The Complexity of *Morchella* Systematics: A Case of the Yellow Morel from Israel

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Figure 1. Fruiting body morphotypes examined in this study. (A) MS1-32, (B) MS1-34, (C) MS1-52, (D) MS1-106, (E) MS1-113. Fruiting bodies were similar in height, approximately 6-8 cm.

Abstract

Individual morel mushrooms are highly polymorphic, resulting in confusion in their taxonomic distinction. In particular, yellow morels from northern Israel, which are presumably *Morchella esculenta*, differ greatly in head color, head shape, ridge arrangement, and stalk-to-head ratio. Five morphologically distinct yellow morel fruiting bodies were genetically characterized. Their internal transcribed spacer (ITS) region within the nuclear ribosomal DNA and partial LSU (28S) gene were sequenced and analyzed. All of the analyzed morphotypes showed identical genotypes in both sequences. A phylogenetic tree with retrieved NCBI GenBank sequences showed better fit of the ITS sequences to *M. crassipes* than *M. esculenta* but with less than 85% homology, while LSU sequences, showed more then 98.8% homology with both species, giving no previously defined species definition according the two sequences.

Keywords: ITS region, *Morchella esculenta*, *Morchella crassipes*, phenotypic variation.

Introduction

Morchella sp. fruiting bodies (morels) are highly polymorphic. Although morphology is still the primary means of identifying mushrooms, this is a difficult task in morels. Morels are polymorphic with respect to head shape, stalk-to-head ratio, immature and mature color, taste and edibility (Weber, 1997; Kuo, 2005; Kellner, 2009). The polymorphic nature of *Morchella* spp. has contributed to taxonomic confusion, with the reported number of species ranging from three (Weber, 1997; Bunyard et al., 1994) to 50 (for review, see Bunyard et al., 1994). Morel collectors, on the other hand, distinguish three groups: the black morel, the yellow morel and the half-free morel. Different scientific names are used, including *Morchella conica, M. angusticeps, M. elata, M. vulgaris, M. rotunda, M. esculenta, M. crassipes,* and *M. deliciosa* (Weber, 1997; Kellner et al., 2005; Kuo, 2005). However, these designations show some overlap, with the same name potentially referring to different species.

Due to the polymorphic nature of morel ascocarps, morphologically based classification is considered unreliable. Therefore, phylogenetic resolution of *Morchella* spp. using molecular methods holds greater promise for their accurate classification. Various methods have been used to clarify morel systematics: Yoon et al. (1990) used starch gel electrophoresis, Jung et al. (1993) used enzyme-linked immunosorbent assay and Bunyard et al. (1994) used restriction-length polymorphism (RFLP) analysis of the 28S and 18S ribosomal RNA genes for phylogenetic resolution of the family Morchellaceae. More recently, sequences of the internal transcribed spacer (ITS) region of genomic DNA have been used for this goal. Buscot et al. (1996), Wipf et al. (1996) and O'Donnell et al. (2003) also used 28S gene sequences for phylogenetic analysis of *Morchella* species.

In Israel, several species of morel have been reported—mainly the yellow morel *M. esculenta* and the black morel *M. conica* —both of which are rare (Binyamini, 1984; Goldway et al., 2000; Masaphy et al., 2009). A recent report has added *M. elata* and *M. vulgaris* to the list (Barseghyan and Wasser, 2008). In the last few years, a study on morel species distribution in Israel has been conducted in our lab at MIGAL Institute, in which morels located in different sites throughout Israel were subjected to genetic characterization. In the present work, the molecular characterization of five morphologically distinct mushrooms, all presumed to be *M. esculenta,* located in the Galilee region in northern Israel were studied by sequence analysis of the ITS region of the nuclear ribosomal DNA and the partial LSU (28S rRNA) gene, in order to resolve their taxonomy.

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Materials and Methods

Fruiting bodies: Fruiting bodies used in this study were collected from the Galilee region in Israel in the 2003-2007 seasons. All were found in healthy Mediterranean groves, and each fruiting body was photographed in its natural growing site before collection. Five morphologically distinct fruiting bodies were used for molecular characterization (Fig. 1). Mycelial biomass was obtained after spores culturing on PDA medium. Specimens are preserved in a collection at MIGAL Institute (Kiryat Shmona, Israel).

Molecular analyses: The partial LSU gene and the ITS region (nrDNA) were used for phylogenetic analyses of the different fruiting bodies according to Kellner (2009). Freeze-dried mycelial biomass (50 mg for each sample) was ground in liquid nitrogen. DNA was extracted by phenol/chloroform procedure. The ITS region was amplified by ITS1/ITS4 primer pairs (Kellner, 2005; Wipf et al., 1996) and the partial LSU gene was amplified using LROR/LR6 primer pairs (Campbell et al., 2003; Kellner, 2009). PCR amplifications were carried out using a Flexigene thermocycler (Techne, UK) under the conditions described in Wipf et al. (1996). Amplification products were sequenced by HyLabs (Israel). Other ITS and LSU sequences were retrieved from GenBank (NCBI—*National Center for Biotechnology Information)* and used for comparison with the sequences obtained in this work.

Sequence alignments and phylogenetic analysis: The sequences of the ITS region and the partial LSU gene of one of the fruiting bodies, designated MS1-32, were submitted to GenBank (accession numbers GU589858 and GU589859, respectively). Sequences were assembled and edited by SeqMan program and aligned, and a phylogenetic tree was constructed using the programs Lasergene MegAlign (DNASTAR, USA) and MEGA 4 (Tamura et al., 2007). The relationship between the sequences of the different morphologically distinct fruiting bodies and other *Morchella* spp. sequences retrieved from the GenBank database (NCBI) (for a list of previously published sequences, see Table 1), were studied. Members of other genera of the Morchellaceae (representative species of genus *Verpa* and genus *Disciotis*) were used as outgroup candidates (Table 1). The phylogenetic tree was based on multiple sequence alignments and cluster analyses. Two different dendrograms (comparing the ITS regions or the LSU) were created along with 2000 bootstrap repeat test of phylogeny using the Neighbor-Joining algorithm (Saitou and Nei, 1987).

Results and Discussion

The fruiting bodies analyzed in this study differed from each other in several morphological features: head shape (conical or round) and color, ridge arrangement, and depth and density of the pits (Fig. 1). In general, all of the examined morels were spotted in healthy-tree-bearing sites. Each of the fruiting bodies had uniformly colored ridges and pits, with the latter generally being elliptical-rounded. All of the examined fruiting bodies were of the

Table 1. Sequence analyses of ITS region and partial LSU gene of MS1-32 as representative of the five examined morel morphotypes with other species of the genus *Morchella*; species of *Verpa* and *Disciotis* (family Morchellaceae) were used as outgroups.

Putative species	GenBank accession number	Location	Sequence size	Matched sequence length*	Degree of similarity (%)**
ITS region					
MS1-32	GU589858	Israel	1143		
MS1-34			1117	1119	99.6
MS1-52			1119	1123	99.0
MS1-106			1079	1080	99.8
MS1-113			1079	1080	99.8
M. crassipes	EU701002	Rwanda (Degreef et al., 2009	1209	1145	99.7
Morchella sp.	AJ539479	India (Kellner et al., 2007)	1207	1144	99.3
M. crassipes	AJ539480	Germany (Kellner et al., 2005)	1226	1175	84.8
M. esculenta	AJ543741	Germany (Kellner et al., 2007)	1138	1152	83.6
M. esculenta	Meu51851	France (Wipf et al., 1996)	1133	1152	83.6
M. esculenta	DQ257342	China (Hu and Fan, 2005)	1138	1152	83.3
M. spongiola	AJ 539476	Germany (Kellner et al., 2007)	1186	1155	82.5
M. conica	AJ544194	Germany (Kellner et al., 2007)	737	841	60.5
M. elata	AJ544200	India (Kellner et al., 2007)	737	841	59.8
Partial LSU sequence					
MS1-32	GU589859	Israel	1003		
MS1-34			972	970	100
MS1-52			984	980	100
MS1-106			984	980	100
MS1-113			971	971	100
Morchella sp.	AJ698464	India (Kellner et al., 2007)	1114	1004	99.3
M. esculenta	AY533016	USA (Buschbom and Mueller, 2004).	1286	991	99.3
M. crassipes	AJ698462	Germany (Kellner et al., 2007)	1114	1004	98.8
M. elata	AJ698469	India (Kellner et al., 2007)	1118	1006	97.2
M. conica	AJ698468	Germany (Kellner et al., 2007)	1117	1006	97.0
Verpa conica	AJ698470	Germany (Kellner et al., 2007)	1114	1006	94.7
Disciotis venosa	AJ698472	Germany (Kellner et al., 2007)	1115	1006	94.0
Verpa bohemica	FJ176853	USA (Schoch et al., 2009)	886	871	93.1

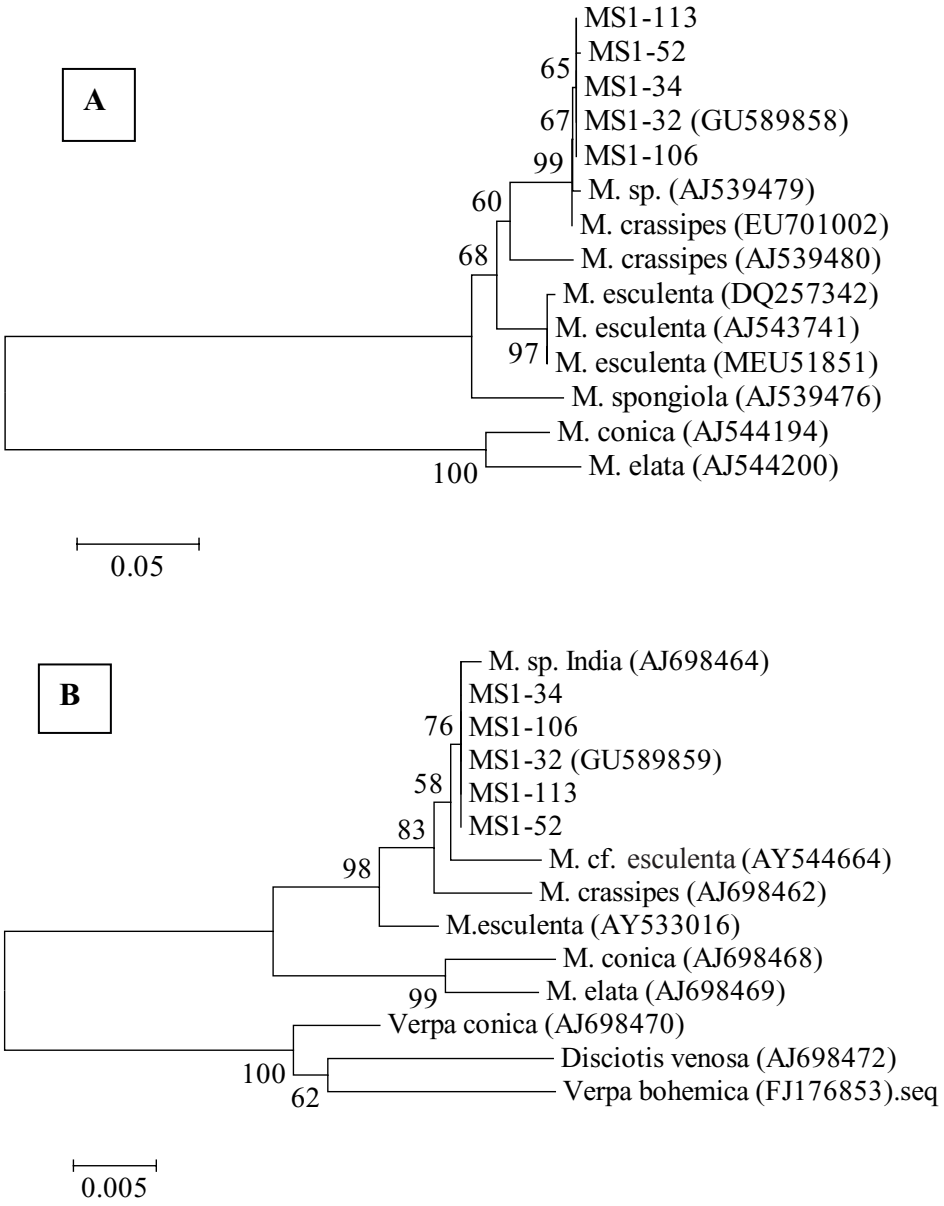
****Degree of homology as % base pair identity with best match.

same height, approximately 6-8 cm, with the stalk being shorter than the head (approx. one-third of the whole fruiting body).

Genetic analyses of all five mor-photypes resulted in ITS fragment lengths of 1079 to 1143 base pairs (bp) and LSU fragments of 971 to 1003 bp (Table 1). Comparison of the sequences obtained by PCR for the partial LSU fragment from all five morphotypes showed 100% similarity, with a matched sequence of 970 to 980 bp in length (Table 1). ITS region sequences showed between 99 and 99.8% similarity, with matched-sequence sizes of 1144 to 1180 bp of the aligned length.

After showing that all morphotypes were identical with respect to LSU sequences and more than 99% homologous for ITS sequences, the ITS region and partial LSU sequences were aligned with those of other morel sequences retrieved from the GenBank database to determine phylogeny (Fig. 2). The fragment sequences of morphotype MS1-32 were subjected to multiple sequence alignment with several morel ITS and LSU sequences and cluster analyses. This placed the examined Israeli morel genotype in the *M. crassipes* neighborhood, with highest similarity (99.7%) of the ITS region sequence to that of a *M. crassipes* designated "African *M. crassipes*" from Rwanda (Degreef et al., 2009), and second highest similarity (99.3%) to a *Morchella* sp. from India (Kellner et al., 2007). The third best match was with a *M. crassipes* from Germany, with only 84.8% similarity. All other yellow morel species found in the GenBank sequence list exhibited lower similarities (82.5 to 84%) to morphotype MS1-32, while the black morels exhibited only 59.8 to 60.5% similarity. Comparison of the LSU sequences with sequences retrieved from GenBank showed a neighborhood pattern differ from that of the ITS sequence, i.e. highest similarity to the Indian *Morchella* sp., followed by *M. esculenta* of AY533016, and only then the *M. crassipes* from Kellner et al. (2005), with *M. conica* and *M. elata* showing lower similarities.

Figure 2. Dendrogram showing the relationships and degrees of similarity in (A) ITS region and (B) partial LSU (28S) sequence among the five different morphotypes examined and several representative morels retrieved from GenBank, as well as several outgroup species of *Verpa* and *Disciotis*. Numbers in parentheses are the GenBank accession numbers of the various sequences. The numbers shown next to the branches, indicate the percentage of bootstrap replicates in which the associated species clustered together.



The ITS and LSU sequences of the Israeli morel studied were specifically compared to the sequences reported by Keller et al. (2005, 2007, 2009) in order to place the Israeli genotype within the phylogenetic tree that was already constructed in Kellner's work. The studied Israeli morel exhibited highest homology of its ITS sequence to morel sequences that differed from the European *M. cassipes*, based on the work of Kellner (2005, 2007). The distance between the ITS genotype of the Israeli morel and that of the European *M. crassipes* was higher than that between the European *M. crassipes* and *M. esculenta* (Table 1), but this was not the case for the LSU sequence. This recalls the debate on how much genetic distance should exist to define a species. The Israeli type may be so distinct as to be considered a new species, with the Indian *Morchella* sp. and the "African *M. crassipes,*" which would then need to be reclassified. Alternatively, the Israeli species, like the Indian and African morels, could be regarded as *M. crassipes*, distinct from the European *M. crassipes*. However, the Israeli yellow morel definition according to genetic information could be resolved only when more phylogenetically informative data are available for comparison.

The complexity involved in defining taxonomic distinctions within the yellow morels is reflected by the different studies, which have applied different methods to resolve the species identification. In general, many yellow mushrooms are considered to be *M. esculenta* based on their morphology. However, the group of *M. esculenta* has been found to include distinct genotypes. Kellner et al. (2005), using ITS region sequence analysis, showed that *M. esculenta* from Germany and France includes three distinct species: *M. esculenta*, *M. crassipes* and *M. spongiola,* while Dalgleish and Jacobson (2005) used RAPD-PCR to show high genetic variations among *M. esculenta* populations located in three separate sites in the United States.

The morphologically different fruiting bodies examined in this study showed high homology by LSU sequence, but slight differences were found in the ITS sequences. The ITS differences will be closely examined in future studies to find their relation to polymorphism within species populations. While ITS and LSU sequencing is increasingly being used for morel classification, the information is not sufficient to designate different species. There is a strong need for comprehensive work by morel molecular taxonomists worldwide to resolve the uncertainties regarding the number of species and their ecology.

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