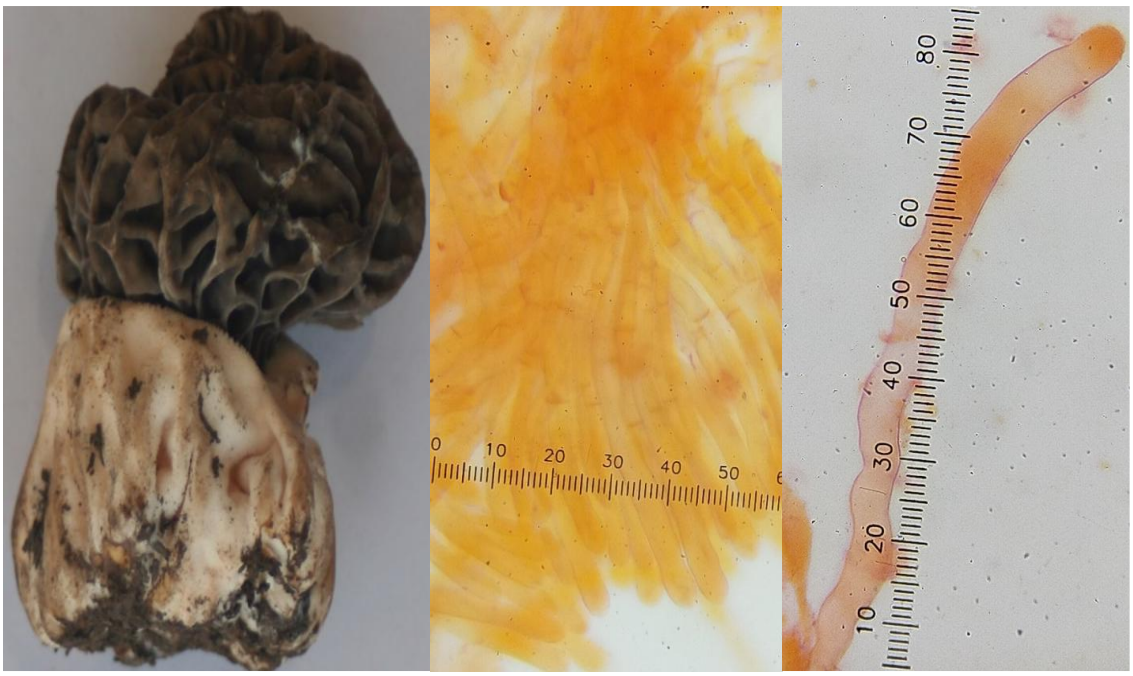

Figure 7. *Morphological and anatomical characters of Morchella (unidentified) collected from Kalam, District Swat, Khyber Pakhtunkhwa, Pakistan during 2018-2019*

Molecular characterization

The edited ITS region (including ITS-1, 5.8S, ITS-2) of the voucher specimen *UMH*Ch-7 composed of a total of 720 nucleotides sequence. The modified sequence's nucleotide BLAST search revealed a 99% match with *Morchella* elata (*Table 2*). A 99% alignment score was obtained from multiple alignments (using clustal W omega) across

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35 sample sequences that were downloaded from the NCBI Gene Bank. These sequences included M. elata (EF062475.1, KP670929.1), M. conica (GQ304964.1), M. deliciosa (LC028422.1), M. brunnea (LC028422.1, MG976329.1, MH198756.1), and M. septentrionalis (MT373944.1). With our sample sequence, there was not a single 100% alignment score. Three techniques were used for the phylogenetic analyses: maximum parsimony, neighbor joining, and greatest likelihood. Using a total of 36 ITS nucleotide sequences, the phylogenetic connection was inferred, and Verpa bohemica (MH423878.1) and Gyromitra esculenta (MT373906.1) were employed as outgroup taxa. Maximum Likelihood was the best evolutionary model built using the aligned data. Our sample sequence had a distinct lineage that was more closely related to the *Morchella* brunnea group, according to the analysis that revealed multiple clades. Additionally, the morphological characteristics agreed with the molecular data (*Fig. 8*).

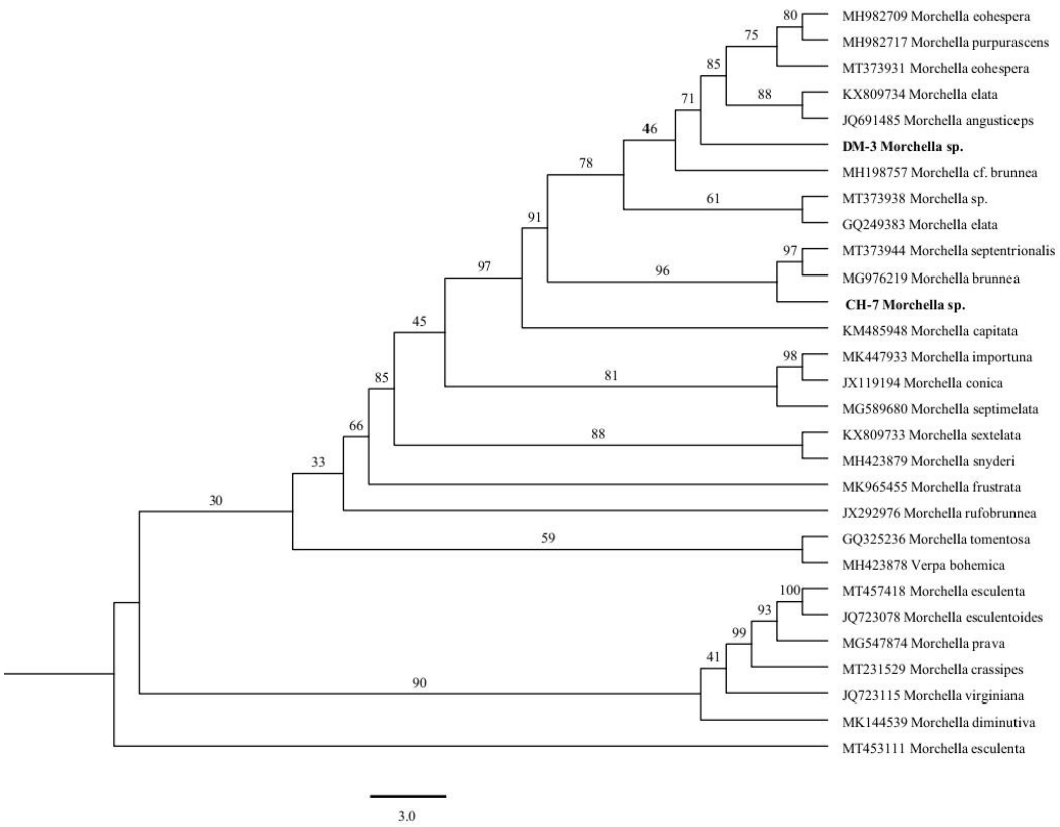

Figure 8. *Phylogenetic analyses of M. angusticeps and M. brunnea using maximum likelihood analysis through MegaX using Tamura-Nei model*

A total of 682 nucleotides made up the modified ITS region (containing ITS-1, 5.8S, and ITS-2) from the voucher specimen UMHMd-3. The modified sequence's nucleotide BLAST search analysis revealed 99% matches to both *Morchella* esulenta and *Morchella* elata (*Table 2*). Upon downloading 27 sequences from the NCBI Gene Bank, multiple alignment data for the samples revealed a 100% alignment score for 4 sequences: M. eohespera (MH982709.1), M. elata (GQ22847.1), and M. angusticeps (JQ691485.1). Three techniques, including maximum parsimony, neighbor joining, and maximum likelihood, were used to conduct phylogenetic studies. Maximum Likelihood was the most suitable evolutionary model as determined by the aligned sequence data.

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Using a total of 28 ITS nucleotide sequences, the phylogenetic connection was inferred, and Verpa bohemica (MH423878.1) was employed as an outgroup taxon. The final dataset contained 1408 locations in total. Several clades were identified by the study; however, our sample included a distinct lineage that was most likely connected to *Morchella* angusticeps. The morpho-anatomical characteristics of the specimens that were gathered provided additional support for the sample data (*Fig. 8*).

Table 2. *BLAST alignment with matching taxa*

Matching taxa	Accession No	Total score	Query Cover	E value	Per. Identity
Morchella sp. Mjb.	MT373929.1	1291	100%	0.0	99.03%
Morchella elata	EF080996.1	1288	100%	0.0	99.03%
Morchella angusticeps	AJ544203.1	1282	99%	0.0	98.89%
Morchella brunnea	MG547873.1	1280	100%	0.0	98.75%
Morchella elata	GQ228470.1	1280	99%	0.0	98.88%
Morchella esculenta	MH014730.1	1166	99%	0.0	99.85%
Morchella elata	GQ228471.1	1249	99%	0.0	99.85%
Morchella elata	EF017946.1	1247	99%	0.0	99.85%
Morchella eohespera	KT819370.1	1251	99%	0.0	99.85%
Morchella purpurascens	GU551438.1	1251	99%	0.0	99.85%

Discussion

The current study's findings advanced scientific knowledge of the richness and taxonomy of *Morchella* species in Pakistan's Khyber Pakhtunkhwa District Swat. The goal of this study was to gather, classify, and conserve a large number of *Morchella* species that were flourishing in the District Swat region. Fifteen specimens in total were gathered from the three principal locations located in the Upper and Lower Swat areas. To identify the gathered species, morphological traits particularly taxonomic traits were utilized. Five distinct species were found in the current exploratory study. These species are classified into two distinct clades: the Esculenta clade (M. esculenta, M. vulgaris, & M. esculenta clade), usually known as yellow morels, and the Elata clade (M. brunnea & M. angusticeps), often known as black morels.

Certain physical and ecological characteristics of the *Morchella* species that were examined in this study point to important taxonomic characteristics that are useful for identifying different species. Several important morphological and ecological (habitat and habit) characteristics of the species were recorded in the current investigative study. Our findings showed that the taxonomic characteristics previously described in the published study served as the primary basis for the identification of morphological species (Kanwal et al., 2011). Our findings were in line with the published study, which demonstrated that several characteristics—such as pileus structure, color, ridge pattern, pit depth, stipe texture, and attachment to the cap were useful in describing the identification of *Morchella* species (Masaphy, et al., 2010).

According to earlier research, a number of species' phenotypic characteristics are largely determined by micro morphological features, including the spores' shape and surface, paraphyses, apex and septation, sterility of the ridges, and stalk cortex (Kuo, 2017; Loizides, et al., 2015). Our findings also revealed that the sizes, shapes, and paraphyse septation of collected specimens are very helpful in their identification.

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According to a previous study, the length of *Morchella* species' spores ranged from 20 to 25 μm, and occasionally, different sizes of the same species were found when they were collected from different sites (Clowez, 2014). We also observed in this investigation that there was a noticeable difference in the forms, sizes, and wall thicknesses of spores within the same species. The length of each spore ranged from 25.7 to 27.7 μm. The findings on spore sizes showed that specimens with voucher number UMHKm-1 have spores that range in length from 14.25 to 27.2 μm, which is comparable with spore sizes described in earlier studies (Loizides et al., 2016).

Based on morphological characteristics, Clowez (2014) identified just two major species of *Morchella*: *Morchella esculenta* and *Morchella angusticeps*. After comparing their published sequences with those obtained from the Gene Bank, Richard et al. (2015) conducted a phylogenetically analysis of the 47 esculenta and 60 elata clade collections. Six of them were synonyms for previously identified *Morchella* species. Numerous research have indicated that the ecological parameters are important for morel species identification (Loizides, 2020). The total diversity and distribution of the mushroom species are also influenced by the surrounding vegetation and geographic location. It was discovered that morels were closely related to the Pinus, Quercetum frainetto-cerris, and Calamintho grandiflorae-Fagetum groups (Karadelev et al., 2009). According to certain studies, certain mycorrhizal species, such as morels, have demonstrated a strong relationship with vascular plant species (Chauhan et al., 2014; Raut et al., 2019). According to our findings, within the two sampling localities, the morel species exhibited close associations with Taxus baccata, Abies spindrow, Juglans regia, and Pinus wallichiana; in Site-1 (Chinar, Swat), however, associations were also reported by Hussain et al. (2020) with Quercus dilatata, Diospyros kaki, and Populus spp.

Ascocarp morphology is the primary basis for the traditional methods used to identify species in the *Morchella*ceae family, yet they have shown to be extremely confusing. Consequently, it was thought that phylogenetic analysis offered a more trustworthy approach to identifying species. Current phylogenetic analyses have incorporated the variance observed in ribosomal RNA gene (rDNA) sequences, particularly in the ITS region (Loizides, et al., 2015). Two of the collected species were identified by unique morphological characteristics and ITS-based genetic sequences.

Loizides et al. (2016) have reported using this method to demonstrate the diversity of *Morchella* species in Cyprus and throughout the Mediterranean region. Eleven species were confirmed by molecular research; of these, eight are members of the Elata clade, two are of the esculenta clade, and one is placed in the rufobrunnea lineage. The high degree of physical variation and variations in the macro and micro morphological characteristics observed during their life cycle and fruiting maturation, as noted by traditional taxonomic literature, frequently highlight the difficulties encountered during the identification process (Clowez, 2014; Loizides et al., 2015).

According to Buscot et al. (1996), the ITS region of the rRNA gene differentiates itself from interspecific species and showed a significant degree of variation in its taxonomically defined species. According to a reported investigation, the ITS gene data was also utilized to differentiate between the known phylogenetic species of *Morchella*; nevertheless, the ITS region is unable to satisfy the requirements for intraspecific modification (Du, et al., 2012). The inability to distinguish the closely related species was indicated by the low variability in the ITS region. This is because, whereas multiple

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locus base sequences have consistently shown success in species identification, single locus sequences can present challenges in certain cases (Roe, et al., 2010).

Conclusion

The genus *Morchella* has gained significant global relevance due to its great commercial and scientific value. In 2018 and 2019, a total of 15 distinct *Morchella* species were collected from three localities (Chinar, Miandam, and Kalam) in District Swat, Khyber Pakhtunkhwa, Pakistan, for the present study, which was planned due to its high medicinal value. Understanding the taxonomy of morel species may be aided by phylogenetic analyses based on DNA sequences, given the significant physical variability seen in *Morchella* species. Based on morpho-anatomical traits, four species of collected morel specimens (M. esculenta (Linn.) Pers., M. vulgaris (Pers.) Boud., M. angusticeps Peck., and M. brunnea M. Kuo, sp. nov) were identified.

However, the internal transcribe spacer sequences (ITS-1 and ITS-2) based on molecular approach were used to identify the two morphologically distinct species (*Morchella* (unidentified) and M. esculenta clade). The current study aims to identify certain locally growing morel ecotypes based on morpho-anatomical traits and ecological criteria, as there has been limited work done in Pakistan recently.

Competing interests. All the authors declare no conflict of interest.

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