



# Genetic Diversity of Natural *Morchella* spp. from the Southern Gansu Province Based on rDNA-ITS, China

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## ABSTRACT

**Background:** China is a mainland country rich in natural *Morchella* spp. resources. At present, about half of the natural *Morchella* spp. in the world has been recorded in China. The current study was aimed at the classification and cultivation of *Morchella* spp. This study provided a more accurate molecular trait for the systematic classification of *Morchella* spp. in southern Gansu Province, China.

**Methods:** From April to May 2019. Based on the molecular biological analysis of 16 natural *Morchella* spp. strains collected from the southern Gansu province, China. The ITS fragments between transcripts of the rDNA gene were amplified by PCR and sequenced.

**Result:** The results of BLAST comparison according to GenBank showed that the 16 tested strains of *Morchella* spp. were classified into 5 species, which were *M. angusticeps*, *M. esculenta*, *M. elata*, *M. crapssipes* and *M. conica*. According to the molecular evolutionary trees constructed by the Maximum-Parsimony method (MP) and the Neighbour-Joining method (NJ), the topological structures of the two molecular evolutionary trees were similar and the Bootstrap verification had a high support rate, indicating that the phylogenetic relationship had high credibility.

**Key words:** Genetic diversity, *Morchella* spp., rDNA-ITS, Sequence analysis.

## INTRODUCTION

Morel, It is the general name of all species of *Morchella* in Morchaceae. It belongs to a kind of rare natural edible and medicinal fungi with high economic value. It has high nutritional and medicinal value and is highly recognized in the world (Hawksworth, 2004). According to the relevant data, it contains a variety of physiologically active substances, especially the protein content is very rich, but also contains all kinds of essential amino acids, vitamins and iron, zinc and other mineral elements (Pilz *et al.* 2007, Kuo, 2008). However, in the taxonomic study of *Morchella* spp., because its morphological characteristics are very malleable and artificial, it brings a lot of difficulties to the classification research and it is difficult to solve the systematic problems of *Morchella* spp. only by relying on the traditional morphological classification. The rapid development of molecular biology provides a new technical method and research platform for solving the systematic problems of *Morchella* spp. at the molecular level (Du *et al.* 2015, Zhao *et al.* 2009, Wu *et al.* 1996). At present, in the fungal index database (<http://www.indexfungorum.org/Names/Names.asp>), there are a total of 327 valid record species (including subspecies and varieties) of *Morchella* spp. in the world. According to the color morphology of the fruit body, *Morchella* spp. can be divided into black morel group, yellow morel group, red-brown morel group and half-free morel group (Kuo *et al.* 2012).

The southern region of Gansu province, China. (100°46'~104°44' E, 33°06'~36°10'N) is the water culvert area of the Yangtze River and the Yellow River, with many rivers and unique hydropower resources. The Yellow River, Bailong River, Tao River, Daxia River, and more than 120 tributaries across the whole territory. However, for the

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southern region of Gansu, which is one of the main producing areas of natural *Morchella* spp. in China, except for the research reports on resources of *Morchella* spp. by some scholars in the 1980s (Gu, 1984, Luo, 1986), there are few studies on the molecular systematics of *Morchella* spp. in this area. Given this problem, this study attempted to explore the phylogenetic relationship of natural *Morchella* spp. in southern Gansu from morphological taxonomy and molecular systematics. it will be helpful to protect and develop the biodiversity of this famous edible fungus in the ecological key area of China.

## MATERIALS AND METHODS

### Collection of *Morchella* spp.

Sixteen fresh *Morchella* spp. were collected under a broad-leaved forest below 2300 m altitude in the southern Gansu province from April to May 2019. Details of each collection,

including information on the serial numbers, locus, and habit/dominant plants were listed in Table 1.

### Morphological description

A small part of the pileus from each sample was removed and placed on a slip glass for 5 min, immersed in 100  $\mu$ L water and then sliced to 10~15 nm thick particle size by hand. The sliced samples were transferred to new slip glasses and covered with a thin cover, which was pressed to spread the sample. The morphological assessment focused on paraphyses, septate orientation, spore, asci and the number of ascospores. Images were taken with an Olympus microscope (Olympus Ltd., Nanjing China) at 40 $\times$  and 100 $\times$  magnifications.

### DNA extraction

Refer to the method of Bai *et al.* (2011). To obtain pure mycelium, After many improvements and verification, the DNA extraction technology suitable for this experiment was explored. The details were as follows: (1) the lid of the fruit body of *Morchella* spp. which was soaked, washed and sterilized about 0.5 g, chopped and placed in an aseptic pre-cooled mortar, added the appropriate amount of liquid nitrogen and grind to a powder. (2) quickly transferred it into the centrifuge tube of 2 mL, added CTAB (pH 8.0) pyrolysis buffer preheated by 1.5 mL to 65°C, slowly reversed and mixed, as for water bathed at 65°C for 1.5 h, mixed once every 10 mins. (3) after 12000 r/min centrifugation for 15 mins, the supernatant was transferred to a new centrifuge tube. (4) added the same volume of phenol: chloroform, shake slowly, a centrifuge with 12000 r/min for 10 mins and moved the supernatant to a new centrifuge tube. (5) added the same volume of chloroform: isoamyl alcohol (24 pur), shake slowly, a centrifuge with 12000 r/min for 10mins, and moved the supernatant to a new centrifuge tube. (6) added 2 times volume of precooled anhydrous ethanol, mixed it gently, put it at -20°C for 3.0 hours, centrifuged for 10 mins,

discarded the supernatant, and left DNA to precipitate. (7) washed DNA precipitate twice with 75% ethanol and discarded the supernatant. (8) DNA was obtained by centrifugation and vacuum drying. The proper amount of aseptic water was dissolved and precipitated in each tube and stored at low temperature at -20°C.

The primers used to amplify the ITS region were as follows: Upstream primer ITS1: 5'-TCCG TAGGTGAACCTG CGG-3', Downstream primer ITS4: 5'-TCCTCCGCTTATTGA TATGC-3' (synthesized by Shanghai Sheng Gong Biotechnology Service Co., Ltd. China).

### PCR amplification and sequencing

All extracted DNA samples were used as substrates of PCR amplification. The PCR amplification system is 50  $\mu$ L, including 10 $\times$ PCR buffer 5.0 $\mu$ L, dNTPs 1.0  $\mu$ L, template DNA 1.0  $\mu$ L, ITS1 1.0  $\mu$ L, ITS2 1.0  $\mu$ L, Taq polymerase 1.0  $\mu$ L, double distilled water 40.0  $\mu$ L. PCR amplification program: pre-denaturation at 94°C, denaturation at 94°C, denaturation at 40°C, annealing at 55°C, annealing at 60°C, the extension of 100 mins at 72°C for 35 cycles and leveling at 72°C for 10mins. The termination temperature was 4°C. The ITS-PCR amplification products were detected by 0.8% agarose gel electrophoresis and the gel imaging system was used to take pictures and record the electrophoretic bands.

The PCR products were recovered and purified using the UNIQ-10 column DNA recovery kit provided by Shanghai Jikang Biotechnology Co., Ltd. and the samples were submitted to Shanghai Jikang Biotechnology Co. Ltd. for sequencing. The sequence results of the strains were analyzed by BLAST on GenBank and the deletion sites and incomplete sites were deleted by the software CLUSTALX (version 1.81) and then the homology was compared. Finally, the molecular phylogenetic trees were constructed by the maximum-parsimony method (MP) and the neighbour-joining method (NJ) by the software MEGA6.06 to clarify

**Table 1:** Collections of *Morchella* spp. in this study.

Serial numbers	Locus	Habitats/dominant plants
GN-M1	Kagaman village/Hezuo	<i>Populus simonii</i>
GN-M2	Kagaman village/Hezuo	<i>Populus simonii</i>
GN-M3	Kagaman village/Hezuo	<i>Populus simonii</i>
GN-M4	Dangzhou grassland/Hezuo	<i>Juglans regia</i>
GN-M6	Dangzhou grassland/ Hezuo	<i>Juglans regia</i>
GN-M9	Lianhua mountain/Lintan	<i>Quercus aliena</i>
GN-M10	Lianhua mountain/Lintan	<i>Quercus aliena</i>
GN-M12	Lianhua mountain/Lintan	<i>Quercus aliena</i>
GN-M14	Yeliquan/Lintan	<i>Larix principis-rupprechtii</i>
GN-M17	Yeliquan/Lintan	<i>Larix principis-rupprechtii</i>
GN-M19	Yeliquan/Lintan	<i>Larix principis-rupprechtii</i>
GN-M22	Yeliquan/Lintan	<i>Larix principis-rupprechtii</i>
GN-M25	Gahai Lake/Luqu	<i>Pinus densiflora</i>
GN-M27	Gahai Lake/Luqu	<i>Pinus densiflora</i>
GN-M31	Gahai Lake/Luqu	<i>Pinus densiflora</i>
GN-M32	Gahai Lake/Luqu	<i>Pinus densiflora</i>

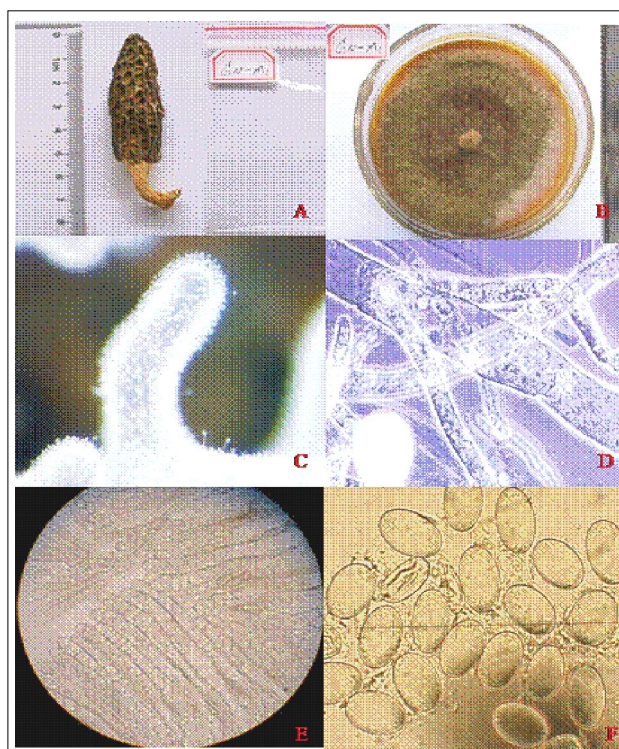
the species and taxonomic status of the tested strains (Richard, 2015, O'Donnell *et al.* 2011, Taşkin *et al.* 2015)

## RESULTS AND DISCUSSION

### Morphological descriptions of *Morchella* spp. (Voucher GN-M1)

The fruit body was generally large, height was 6.05~8.10 (6.90) cm, the diameter of the cap was 3.5-4.5 (3.88) cm,

long conical or oval, light loess, or yellowish-brown and its longitudinal veins were long. The inside of the stalk was hollow up to the lid, and the fungus meat was thin (Fig 1A). When cultured on compound PDA medium at 23°C for 7 days, the color of mycelium gradually deepened, from white to light yellow at first and finally to dark brown, in the middle and later stage of mycelium growth, the brown pigment secreted by mycelium made the compound PDA medium



**Fig 1:** Morphology of *Morchella* spp.

(A): Ascomycete, (B): Mycelium of *Morchella* spp. in PDA, (C): A coronal process at the tip of a mycelium, (D): Microstructure of mycelium, (E): Eight paralleled ascospores in each matured ascus, (F): Ascospores.

**Table 2:** The ITS sequencing results of 16 strains of *Morchella* spp.

Lanes	No.	ITS sequences (bp)	GC percentage (%)	Strains
1	GN-M1	732	50.33	<i>Morchella angusticeps</i>
2	GN-M2	738	50.14	<i>Morchella angusticeps</i>
3	GN-M3	1138	55.33	<i>Morchella esculenta</i>
4	GN-M4	1138	56.80	<i>Morchella esculenta</i>
5	GN-M6	736	50.64	<i>Morchella angusticeps</i>
6	GN-M9	1138	55.85	<i>Morchella esculenta</i>
7	GN-M10	675	50.92	<i>Morchella angusticeps</i>
8	GN-M12	1138	55.61	<i>Morchella esculenta</i>
9	GN-M14	679	50.29	<i>Morchella elata</i>
10	GN-M22	675	50.19	<i>Morchella elata</i>
11	GN-M17	675	50.61	<i>Morchella elata</i>
12	GN-M19	673	50.53	<i>Morchella elata</i>
13	GN-M25	1131	55.41	<i>Morchella crassipes</i>
14	GN-M27	737	50.70	<i>Morchella conica</i>
15	GN-M31	1209	55.13	<i>Morchella crassipes</i>
16	GN-M32	738	50.38	<i>Morchella conica</i>



turned dark brown (Fig 1B). The hyphae were colorless and transparent in the early stage, brown and transparent in the middle stage, with a smooth surface and a diameter of 2.6~11.3  $\mu\text{m}$ . The hyphae were elongated, bamboo-like, septate, branched and intertwined into a network (Fig 1C, D), the ascus was nearly cylindrical, generally containing 8 spores (Fig 1E), the spores were oval, colorless and smooth, 18.7~24.5  $\mu\text{m} \times 10.0 \sim 13.0 \mu\text{m}$  (Fig 1F), the lateral filaments were slender and thicker at the top.

### Phylogenetic analysis based on ITS rDNA

The changes of ITS sequence length and GC content of 16 *Morchella* spp. strains were shown in Table 2. Logged in the Genbank and carried out BLASTN alignment analysis of the obtained sequences. The results showed that the nucleotide sequences of GN-M1, GN-M2, GN-M6 and GN-M10 were 99% similar to the ITS sequence of *M. angusticeps* with the accession number JQ691485, DQ257338, AJ698476, JX069625. The sequence similarity of GN-M3, GN-M4, GN-M9 and GN-M12 to the ITS sequence of *M. esculenta* was 99% with the accession number U51851 and AJ543741. The sequence similarity of GN-M14, GN-M22, GN-M17 and GN-M19 to the ITS sequence of *M. elata* with accession number GQ249378, GQ249383, EU83482 reached 97%~98%. The sequence similarity of GN-M25 and GN-M31 to the ITS sequence of *M. crassipes* with accession number EU701002 reached 99%. The sequence similarity of GN-M27 and GN-M32 to the ITS sequence of *M. conica* with accession number AM269501 reached 97%~98%.

### Construction of systematic tree based on ITS sequence

To better explore the phylogenetic relationship of natural *Morchella* spp. in southern Gansu, China. Three ITS sequences of *verpa* of Morchaceae (accession numbers GU551670, GU551672 and JN043311) and *M. vulgaris*, *M. spongiosa*, *M. semilibera* and *M. rufobrunnea* (accession numbers AF000971, JQ691480, AF008233 and DQ355921) were downloaded from GenBank. After BLASTN alignment analysis, the molecular evolution tree was constructed by neighbour-joining (NJ) and maximum parsimony (MP), as shown in Fig 2 and Fig 3.

In Fig 2 and 3, the topological structure of the phylogenetic tree constructed by NJ and MP is similar and each branch of the Bootstrap verification system tree has a high support rate, indicating that the credibility of the system relationship was high. Combined with the analysis of sequencing results, 16 strains of *Morchella* spp. in this experiment. It was finally classified into 5 species after molecular identification. They were *M. angusticeps* (GN-M1, GN-M2, GN-M6, GN-M10), *M. esculenta* (GN-M3, GN-M4, GN-M9, GN-M12), *M. elata* (GN-M14, GN-M22, GN-M17, GN-M19), *M. crassipes* (GN-M25, GN-M31) and *M. conica* (GN-M27, GN-M32). These five species of *Morchella* spp. were mainly classified into the yellow morel group (*M. esculenta*, *M. crassipes*) and the black Morel group (*M. conica*, *M. angusticeps*, *M. elata*).

Through the analysis of the phylogenetic tree, it was

found that two species of *Verpa* with accession numbers GU551670 and GU551672 were clustered into one branch, but one strain of *Verpa* with accession number JN043311

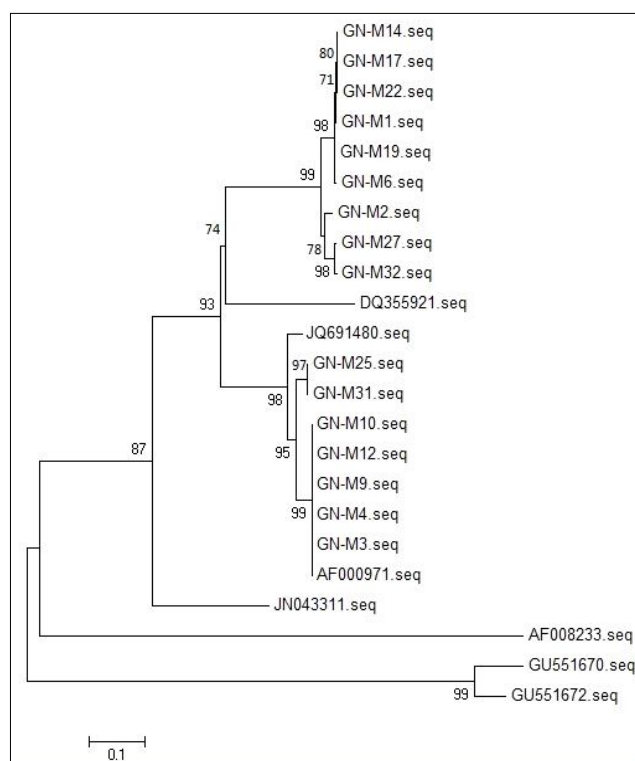


Fig 2: The phylogenetic tree constructed by NJ (Bootstrap, 1000 repeated).

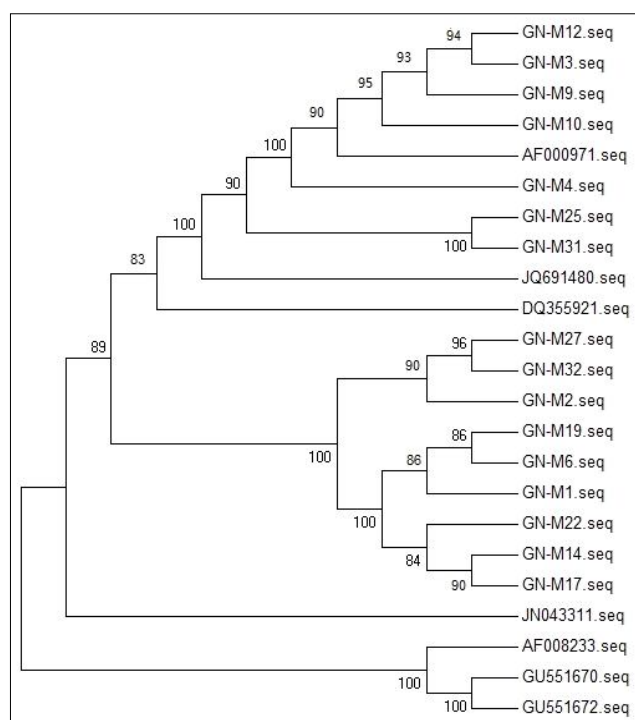


Fig 3: The phylogenetic tree constructed by MP (Bootstrap, 1000 repeated).

was clustered into the same group as *Morchella* spp. *M. vulgaris* (accession number AF000971) and *M. spongiosa* (accession number JQ691480) were closely related to *M. esculenta* (GN-M3, GN-M4, GN-M9, and GN-M12) and *M. angusticeps* (GN-M1, GN-M2, GN-M6, GN-M10) and could be grouped into one group. *M. rufobrunnea* with accession number DQ355921 was roughly grouped with *M. conica* (GN-M27, GN-M32) and *M. crapssipes* (GN-M25, GN-M31). *M. semilibera* with accession number AF008233 was far away from other *Morchella* spp. and seemed to be clustered into the same group with two strains of *Verpa* (GU551670 and GU551672). The reasons need to be further studied.

## CONCLUSION

The evaluation of *Morchella* spp. species diversity is often complicated by the plasticity of macro-and micro morphological characteristics. Genomics is therefore important for aiding in species recognition, and they are often used instead of the morphology to identify these cryptic species (Chenna *et al.* 2003). In this study, 16 strains of natural *Morchella* spp. from southern Gansu, China were classified into 5 species. It was finally classified into 5 species after molecular identification. They are *M. angusticeps* (GN-M1, GN-M2, GN-M6, GN-M10), *M. esculenta* (GN-M3, GN-M4, GN-M9, GN-M12), *M. elata* (GN-M14, GN-M22, GN-M17, GN-M19), *M. crapssipes* (GN-M25, GN-M31) and *M. conica* (GN-M27, GN-M32). These five species of *Morchella* spp. were mainly classified into the yellow morel group (*M. esculenta*, *M. crapssipes*) and the black morel group (*M. conica*, *M. angusticeps*, *M. elata*). According to the molecular evolutionary tree, the topological structure of the phylogenetic tree constructed by the maximum-parsimony method (MP) and the Neighbour-Joining method (NJ) was similar and the Bootstrap verification of each branch of the phylogenetic tree has a high support rate, indicating that the phylogenetic relationship has high credibility. At the same time, it also showed that this study has provided a clear molecular basis for the systematic classification of *Morchella* spp. in this area.

In this paper, the results of the molecular identification of *Morchella* spp. were different from those of morphological classification, which might be due to the morphological variation of the fruit body of *Morchella* spp. in this region. the morphological characteristics and physiological indexes of *Morchella* spp. species were affected by a variety of external factors, which has great restrictions on its morphological classification. As for how many species of *Morchella* spp. were still distributed in southern Gansu, more fresh samples need to be collected for isolation and identification in the future. The development of this work can not only enrich the strain resource database of *Morchella* spp. but also provide scientific theory and the basis for local artificial cultivation and future development and utilization of *Morchella* spp.

Nevertheless, molecular phylogenetic studies have indicated that many epithets may be synonymous species, homonymous species, or incorrectly named species, given

that the majority of *Morchella* spp. species appear to exhibit high continental endemism and provincialism in the Northern hemisphere. Initially, the Internal Transcribed Spacer (ITS) rDNA region was used as the sole locus in most studies for assessing *Morchella* genetic diversity. Although ITS sequences were useful for identifying 77.4% of the known phylospecies, at least 66% of the named *Morchella* sequences in GenBank were misidentified (Du, 2012). Thus, the use of multilocus DNA sequence datasets and phylogenetic species recognition based on genealogical concordance and nondiscordance was initiated and accepted by academia (Taylor, 2000, Taşkın *et al.* 2010). Currently, 61 phylospecies, including from China, Europe, and North America, respectively, have been resolved by employing maximum parsimony and maximum-likelihood frameworks based on genealogical concordance phylogenetic species recognition (GCPSR) (Du *et al.* 2012, Rajalaxmi, 2020, Chitdeshwari *et al.* 2020, Burcu and Volkan, 2020). Therefore, a uniform recognition of this cryptic species using GCSPR methods will be highly necessary.

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## Author contributions

L.Wang performed experiment investigations, data analysis, and wrote the original manuscript. Q.P. Zhou conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper. J.M. Liu and G.H. Shi provided parts of resources and materials. All authors reviewed the manuscript.

## Competing interests

The authors declare no competing interests.

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