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Original Research

Molecular Identification of New Recorded Morels from Kashmir, Western Himalayas

Tariq Saiff Ullah[1, 2*], Syeda Sadiqa Firdous[2], Muhammad Manzoor[3], Syed Waseem Gillani[3], Wayne Thomas Shier[4], Muhammad Nauman Khan[5, 6], Alevcan Kaplan[7], Hesham S. Almoallim[8], Mohammad Javed Ansari[9]**

[1]Department of Botany, University of Kotli, Azad Jammu and Kashmir, Kotli, 11100, Pakistan
[2]Department of Botany, University of Azad Jammu and Kashmir (UAJK), Muzaffarabad, 13100, Pakistan
[3]Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan
[4]Department of Medicinal Chemistry, College of Pharmacy, University of Minnesota, 308 Harvard St., SE, Minneapolis MN 55455, USA
[5]Department of Botany, Islamia College Peshawar, 25120 Peshawar, Pakistan
[6]University Public School, University of Peshawar, 25120 Peshawar, Pakistan
[7]Batman University, Department of Crop and Animal Production, Sason Vocational School, Batman, 72060, Turkey
[8]Department of Oral and Maxillofacial Surgery, College of Dentistry, King Saud University, PO Box-60169, Riyadh -11545, Saudi Arabia
[9]Department of Botany, Hindu College Moradabad (Mahatma Jyotiba Phule Rohilkhand University Bareilly) -244001, India

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Abstract

Morels are edible fungi collected for food and medicines worldwide. In the present study, samples of *Morchella* were collected during consecutive fungal surveys in 2017-2021 from Neelum Valley, Azad Jammu, and Kashmir. The specimens were identified as *Morchella costata* Pers., *Morchella conica* Krombh., Naturgetr. Abbild. Beschr., *Morchella deliciosa* Fr., *Morchella elata* Fr., and *Morchella tridentina* Bres., after phylogenetic sequencing using the nuclear ribosomal DNA (*nr*DNA) barcoding technique along with morpho-anatomical analyses. The highest stipe length was found in *Morchella elata* (6-8.8 cm), and the lowest was noted in *Morchella deliciosa* (2-3.5 cm). The highest pileus length was found in *Morchella costata* (6.9-12 cm), and the lowest was noted in *Morchella conica* (2.5-4 cm). The highest spore size was observed in *Morchella costata* (26.5-33 × 12-16 µm), and the lowest was observed in *Morchella conica* (7.2-7.5 × 12.5-14.6 µm). This is the first detailed study on morels, based on molecular phylogeny from the State of Azad Jammu and Kashmir (AJK), Pakistan. Variations have been observed in both qualitative and quantitative characters. The results reinforced the significance of the morpho-anatomical features of the genus *Morchella* and will be used as an aid for valuable taxonomic tools in the systematics of morels. The accurate identification of morels species is very crucial for the isolation of bioactive compounds, and these compounds play a vital role in the drug

*e-mail: tariqbot89@gmail.com;
**e-mail: sgillani@bs.qau.edu.pk

discovery. So, we recommend a comprehensive exploration of Morels in the Western Himalayan Forests by using advanced molecular approaches and the isolation of bioactive compounds because this genus is still unexplored in the context of pharmaceutics.

Keywords: Ascomycetes, morels, M*orchella*, Neelum Valley, Western Himalayas

Introduction

Morels (*Morchella* spp.) are edible macro-fungi, well known due to their proteins, fibers, minerals, and bioactive contents [1, 2]. They are characterized by a distinct honeycomb-like hymenium on the hollow fruiting body and typically fruit in the spring (at least in the temperate zone), except for some autumn species [3]. Morels are important because of their scientific and economic worth, in addition to their nutritional and therapeutic qualities [4-6]. Morels occur in a variety of environments worldwide. In Asian countries, mature fruiting bodies of morel are used in traditional herbal medicines [7]. Morels are a great source of immunostimulants and anticancer agents and are used extensively in soups and cookeries because of their distinct flavor and high nutritional content [8].

Taxonomically, *Morchella* is a polymorphic and problematic genus within the Pezizales. Some authors have identified more than 50 species within the genus *Morchella*, whereas others consider only three species [9]. Consequently, the genus is currently being studied using integrative scientific techniques that combine molecular data with morphological, ecological, and distributional information [10]. Recent research has shown that many morel species are endemic, frequently restricted to certain regions or continents, in contrast to earlier theories that the genus is made up of a small number of worldwide species. All currently known morel species are included in three major categories: the Rufobrunnea clade (sect. *Rufobrunnea*), the Esculenta clade (sect. *Morchella*), and the Elata clade (sect. *Distantes*) [11]. Numerous aspects of morel biology, such as their reproductive methods, interspecific hybridization, nuclear and mitochondrial genome analysis, transcriptome analysis, physiological and biological traits, trophic modes, and the dynamics of the microbial community related to morel cultivation, have recently yielded significant new insights [3]. Morels were used to cure a range of stomach issues in China, as documented in the renowned pharmacological treatise "Compendium of Materia Medica," penned by Li Shizhen during the Ming Dynasty. Some of the most significant fungi in the Himalayan range from an economic, sociological, and ethnomycological standpoint are morels, also known as "Guchhi" in the Indian market [12].

Therefore, molecular methods were used in addition to morphological characterization to support the traditional taxonomy of fungi. The best methods for identifying mushrooms to begin phylogenetic investigations have been found to be sequencing the ITS area, which is composed of the 5.8 s rRNA gene, along with, SSU, LSU, RNA polymerase, and RAPD-PCR study [8]. Molecular phylogeny has confirmed more than 70 species of the genus *Morchella* worldwide [13]. Prominent and taxonomically important features of *Morchella* include the color and configuration of the ridges, pits, discoloration, bruising, the attachment of the cap to the stem (sinus), and the length of the stem. Important microscopic characters are spore size, shape, and ornamentation, the paraphyses, the ectal esxcipulum, and the stipe hairs [14-16]. Monophyletic status for Morchellaceae and the genus *Morchella* has been confirmed by O'Donnell et al. [17] using *nr*DNA. Phylogenetic studies, including sequencing of Internal Transcribed Spacer (ITS1 & ITS4) regions, small subunit-coding sequences (SSU), large subunit-coding sequences (LSU), and random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) are suitable techniques for morel identification [18-20].

In Pakistan, only five species of *Morchella* have been confirmed by DNA sequencing: *Morchella pakistanica* [21], *Morchella pulchella* [22], *Morchella crassipes, Morchella spongiola*, and *M. elata* [23]. In the past, different *Morchella* species, namely *M. crassipes* (Vent.), *Morchella esculenta* (L.) Pers., *M. elata*, and *Morchella semilibera* DC. have been reported solely based on morphological identifications [24, 25]. In-depth research on the genetic diversity and identification of morels growing in the western Himalayan region of Kashmir Forest has not been done. The major objective of the present research was to identify the morels from the Western Himalayan region AJK, by using a morphoanatomical and phylogenetic approach. In this study, we report and describe *M. conica*, *M. costata*, *M. elata*, *M. deliciosa*, and *M. tridentina* for the first time from the state of Jammu and Kashmir based on their morpho-anatomical and molecular bases.

Materials and Methods

Sampling Sites

The study area lies in District Neelum Valley, Western Himalayan region, Azad Jammu and Kashmir, Pakistan, within 32º 23″ to 32º 87″ North latitude and 74º 10″ to 74º 81″ East longitude at an altitudinal range of 1400 m in the south to 5200 m in the north. The area is entirely hilly and covered with coniferous forests. The vegetation of the study area consists of blue pine (*Pinus wallichiana* A.B Jacks.), fir, (*Abies pendrow* Royle ex D. Don), Deodar (*Cedrus deodara* Roxb. G.

Don.), Bird charry, (*Celtis eriocarpa* Decne, *Aesculus indica* (Wall. Ex Cambess.), Hook and shrubs, *Viburnum grandiflorum* (Wall. Ex DC.), and *Indigofera heterentha* (Wall.) [26, 27].

The average annual rainfall throughout the region is 98 mm. The average snowfall in winter is 54-48 cm in the upper regions of the valley [28]. Samples of morels were collected from the selected sites at Dawarian, Surgon, Sharda, Arang Kel, and Taobut during 2017-21 through consecutive field surveys (Fig. 1).

Morpho-Anatomical Characterization

Samples of *Morchella* species were collected and tagged during the collection. Each sample was photographed using Digital Camera (Minolta, 20 MP with 35X lens donated by IDEA WILD, USA). Each spaceman was packed into a separate bag to avoid spore contamination. Macro-morphological characters, shape, size, and color of ascomata (stipe, stalk, etc.) were recorded. After cleaning, the fruiting bodies were dried for anatomical and molecular studies. Fruiting bodies with more moisture were dried by making small pieces. For the anatomical study, slides were prepared in 5% KOH (w/v) and 1% Congo red. Anatomical features, size, shape, and color of ascospores, asci, and excipulum were recorded using a light microscope (MX4300H, Japan) in Fungal Biology and Systematic Research Laboratory, Institute of Botany University of Punjab, Lahore, Pakistan. Plant and fungi names with authorities were retrieved from the International Plant Name Index (http;//www.ipni.org) and Index Fungorum (http;//www.indexfungorum.org), respectively. Specimens studied during this research work have been deposited, after freezing treatment at –80ºC for 15 days, in the herbarium of the University of Kotli Azad Jammu and Kashmir.

DNA Analysis and Phylogeny

DNA extractions were performed from dried fruiting bodies using 2% CTAB buffer with slight modifications [29]. DNA extraction was confirmed through Gel Electrophoresis using 1% Agarose gel for 30 min at 70V [30]. Further amplification of *nr*DNA and the internal transcribed spacer region (ITS) by PCR using a primer combination. ITS1F (5, -CTTGGTCATTTAGAGGAAGTAA-3,), and ITS4 (5,-TCC TCCGCT TAT TGA TAT GC-3,) were proposed by Janowski et al. [31]. Both the forward and reverse sequences were used to construct the final sequence using BioEdit ver. 7.2.5 [32]. Most similar sequences were retrieved from the National Center for Biotechnology Information (NCBI) to compare the sequences generated in the study [33] by using the Basic Local Alignment Search Tool (BLAST). All the sequences were included to construct the final phylogram [34-36]. Multiple sequences were aligned using the online MUSCLE alignment tool

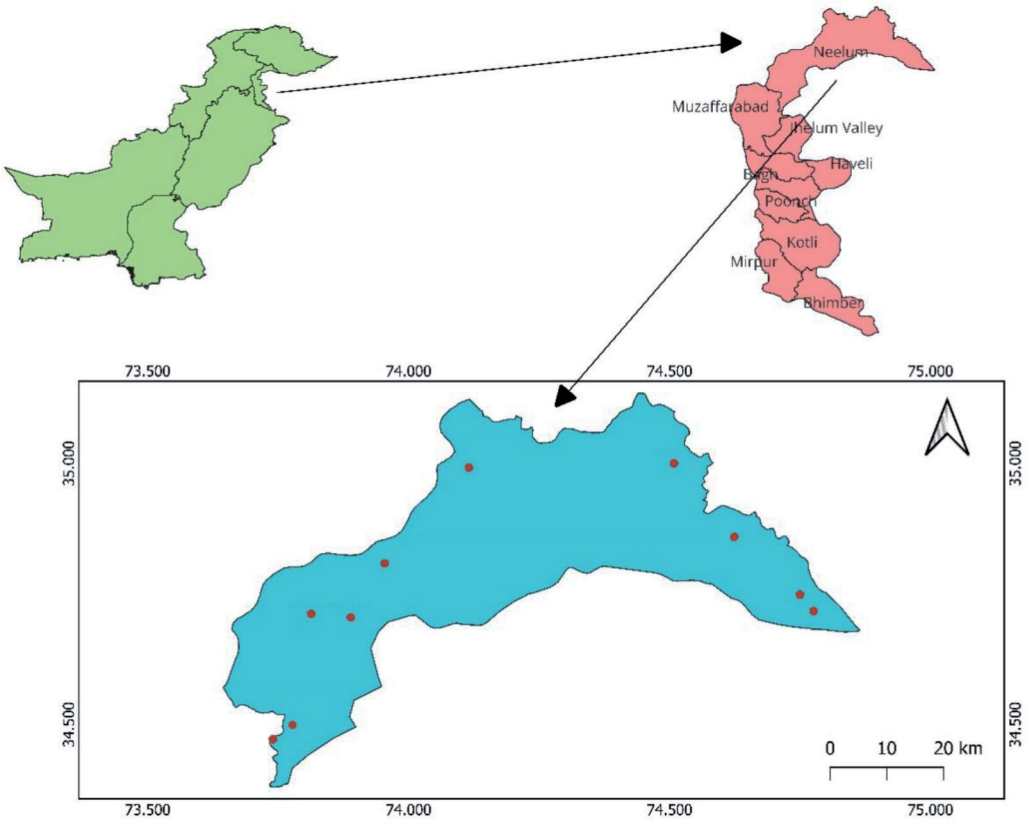

Fig. 1. Map of study area and collection sites (Neelum Valley, AJK).

Table 1. List of Morels species with collection sites, collection date, and accession numbers.

Sr. No	Name of species	Habitat	Collection site	Altitude (m)	Date of collection	Gene Bank Accession Numbers
1.	*Morchella conica* Krombh., Naturgetr. Abbild. Beschr.	Gregarious on soil, under *Pinus wallichiana,* and at decayed hardwood and organic matter.	Azad Jammu and Kashmir Dawarian (Western Himalayan part)	1996	12-04-2017	MT950315
2.	*Morchella costata* Pers.	Growing under *Cedrus deodara* and *Quercus incana,* Sporophores in groups and scattered, late April-June	Azad Jammu and Kashmir Dawarian and Surgon	1981, 2205	24-05-2018, 25-05-2018	Under Process
3.	*Morchella deliciosa* Fr.	Fruiting bodies grow under conifer forests and *Quercus* trees during April-June.	Azad Jammu and Kashmir Dawarian	1959	24-05-2018	MW558089
4.	*Morchella elata* Fr.	Scattered and alone, grow under mixed conifer forests during April-May	Azad Jammu and Kashmir Dawarian, Arang Kel (Western Himalayan part)	1936, 2034	12-04-2017, 08-05-2017	MT584846 & MT977069
5.	*Morchella tridentina* Bres.	Mycorrhizal, alone, scattered, or gregariously under hardwoods, specifically under *Pinus wallichiana,* during late spring	Azad Jammu and Kashmir, Surgon and Dawarian	1898, 1956	28-04-2017, 12-05-2018	MT584841 & MT957957

(http://www.ebi.ac.uk/Tools/msa/muscle) [37]. The phylogram was constructed several times in RAxML-HPC2 using the XSEDE tool (8.2.10) at 1000 bootstrapping to get the best bipartition results.

Statistical Analysis

For the visualization of the phylogram, Figtree ver. 1.4.2. software was used. Further tree annotations were added by using Adobe Illustrator CS10 [38]. DNA (ITS sequences) were deposited in Genbank, and accession numbers were obtained (Table 1).

Results

Morpho-anatomical features of *Morchella* species recorded during the study are given below.

Taxonomical Description and Phylogenetic Analysis of *M. conica*

Pileus: 2.5-4 cm long and 3-4.8 cm wide, conical, blunt to pointed cap, pits regular, medium to long and broad, sometimes irregular. Ridges are longitudinal and parallel from the base to the top. Stalk: thick, short, and hollow; 2-4.5 cm wide; 3.5-4.5 cm long. Ascospores: [50/1/1], 7.2-7.5×12.5-14.6 µm, ellipsoidal, smooth contents, homogenous, hyaline in 5% KOH, and light brown in Congo red solution. Asci: 247.3-258.3×10-12.5 narrow, long, 8 spores, cylindrical, blunt, narrow base, light brown to dark in 5% KOH, and Congo red. Paraphyses: 8.5-13 µm wide, 11.2-21.7 µm long, septate, filamentous, flattened, hyaline in 5% potassium hydroxide (Fig. 2A, B, C).

The results of DNA analysis have shown that the amplification of internal transcribed regions (ITS1 & ITS4) primers produced 666 bp (T-18) sequence. ITS sequence analysis compared with the NCBI database revealed that these sequences showed 100% query coverage and 99% similarity with *M. conica* (GQ304964) and 98% *M. conica* (LC069378). Clade *M. conica* with a 100% bootstrap value contains *M. conica* (MT950315, T-18), closely matched with (AM269501, MG431337, and GQ304964). Accession number MT950315 was obtained after the submission of the nucleotide sequence to the Genbank database. Sequences used in the construction of the phylogenetic tree showed maximum similarity with the sequence (MT950315). Combined with the morphological characteristics of the fruiting body and phylogenetic analysis, the result showed that this strain is *M. conica*. *Verpa bohimica* (AJ698479) was used as an outgroup taxon in the ITS dataset.

Taxonomical Description and Phylogenetic Analysis of *M. elata*

Pileus: 6.1-7.3 cm long, 4.9-6 cm wide, globular, elongated to cylindrical, and sometimes slightly pointed

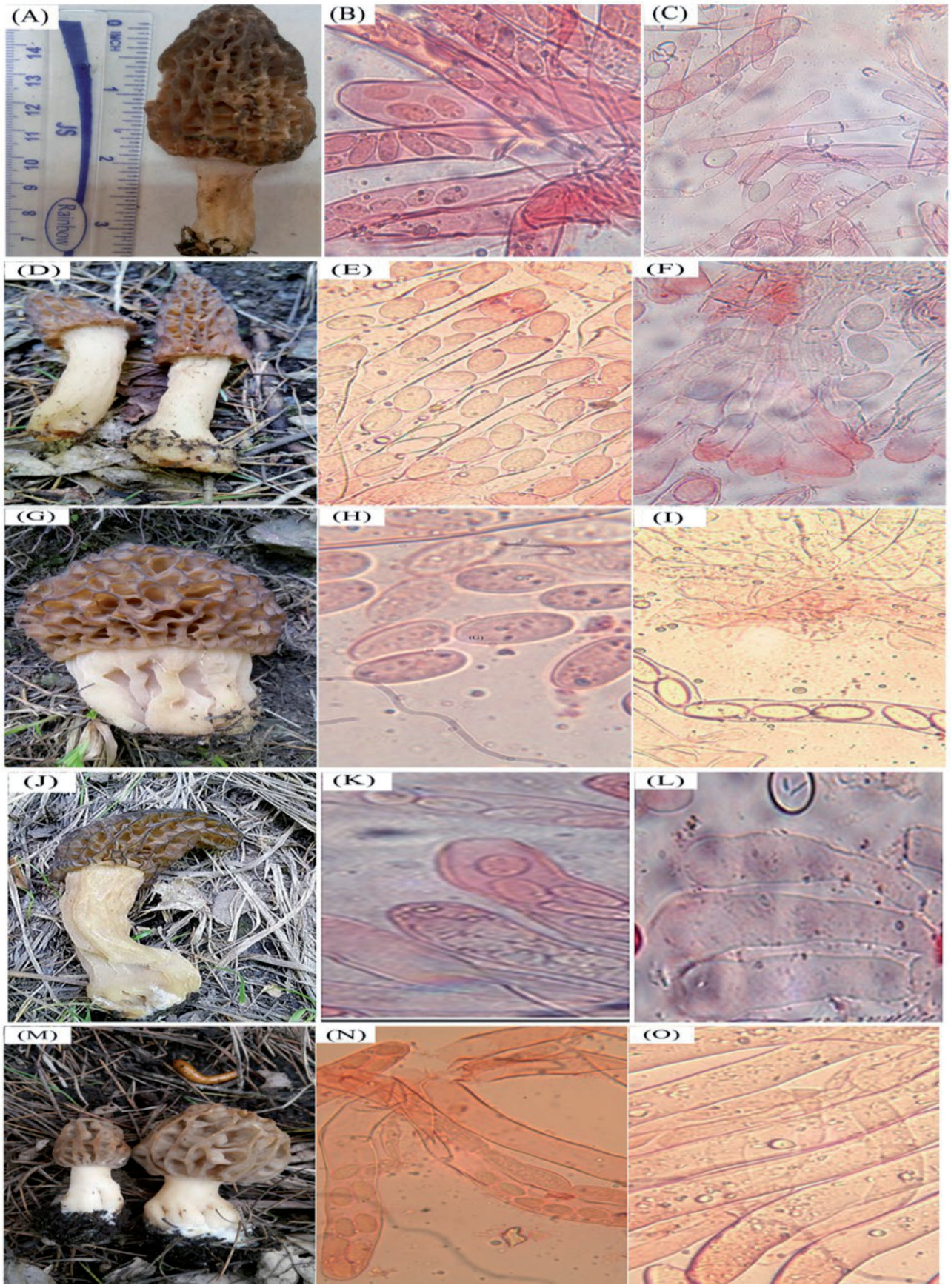

Fig. 2. **(**A**)** A fruiting body of M. conica T-18, (B) ascospores, (C) paraphyses, (D) the fruiting bodies of M. elata T-02 & T-09, (E) Asci with ascospores, (F) Excipulum, (G) the fruiting body of M. deliciosa T12, (H) Ascospores, (I) Ascus with ascospores, Scale Bars; A = 4.5 cm, B = 12 µm, C = 8.8 µm, D = 11.2 µm, (J) the fruiting bodies of M. costata T04, (K) Asci with ascospores, (L) Excipulum, Scale Bars; A = 5 cm, B = 4.3 cm, C = 12µm, D = 11.6 µm, (M) the fruiting bodies of M. tridentina T-05 and T-06, (N) Asci, (O) paraphyses.

cap. The main ribs of the pileus are thin, parallel, and connected by narrow, transverse, and oblique ridges. Elongate, irregular, deep, and tetragonal pits are present between the ribs. The stalk is usually inflated into a bulbous base, 2-8 cm thick and 6-8.8 cm long. The color was noted as brown to reddish-purple. The taste is pleasant. Ascospores (1/1/40). 26.5-33×12-16 µm, broadly ellipsoidal, and smooth with homogeneous contents, hyaline in 5% potassium hydroxide. Asci 198.7-210×10-15.5 µm; 8 spores cylindrical to narrow long base, hyaline in 5% KOH. Paraphyses: 4.8-6.1 µmwide, 125-167.3 µm long, filamentous, and septate, narrow to clavate at the base; hyaline in 5% potassium hydroxide.

The molecular amplification of internal transcribed regions (ITS1 & ITS4) of the samples T-02 and T-09 had primers that produced about 680 bp and 745 bp sequences, respectively. The ITS sequence match of the sequences generated in the study (MT584846, MT977069) has shown 100% query coverage and similarity with *M. elata* (GQ228471). Clade *M. elata* with a 100% bootstrap value contains (T-02: MT584846 and T-09: MT977069) along with its closet matching sequences (DQ257329, GQ228469, and KX809734). Sequences used in the construction of the phylogenetic tree showed maximum similarities with the sequences generated in this study (Fig. 2 D, E, and F).

Taxonomical Description and Phylogenetic Analysis of *M. deliciosa*

Pileus: 5.5-6.7×4.8-5.2 cm, elongated, bulbous, pits smaller, light brown cap. Stalk: short, thick, fine-rigged, elongated, and hollow, 2-3cm wide, 2-3.5cm long. The taste and odor were pleasant (Fig. 2 G, H, and I). Ascospores [40/1/1] 10.2-15.5 µm×8.5-11 µm, ellipsoidal, smooth contents homogenous, hyaline in 5% KOH, and light brown in Congo red solution. Asci 242.3-258.3× 63-82.6 narrow long up to 298 µm, wide up to 11.8µm, 8 spores, cylindrical, light brown to dark in 5% KOH and Congo red. Paraphyses, 8.5-13 µm, 35.5×48.5 µm wide up to 117.2 µm long, septate, filamentous, flattened, hyaline in 5% KOH.

Amplification of the LSU region of DNA produced a 657 bp sequence. The LSU sequence match with NCBI has shown 100% similarity with *M. deliciosa* (KM588022) and 99% *Morchella* sp. (LC028412) with a query cover of 100%. DNA sequence data were submitted to GenBank, and the accession number was obtained: MW558089. Clade *M. deliciosa* with a 100% bootstrap value contains *M. deliciosa* (LC028412, LC028418, and KM588022). The sequence generated in the present study has shown maximum similarity with the sequences in the clade.

Taxonomical Description and Phylogenetic Analysis of *M. costata*

Pileus, 3-6.5 cm long, 2-3.5 cm wide, conical, bulbous, broad, pits longer, light brown during the early

Table 2. Qualitative and Quantitative morphological characteristics of different species of the genus *Morchella.*

Sr. No	Name of species	Color of fruiting bodies	Shape of Pileus	Spore Morphology	Pileus (length× width) cm	Stipe (length) cm	Ascus Length and width µm	Size of spores µm
1.	*Morchella conica*	Black	Conical or blunt to pointed cap	Uniseriate, ellipsoidal, smooth	2.5-4×3.9-4.8	4.5-6	247.3-258.3×1012.5	7.2-7.5×12.5-14.6
2.	*Morchella costata*	Black	Conical, bulbous, broad, and spiral pits	Uniseriate, broadly ellipsoidal, smooth	6.9-12×4.5-5.4	5.8-7.4	198.7-210×10-15.5	26.5-33 × 12-16
3.	*Morchella deliciosa*	Light brown	Elongated, bulbous, pits smaller	Uniseriate ellipsoidal, smooth	5.5-6.7×4.8-5.2	2-3.5	242.3-258.3×63-82.6	10.2-15.5×8.5-11
4.	*Morchella elata*	Brown to reddish brown	Globular, cylindrical, slightly pointed	Uniseriate broadly ellipsoidal, smooth	6.1-7.3×4.9-6.4	6-8.8	198.7-210×10-15.5	26.5-33×12-16
5.	*Morchella tridentina*	Light brown to yellow	Elongated and cylindrical, with vertically arranged conical pits	Uniseriate, smooth, elliptical, contents homogeneous	5.6-9.8×5.1-6.3	4.5-6.5	22.5 to 33×198-215	18.5-25×12-18

stages of development, and dark brown at maturity. Stalk: thick, multi-rigged, short and hollow, 2-3.5 cm wide, 1.5-3 cm long. The taste and odor were pleasant. Ascospores; [45/2/2] 7-7.5×3.1-4.8 µm broadly rounded to ellipsoid, rough, and thickened contents heterogeneous, dark brown in 5% KOH. Asci, 66.7-79.5×11.2-13.9 µm, 8 spores, cylindrical, having a blunt, narrow base; light brown to dark in 5% KOH. Paraphyses, 9 to 14 µm wide, up to 58.5-71.5µm long, septate, filamentous, apices slightly curved, becoming hyaline in 5% KOH (Fig. 2 J, K, L).

The results of DNA analysis have shown that the amplification of internal transcribed regions (ITS1 & ITS4) by primers produced 892bp and 874bp sequences. ITS sequence match with NCBI has shown 99% similarity with *M. costata* (DQ257372) and 98% *Morchella* sp. (JQ691493). Clade *M. costata,* with 100% of the bootstrap value, made an association with DQ257332 and EF080997.

Taxonomical Description and Phylogenetic Analysis of *M. tridentina*

Pileus, 5.6-9.8×3.1-6 cm, wide, conical, elongated, and cylindrical; vertically arranged conical pits; medium ridges; slightly flattened at an early stage of growth; brownish grey to light brown and whitish; when mature, yellowish ridges; pale tan to pinkish-tan; attached to the stalk. Stalk: 1.5-4.5 cm high and 1.5-3.5 cm wide, equal, sometimes swollen at the base, whitish, bald, and hollow. Ascospores [50/2/1], 18.5-25x12-18 µm; elliptical and ovoid; smooth; contents homogeneous. Asci, 16.5-29 µm wide, up to 250-315 µm long, flattened, 8 spored. Paraphyses are polymorphic, cylindrical, with clavate to rounded apices, septate, and brownish in KOH. Paraphyses: 19 to 33 µm wide, up to 215 µm long, septate (1-2 septa at base), filamentous with slightly curved apices, and hyaline in 5% KOH (Fig. 2 M, N, O).

The amplification of the ITS1 and ITS4 regions of DNA produced 892 bp and 874 bp sequences. In initial BLAST through GenBank, our *Morchella* sequences generated in the study (T05 & T06) have shown 100% identity with the sequences of *M. tridentina* from Spain (KM587952) and (KM587964). DNA sequence data were submitted to GenBank, and accession numbers T-05 (NT584841) and T06 (MT957957) were obtained.

Discussion

All the studied *Morchella* species from the sampling sites in the District Neelum (34°37’ N and 73°56’ E) were found at an elevation between 1900 to 2300 m in temperate coniferous forests. The temperate forests of Neelum valley are spread over a larger area in western Himalayas and well-known hub for biodiversity [39].

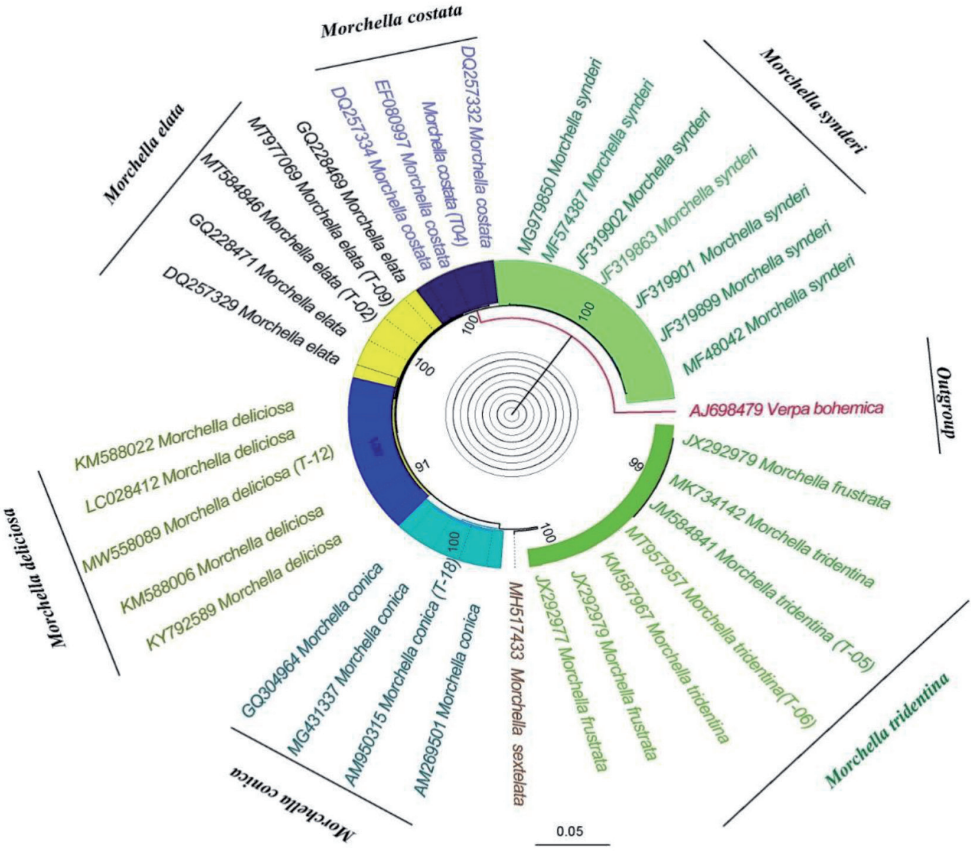

Fig. 3. A Phylogenetic tree separated the Morels into distinct clades based on molecular data.

These temperate forests are consisting of dominant plant species i.e., *Cedrus deodara, Abies pindrow, Pinus wallichiana, Aesculus indica, Acer caesium, Prunus padus, Parrotiopsis jacquemontiana, Viburnum grandiflorum, Picea smithiana, Taxus wallichiana, Quercus incana*, and *Berberis lyceum* [40, 41]. These forests provide a suitable habitat for the diversity of fungi including morel species. The state of Azad Jammu and Kashmir is blessed with various geographical regions and environmental conditions. Morels can be found in a range of forest types and exhibit symbiotic and saprotrophic mycelial dynamics [7].

In the present study, five species of *Morchella* have been described for the first time in the State of Jammu and Kashmir. Among these, *M. elata* has ascospores, 26.5-33×12-16 µm, broadly ellipsoidal with asci up to 198.7 µm, width up to 16.5 µm, cylindrical to narrow long base. It has slight differences from the *M. elata* studied by Ali et al. [23] in the Hindu Kush region of Pakistan. The specimens studied by Ali et al. [23] have a large-sized ascoma 6-9 cm higher than large-size hymenophores. The black morel of Kashmir has slight differences in morpho-anatomical characteristics from the previously described *Morchella* species by Kanwal et al. [42] from India. The difference observed could be due to variations in geographic, environmental, and edaphic characteristics, soil organic matter, and soil fertility. More variations occur among the same species of *Morchella* in a geographically isolated population [43]. *M. crassipes*, *M. esculenta*, and *M. deliciosa* produces white or yellow fruiting bodies, while *M. angusticeps*, *M. eleta*, and *M. conica* produce black fruiting bodies [42]. Various recent studies used molecular approaches for the correct identification of Morels because, due to slight differences in soil properties and environmental conditions, the morphology of Morels significantly varied among individuals of the same species [44]. Many morel species, including *M. rotunda*, *M. esculenta*, *M. elata*, *M. deliciosa*, *M. semilibera*, and *M. conica* have been described from various parts of Pakistan based on their morphological features [45]. On the other hand, *M. pulchella* has been reported based on molecular methods [46]. In this study, *M. tridentina* is characterized by its cap, 5.6-9.8×3.1-6 cm, wide, conical, brownish grey to light brown and whitish in color; ascospores, 18.5-25x12-18 µm; elliptical and ovoid; smooth; and asci, 16.5-29 µm wide, up to 250-315 µm long (Table 1). The results of the present study agree with the previous study on *M. tridentina* by Loizides et al. [13].

The taxonomic status of *M. tridentina* and its synonyms have been confirmed through combined morpho-anatomical and phylogenetic studies. The name *M. tridentina* was first proposed by Machuca [47] from Chile, and it is a poorly known name because it doesn't appear in any influential works of the twentieth century [48, 49]. Their material, studied from different regions, was analyzed by Richard et al. [50], Machuca et al. [51], and Clowez [52] through DNA sequence analysis. All the sequences submitted in the GenBank database named *M. frustrata* and *M. elatoides* are like *M. tridentina*. These taxa are synonyms of *M. tridentina* [50, 13]. Compared with the previous study on *M. tridentina*, morph-anatomical and molecular features [13] affirm this taxon is the first time reported in the western Himalayan region of the State of Jammu and Kashmir.

Previously, *Morchella pulchella* and *M. pakistanica* have been added to the fungi of Pakistan, identified based on a combined morpho-molecular approach [22, 23]. *M. elata*, *M. crassipes*, and *M. spongiola* are *Morchella* spp., which are the first time identified (molecular and micromorphological) species from Pakistan by Ali et al. [13]. Based on our morphology study in *M. costata,* ascospores are 7-7.5×3.1-4.8 µm broadly rounded to ellipsoid, with asci 66.7-79.5×11.2-13.9 µm cylindrical, having a blunt, narrow base. In *M. deliciosa,* ascospores are 10.2-15.5 µm×8.5-11 µm, ellipsoidal, smooth, having asci 242.3-258.3 µm×63 -82.6 µm, narrow long up to 298 µm, wide up to 11.8µm, and cylindrical (Table 2). Based on our morphology study in *M. costata*, ascospores are 7-7.5×3.1-4.8 µm broadly rounded to ellipsoid, with asci 66.7-79.5×11.2-13.9 µm cylindrical, having a blunt, narrow base. In *M. deliciosa,* ascospores are 10.2-15.5 µm×8.5-11 µm, ellipsoidal, smooth, having asci 242.3-258.3×63-82.6 narrow long up to 298µm, wide up to 11.8µm, and cylindrical (Table 2).

The phylogenetic tree (Fig. 3) separated the black morels *M. elata*, *M. conica*, and *M. costata* from *M. tridentina* and *M. deliciosa* into distinct clades. *Verpa bohemica* was used as an out-group taxon. A phylogenetic tree constructed based on ITS sequence analysis confirms the position of studied morels with their nearest match species in the database. The findings of the present study are also supported by previous studies, i.e., [3, 53-57]. The identification of *Morchella* species through morphological and anatomical characters using microscopic and phylogenetic techniques results in the addition of these morels in the western Himalayan region of Kashmir. Now, the number of *Morchella* species confirmed through molecular tools in the State of Jammu and Kashmir has increased to five.

The current research is the first attempt to observe the morphological and anatomical characters of the genus *Morchella* using microscopic and phylogenetic techniques from the state of Jammu and Kashmir. Variations among species have been observed in both qualitative and quantitative characters. Variations in these characters play a significant role in solving problems in the classification of species within the genus. The *Morchella* genus is still unexplored in terms of pharmaceutics. Correct identification of morel species is important to use in the isolation of bioactive contents to develop drugs in the future.

Conclusions

The temperate forests of Western Himalaya are hub of biodiversity as well as rich habitats for the morels. Different field visits were made to collect morels from various unexplored regions of the District Neelum from 2017 to 2021. All the species of morels were collected during the spring season. The collected species of the genus *Morchella* were identified by investigating morphological and anatomical features using microscopic and phylogenetic techniques. Significant variations were observed among the investigated species of *Morchella*, and these variations play a significant role in the correct identification of species in fungal taxonomy by using advanced molecular approaches. The highest value of Pileus length and width was observed in *M. costata* (6.9-12×4.5-5.4 cm) while the lowest value was observed in *M. conica* (2.5-4×3.9-4.8 cm). The largest stipe length was calculated in *M. elata* (6-8.8 cm), whereas the lowest value was calculated in *M. deliciosa* (2-3.5 cm). The accurate identification of Morels species is very crucial for the isolation of bioactive compounds, and these compounds play a vital role in drug discovery. So, we recommend a comprehensive exploration of Morels in the Western Himalayan Forest by using advanced molecular approaches and the isolation of bioactive compounds because this genus is still unexplored in the context of pharmaceuticals.

Ethics Approval and Consent to Participate

All the experiments were performed in accordance with relevant guidelines and regulations.

Author Contributions

Conceptualization, Tariq Saiff Ullah, Wayne Thomas Shier and Syeda Sadiqa Firdous; Data Data curation, Syed Waseem Gillani; Formal analysis, Muhammad Manzoor, Alevcan Kaplan; Investigation, Tariq Saiff Ullah; Methodology, Syeda Sadiqa Firdous, Syed Waseem Gillani and Muhammad Manzoor; Project administration, Tariq Saiff Ullah; Resources, Muhammad Manzoor; Software, Wayne Thomas Shier, Alevcan Kaplan and Muhammad Nauman Khan; Validation, Syed Waseem Gillani; Writing – original draft, Tariq Saiff Ullah, Syeda Sadiqa Firdous; Writing-review & editing, Wayne Thomas Shier, Alevcan Kaplan and Muhammad Nauman Khan. All authors contributed significantly, have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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