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# Extended phylogeography of the ancestral *Morchella anatolica* supports preglacial presence in Europe and Mediterranean origin of morels

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## ABSTRACT

Over 80 species are recognized in the commercially important genus *Morchella*, many of them endemic to specific regions or continents. Among them, *M. anatolica* and *M. rufobrunnea* are the earliest diverging lineages and are key in decoding the evolutionary history, global biogeography, and ecological trends within this iconic genus. Early ancestral area reconstruction (AAR) tests postulated a western North American origin of morels but had not included in the analyses *M. anatolica*, whose phylogenetic identity remained at the time unresolved. Following new collections of *M. anatolica* and *M. rufobrunnea* from the Mediterranean islands of Cyprus, Kefalonia, Lesvos, Malta, and Zakynthos, we performed revised AAR tests to update the historical biogeography of the genus. Our results, inferred from multilocus analysis of an expanded data set of 79 phylopecies, challenge previous reconstructions and designate the Mediterranean basin as the most likely place of origin for morels. Detailed morphoanatomical analyses demonstrate that ascocarp rufescence, the nondarkening apothecial ridges, the absence of a sinus, and the stipe pruinescence are all stable synapomorphic features of sect. *Rufobrunnea*, which could be interpreted as ancestral for the genus. The saprotrophic mode of nutrition, suggested by the prolific in vitro growth of *M. anatolica*, might also be an ancestral trait. Emended descriptions, including extensive imagery and scanning electron microscopy, are provided, and a new evolutionary hypothesis of the genus is proposed.

## KEYWORDS

Ancestral area reconstruction; evolution; historical biogeography; island biogeography; Morchellaceae; morphological analysis; Pezizales; refugia; systematics

## INTRODUCTION

True morels (*Morchella* Dill. ex Pers.) are gastronomically esteemed and commercially important ascomycetous fungi, widely distributed in temperate latitudes of the Northern Hemisphere. Due to the sometimes extreme polymorphism of their ascocarps, morels have long been in the midst of taxonomy debates, particularly over the number of taxa present in the genus and their distributional patterns (Boudier 1897; Groves and Hoare 1953; Dennis 1978; Jacquetant 1984). A number of molecular studies in recent years have confirmed that *Morchella* is a species-rich genus encompassing ~80 phylopecies worldwide, many of them confined to specific regions or continents (Taşkın et al. 2010, 2012; O'Donnell et al. 2011; Du et al. 2012a, 2012b, 2019; Loizides et al. 2016; Baroni et al. 2018). However, our understanding of the global biogeography of morels remains fragmentary, while updated morphological

descriptions for most taxa are still lacking, leading to difficulties in species identification by traditional morphological methods. At the same time, an increasing number of previously assumed endemic taxa have been shown to occur in more than one continent, resulting in controversies concerning the origins of such transcontinental distributions, but also with regard to the evolutionary history of the genus (O'Donnell et al. 2011; Taşkın et al. 2012; Richard et al. 2015; Loizides et al. 2015, 2016).

Phylogenetically, *Morchella* is naturally divided into three well-defined clades, currently arranged as sections *Rufobrunnea*, *Morchella*, and *Distantes* (Clowez 2012). The early diverging clade of *Rufobrunnea* (sect. *Rufobrunnea*) is characterized by species producing acutely conical and often rufescent ascocarps lacking a sinus, featuring predominantly longitudinally arranged apothecial ridges that remain persistently pale

at maturity. Section *Distantes*, on the other hand, is composed of species with a sinus and more or less longitudinally arranged ridges that typically darken at maturity, whereas sect. *Morchella* accommodates species with yellowish apothecial colors and irregularly arranged ridges and pits (Loizides 2017).

Just two species are presently known in the *Rufobrunnea* clade, which are estimated to have emerged in the late Jurassic (Du et al. 2012b): *Morchella rufobrunnea* Guzmán & F. Tapia, originally described from Mexico (Guzmán and Tapia 1998), and *M. anatolica* Işiloğlu, Spooner, Allı & Solak, described from Turkey (Işiloğlu et al. 2010). Although *M. rufobrunnea* was initially assumed to be endemic to the Americas (Kuo 2008; O'Donnell et al. 2011), subsequent collections from Israel (Masaphy et al. 2009), Cyprus (Loizides 2011), and Australia (Elliott et al. 2014) have since challenged this notion, documenting a transcontinental distribution for this species in Mediterranean and subtropical regions (Loizides et al. 2015). In stark contrast, *Morchella anatolica* is known just from three localities in the Mediterranean basin: the type locality of Muğla in the Aegean region of Turkey, and the Cádiz and Córdoba provinces of Andalusia in Spain (Taşkın et al. 2012; Richard et al. 2015; Palazón Pemán et al. 2017).

Because of the topological position *M. anatolica* and *M. rufobrunnea* occupy in global morel phylogenies, understanding their distributional patterns and evolutionary origins is of critical importance in inferring the genus' historical biogeography. To decipher the evolutionary history of the genus, therefore, more collections and data from these early diverging species are needed. New collections of *M. anatolica* from six different localities in the Mediterranean basin are reported in this paper, expanding its distributional range to the island of Cyprus and insular Greece, while the presence of *M. rufobrunnea* in the Maltese islands is confirmed by molecular data for the first time. To better understand the complex ecology of these archaic morels, pure cultures of *M. anatolica* were successfully isolated and the first in vitro description, as well as an emended morphological description, of this poorly known taxon is provided. In light of the newly available data, we also performed revised ancestral area reconstruction tests using an updated data set of 79 phylopecies, in order to shed light on the historical biogeography of the genus and confirm or reject previous reconstructions. Following the results of our analyses, the hypotheses with regard to the evolutionary origins of morels are further debated and a new evolutionary scenario is proposed.

## MATERIALS AND METHODS

**Studied collections and fungarium material.**— Nine collections from the Mediterranean islands of Cyprus, Kefalonia, Lesbos, Malta, and Zakynthos, respectively identified as *Morchella anatolica* and *M. rufobrunnea*, were analyzed using sequencing of four phylogenetically informative loci (internal transcribed spacer [ITS], the largest and second largest subunits of the RNA polymerase II [*rpb1*, *rpb2*], and the translation elongation factor 1 alpha [*tef1*]), macro- and microanatomical studies, scanning electron microscopy, in vitro culture studies, and finally ancestral area reconstruction (AAR) tests. The ascocarps of each collection were photographed in situ, and the altitude, surrounding vegetation, and substrate composition were documented. Exsiccatae are kept at CEFÉ (Centre d'Ecologie Fonctionnelle et Evolutive, Montpellier, France) and the Collection of Dried Fungal Specimens at the ATHUM (National and Kapodistrian University of Athens, Greece) Mycetheca, with duplicates in the private collections of the authors. Herbarium abbreviations follow Thiers et al. [continuously updated]. Phylopecies not yet formally described or taxonomically clarified are respectively designated as “*Mel*” for the *Elata* clade and “*Mes*” for the *Esculenta* clade followed by a serial number for each species, based on the coding system introduced by Taşkın et al. (2010) and O'Donnell et al. (2011).

**Morphological analyses.**— Macroanatomical features were observed and documented in detail from fresh ascocarps in various developmental stages, based on methods described in Loizides et al. (2015, 2016). Four optical microscopes were used for microanatomical studies: an AM T360B Infinity plan achromatic (USA), a Leica BM E (Wetzlar, Germany), an Optika B-500 Ti (Ponteranica, Italy), and a Zeiss Axioimager (Oberkochen, Germany) with a Zeiss AxioCam MRC digital camera, at  $\times 40$ ,  $\times 100$ ,  $\times 400$ , and  $\times 1000$  magnifications. Ascospores from three ascocarps of *M. anatolica* and two of *M. rufobrunnea* were obtained from naturally discharged prints on a glass slide when possible, or from deposits on the stipe surface. At least 40 spores for each species were measured in water and the Me (average length and width), Q (minimum and maximum length/width ratios), and Qm (average length/width ratio) values were calculated. Spore ornamentation was observed from dry sections of the hymenium mounted in heated lactophenol cotton blue following rehydration for a minimum of 2 h (Baroni et al. 2018) and further evaluated under a Hitachi S-3000-N scanning electron microscope (SEM; Krefeld, Germany) following gold

**Table 1.** GenBank accession IDs of newly sequenced collections.

| Species                      | Specimen voucher | Country | Locality         | GenBank accession IDs |             |             |             |
|------------------------------|------------------|---------|------------------|-----------------------|-------------|-------------|-------------|
|                              |                  |         |                  | ITS                   | <i>rpb1</i> | <i>rpb2</i> | <i>tef1</i> |
| <i>Morchella anatolica</i>   | ML81331MA2       | Cyprus  | Lythrodontas     | MT036037              | MT232931    | MT232939    | MT232947    |
| <i>Morchella anatolica</i>   | ML81331MA        | Cyprus  | Moni Lemesou     | MT036038              | MT232932    | MT232940    | MT232948    |
| <i>Morchella anatolica</i>   | GF813421MA       | Greece  | Lesvos island    | MT036039              | MT232933    | MT232941    | MT232949    |
| <i>Morchella anatolica</i>   | ATHUM9661        | Greece  | Zakynthos island | MT036040              | MT232934    | MT232942    | MT232950    |
| <i>Morchella anatolica</i>   | ML9141MA         | Cyprus  | Moni Lemesou     | MT036042              | MT232935    | MT232943    | MT232951    |
| <i>Morchella anatolica</i>   | ML914MAK1        | Greece  | Kefalonia island | MT036043              | MT232936    | MT232944    | MT232952    |
| <i>Morchella anatolica</i>   | ML914MAK3        | Greece  | Kefalonia island | MT036044              | MT232937    | MT232945    | MT232953    |
| <i>Morchella rufobrunnea</i> | ML91291M         | Cyprus  | Lemesos          | MT036045              | MT232938    | MT232946    | MT232954    |
| <i>Morchella rufobrunnea</i> | CS789            | Malta   | Verdala area     | MT036041              |             |             |             |

coating (Quorum Q150R S Plus; Laughton, UK) and imaged at 15 kV (Y. Ruiz, Real Jardín Botánico, Madrid, Spain). Additional microanatomical features were studied in 5% potassium hydroxide (KOH) from both fresh and dried material. Hyphoid hairs (terminal elements) of the ectal excipulum were observed from fine sections obtained from the stipe surface of young ascocarps, preferably the apex. Acroparaphyses were observed from fine sections obtained from intact apothecial ridges of young ascocarps. Terminology of macro- and microanatomical characters follows Loizides et al. (2016). Ascospore measurements are reported as (smallest absolute measurement–)lowest mean measurement–highest mean measurement(–highest absolute measurement), all rounded to the nearest half micron.

**Fungal isolation, culture preparation, and in vitro analyses.**— Pure culture isolations were obtained in potato dextrose agar medium (PDA; Difco, BD, USA), from both ascospores and sterile ascocarp tissue of specimen ATHUM9691. Macro- and micromorphological features were observed from the spore-isolated strain, which was inoculated on PDA and incubated at 25 °C, in both dark and daylight conditions. Microscopic observations, measurements, and imaging were carried out under the Zeiss microscope using bright field, as well as differential interference contrast (DIC). The fungal specimens were mounted in 3% KOH with or without the addition of phloxine. The strain is preserved in the Culture Collection of Fungi at the ATHUM Mycethoteca, under the same accession number as the dried specimen from which it originated. Terminology of the mycelium micromorphological features follows Cléménçon (2004).

**DNA extraction, amplification, and sequencing.**— Dried samples were extracted with the REExtract-N-Amp Plant PCR Kit (Sigma-Aldrich, St. Louis, Missouri), following the manufacturer's instructions. The internal transcribed spacer region of the nuclear rDNA locus (ITS), the largest and second largest

subunits of the RNA polymerase II (*rpb1* and *rpb2*, respectively), and the translation elongation factor 1 alpha (*tef1*) were polymerase chain reaction (PCR)-amplified using, respectively, primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993), gRPB1A/aRPB1C (Matheny et al. 2002), 9F/3R (Liu et al. 1999), and EF526F/EF3AR (Rehner and Buckley 2005), following Richard et al. (2015). Sequencing was performed at Eurofins Genomics (Ebersberg, Germany). Sequences were edited and assembled under Codon Code Aligner 4.1.1 (CodonCode Corp., Centerville, Massachusetts) and deposited in GenBank under accession numbers listed in TABLE 1.

**Phylogenetic analyses.**— Two distinct data sets were assembled and phylogenetically analyzed. Data set 1 includes *rpb1*, *rpb2*, and *tef1* sequences from eight novel collections of *M. anatolica* and *M. rufobrunnea*, together with sequences of 26 collections belonging in sect. *Rufobrunnea* ( $n = 1$ ), sect. *Morchella* ( $n = 8$ ), and sect. *Distantes* ( $n = 17$ ). Data set 2 includes the nine ITS sequences generated for the present study and all published ITS sequences of *M. anatolica* ( $n = 4$ ) and *M. rufobrunnea* ( $n = 25$ ). Multiple sequence alignments were performed online at [www.phylogeny.fr](http://www.phylogeny.fr) (Dereeper et al. 2008) with MUSCLE 3.7 (Edgar 2004), following concatenation for data set 1. Maximum likelihood phylogenies were inferred with PhyML 3.0 (Guindon et al. 2010), using the GTR+I+ $\Gamma$  model of evolution and the Shimodaira-Hasegawa version of the approximate likelihood-ratio test (SH-aLRT) of branch support (Anisimova et al. 2011), which has been estimated to be statistically significant when  $>0.8$  (Bellanger et al. 2015). Bayesian inference of phylogeny was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), on the CIPRES science gateway (Miller et al. 2010). Two runs of four Monte Carlo Markov chains were performed for 1 000 000 generations, with tree and parameter sampling every 1000 generations. The initial burn-in was set to 25% (250 trees). Stationarity was achieved, as assessed by potential scale reduction factor

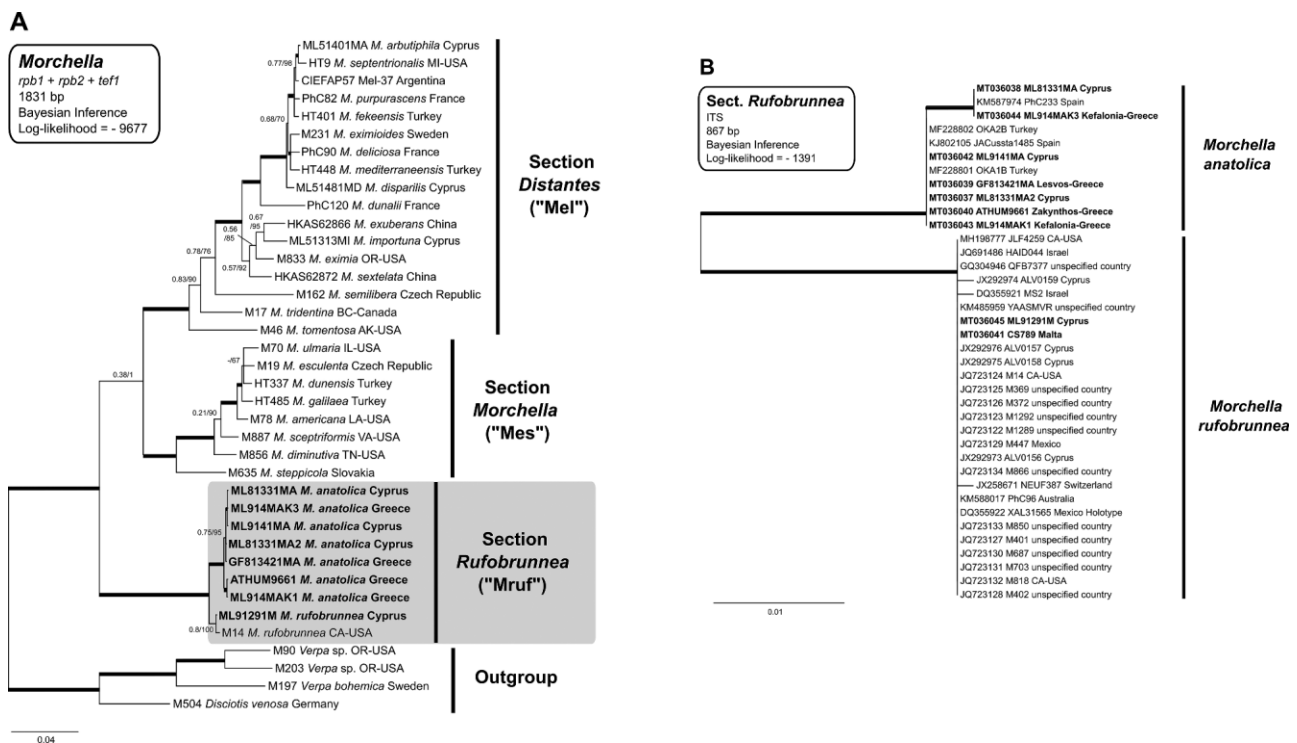
(PSRF; Gelman and Rubin 1992) of parameters between 0.997 and 1.002. A 50% majority-rule consensus tree was computed, and the proportions of this tree correspond to Bayesian posterior probabilities (BPPs) that are reported (as percentages) on branches of the phylograms depicted in FIG. 1. Trees were built using TreeDyn 198.3 (Chevenet et al. 2006) and edited with Inkscape 0.91 (<https://inkscape.org/fr>).

**Ancestral area reconstruction tests.**— To estimate the geographic origin probabilities of *Morchella*, we used Lagrange reconstruction implemented in the BioGeoBEARS R packages (Matzke 2013). First, we collected geographic data out of published and unpublished sequences of *Morchella* species, coding origins as the following eight ecoregions depicted on the map in FIG. 2: South and Central America (S-A), West North America (WNA), East North America (ENA), Africa excluding the Mediterranean area (Afr), Europe excluding the Mediterranean area (Eur), Asia excluding the Mediterranean area (Asi), the Mediterranean area as defined in, e.g., Quézel and Medail 2003 (Med), and Oceania (Oce). Second, we built a comprehensive

multilocus phylogeny of 79 species at the genus scale, performed as described above for data set 1. Then, we found the best DEC (dispersal-extinction cladogenesis) model (scripts and parameters available in SUPPLEMENTARY DATA) given the phylogeny and the distribution of sampled *Morchella* species. Finally, we used the best DEC model to compute the ancestral distribution of each internal node in the form of probabilities regarding each species' presence in each region, except two Mediterranean species, *Mes-28* and *M. castaneae*, which could not be included in the analysis because only their ITS sequences are currently available in public databases.

## RESULTS

The phylogenetic analysis of our multilocus data set (data set 1,  $n = 34$  collections sequenced at the *rpb1*, *rpb2*, and *tef1* loci, of which 8 were newly generated for the present study) confirms that *Morchella* consists of three distinct and strongly supported lineages (FIG. 1A). Section *Rufobrunnea*, including for the first time *M. anatolica* in a multilocus phylogeny, displays maximal branch support in maximum likelihood and



**Figure 1.** Phylogeny of *Morchella* sect. *Rufobrunnea*. A. Bayesian inference (BI) analysis of a multilocus (*rpb1* + *rpb2* + *tef1*) sequence data set spanning the entire *Morchella* genus. B. Bayesian inference (BI) analysis of an ITS sequence data set including all available sequenced collections belonging to sect. *Rufobrunnea*. Maximum likelihood (ML) analyses yielded the same tree topologies as those illustrated here, except for *M. galilaea* in A, which is not resolved from the trio *M. dunensis*/*M. esculenta*/*M. ulmaria*. Values by nodes are ML (SH-aLRT) and BI (BPP, as percentage) branch supports. When these metrics are both strongly significant (SH-aLRT > 0.8 and BPP ≥ 95%), they are not indicated but the branch is thickened. Sequences generated for the present study are highlighted in bold.

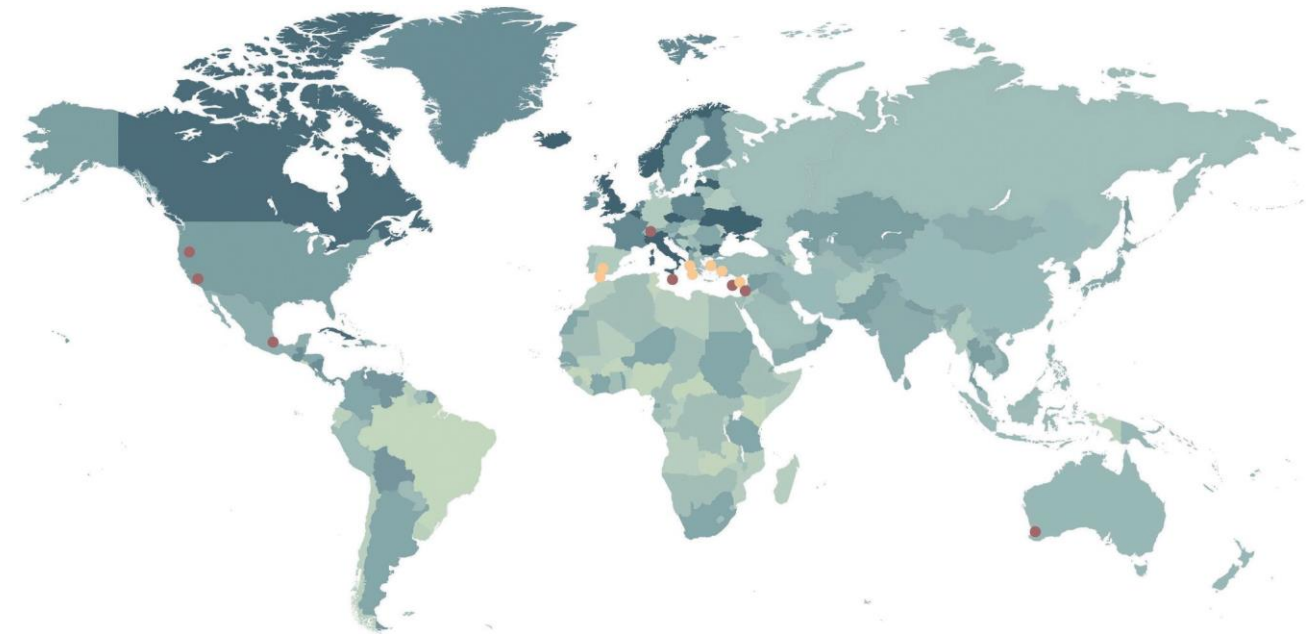
Bayesian inference (SH-aLRT = 1, BPP = 100%, respectively), and the two sister species are also strongly supported as monophyletic (SH-aLRT = 0.97, BPP = 100%, respectively, for *M. anatolica*). Furthermore, the section is firmly established by both analyses as the earliest diverging lineage within the genus, consistent with previous findings for *M. rufobrunnea* alone (O'Donnell et al. 2011; Du et al. 2012b). A later common ancestor for sect. *Morchella* and sect. *Distantes* is resolved by both ML and BI, with markedly different support values though (SH-aLRT = 0.38, BPP = 100%, respectively).

The analysis of our ITS data set focusing on the sect. *Rufobrunnea* (data set 2,  $n = 38$  ITS sequences, of which 9 were generated for the present study) confirms the presence of two strongly supported species in this section (SH-aLRT = 1, BPP = 100% for both species; FIG. 1B). *Morchella rufobrunnea* displays an intercontinental but disjunct distribution and is so far represented in Mexico, the west coast of North America, Australia, Switzerland, Israel, and the Mediterranean islands of Cyprus and Malta. By contrast, *Morchella anatolica* appears to be much more regionalized and so far narrowly endemic to the Mediterranean region. In addition to the previous reports from the Aegean region of Turkey and southern Spain, its presence is here confirmed on the Aegean island of Lesbos, the Ionian islands of Kefalonia and Zakynthos, and the island of Cyprus (FIG. 2).

At the ITS locus, the two species differ by a minimum of 29 substitutions and 11 indels, representing ca. 3.5%

of sequence divergence. Polymorphism within *M. rufobrunnea* is low, but three ITS sequences within *M. anatolica* form a well-supported subclade relative to the core clade, reflecting two substitutions and one length polymorphism in the ITS1 sequence of collections ML81331MA, ML914MAK3, and PhC233 (FIG. 1B). However, this intraspecific topology is not supported by the multilocus analysis, which also displays slight polymorphism but groups together different collections (FIG. 1A). Because of the lack of phylogenetic structure and biogeographic, morphological, or ecological support to these signals, we conclude that they represent natural polymorphism within *M. anatolica* rather than cryptic speciation.

Three of the seven newly sequenced collections of *M. anatolica* (ATHUM9661, GF813421MA, ML9141MA) and both newly sequenced collections of *M. rufobrunnea* (CS789, ML91291M) were found directly under or in close vicinity of *Olea europaea*, a pattern consistent with previous sightings of these species, suggesting a possible biotrophic association with *Oleaceae* (Richard et al. 2015; Loizides et al. 2015; Palazón Pemán et al. 2017). However, the prolific growth of collection ATHUM9661 in vitro, as well as collections ML81331MA and ML81331MA2 found without the presence of *Oleaceae* plants, indicates that this putative association is not obligate and both *M. anatolica* and *M. rufobrunnea* can function as facultative saprotrophs. Detailed morphoanatomical comparisons between collections of the two species



**Figure 2.** Global distribution of the two earliest diverging species in genus *Morchella*, *M. anatolica* (yellow) and *M. rufobrunnea* (red), based on collections confirmed by DNA sequencing.

demonstrate that the section is defined by a stable set of synapomorphic traits, most notably the nondarkening, longitudinally arranged apothecial ridges, the rufescence of the ascocarps, an absence of a sinus at the attachment of the pileus with the stipe, a dark pruinescence on the stipe surface, and, microscopically, longitudinally deeply grooved ascospores and lack of capitate elements in the acroparaphyses (FIGS. 3, 5; see Taxonomy).

Our updated biogeographic data and ancestral area reconstruction (AAR) analysis supports distinct origins for the main *Morchella* clades (FIG. 6). The *Esculenta* clade (sect. *Morchella*) is currently dominated by species occurring in China, and, consistently, our analyses designate Asia as the most likely origin of this lineage, with late and independent migrations or introductions into Europe or the New World. Conversely, the most likely origin of the *Elata* clade (sect. *Distantes*) appears to be western North America (WNA), mainly driven by the early branching of the North American endemic *M. tomentosa*. The origin of the last common ancestor of the *Esculenta* and *Elata* clades is less clear, designated as either Asiatic or western North American in our multilocus reconstruction. Contrary to previous reconstructions, however, the early-diverging sect. *Rufobrunnea* is here supported to have a Mediterranean origin, mainly driven by the narrowly restricted distribution of *M. anatolica* to this ecoregion. As a result, our analyses designate the Mediterranean area as the most likely place of origin for the genus, contradicting previous historical reconstructions (O'Donnell et al. 2011; Du et al. 2012b) that postulated a North American origin of morels.

## TAXONOMY

*Morchella anatolica* Işiloğlu, Spooner, Allı & Solak, Mycologia 102:455. 2010. FIGS. 3–6

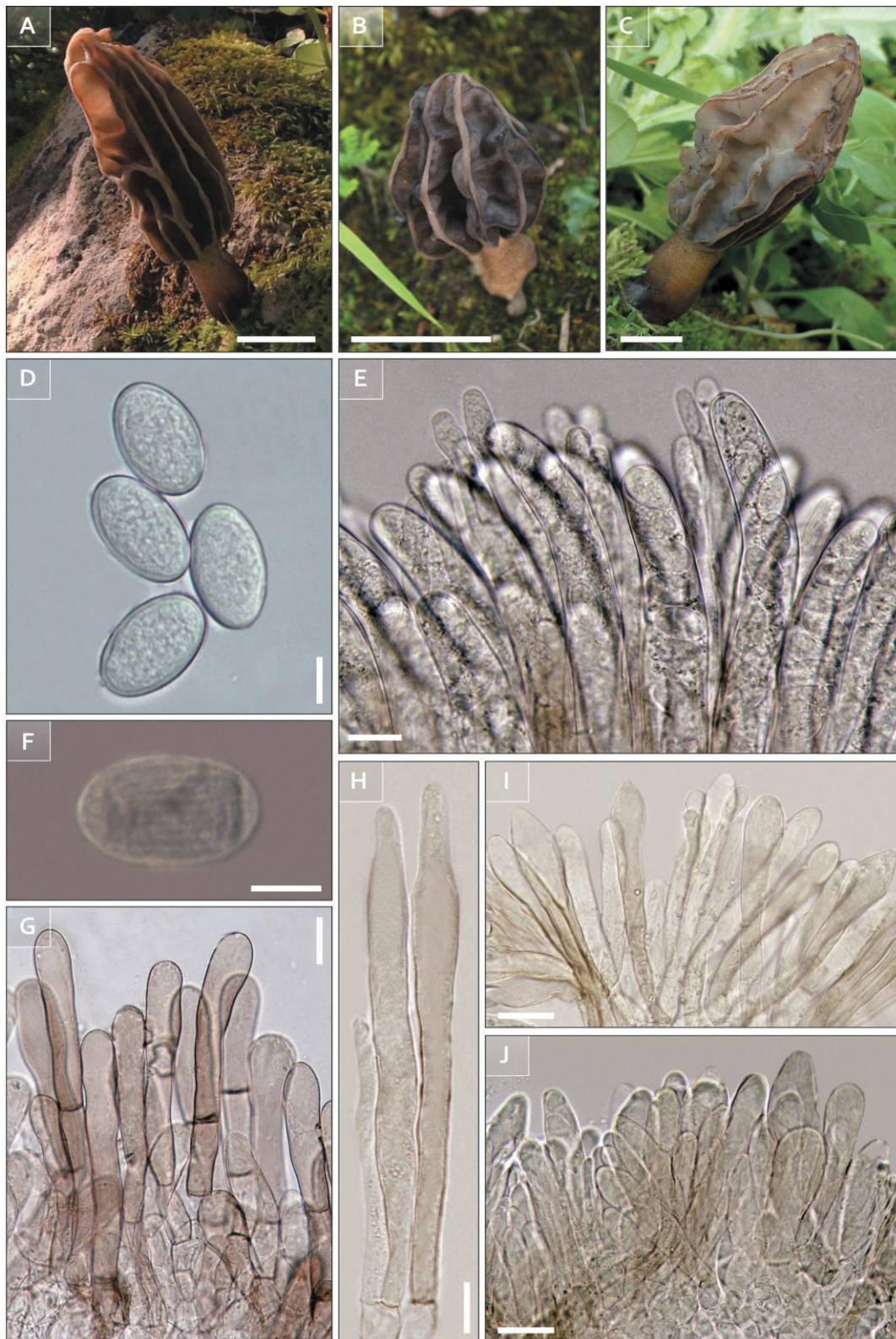
= *Morchella lanceolata* Clowez & Illescas in Clowez, Bull Soc Mycol France 126(3–4):282. 2012, ad int. (nom. inval., type not designated).

Pileus 2–4(–6) cm high × 1–1.5 cm wide, slenderly elongated to acutely conical, attached to the stipe without a sinus. Longitudinal primary ridges distant, 12–18, more or less parallelly arranged to somewhat sinuous, with or without sparse anastomoses, tomentose and silvery white to buff when young, not darkening at maturity, but often staining reddish-orange or reddish-brown and moderately eroding with age. Transverse secondary ridges usually absent but sparse, isolated transverse ridges may occasionally form as anastomoses of the primary ridges. Pits large and vertically elongated, often stretching from the base of the pileus all the way to the apex; surface weakly lobed to somewhat blistered,

charcoal gray to black when young and creating a sharp contrast with the pale ridges, gradually paler and eventually concolorous with the ridges in fully maturity, buff to pale grayish-brown, sometimes with dull purplish-lilac tinges. Secondary pits absent. Stipe usually  $\frac{1}{4}$  to  $\frac{2}{3}$  the length of the pileus, measuring 1–3(–4) × 0.7–1.5 cm, cylindrical but inflated at the base, smooth or weakly wrinkled, initially covered in a distinct gray or brownish-gray pruinescence often fading in mature ascocarps; stipe surface furfuraceous and often rufescent. Flesh elastic, rather tough; odor weak, faintly spermatoc or indistinct. Spore mass ochraceous.

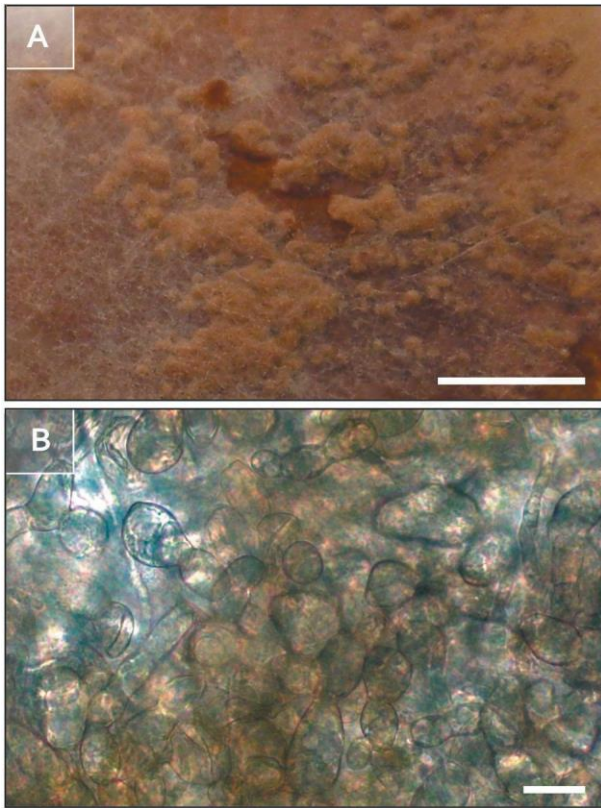
*Micromorphological description:* Ascospores (22.5–)24–27(–32) × (12–)13–17(–20) µm, Me = 25.6 × 15.8 µm, Qm = 1.64 [*n* = 40], elliptical, hyaline, thick-walled, frequently with lipid bodies (guttules) externally attached to the poles; spore surface appearing smooth in water but deeply longitudinally channeled in lactophenol cotton blue, with multiple depressed interconnecting ridges. Asci 266–345 × 20–26(–35) µm [*n* = 20], cylindro-clavate, hyaline, uniseriate or rarely irregularly biseriate, 8-spored, simple-septate at the base, inamyloid. Paraphyses hyaline, bifurcate or locally fasciculate, slenderly cylindrical or slightly inflated in parts, measuring 150–250 × 8.5–12 µm [*n* = 20], 1–2(–3)-septate in the lower third and with diffused, brownish-gray intracellular pigment when young, not or only slightly constricted at the septa; apices rarely enlarged, usually rounded to attenuated, occasionally also ophiomorphous (snakehead) and enlarged to 15–18 µm wide. Heteroparaphyses not seen. Acroparaphyses measuring 60–217 × 14–30 µm [*n* = 20], fasciculate, polymorphic, ranging from cylindrical, clavate, fusiform, lanceolate or occasionally ophiomorphous, thick-walled, locally encrusted and often gray-pigmented, 1–4-septate in the lower third and usually catenulate at the base, arising from an intricate hyphal structure resembling a *textura angularis/globosa*, with some elongated elements intermixed; capitate elements in the acroparaphyses apices absent or extremely rare. Stipe cortex a *textura globosa*, giving rise to abundant, fasciculate, subhyaline to pale gray and subcapitate, clavate or pyriform hyphoid hairs measuring (64–)89–174(–222) × 23–40 µm [*n* = 20], 1–3-septate, thick-walled and often markedly constricted at the septa.

*Ecology and distribution:* Appearing solitary or in small groups from late March to mid-April, in low to middle range altitudes rarely exceeding 500 m, growing on streambanks, among mosses and gravel, or on rocky banks, sometimes in the presence of *Fraxinus angustifolia*, *Olea europaea*, and other Mediterranean vegetation. Restricted to the Mediterranean basin and so far confirmed with DNA evidence from Muğla (Turkey), Cádiz



**Figure 3.** Macro- and micromorphological aspect of *M. anatolica*. A. ATHUM9661, semimature ascocarp, ~50% life cycle. B. ML914MAK1, young ascocarp, ~25% life cycle. C. GF813421MA, mature ascocarp, ~80% life cycle. D. Naturally ejected ascospores in water. E. Palisade of asci and paraphyses in KOH. F. Ascospore in heated lactophenol cotton blue revealing longitudinal striations. G. Clavate to subcapitate terminal elements (hyphoid hairs) of the ectal excipulum of the stipe in KOH. H. Bifurcate paraphyses in KOH. I, J. Palisade of acroparaphyses in KOH showing a mixture of fusiform, clavate, cylindrical, and ophiomorphic elements. Bars: A-D, F, H = 10 mm; E = 20  $\mu$ m; G, I, J = 30  $\mu$ m.





**Figure 4.** Culture of *M. anatolica* (ATHUM9661) growing on PDA. A. Primary isolation in pure culture from spores, 20 d old. B. Mycelial crusts in a 60-d-old culture. C. Cuticular hyphae of mycelial crusts. Bars: B = 5 mm; C = 20  $\mu$ m.

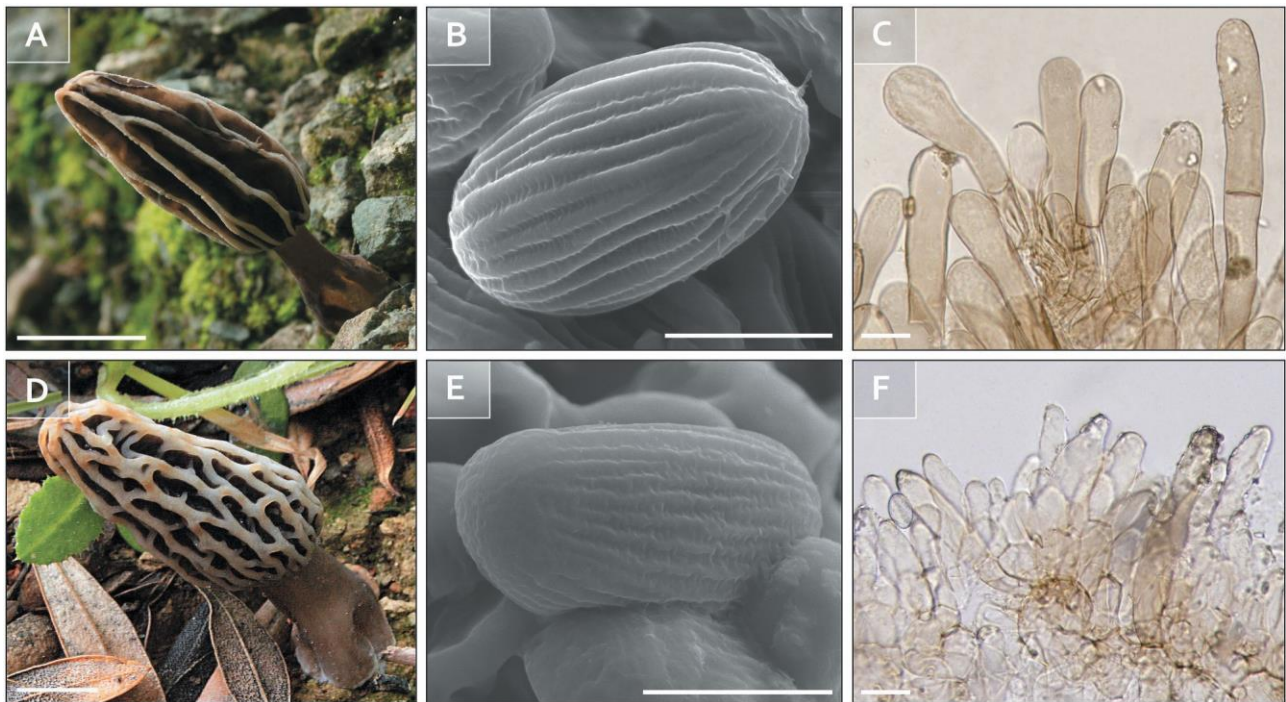
and Córdoba (Spain), the islands of Kefalonia, Lesvos, and Zakynthos (Greece), and the island of Cyprus.

**Culture description:** Colony quite fast-growing, covering the 90-mm Petri dishes in almost 20 d, with slower growth after successive reculturings finally reaching a diameter of 30–40 mm. Mycelium homogeneous, buff-colored, rather felty and adhered to medium, heterogeneous in most recent cultures with a compact center area, buff to paler beige, with concolorous sparse aerial hyphae and sometimes forming irregularly shaped mycelial crusts, mainly at the colony margin (FIG. 4A–B). Reverse colony color brownish to pale brown, soluble exudate in medium buff-yellowish. Vegetative hyphal system diverse with sparsely branched, thick-walled hyphae with intraparietal brown pigment, 8–14.5  $\mu$ m diam; hyphae hyaline, slightly thick-walled; leading hyphae thin to slightly thick-walled, hyaline or light brownish, rather straight, 5–7.5  $\mu$ m; and interlacing hyphae thin- to slightly thick-walled, hyaline or light brownish, less straight to tortuous, 2.5–5  $\mu$ m diam; the latter three types of hyphae with even walls, but also quite often incrustated and with branches usually in more or less right angles. Unusual secondary formation of hyphae

developing within empty ones. Mycelial crusts with interlocking cuticular cells and hyphae.

**Specimens examined:** *Morchella anatolica*—CYPRUS. Limassol District: Moni, circa 50 m above sea level (a.s.l.) on rocky bank among gravel and mosses, 12 Mar 2018, M. Loizides ML81331MA; Ibid., circa 50 m a.s.l. under *Olea europaea*, 1 Apr 2019, M. Loizides ML9141MA; Nicosia District: Lythrodontas, circa 400 m a.s.l. on mossy stream bank near *Nerium oleander* and other vegetation, 13 Mar 2018, M. Chelides ML81331MA2; GREECE. Kefalonia: Kefalonia District, Ayia Irini circa 85 m a.s.l. on rocky stream bank near *Ulmus minor*, *Platanus orientalis*, *O. europaea*, and other vegetation, 31 Mar 2019, P. Drakopoulos ML914MAK1; Ibid., 23 Apr 2019, P. Drakopoulos ML914MAK3; Lesvos: Mytilene District: Ippeios, circa 15 m a.s.l. under *O. europaea*, with *Ulmus canescens*, *Celtis australis*, and other vegetation present in the vicinity, 24 Mar 2018, Giorgos Fransuas GF813421MA; Zakynthos: Xyrokastello, Longos Agiou, among mosses, near *Quercus coccifera* with *Cupressus sempervirens*, *O. europaea*, and other vegetation in the surrounding area, Mar 2014, K. Poirazidis & A. Martinis ATHUM9661. *Morchella rufobrunnea*—CYPRUS. Limassol District: Lemesos, 30 m a.s.l. under *O. europaea*, 19 Feb 2019, M. Loizides ML91291M; MALTA. Siggiewi District: Verdala area (Buskett), circa 175 m a.s. l. near *Olea europaea* and conifers, 10 Jan 2015, C. Sammut CS789.

**Notes:** Section *Rufobrunnea* is defined by a stable set of characters and accommodates two closely related but phylogenetically, morphologically, and phenologically well-delimited species. Both species form acutely conical and rufescent ascocarps with persistently pale longitudinally arranged apothecial ridges, lack a sinus at the attachment of the pileus with the stipe, and have a stipe surface covered in a distinct gray-brown pruinescence formed by clusters of extensively encrusted terminal hyphae with gray-brown intracellular pigment. Despite these similarities, however, discriminating between the two species is usually straightforward: *Morchella anatolica* produces smaller and often acutely slender ascocarps with very few or no transverse interconnecting apothecial ridges (FIGS. 3A–C, 5A), whereas *M. rufobrunnea* ascocarps are typically more robust and have several interconnecting ridges forming multiple longitudinally arranged pits (FIG. 5D). Microscopically, both species are characterized by predominantly fusiform elements in the acroparaphyses, with entirely absent or extremely rare capitate elements (FIG. 3I, J), and have longitudinally striate ascospores when viewed in SEM (FIG. 5B, E), lactophenol cotton blue (FIG. 3F), or sometimes KOH. However, the ascospores of *M. anatolica* are slightly larger on average,



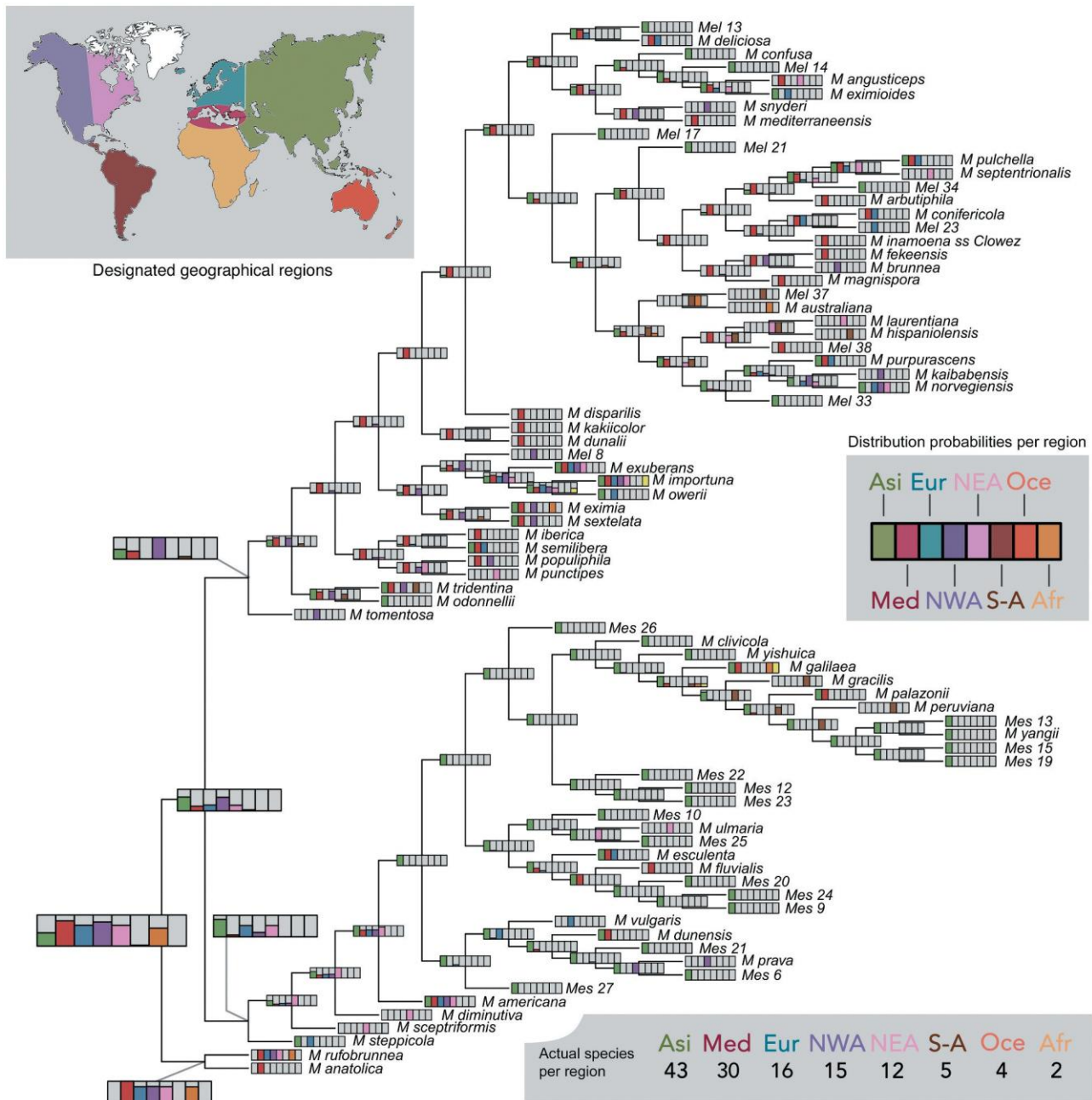
**Figure 5.** Comparison of key characters between *Morchella anatolica* (top row) and *M. rufobrunnea* (bottom row). A, D. Ascocarps in situ displaying differences in gross morphology and contrasting anatomy of apothecial ridges. B, E. Ascospore ornamentation imaged under scanning electron microscope (SEM) displaying deeper longitudinal channels in the spores of *M. anatolica*. C, F. Differently shaped and sized terminal elements (hyphoid hairs) of the ectal excipulum. Bars: A, B, D, E = 10 mm; C, F = 20  $\mu$ m.

usually ranging between 24 and 28  $\mu$ m in length (FIG. 5B), whereas those of *M. rufobrunnea* mostly range between 22 and 26  $\mu$ m (FIG. 5E). It nonetheless must be noted that careful study of several specimens revealed that large-spored ( $\sim$ 30  $\mu$ m) collections of both species can sometimes occur; therefore, spore size alone cannot reliably discriminate between them. Instead, the two species have differently shaped and sized terminal hyphae in the ectal excipulum of the stipe: distinctly long and clavate to subcapitate in *M. anatolica*, frequently exceeding 100–200  $\mu$ m (FIGS. 3G, 5C); shorter and cylindrical to fusiform in *M. rufobrunnea*, rarely exceeding 100  $\mu$ m (FIG. 5F). Even though both species are often found in the presence of *Oleaceae* plants, they do not appear to be strict biotrophs and, at least in the Mediterranean region, exhibit distinct phenological and ecological patterns. *Morchella rufobrunnea* fruits very early in the season from mid- or late winter to early spring (January–March) and frequently even in the autumn months in urban and suburban areas (Masaphy et al. 2009; Loizides et al. 2011, 2015; Sammut 2015), whereas *M. anatolica* fruits later, from mid-March to mid-April in rural areas, usually on rocky, well-drained substrates and streambanks. Judging from its distinctive morphology, it is believed that the gray-stemmed unidentified morel depicted in Kyriakou et al.

(2009) also represents *M. anatolica*, although we were not able to sequence this collection.

## DISCUSSION

The origins and evolutionary history of morels have been controversial. Early ancestral area reconstruction (AAR) tests (O’Donnell et al. 2011) estimated the divergence of *M. rufobrunnea* from its closest genealogical relatives to have occurred in the early Cretaceous within western North America, some 129 million years ago (Mya). This preliminary estimate was later revised by Du et al. (2012b), who also postulated a North American origin of the genus but pushed the date back to the late Jurassic at  $\sim$ 154 Mya. None of these studies, however, had included *M. anatolica* in their analyses, which at the time remained phylogenetically unresolved. Moreover, both studies assumed its sister species *M. rufobrunnea* to be an American endemic. This assumption later revealed to be doubtful, as sequenced collections from Israel (Masaphy et al. 2009), Australia (Elliott et al. 2014), Cyprus (Loizides et al. 2015), and Malta (this study) have provided evidence of an intercontinental distribution for this species in Mediterranean and subtropical regions. The phylogenetic placement of the isotype



**Figure 6.** Multilocus *rpb1* + *rpb2* + *tef-1* ancestral area reconstruction (AAR) of the *Morchella* genus, using 79 *Morchella* species and the DEC (dispersal-extinction cladogenesis) model in BioGeoBEARS R package (Matzke 2013). For each node, the ancestral distribution is depicted by the probabilities for the ancestor to inhabit each color-coded region. Note that branch length is not informative in this phylogeny.

collection of *M. anatolica* in the *Rufobrunnea* clade should have called for revised AAR tests to incorporate this critical new data, but instead the presence of this early diverging lineage in Turkey was controversially attributed to accidental anthropogenic introductions from North America (Taşkın et al. 2012), even though no collection of this species is known from the American continent. We consider such a scenario extremely unlikely, not only because this species is apparently absent

from North America, but also because all evidence so far points to a paleoendemic presence of *M. anatolica* in the Mediterranean (FIG. 2), where it occurs within native secluded habitats often in association with indigenous flora.

The new collections reported here strongly support *M. anatolica*'s endemism in the Mediterranean region, and our revised multilocus AAR tests designate the Mediterranean basin as the most likely place of origin

for morels (FIG. 6). Algorithms underlying AAR analyses are heavily influenced by the topology of the phylogenetic tree at the deepest nodes for each lineage, which likely accounts for the differences between previous and present reconstructions, since *M. anatolica* is one of the two earliest diverging lineages in the genus. The sensitivity of AAR analyses to deep-node topologies is further exemplified by present results regarding the origins of the *Distantes* clade (FIG. 6). The topological position of the American endemic *M. tomentosa* within this clade has indeed a profound impact on the inferred geographic origin of sect. *Distantes*, which is predicted to be western North America (WNA) despite a proliferation of contemporary *Distantes* species occurring in Asia.

These strengths and limitations of AAR inferences call for cautious interpretations of the results and careful consideration of complementary evidence, such as ecological or biogeographic constraints and estimated abundance of species in each continent, especially for the early diverging lineages. For instance, the earliest diverging lineage of the *Esculenta* clade, *M. steppicola*, is absent from North America and at the same time

widespread in temperate grasslands and arid steppes of central Eurasia and the Balkans (Yatsiuk et al. 2016, unpubl. data). This fact in itself challenges the previous hypothesis postulating a North American origin for this species, followed by its assumed dispersion into Eurasia through the Thulean North Atlantic land bridge (O'Donnell et al. 2011), and our revised AAR analyses indeed support an Asian origin for *M. steppicola*. In contrast, the second earliest diverging species in sect. *Distantes*, *M. tridentina*, exhibits a transcontinental but disjunct distribution, having been molecularly verified in no fewer than 10 countries on four continents and is linked to an exceptional number of putative tree hosts (Loizides et al. 2015; Loizides 2017). The fragmented biogeography of this species makes its place of origin less clear in our ancestral reconstructions (FIG. 6), although its disjunct distribution is more likely the result of climatic refugia from a wider, originally pantropical population (Loizides et al. 2015, 2016), rather than recent anthropogenic introductions (Taşkın et al. 2012).

Hot spots of endemic or range-restricted species are strongly linked to long-term climatic stability (Fjeldså et al. 1997; Jansson 2003; Feng et al. 2016; Sandel et al. 2016; Harrison and Noss 2017). Periodical changes in Earth's orbit have triggered dramatic climate fluctuations in the past, the so-called "Milankovitch climate oscillations," believed to have been a cause for mass extinctions and major shifts in the distribution of species (Berger 1988; Dynesius and Jansson 2000). During recurrent cycles of ice-sheet expansion and contraction,

warm and climatically stable regions have served as refuge for a large number of species, enabling their survival in isolated, often disjunct pockets of populations (Fjeldså and Lovett 1997; Hewitt 2000; Keppel et al. 2012; Gavin et al. 2014). All but the Swiss collection of *M. rufobrunnea* and all known collections of *M. anatolica* so far originate from lower-latitude biodiversity hot spots that lack histories of glaciation, suggesting that they represent climatic relicts of old and once wider populations that perhaps became fragmented during the Pleistocene (Loizides et al. 2015, 2016). Such fragmentation of an initial population followed by secondary contacts of genetically divergent subpopulations has been demonstrated in a few lineages in sect. *Distantes* through recombination events at highly polymorphic loci (Du et al. 2016) and may be further reflected in the genetic polymorphism seen in *M. anatolica* in our analyses (FIG. 1).

Also notable is the fact that all nine collections of *M. anatolica* and *M. rufobrunnea* reported here originate from insular ecosystems (FIG. 2). Mediterranean islands, especially those characterized by topographic diversity and altitude, are well-known reservoirs of endemic and range-restricted species, harboring many climatic relicts of animals, plants, and fungi (Honer and Greuter 1988; Médail and Vidal 1998; Norstedt et al. 2001; Kier et al. 2009; Médail and Diadema 2009; Médail 2013). Such refugia hot spots have not only been critical in preserving biodiversity during cycles of globally adverse climate but also functioned as nuclei for subsequent radiations when regional conditions improved (Paulay 1994; Hewitt 2000; Gavin et al. 2014; Médail 2017). Nowhere is this pattern more apparent than the island of Cyprus, currently accommodating the highest known *Morchella* diversity per standard area in the world (Loizides et al. 2016), including ancient relictual species (*M. anatolica*, *M. rufobrunnea*, *M. tridentina*), regional endemics (*M. disparilis*, *M. dunalii*), as well as recently evolved narrowly endemic taxa (*M. arbutiphila*, *Mel-38*, *Mes-28*).

Ecological specializations and trophic niches vary greatly among morel species. The association of some morels with plants of the *Oleaceae* family had indeed been hypothesized as early as 1865, after morel mycelia were seen adhering to the roots of *Oleaceae* plants (Robert 1865). However, the prolific growth of some *Morchella* mycelia on simple culture nutrients such as starch, sugar, or nitrates had led other authors to assume that the genus was entirely composed of saprotrophs (Robbins and Hervey 1959; Dennis 1978; Hobbie et al. 2001). Strong links between most species of *Morchella* and vascular plants are nowadays widely accepted and have in some cases been experimentally verified (Buscot

and Kottke 1990; Buscot 1994; Dahlstrom et al. 2000; Stark et al. 2009; Baynes et al. 2012). A wide spectrum of observed or hypothetical mutualisms have been reported, such as a secondary mycorrhizal association formed by the opportunistic use of mycorrhizae established by other fungi (Buscot 1992), an ectendomycorrhizal association where some hyphae of the ectomycorrhizal mantle penetrate into the root cells of the host plant (Dahlstrom et al. 2000), or an endosymbiotic association where the entire mycelium colonizes living plant cells as an endophyte (Baynes et al. 2012; Baroni et al. 2018). A number of morel species may also be able to alternate between a biotrophic mode of nutrition associated with living host cells and a saprotrophic ecology associated with dead organic matter and are perhaps best defined as “weak, facultative biotrophs” (Tedersoo et al. 2010; Smith et al. 2017). Such presumably dual ecology may have emerged quite early in the genus’ evolutionary history and may still be present in the two species of the *Rufobrunnea* clade, frequently encountered in the vicinity of *Oleaceae* plants, but also in treeless, sometimes disturbed environments (Kuo 2008; Loizides et al. 2011, 2015; Palazón Pemán et al. 2017). The prolific in vitro growth of *M. anatolica* suggests that like its commercially cultivated sister species *M. rufobrunnea* (Ower et al. 1986, 1988), it too can probably grow as a saprotroph.

Summing up, our results provide a fresh look at the evolutionary history of morels, inferred from revised AAR analyses of an expanded data set, and, equally importantly, an improved understanding of the biogeographic distribution of the two earliest diverging species in the genus, especially the rare *M. anatolica*. Considering the reliance of AAR analyses on deep-node topologies, it is plausible that the hypothetical discovery of an ancestral lineage in Asia or WNA in the future might yield different results. As such, ancestral reconstructions should not be viewed as definitive or immutable, but more as a work-in-progress toward a deeper understanding of the genus at a global scale. Taking all available evidence into consideration, however, we can infer that a Mediterranean origin of morels is currently the most likely evolutionary scenario because (i) the Mediterranean area harbors high concentrations of both ancient relictual (paleoendemic) species as well as recent and rapidly diverging (neoendemic) lineages of *Morchella*; (ii) both species of the *Rufobrunnea* clade are present in the Mediterranean basin; (iii) *M. anatolica* further appears to be narrowly restricted in the Mediterranean and at the same time absent from both Asia and North America; (iv) nearly all records of *M. anatolica* and *M. rufobrunnea* originate from refugia hot spots that remained unglaciated during

the Pleistocene; (v) both species appear to be facultatively associated with endemic flora based on above-ground observations; (vi) the early diverging *M. steppicola* is also a Eurasian endemic; (vii) the early diverging *M. tridentina* is also abundantly present in refugia hot spots and indeed across the entire Mediterranean region; (viii) there is presently no convincing evidence that any of these species have been anthropogenically introduced in Europe; (ix) all of the above are supported by multilocus AAR tests designating the Mediterranean region as the most likely place of origin of morels; and (x) a Mediterranean origin is more parsimonious than a North American or Asian hypothesis. As a final note, and taking into account the rarity, restricted distribution, and evolutionary significance of *M. anatolica* in the current context of climate change, we recommend that this ancient climatic relict be regarded as critically endangered and placed in National Red Lists, the European List of Threatened Fungi (Dahlberg and Croneborg 2006), the Global Fungal Red List Initiative ([http://iucn.ekoo.se/iucn/species\\_list/#tab-species-list-thumbs](http://iucn.ekoo.se/iucn/species_list/#tab-species-list-thumbs)), and the International Union for Conservation of Nature Red List (IUCN 2018).

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