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Morchella tridentina (Ascomycota) from southwestern Turkey based on morphological and molecular evidences

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Abstract

Morchella is a species-rich genus in the family *Morchellaceae*, but phenotypic variations among species and subgeneric groupings are poorly understood. New collections of *Morchella tridentina*, a taxon with a problematic taxonomic history, are reported from Denizli province in southwestern Turkey based on morphology and molecular phylogeny. This is a new locality record for the Turkish mycobiota. Description of the morphological characteristics, including macro- and microphotographs are provided. Additionally, phylogenetic data, using the nrITS locus, and infrageneric relationships of *M. tridentina* within the *Distantes* section are given. Analyses of the nrITS showed that the lowest sequence divergence among in-group taxa was observed between *M. angusticeps* and *M. eximioides*, 0.002% (SE = 0.001). The highest sequence divergence with in-group taxa was found between *M. tomentosa* and *M. semilibera*, 0.956% (SE = 0.167), and between *M. semilibera* and *M. tridentina*, 0.866% (SE = 0.147). Finally, it was determined that *M. exuberans* was genetically the closest species to *M. tridentina*, 0.398% (SE = 0.054).

Key words – genetic distance – morel – nrITS – phylogeny – taxonomy – Turkish mycobiota

Introduction

Morchella Dill. ex Pers. is a taxonomically challenging genus within *Pezizales* J. Schröt. (Barseghyan et al. 2012, Du et al. 2012). *Morchella* has been the focus of attention of many researchers all over the world due to its economical aspects (Barseghyan et al. 2012) and the specific diversity (O'Donnell et al. 2011, Richard et al. 2015). The distinguishing characteristics of the genus *Morchella* are based on numerous features, i.e. the ridges, pits, colour of the pileus, size and shape of the ascospores and paraphyses, texture of the flesh, typology of connection between the pileus and the stipe and colour change on bruising (Barseghyan et al. 2012). Researchers have studied the molecular phylogeny, taxonomy and biogeography of *Morchella*, and in the last decade several new species have been described from Australia (Elliott et al. 2014), France (Clowez 2012), Canada (Voitk et al. 2016), Colombia (Pinzón-Osorio & Pinzón-Osorio 2017), Cyprus (Loizides et al. 2016), North America (Kuo 2008, Kuo et al. 2012), Spain (Clowez et al. 2014, 2015) and Turkey (Işıloğlu et al. 2010, Taşkın et al. 2016). Recent multigenic DNA studies have demonstrated the existence of forty species of *Morchella* throughout the world (Wijayawardene et al. 2017), of

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which thirty-four occurring in Europe, thirty-two in Asia and twenty-one in North America (Loizides 2017). Some species of *Morchella* show a high degree of continental endemism or provincialism (O'Donnell et al. 2011), while many others are found on more than one continent (Du et al. 2012, Richard et al. 2015, Loizides et al. 2016, Loizides 2017).

Many *Morchella* species are considered to be in symbiotic or endophytic relationships with trees or plants, while others are saprotrophs (Loizides 2017). Yellow morels occur usually in cool northern and polar regions with deciduous trees, black morels grow often in southern and Mediterranean areas with conifers (Loizides 2017), and white morels are mostly found on treeless, disturbed substrates and wood chippings in the Mediterranean basin, and also under olive trees (Loizides et al. 2015).

Morchella tridentina Bres. was previously recorded from Muğla, Uşak and Aydın provinces of Aegean Region of Turkey (Taşkın et al. 2012, Doğan et al. 2016). Our collections of *M. tridentina* from Denizli province, is a new locality record for the Turkish mycobiota. The purpose of this study is to present current information on *M. tridentina* identified from southwestern region of Turkey, based on their morphology and molecular data.

Materials & Methods

Samples collection

Morchella specimens were collected from Denizli province, southwestern Turkey in 2010 and 2019. The morphological features and ecological notes were recorded from young to mature, fresh and dry fruiting bodies and ascomata. They were photographed in their natural habitats.

Morphological studies

The macroscopical descriptions and images of the ascomata were obtained by observation of fresh or dried specimens. For microscopical analyses, the dried materials were rehydrated in 5% KOH, and subsequently stained with Congo Red or Melzer's solution. The following abbreviations are used in the descriptions: L^m for the average length of all the measured ascospores, W^m for the average width of all the measured ascospores, Q for the quotient of length and width of all the measured ascospores, and Q^m for the average of all calculated Q values for all ascospores measured. At least thirty mature ascospores from each ascoma were measured. The exsiccata were deposited in the personal fungarium of the first author at Isparta University of Applied Sciences (Turkey).

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from fresh or dried material utilising the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo research, Irvine, CA, USA). The PCR amplification was used for ITS region by universal primers ITS1F/ITS4B (Gardes & Bruns 1993). The amplifications were performed in a 25 µl reaction volume containing 1 µl of DNA template, 12.5 µl (1X final concentration) of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs, Ipswich, MA, USA), 1 µl of each primer (forward and reverse) at 10 µM and 9.5 µl ddH_2O. The amplification condition for ITS consisted of initial denaturation at 95°C for 5 min; followed by 35 cycles of 45 s at 94°C, 35 s at 54°C and 1 min at 72°C, and a final extension period of 10 min at 72°C. All PCR products were sequenced in Sanger DNA sequencing service (Source Bioscience, Berlin, Germany), with the same primers used in the PCR reactions. The raw DNA sequencing files were edited and assembled with the help of CLUSTAL X (Thompson et al. 1997) and MEGA X v.10.0.5 (Kumar et al. 2018). The edited sequences were then used for BLAST search in the GenBank (www.ncbi.nlm.nih.gov/genbank/). The newly generated sequences were deposited in GenBank with corresponding accession numbers (MK734141-MK734143 and MK758080-MK758081).

Sequence alignment and phylogenetic analyses

For this study, five new sequences of nrITS were generated. Further sixty-two related sequences used in phylogenetic analysis were downloaded from the NCBI (National Center for Biotechnology Information, Rockville Pike, Bethesda MD, USA) database. All sequences were aligned by CLUSTAL X and the MAFFT (/version 7.110) programs (Katoh & Standley 2013). In addition, final alignments were manually corrected via BioEdit version 7.2.3 (Hall 1999) and MEGA X. In both Bayesian Inference (BI) and Maximum Likelihood (ML) analyses, *Gyromitra gigas* (Krombh.) Cooke (MH938669) and *Gyromitra ticiniana* Littini (MH938674) were used as the outgroup taxon.

Phylogenetic tree inference was performed for the ITS dataset by both BI and ML methods. The BI was carried out using Markov Chain Monte Carlo (MCMC) methods with MrBayes version 3.2.2 (Ronquist et al. 2012). Markov chains were run for 10^6 generations, saving a tree every 1000^{th} generation, with two runs per analysis. The initial 25% trees recovered were excluded as a burn-in, and a 50% majority consensus tree of the remaining trees was then used to calculate the posterior probabilities (PP) of the group. The ML analysis was performed through the Cipres Science Gateway v.3.3 interface (http://www.phylo.org/portal2/) (Miller et al. 2010) using RAxML v.8.2.10 (Stamatakis 2014) employing the GTRGAMMA model with 1000 ML bootstrap replicates and default settings for other options. Only Bayesian posterior probability (BPP) values ≥ 0.85 and Maximum likelihood bootstrap (MLB) values of ≥ 90 are reported in the resulting tree (Fig. 1). Branch lengths were estimated as mean values over the sampled trees. The phylogram inferred from both analysis were displayed with FigTree v.1.4.3 (Rambaut 2016).

Calculation of genetic distances

Evolutionary divergence analyses based on the selected nrITS sequence set were conducted using MEGA X. The estimation of phylogenetic distances was conducted using the Maximum Composite Likelihood method (Tamura et al. 2004) which is used to calculate by averaging over all sequence divergences between the inferred species and is expressed as numbers of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair (pairwise deletion option). Standard error estimates were got by a bootstrap procedure (500 replicates).

Results

Molecular phylogeny

Our phylogenetic analysis contains a total of sixty-seven sequences. The alignment contained 1067 nucleotide sites (including gaps), of which 359 characters are constant, 48 parsimony-uninformative and 660 parsimony-informative. The phylogram obtained using Bayesian (MCMC) method, showing the Bayesian posterior probability (BPP), displayed similar topology to the phylogram obtained using Maximum likelihood (ML) analyses in RAxML. Therefore, only the BI phylogenetic tree with both Bayesian posterior probability (BPP) and Maximum likelihood bootstrap (MLB) values have been indicated in Fig. 1.

The genetic divergence matrix of the fourteen taxa studied depending on ITS sequences was estimated according to the Maximum Composite Likelihood method (Table 1). The lowest sequence divergence within in-group taxa was observed between *M. angusticeps* Peck and *M. eximioides* Jacquet., 0.002% (SE = 0.001). The highest sequence divergence with in-group taxa was found between *M. tomentosa* M. Kuo and *M. semilibera* DC., 0.956% (SE = 0.167). Genetically, *M. exuberans* Clowez, Hugh Sm. & S. Sm. is the closest species to *M. tridentina*, 0.398% (SE = 0.054). This also confirms that, as the phylogenetic tree shows, these taxa are members of different species (Fig. 1).

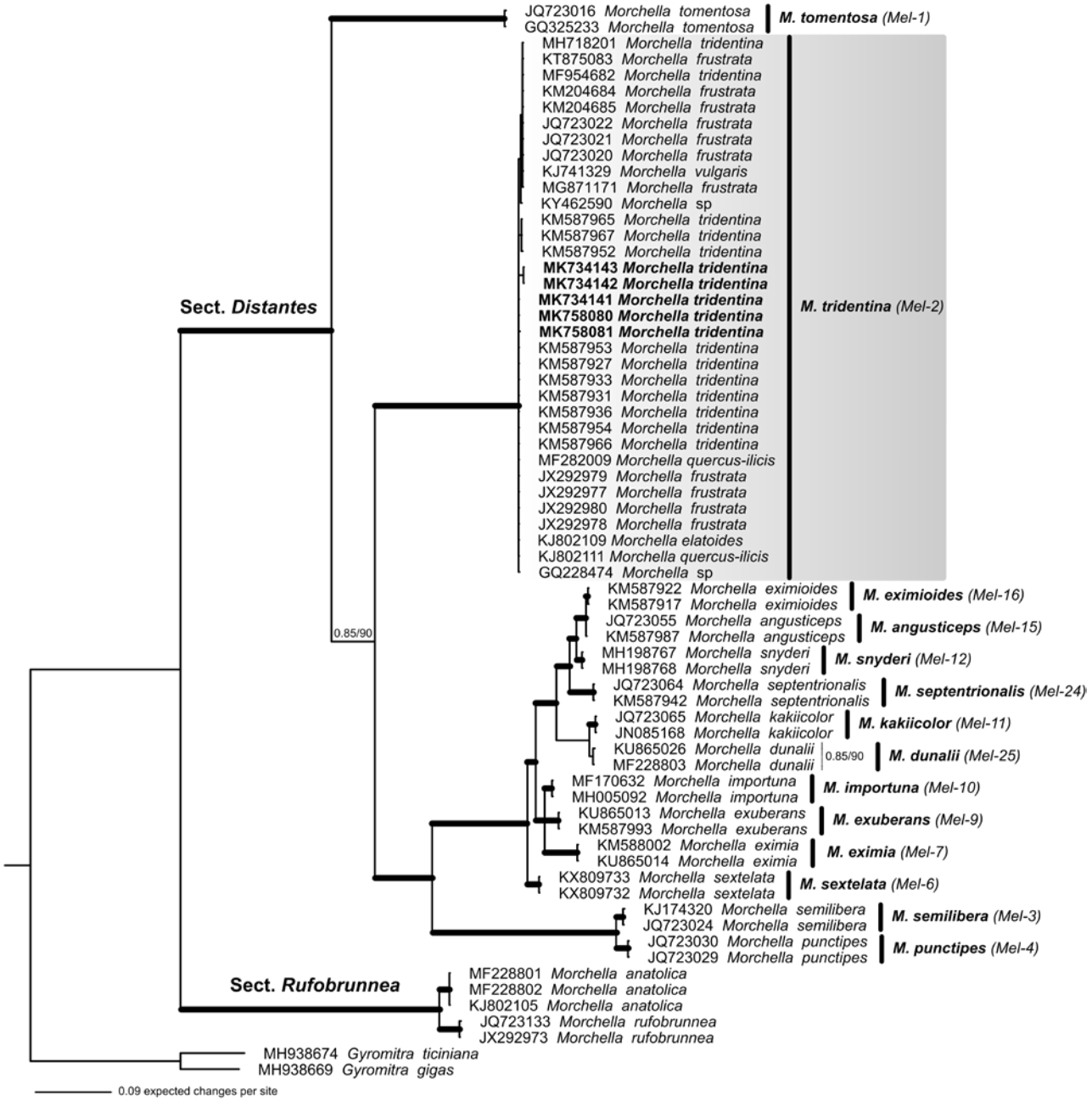

Fig. 1 – Phylogenetic tree obtained from Bayesian inference of the nrITS dataset. *Gyromitra gigas* (MH938669) and *Gyromitra ticiniana* (MH938674) were used as the outgroup taxon. Support values (Bayesian posterior probability – BPP ≥ 0.85 / Maximum likelihood bootstrap – MLB ≥ 90 %) are shown above individual branches. The branches are bold when BPP ≥ 0.95 and MLB ≥ 90 %. Newly generated sequences from Turkey are marked in bold.

Table 1 Genetic distance estimation based on Maximum Composite Likelihood Model method of selected species within section *Distantes*.

Taxa No	Divergence (%, lower left) / Standard error (upper right)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1		0.147	0.095	0.076	0.066	0.054	0.074	0.090	0.074	0.073	0.082	0.091	0.070	0.140
2	0.866		0.005	0.068	0.049	0.054	0.067	0.075	0.069	0.060	0.069	0.068	0.064	0.167
3	0.614	0.021		0.063	0.053	0.057	0.066	0.077	0.070	0.065	0.070	0.073	0.068	0.127
4	0.529	0.480	0.450		0.008	0.008	0.012	0.013	0.014	0.012	0.014	0.015	0.014	0.094

Table 1 Continued.

Taxa No	Divergence (%, lower left) / Standard error (upper right)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
5	0.468	0.370	0.391	0.044		0.006	0.011	0.015	0.016	0.013	0.017	0.013	0.013	0.072
6	0.398	0.393	0.403	0.048	0.027		0.012	0.016	0.016	0.014	0.016	0.015	0.014	0.090
7	0.494	0.443	0.439	0.080	0.058	0.062		0.021	0.021	0.018	0.020	0.021	0.019	0.093
8	0.606	0.514	0.518	0.084	0.086	0.100	0.138		0.001	0.005	0.009	0.013	0.013	0.094
9	0.499	0.472	0.478	0.090	0.094	0.098	0.136	0.002		0.005	0.009	0.015	0.013	0.094
10	0.502	0.431	0.455	0.079	0.077	0.088	0.123	0.018	0.018		0.008	0.011	0.011	0.075
11	0.519	0.476	0.475	0.086	0.101	0.098	0.129	0.046	0.044	0.048		0.017	0.015	0.097
12	0.605	0.496	0.521	0.101	0.085	0.095	0.135	0.085	0.093	0.076	0.103		0.003	0.085
13	0.471	0.465	0.486	0.096	0.087	0.090	0.128	0.090	0.090	0.074	0.097	0.009		0.080
14	0.839	0.956	0.736	0.588	0.466	0.559	0.568	0.594	0.583	0.487	0.593	0.546	0.513	

1- *M. tridentina* (MK734143), 2- *M. semilibera* (KJ174320), 3- *M. punctipes* (JQ723030), 4- *M. sextelata* (KX809733), 5- *M. importuna* (MF170632), 6- *M. exuberans* (KM587993), 7- *M. eximia* (KM588002), 8- *M. eximioides* (KM587922), 9- *M. angusticeps* (JQ723055), 10- *M. snyderi* (MH198768), 11- *M. septentrionalis* (KM587942), 12- *M. kakiicolor* (JN085168), 13- *M. dunalii* (MF228803), 14- *M. tomentosa* (GQ325233).

Taxonomy

Morchella tridentina Bres., Fungi Tridentini 2(11-13): 65 (1898) Figs 2–4

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Heterotypic synonyms – *Morchella conica* var. *pseudoeximia* Clowez, Bulletin de la Société Mycologique de France 126 (3-4): 308 (2012); *Morchella elatoides* Jacquet., Les Morilles: 103 (1984), inval.; *Morchella elatoides* Jacquet., Documents Mycologiques 14 (56): 1 (1985); *Morchella elatoides* var. *elegans* Jacquet., Les Morilles (Paris): 103 (1984); *Morchella elatoides* var. *elegans* Jacquet., in Jacquetant & Bon, Documents Mycologiques 14 (56): 1 (1985); *Morchella frustrata* M. Kuo in Kuo et al., Mycologia 104 (5): 1167 (2012); *Morchella quercus-ilicis* Clowez, L. Ballester & L. Romero, in Clowez, Bulletin de la Société mycologique de France 126 (3-4): 318 (2012).

Description – Ascomata 50–90(–100) mm high. Hymenophore 35–85 mm high, 20–35(–45) mm wide, elongated and acutely conical or broadly conical, at first homogeneously grey or brownish-grey, later pale greyish-brown to greyish-beige or beige, attached to the stipe forming a shallow sinus. Longitudinal primary ridges moderately spaced to crowded, more or less parallel, usually split and anastomosed, thick, fleshy, concolorous or paler than the pits. Transversal secondary ridges conspicuous, numerous, and forming a ladder-like arrangement with age, almost concolorous with the pits. Pits elongated, dull grayish to pale yellowish or nearly whitish when young, becoming concolorous with the ridges or slightly paler at maturity, often longitudinally arranged. Stipe 18–40 × 15–30 mm, cylindrical and usually enlarged at the base, hollow, pure white, whitish or ochraceous-white. Context whitish, firm, waxy, elastic. Smell slightly spermatic with a sweet. Ascospores uniseriate or rarely irregularly biseriate, (18.2–)21.0–24.3(–25.5) × (12.7–)13.0–16.3(–16.5) µm, $L^m \times W^m$ = 22.3 × 14.6 µm, Q = (1.3–)1.4–1.7(–1.8) µm, Q^m = 1.5 µm, ellipsoid, thick-walled, hyaline, inamyloid, sometimes microguttulate at the poles. Asci 230–350 × 15–25 µm, cylindrical to clavate, hyaline, inamyloid, 8–spored. Paraphyses 100–250 × 12–35 µm, usually clavate to subclavate, some moniliform or with subcapitate apices, most branching and septate with 2–3(–4) items per branch, with an ochraceous-yellow parietal pigment in the lower part of the terminal element. Elements on sterile ridges 70–110 × 12–19 µm, cylindrical to clavate, thick-walled, hyaline or brownish pigmented. Stipe cortex of a textura globulosa, composed of variously sized, irregularly arranged rounded cells, mixed with long-cylindrical to clavate, or subcapitate terminal elements, with 2–3 septa, measuring 55–110 × 12–15 µm.

Specimens examined – TURKEY, Denizli province, Acıpayam district, around Kuzören town, under *Pinus nigra* J.F. Arnold, on calcareous soil, elev. 1100 m, 08.04.2010, coll. and det. by O. Kaygusuz (MK758080, OKA-TR-T4); Acıpayam district, around Kuzören town, under *P. nigra* on calcareous soil, elev. 1110 m, 09.04.2010, coll. and det. by O. Kaygusuz (MK758081, OKA-TR-T5); Denizli province, Bağbaşı district, around Zeytin Upland, under *Quercus coccifera* L. on calcareous soil, elev. 920 m, 07.04.2019, coll. and det. by O. Kaygusuz, (OKA-TR-T6); Denizli province, Bağbaşı district, around Zeytin Upland, under *Juniperus excelsa* Bieb. and *Q. coccifera* on calcareous soil, elev. 1205 m, 15.04.2017, coll. and det. by O. Kaygusuz, (MK734143, OKA-TR-T3); Denizli province, Bağbaşı district, around Zeytin Upland, under *J. excelsa* and *Q. coccifera* on calcareous soil, elev. 1195 m, 22.04.2017, coll. and det. by O. Kaygusuz, (MK734142, OKA-TR-T2); Denizli province, Bağbaşı district, around Zeytin Upland, under *J. excelsa* and *Q. coccifera*, on calcareous soil, elev. 1220 m, 23.04.2017, coll. and det. by O. Kaygusuz, (MK734141, OKA-TR-T1).

Fig. 2 – *Morchella tridentina*. Macroscopical features: A ascomata in various stages of maturity. B very pale mature ascomata. C ascomata at various stages of development. D–E very mature ascomata. F young ascomata. G–H ascomata dried in their natural habitat. Scale bars = 20 mm. Photos by O. Kaygusuz.

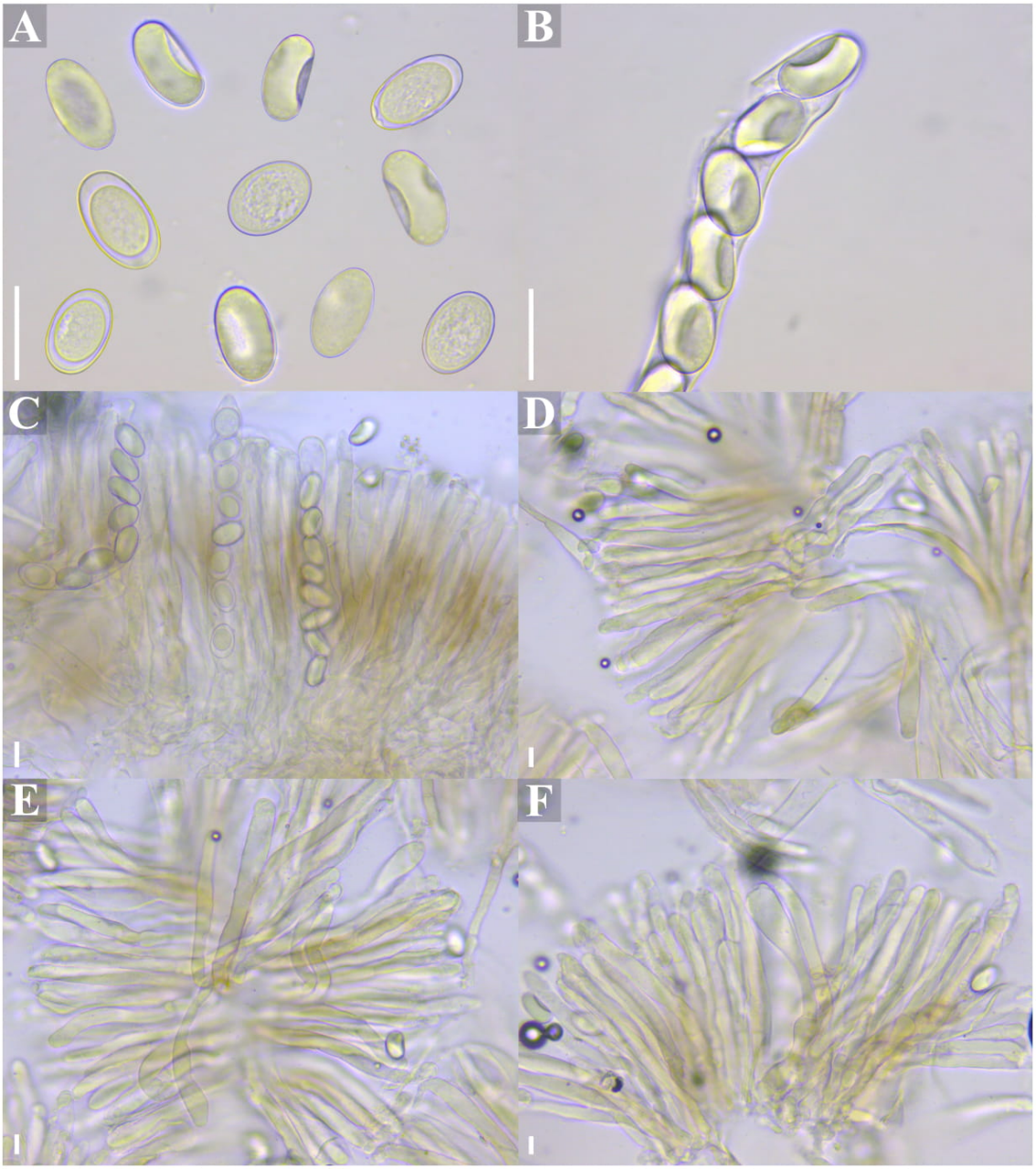

Fig. 3 – *Morchella tridentina*. Microscopical features: A ascospores. B–C asci and paraphyses. D–F acroparaphyses. Scale bars = 20 µm. Photos by O. Kaygusuz.

Ecology – Characteristically appearing in warm periods between late March and early April, usually present at over 900 m of elevation. The fruiting bodies appear solitary or in small groups, on mossy places with highly calcareous soil, in mixed forests. Found under or in the close vicinity of *Juniperus excelsa*, *Pinus nigra* and *Quercus coccifera*.

Notes – *Morchella tridentina* was originally described by Bresadola (1898) from the north Italy. This species is characterized by a relatively smooth and pure white stipe, the distinct rufescence coloration of the ascocarps, the ladder-like arrangement of pits like those of black morel, the non-darkening sterile ridges at maturity and the much less developed horizontal ridges (Roberts 2015, Loizides 2017). Recent phylogenetic and morphological studies clarified the taxonomy of *M. tridentina*, a cosmopolitan and widespread species described under many names, i.e. *M. conica* var. *pseudoeximia*, *M. elatoides* var. *elegans*, *M. elatoides*, *M. frustrata* and *M. quercus-ilicis* (Loizides et al. 2015, Loizides 2017).

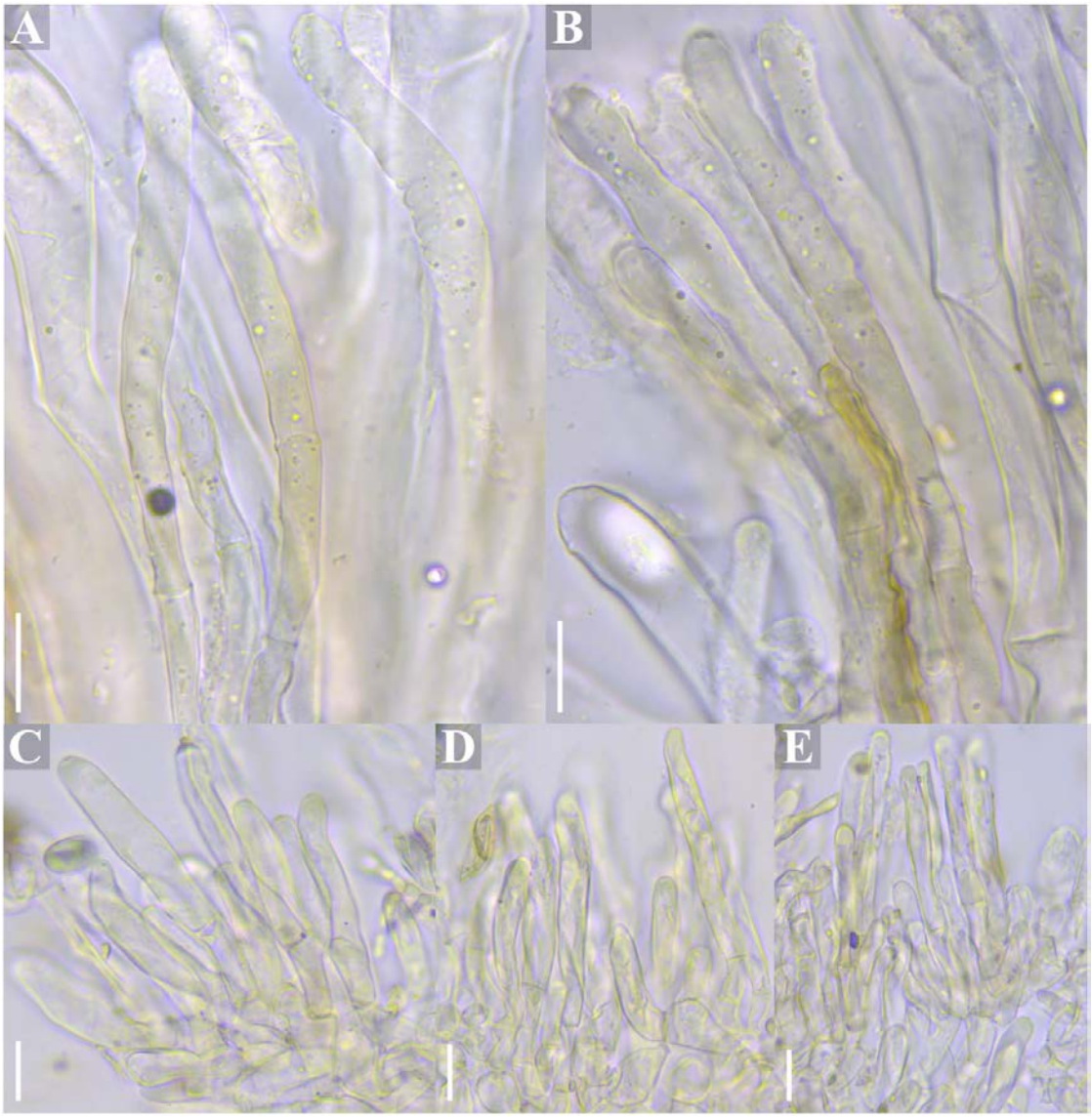

Fig. 4 – *Morchella tridentina*. Microscopical features: A–B paraphyses. C–E hyphae from the stipe. Scale bars = 20 μm. Photos by O. Kaygusuz.

Morphologically *M. tridentina* can be very similar to *M. rufobrunnea* Guzmán & F. Tapia, another cosmopolitan species with pale coloration and rufescent ridges, but the former usually has a uniformly coloured pileus at all stages of growth, attached to the stipe with a shallow sinus, whitish stipe (while *M. rufobrunnea* has a strongly lacunose, gray pruinose stipe), vertical ladder-like and split ridges (Loizides et al. 2015, 2016). Microscopically it has usually clavate or moniliform paraphyses with an ochraceous-yellow parietal pigment. While *M. tridentina* is found in broadleaved, coniferous or mixed woodland and matorral inhabitant, *M. rufobrunnea* is frequently found in under old olive trees on the urban and suburban coastal areas, or without the presence of a tree. Furthermore, *M. tridentina* usually appears later in the season, from mid-March to early May, mostly at elevation over 500 meters, while *M. rufobrunnea* appears in late winter, between mid-February to mid-March, at elevation scarcely exceeding 200 meters (Loizides et al. 2015). Due to its similar light coloured hymenophores, *M. tridentina* may also be confused with *M. esculentoides* M. Kuo, Dewsbury, Moncalvo & S.L. Stephenson, *M. esculenta* (L.) Pers. and *M. americana* Clowez & Matherly, but it can be distinguished by the vertically arranged pits. *M. tridentina* can appear similar to *M. dunalii* Boud., *M. kakiicolor* (Clowez & L. Romero) Clowez, L. Romero, P. Alvarado & Loizides, *M. purpurascens* (Krombh. ex Boud.) Jacquet. and *M. snyderi* M. Kuo & Methven, but it can be distinguished with non-darkening ridges at maturity (Loizides et al. 2015,

Loizides 2017). *M. tridentina* is the only black morel having the edge of the ribs that turns orange like yellow morels.

Although the general morphological characteristics of *M. tridentina* are similar to those of *M. rufobrunnea*, *M. esculentoides*, *M. esculenta*, *M americana*, *M. dunalii*, *M. kakiicolor*, *M. purpurascens* and *M. snyderi*, our ITS phylogeny with high statistical support in both analyses (BPP = 0.99, MLB = 98%, Fig. 1) and that of Loizides et al. (2015), Richard et al. (2015) and Clowez & Moreau (2018) placed *M. tridentina* into the *Morchella* sect. *Distantes*.

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