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Phylogeny and historical biogeography of true morels (*Morchella*) reveals an early Cretaceous origin and high continental endemism and provincialism in the Holarctic

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SUMMARY

True morels (*Morchella*, Ascomycota) are arguably the most highly-prized of the estimated 1.5 million fungi that inhabit our planet. Field guides treat these epicurean macrofungi as belonging to a few species with cosmopolitan distributions, but this hypothesis has not been tested. Prompted by the results of a growing number of molecular studies, which have shown many microbes exhibit strong biogeographic structure and cryptic speciation, we constructed a 4-gene dataset for 177 members of the Morchellaceae to elucidate their origin, evolutionary diversification and historical biogeography. Diversification time estimates place the origin of the Morchellaceae in the middle Triassic 243.63 (95% highest posterior density [HPD] interval: 169.35–319.89) million years ago (Mya) and the divergence of *Morchella* from its closest relatives in the early Cretaceous 129.61 (95% HPD interval: 90.26–173.16) Mya, both within western North America. Phylogenetic analyses identified three lineages within *Morchella*: a basal monotypic lineage represented by *Morchella rufobrunnea*, and two sister clades comprising the black morels (Elata Clade, 26 species) and yellow morels (Esculenta Clade, 16 species). *Morchella* possesses a Laurasian distribution with 37/41 species restricted to the Holarctic. All 33 Holarctic species represented by multiple collections exhibited continental endemism. Moreover, 16/18 North American and 13/15 Eurasian species appeared to exhibit provincialism. Although morel fruit bodies produce thousands of explosively discharged spores that are well suited to aerial dispersal, our results suggest that they are poorly adapted at invading novel niches. Morels also appear to have retained the ancestral fruit body plan, which has remained remarkably static since the Cretaceous.

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1. Introduction

Of the world's 1.5 million estimated species within kingdom Fungi (Hawksworth, 2004), true morels (*Morchella*, phylum Ascomycota) are arguably the most charismatic and widely recognized wild edible fungi intensively collected by mycophiles (Kuo, 2005). The ~100 described morphospecies within this genus of haploid macrofungi are largely restricted to temperate regions of the Northern Hemisphere where their ephemeral fruiting season is limited to a few weeks each spring. Due to the ever-increasing demand by gourmet chefs, annual large-scale commercial harvesting has become a multimillion-dollar industry within the United States and in other morel-rich countries (Pilz et al., 2007). As a result, it is now possible to purchase dried morels year-round in local supermarkets and via the Internet. In addition, the successful commercial cultivation of morels has also made it possible to purchase fresh morels throughout the year (Ower et al., 1986).

Morphological synapomorphies that unite *Morchella* with two other epigeous genera (i.e., *Discioitis* and *Verpa*) within the family Morchellaceae include explosively discharged multinucleate, eguttulate ascospores (haploid meiospores), which like those in many ascomyceteous fungi possess a drag-minimizing ellipsoidal shape (Roper et al., 2008). Although molecular phylogenetic analyses have provided strong support for Morchellaceae monophyly (O'Donnell et al., 1997; Hansen and Pfister, 2006), the few molecular systematic

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studies conducted to date on morels (Bunyard et al., 1995; Buscot et al., 1996; Wipf et al., 1999; Kellner et al., 2005) have not focused on developing a robust phylogenetic hypothesis for *Morchella* or on elucidating its historical biogeography. Field guides for morels in North America (Arora, 1979; Weber, 1988; Kuo, 2005) and Asia (Imazeki et al., 1988) typically use names based on European collections, implying that these species are cosmopolitan in their distribution. We hypothesized the assumption that 'everything is everywhere' was likely to be fallacious for *Morchella*, based on the growing body of evidence that diverse microbes have been shown to exhibit strong biogeographic structure when phylogenetic species recognition is employed (Whitaker et al., 2003; Taylor et al., 2006; Giraud et al., 2008). Results of our preliminary assessment of *Morchella* species diversity in Turkey (Taskin et al., 2010), which identified high species endemism, appear to support this hypothesis.

To obtain an initial estimate of *Morchella* species diversity and their geographic distribution, we generated partial DNA sequence data from one-to-two single-copy nuclear genes, RNA polymerase largest (*RPB1*) and second largest subunit (*RPB2*) or translation elongation factor 1-alpha (*EF-1α*), to screen a collection of 590 specimens. Based on the results of this screen, we selected 177 specimens, representing the global genetic diversity sampled, for which we generated ~3.7-kb of DNA sequence data from portions of four nuclear genes. The primary objectives of this study were to (i) investigate species limits within *Morchella* using genealogical concordance phylogenetic species recognition (GCPSR; Taylor et al., 2000); (ii) estimate divergence times for the origin and diversification of *Morchella* and the Morchellaceae from the molecular data; and (iii) develop a model-based historical biogeographic hypothesis for the evolutionary diversification of the genus *Morchella*.

2. Methods

2.1. DNA isolation

Prior to extracting total genomic DNA, herbarium specimens were examined under a dissecting microscope to insure that the region sampled was free of contaminating debris. If debris was observed in the region to be sampled, it was removed by lightly brushing with a toothbrush. Approximately 50–100 mg of the pileus was excised and pulverized in a 1.5 ml eppendorf tube with a 200 μL pipette tip (Rainin Instrument Co., Oakland, CA). The samples were suspended in 700 μL of CTAB (hexadecyltrimethylammonium bromide; Sigma Chemical Co, St. Louis, MO) extraction buffer (100 mM Tris–Cl pH 8.4, 1.4 M NaCl, 25 mM EDTA, 2% CTAB) and then incubated from 1 h to overnight at 60 °C. Once samples cooled to room temperature, 700 μL of chloroform was added to each tube and then they were shaken vigorously for a few seconds to obtain an emulsion. Samples were spun for 10 min at 12,300*g* in a microcentrifuge (Savant, Holbrook, NY), and 350 μL of the upper aqueous phase was transferred to a new 1.5 mL eppendorf tube. DNA was precipitated by adding 350 μL of −20 °C isopropanol to each tube and then inverting several times until a homogeneous mixture was obtained. After the samples were spun in a microcentrifuge for 2 min at 12,300*g* to pellet the DNA, the soup was discarded and the pellet was gently washed with 70% ethanol. The samples were spun for 2–3 min at 12,300*g* after which the soup was gently discarded. The pellets were suspended in 200 μL of sterile ultrapure water by heating for 1 h at 60 °C and then they were stored frozen at −20 °C until ready to use. For PCR, 4–8 μL of each DNA preparation was diluted into 200 μL of ultrapure water in a 96 well PCR plate. Total genomic DNA was obtained from lyophilized mycelium from pure cultures grown in yeast-malt broth (3 g malt extract, 3 g yeast extract, 5 g peptone and 20 g dextrose per L) following the same protocol outlined above. The difficulty of obtaining DNA sequence data from the single-copy nuclear genes is reflected by the fact that only 22 of the 177 collections (Supplementary Table S1) were more than 30 years old, with the oldest being collected in 1956.

2.2. Taxon sampling and pure culturing

A portion of one or two nuclear gene fragments were amplified and sequenced using standard methods from a total of 590 collections to obtain an initial estimate of Morchellaceae genetic diversity. This sampling included 562 collections of *Morchella* and 28 outgroup collections of *Verpa* and *Disciotis*. Based on maximum parsimony (MP) phylogenetic analyses of these datasets, 177 collections were selected to represent the full range of genetic diversity sampled (Supporting information Table S1). Of the 177 collections analyzed, 41 from North America were obtained via the Morel Data Collection Project (MDCP) accessible at http://www.mushroomexpert.com/mdcp/ (Kuo, 2006). Due to our inability to apply Latin binomials with confidence to 36 of the 41 phylogenetic species within *Morchella*, species were identified by clade (*Mes* for Esculenta and *Mel* for Elata) followed by an Arabic number. In addition, *Mel*-12-to-24 was used to identify a species-rich subclade comprising 13 species within the Elata Clade.

Ninety of the 177 collections analyzed are available as pure cultures from the ARS Culture Collection (NRRL; http://nrrl.ncaur.usda.gov/TheCollection/Requests.html). These include 31 of the 41 species of *Morchella* identified in this study and five of the seven outgroup species (see Supplementary Table 1; Hawksworth, 2004). Twenty-five of the cultures were obtained from the following culture collections: Centraalbureau voor Schimmelcultures (CBS-KNAW), Utrecht, The Netherlands ($N = 6$); Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany ($N = 6$); Institute for Fermentation (IFO), Osaka, Japan ($N = 3$); and The Pennsylvania State University, Department of Plant Pathology (WC, wild collection; $N = 10$). Sixty-five of the cultures were obtained in the present study by germinating ascospores overnight on 3% water agar (Difco, Detroit, MI) supplemented with approximately 40 U penicillin and 40 μg streptomycin per ml (P4333 Sigma–Aldrich, St. Louis, MO) in 60 mm disposable Petri dishes. Germlings were transferred to a second water agar plate with antibiotics using a sterile hypodermic needle and allowed to grow for several days until mycelial tips at the colony margin could be transferred to a potato dextrose agar plate (Difco, Detroit, MI). Prior to being stored in the ARS Culture Collection, cultures were typed using a partial *RPB1* or *RPB2* gene sequence to make sure they matched sequence data obtained directly from the specimens. This step was determined to be absolutely critical because specimens are frequently contaminated with ascospores of other species when collected in bulk. For this reason, we consider data obtained from ascocarps to be much more reliable than from ascosporic cultures, unless data from both are obtained. Once cultures were determined to be authentic for a collection, they were grown up in yeast-malt broth and then stored in liquid nitrogen vapors at −175 °C in a cryogen consisting of 10% skim milk and 1% DMSO. Where possible we recommend starting cultures from ascospore prints taken from a single ascocarp to avoid interspecies contamination, and because ascospores on prints appear to be viable for decades. By contrast, we were unable to culture ascospores from ascocarps more than five years old.

2.3. PCR amplification and sequencing

PCR and sequencing primers are listed in Supporting information Table S2. Platinum Taq DNA polymerase Hi-Fi (Invitrogen Life Technologies, Carlsbad, CA) was used to PCR amplify portions of the following four nuclear genes for the 177 taxa included in our

analysis: RNA polymerase I (*RPB1*, 744 bp; Matheny et al., 2002), RNA polymerase II (*RPB2*, 873 bp; Liu et al., 1999; Reeb et al., 2004), translation elongation factor 1-α (*EF-1α*, 1464 bp; Rehner and Buckley, 2005), and domains D1 and D2 of the nuclear large subunit (LSU) 28S rDNA, 597 bp; O'Donnell et al., 1997). In addition to the nuclear ribosomal LSU 28S rDNA, the adjacent internal transcribed spacer region (ITS rDNA; White et al., 1990) was sequenced for 51 members of the Elata *Mel*-12-to-24 subclade (1315 bp), yielding 4.6-kb of aligned nucleotide sequence data.

PCR amplifications were performed in a total volume of 40 μl following the manufacturers instructions, and included 1 × High Fidelity PCR buffer (Invitrogen Life Technologies, Carlsbad, CA), 1.25 mM $MgSO_4$, 0.2 mM of each deoxynucleoside triphosphate, 10 pmol of each primer, 0.5 U of Platinum *Taq* DNA Polymerase High Fidelity, and approximately 10 ng of genomic DNA. PCRs were conducted in an Applied Biosystems (ABI) 9700 thermocycler (ABI, Foster City, CA), using the following cycling parameters: 94 °C for 90 s, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 68 °C for 3–5 min, followed by 68 °C for 5 min and a 4 °C soak. Amplicons were size-fractionated in 1.5% agarose gels (Invitrogen) in 1 × TAE buffer (Sambrook and Russell, 2001), stained with ethidium bromide, and then visualized over a UV transilluminator. Amplicons were purified by using $Montage_{96}$ filter plates (Millipore Corp., Billerica, MA) and sequenced using ABI BigDye version 3.1 and the primers listed in Supporting information Table S2. Sequencing reactions were conducted in a 10 μl volume containing 2 μl of ABI BigDye version 3.1 terminator reaction mix, 2–4 pmol of a sequencing primer, and approximately 50 ng of amplicon in an ABI 9700 thermocycler using the following profile: 96 °C for 15 s, 40 cycles of 96 °C for 15 s, 50 °C for 10 s, 60 °C for 4 min, and ending in a 4 °C soak. Sequencing reactions were purified using ABI XTerminator and then they run on an ABI 3730 genetic analyzer.

Raw sequence data were edited and aligned by using Sequencher version 4.9 (Gene Codes, Ann Arbor, MI) and then they were either manipulated manually using TextPad ver. 5.1.0 for Windows (http://www.textpad.com/) to establish positional homology or aligned automatically using MAFFT ver. 6.0 (http://align.bmr.kyushu-u.ac.jp/mafft/software/). Due to the presence of length variable indels within the *EF-1α* introns, 113, 110 and 120 ambiguously aligned nucleotide positions were excluded, respectively, from the Morchellaceae (Table 1), Esculenta clade (Table 2) and Elata clade (Table 3) datasets.

2.4. Phylogenetic analyses

A conditional combination approach revealed that data from the individual data partitions could be concatenated (O'Donnell et al., 2008). The following concatenated four-gene datasets were analyzed via MP: (i) a 49-taxon, 3678 bp Morchellaceae dataset (Fig. 1); (ii) a 73-taxon, 3774 bp Esculenta Clade dataset (Fig. 2); (iii) a 57-taxon, 3809 bp Elata Clade dataset (Fig. 3); and (iv) a 51-taxon, 4613 bp *Mel*-12-to-24 subclade dataset within the Elata Clade (Fig. 4). Analyses were conducted via maximum parsimony (MP) in PAUP* V 4.0b10 (Swofford, 2002) and maximum likelihood (ML) in GARLI V 0.951 (Zwickl, 2006) as previously described (O'Donnell et al., 2008). The GTR + I + Γ model of evolution was identified using ModelTest V 3.8 (Posada, 2006) for the concatenated four-gene 49-taxon Morchellaceae dataset. Clade support was assessed by 1000 MP and ML bootstrap (BS) pseudoreplicates of the data. To assess whether three obligately fire-adapted species of *Morchella* evolved convergently, we compared constrained and unconstrained topologies using the Kishino–Hasegawa test implemented in PAUP* (Swofford, 2002). Sequences have been deposited in GenBank under accession numbers GU550978-GU551723.

2.5. Diversification time estimates

The program BEAST version 1.5.1 (Drummond and Rambaut, 2007) was used to generate divergence time estimates. The 49-taxon Morchellaceae dataset was used as well as five additional species that were included as calibration points for the analysis. These five taxa included *Candida albicans*, *Saccharomyces cerevisiae*,

Table 1
Morchellaceae 49-taxon dataset tree statistics and summary sequence (see Fig. 1).

Locus	# Characters[a]	# MPTs[b]	MPT length	CI[c]	RI[d]	Aut[e]	Syn[f]	PIC/bp[g] (%)
LSU rDNA	597	>2000	182	0.637	0.89	19	78	13.1
RPB1	744	264	689	0.56	0.872	26	254	34.1
RPB2	873	72	854	0.498	0.858	17	267	30.6
EF-1α	1464	8	1610	0.519	0.828	94	476	32.5
Combined	3678	12	2942	0.524	0.851	145	978	26.6

[a] One-hundred-and-thirteen nucleotide characters were excluded as ambiguously aligned within the *EF-1α* and combined datasets.
[b] MPTs, most-parsimonious trees.
[c] CI, consistency index.
[d] RI, retention index.
[e] Aut, autapomorphic or parsimony uninformative character.
[g] PIC/bp, parsimony-informative characters/base pair.

Table 2
Esculenta Clade 73-taxon dataset tree statistics and summary sequence (see Fig. 2).

Locus	# Characters[a]	# MPTs[b]	MPT length	CI[c]	RI[d]	Aut[e]	Syn[f]	PIC/bp[g] (%)
LSU rDNA	598	>2000	84	0.69	0.934	26	37	6.2
RPB1	794	7	307	0.762	0.915	37	169	21.3
RPB2	880	1	318	0.799	0.939	15	191	21.7
EF-1α	1502	52	529	0.762	0.931	37	284	18.9
Combined	3774	9	1250	0.766	0.926	115	681	18

[a] One-hundred-and-ten nucleotide characters were excluded as ambiguously aligned within the *EF-1α* and combined datasets.
[b] MPTs, most-parsimonious trees.
[c] CI, consistency index.
[d] RI, retention index.
[e] Aut, autapomorphic or parsimony uninformative character.
[f] Syn, synapomorphic or parsimony informative character.
[g] PIC/bp, parsimony-informative characters/base pair.

Table 3
Elata Clade 57-taxon dataset tree statistics and summary sequence (see Fig. 3).

Locus	# Characters[a]	# MPTs[b]	MPT length	CI[c]	RI[d]	Aut[e]	Syn[f]	PIC/bp[g] (%)
LSU rDNA	598	82	91	0.703	0.929	4	54	9
RPB1	804	28	437	0.668	0.928	19	232	28.9
RPB2	894	>2000	489	0.638	0.916	12	230	25.7
EF-1α	1513	2	664	0.669	0.914	53	299	19.8
Combined	3809	>2000	1701	0.654	0.917	88	815	21.4

[a] One-hundred-and-twenty nucleotide characters were excluded as ambiguously aligned within the *EF-1α* and combined datasets.
[b] MPTs, most-parsimonious trees.
[c] CI, consistency index.
[d] RI, retention index.
[e] Aut, autapomorphic or parsimony uninformative character.
[f] Syn, synapomorphic or parsimony informative character.
[g] PIC/bp, parsimony-informative characters/base pair.

Magnoportha grisea, *Schizosaccharomyces pombe* and *Aspergillus flavus*. Dates for the divergence times of these taxa were taken from Heckman et al. (2001) and Blair (2009). GenBank accession numbers for these calibration taxa are provided in Supporting information Table S3. The program FINDMODEL (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) identified the Tamura-Nei plus gamma and equal frequencies model as the best fit to the data (Tamura and Nei, 1993). Analyses were run under both a strict molecular clock and an uncorrelated lognormal relaxed molecular clock. The priors for both analyses were the same and included a birth–death speciation process as the tree prior in addition to the fossil-based calibration dates taken from the literature mentioned above. All other priors were set to the default, and operators were allowed to auto-optimize. Chains were allowed to run to convergence, which was considered obtained when effective sample sizes reached values greater than 200; the latter were determined using the program TRACER version 1.4.1 (Rambaut and Drummond, 2007). Model comparison was also conducted using TRACER version 1.4.1 through Bayes factor calculation. Statistical uncertainty of divergence time estimates was assessed through the calculation of highest probability density (HPD) values.

2.6. Historical biogeography

The program LAGRANGE (Ree and Smith, 2008) was used to reconstruct ancestral area ranges from the Maximum Clade Credibility (MCC) phylogeny generated from the BEAST analysis. The latter was identical to the MP and ML phylogenies but differed by having the divergence date values generated using BEAST associated with each node. The geographic distributions for the Morchellaceae were delineated into eight areas: eastern North America, western North America, Europe, Asia, Hawaii, the Dominican Republic, Venezuela and the Canary Islands. The four areas we recognized within the Northern Hemisphere (Europe, Asia, eastern and western North America) correspond to geologically persistent landmasses recognized in the most detailed historical biogeographic analyses of animals (Sanmartín et al., 2001) and plants (Donoghue and Smith, 2004) in the Holarctic. Using the definitions employed by these authors, we considered a species to be endemic if it was restricted to Eurasia or North America. If a species was restricted to Europe or Asia, or eastern or western North America, it was considered to be provincial. Asia was restricted to the mainland, but included Japan because the latter was initially connected to China. The models employed in the analyses varied on the basis of dispersal probabilities between these areas, which were taken from Clayton et al. (2009) and were used to evaluate biogeographic events over the Late Cretaceous (70 Mya) and Early Oligocene (30 Mya) time periods. These probabilities were derived on the basis of continental land mass location as well as the appearance or disappearance of land bridges during these time periods (see references in Clayton et al. (2009)). Models also varied on the basis of the area inferred for an ancestral species at a given node, in which the plausibility of whether a species could inhabit two given areas and no others on the basis of their adjacency was taken into account. Thus, for each set of dispersal probabilities, two distinct models were employed: (i) an unconstrained model that allowed species to inhabit any two areas regardless of whether or not they were adjacent and (ii) a constrained model that allowed species to inhabit areas that were adjacent (under this model, for example, a species would not be allowed to inhabit both the Dominican Republic and Hawaii but it could inhabit the Dominican Republic and Venezuela or the Dominican Republic and eastern or western North America). The resulting likelihood values for each of these models were compared directly (Ree and Smith, 2008; Clayton et al., 2009). To our knowledge, this is the first study where LAGRANGE has been used to reconstruct the range evolution of a fungus.

3. Results

3.1. Phylogenetic species recognition within and molecular phylogeny of Morchella

We constructed a four-gene dataset (*RPB1*, *RPB2*, *EF-1α*, LSU 28S rDNA), comprising approximately 0.74 Mbp of DNA sequence data from a global collection of 177 Morchellaceae to assess species limits within *Morchella*, the genus of true morels. Because the 177-taxon phylogeny was too large to display effectively, MP and ML analyses of the full 177-taxon matrix were used primarily to identify major well-supported lineages, which included the Esculenta (yellow morels) and Elata (black morels) Clades, so that the species limits within each lineage could be investigated further using genealogical concordance phylogenetic species recognition (GCPSR; Taylor et al., 2000). GCPSR-based criteria proposed by Dettman et al. (2003) were used to investigate species limits. Specifically, phylogenetic species were recognized if they were resolved as reciprocally monophyletic in at least one of the bootstrapped individual genealogies and in the combined dataset, and their genealogical exclusivity was not contradicted by bootstrap analyses of any individual data partition. Results of the GCPSR-based analyses were then used to select an exemplar from each species for inclusion in a full phylogenetic analysis of the Morchellaceae (Fig. 1). Although the monophyly of five putative species lineages (*Mes*-10, *Mes*-12, *Mel*-8, *Mel*-11 and *Mel*-17) represented by single collections could not be tested, they were considered to be phylogenetically distinct because they were all genetically divergent from their sisters.

A 49-taxon four-gene dataset was constructed to infer phylogenetic relationships within the Morchellaceae using a concatenated dataset of 3678 aligned nucleotide positions. Maximum parsimony (MP) and maximum likelihood (ML) analyses of the 49-taxon

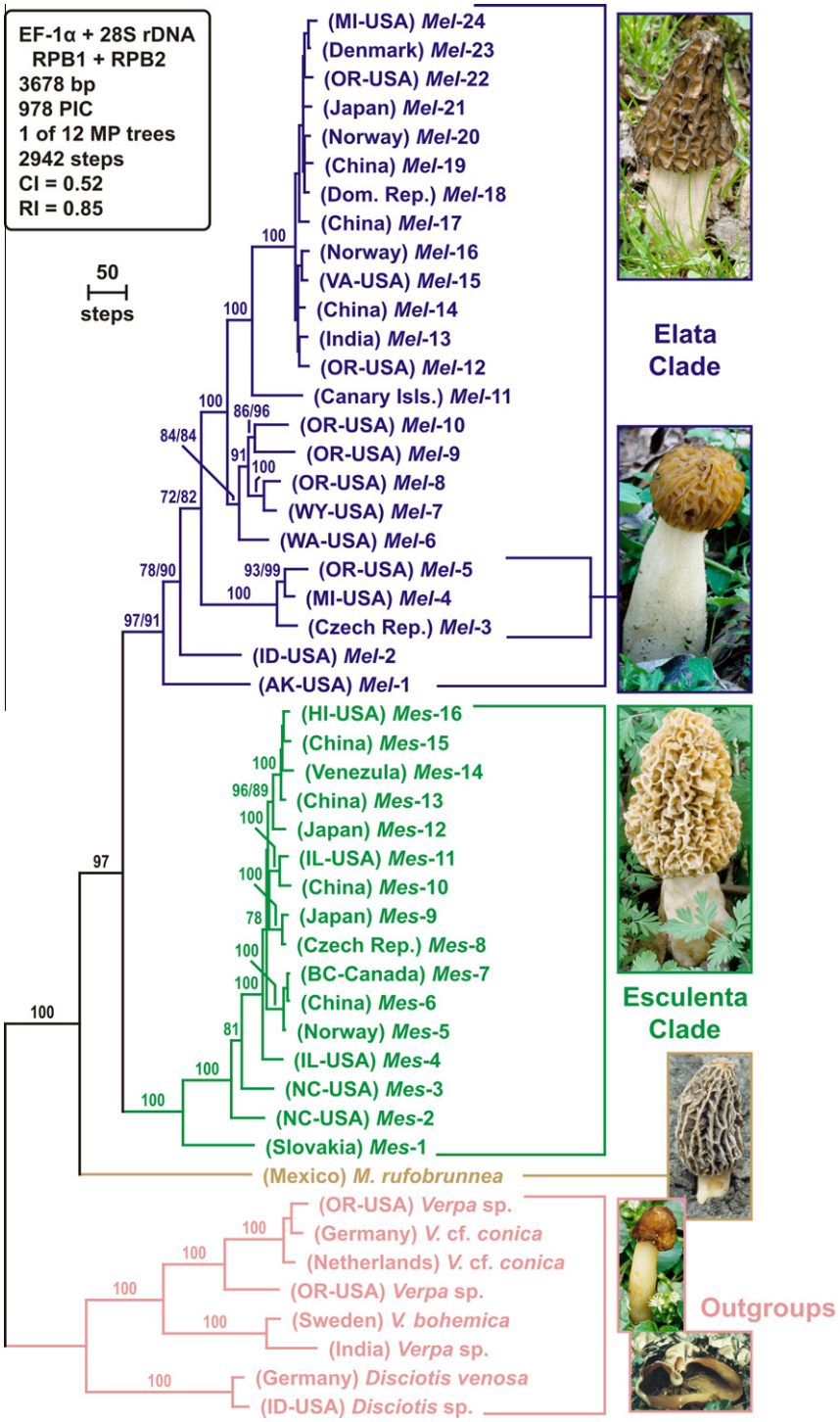

Fig. 1. One of 12 equally parsimonious phylograms depicting phylogenetic relationships for 49 species of Morchellaceae inferred from a four-gene dataset containing 978 parsimony-informative characters. Sequences of *Verpa* and *Disciotis* were used to root the phylogeny. The true morel genus *Morchella* comprises the ingroup, within which three evolutionary lineages were resolved: a basal monotypic *M. rufobrunnea* lineage, and the Elata (black morels) and Esculenta (yellow morels) Clades. The bracket within the Elata Clade identifies three allopatric species comprising the *M. semilibera* subclade. See Supporting information Table S6 for bootstrap values within the Elata *Mel*-12-to-24 subclade. Because Latin binomials are unknown for most of the species within the Elata and Esculenta Clades, species are identified by, respectively, *Mel* or *Mes* followed by an Arabic number. MP bootstrap values based on 1000 pseudoreplicates of the data are indicated above internodes. Bootstrap values ⩾50% are listed as MP/ML only when the ML value differed by ⩾5% of the MP value. Note that the bottom three photos are reproduced at about half the size of the top three. See Supporting information dataset S1 for the NEXUS file.

matrix recovered a nearly fully resolved phylogeny of the Morchellaceae (Fig. 1, Table 1). These analyses strongly support the monophyly of *Morchella* and its epigeous sisters, *Verpa* and *Disciotis*, whose sequences were used to root the phylogeny. The outgroup in the MP and ML analyses always joined the tree with *Morchella rufobrunnea* forming the most basal branch within *Morchella* (MP/ML bootstrap = 100%). The phylogenetic results support a sister-group relationship between the Esculenta (yellow morels) and Elata (black morels) Clades. Because Latin binomials could be applied with confidence to only five of the 41 species

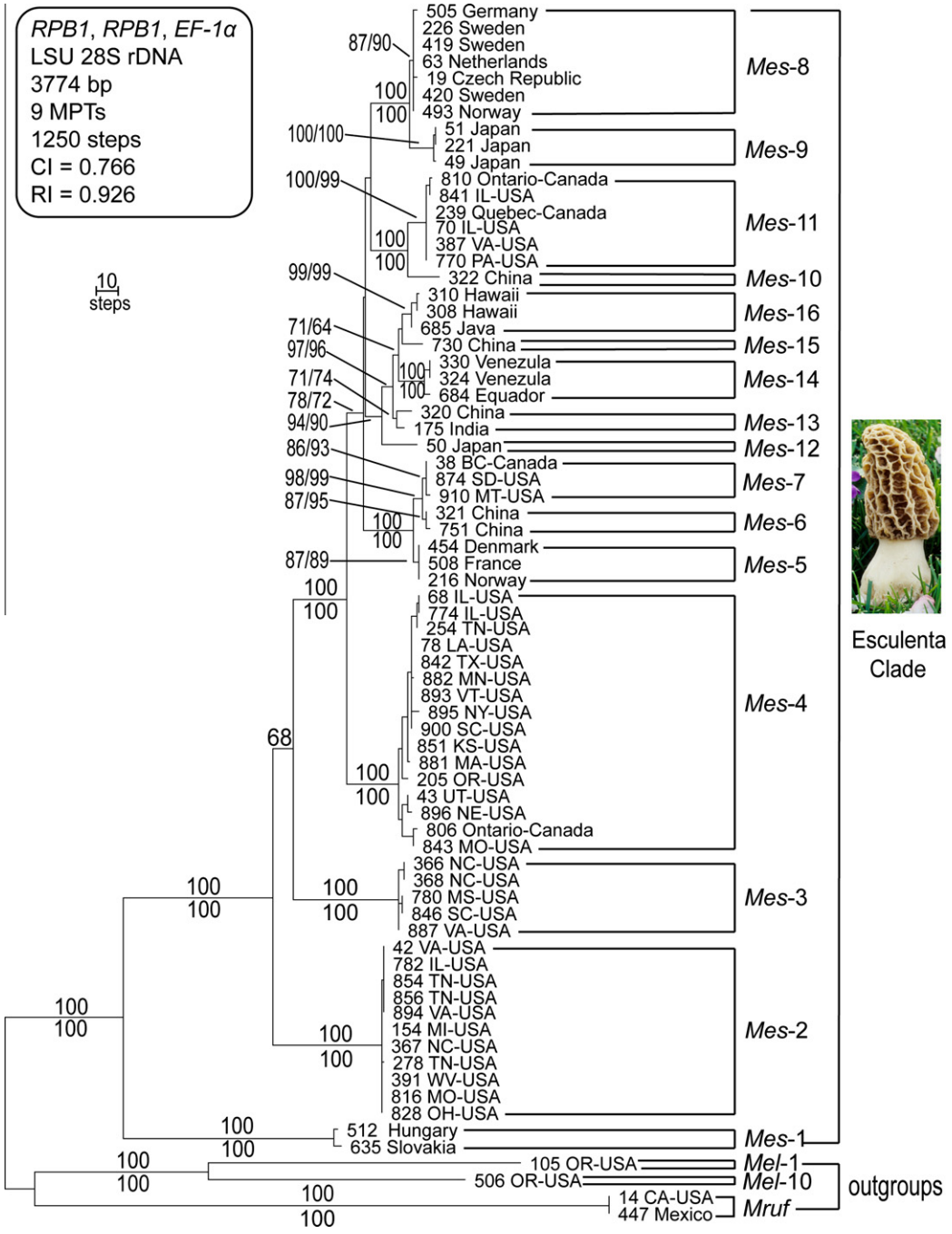

Fig. 2. One of 9 equally parsimonious phylograms inferred from the *Esculenta* Clade (yellow morels) 73-taxon four-locus dataset comprising 3774 bp of aligned DNA sequence data and 681 parsimony-informative characters. The 16 species within this clade are identified by *Mes* followed by a unique Arabic number. *Mes*-1 (*Morchella steppicola*) is the only species within this clade for which a binomial can be applied with confidence. MP and ML bootstrap values based on 1000 pseudoreplicates of the data are indicated above and below nodes respectively. Sequences of two *Elata* Clade species (*Mel*-1 and *Mel*-10) and *M. rufobrunnea* (*Mruf*) were used to root the phylogram. See Supporting Information Dataset S2 for the NEXUS file and Supplementary Table S4 for bootstrap values obtained from the individual data partitions.

we identified within *Morchella*, species were distinguished by clade (*Mes* for Esculenta and *Mel* for Elata) followed by an Arabic number. Phylogenetic analyses also identified a relatively recent and rapid species-rich radiation within the Elata Clade designated the *Mel*-12-to-24 subclade, which comprises close to one-third of *Morchella*. At least three species within the Elata Clade (i.e., *Mel*-1, *Mel*-6 and *Mel*-7) appear to be obligately fire-adapted to post-fire sites in western North America. A topological constraint forcing the monophyly of these three species was 92 steps longer and significantly worse than the unconstrained MP trees ($P < 0.001$ Kishino–Hasegawa likelihood test implemented in PAUP*), suggesting that these adaptive shifts evolved convergently.

Hypotheses of species limits within the 73-taxon Esculenta Clade dataset were tested by phylogenetic analyses of the four individual single-locus data partitions (*RPB1*, *RPB2*, *EF-1α*, and LSU 28S rDNA), and the combined dataset comprising 3774 aligned nucleotide positions (Fig. 2). Tree statistics for the individual and combined data partitions are summarized in Table 2 (see Supporting information

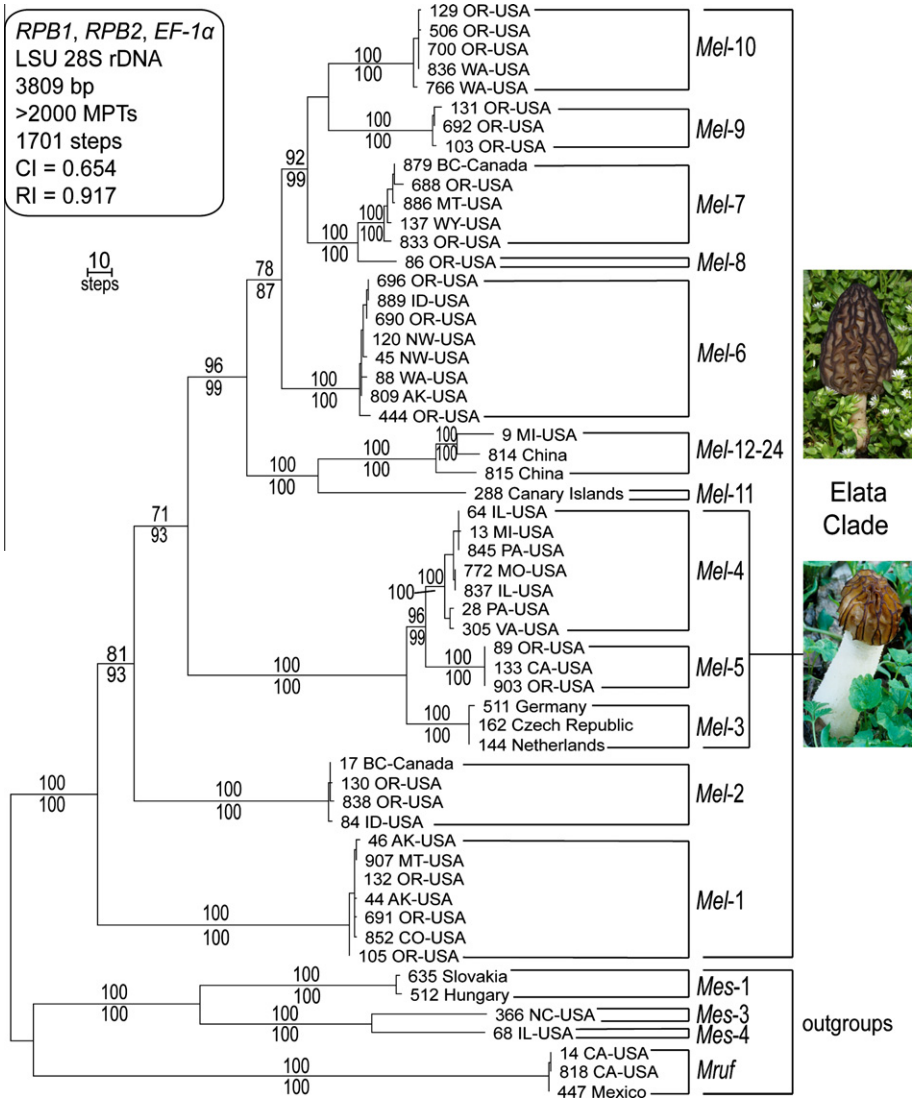

Fig. 3. One of >2000 equally parsimonious phylograms inferred from the *Elata* Clade (black morels) 57-taxon four-locus dataset, using three *Esculenta* Clade species (*Mes*-1, *Mes*-3 and *Mes*-4) and *M. rufobrunnea* (*Mruf*) to root the tree. The combined dataset totaled 3809 bp of aligned DNA sequence data and included 815 parsimony-informative characters. Latin binomials can be applied confidently to *Mel*-1 (*M. tomentosa*; Kuo, 2008), *Mel*-3 (*M. semilibera*), and *Mel*-4 (*M. punctipes*). The base of the cap in the half-free morels (*Mel*-3-to-5 subclade) is detached from the stalk. MP and ML bootstrap values from 1000 pseudoreplicates of the data are indicated, respectively, above and below nodes. See Supporting information dataset S3 for the NEXUS file and Supplementary Table S5 for bootstrap values obtained from the individual data partitions.

Table S4 for a summary of the bootstrap analyses). Sequences of two Elata Clade species (*Mel*-1 and *Mel*-10), and those of the monotypic *M. rufobrunnea* lineage, were used as outgroups to root the phylogeny based on more inclusive analyses (Fig. 1). Of the 16 Esculenta Clade species recognized, two genetically divergent Asian lineages were each represented by a single collection (*Mes*-10 from China and *Mes*-12 from Japan). Therefore, additional sampling is needed to fully assess their species limits. The genealogical exclusivity of three species (*Mes*-5 and *Mes*-8 from Europe, and *Mes*-6 from China) was only weakly supported by bootstrap analyses of the individual data partitions ($\geqslant$65%), however, their monophyly was strongly supported based on analyses of the combined dataset (MP-BS $\geqslant$ 87%, ML-BS $\geqslant$ 89%). Additional support for the provisional recognition of these lineages as phylogenetically distinct species is indicated by the fact that their ranges do not overlap with their sisters. Monophyly of the 10 remaining Esculenta Clade species was supported by one or more of the individual gene genealogies (MP-BS 77–100%) and combined dataset (MP-BS 71–100%, ML-BS 74–100%). MP and ML analyses of the combined four-locus dataset provided a nearly fully resolved phylogeny except for two nodes along the backbone in the terminal part of the tree. The earliest diverging lineage was represented by *Mes*-1 (*Morchella steppicola*), a species known only from the Balkans. Three other lineages were represented by Neartic species, including two restricted to eastern North America (*Mes*-2 and *Mes*-3); the third species (*Mes*-4) was the most common Esculenta Clade species sampled within eastern North America, however, this species appears to be rare in western North America. As discussed later, *Mes*-14 (from Venezuela and Ecuador) and *Mes*-16 (from Hawaii and Java) appear to represent anthropogenic introductions into their current range. The 14 remaining Esculenta Clade species appear to exhibit continental endemism, and all but two (*Mes*-4 and *Mes*-7), which occur in Eastern and Western North America, exhibited provincialism.

Species limits with the Elata Clade were investigated using two separate datasets: a 3.8-kb 57-taxon dataset constructed for the Elata Clade (Fig. 3), which included only three taxa within the species-rich *Mel*-12-to-24 subclade, and a 4.6-kb 51-taxon dataset for the *Mel*-12-to-24 subclade (Fig. 4). These datasets were constructed and analyzed separately because the separate analyses allowed us to add 717 bp of DNA sequence data from the ITS rDNA

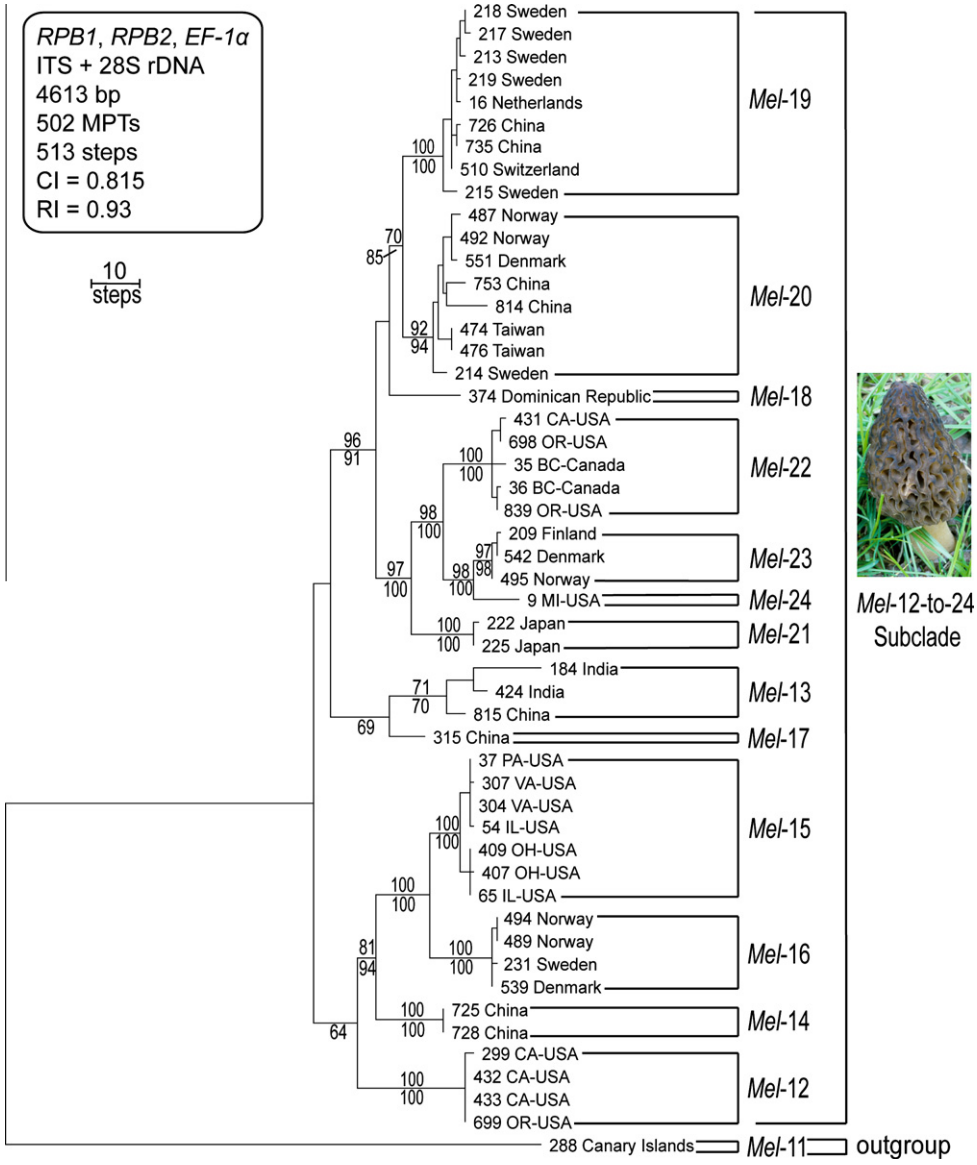

Fig. 4. One of 502 equally parsimonious phylograms inferred from the four-locus dataset for the *Elata Mel*-12-to-24 subclade. The 51-taxon dataset comprised 4613 aligned nucleotide positions and 184 parsimony-informative characters. Sequences of *Mel*-11 were used as the outgroup to root the tree. Numbers above and below nodes represent, respectively, MP and ML bootstrap values based on 1000 pseudoreplicates of the data. See Supporting information dataset S4 for the NEXUS file and Supplementary Table S6 for bootstrap values obtained from the individual data partitions.

region to the *Mel*-12-to-24 data matrix. In contrast to other Elata and Esculenta Clade species, alignment of the ITS rDNA dataset for the *Mel*-12-to-24 subclade only required the insertion of nine indels 1–4 bp in length to establish position homology. Tree statistics for the individual and combined datasets for the Elata Clade and *Mel*-12-to-24 subclade are provided in Tables 3 and 4, respectively. A summary of bootstrap analyses of the single-locus and combined data partitions for the Elata Clade and *Mel*-12-to-24 subclade are provided, respectively, as Supporting information Tables S5 and S6. Based on more inclusive analyses (Fig. 1), sequences of three Esculenta Clade species (*Mes*-1, *Mes*-3 and *Mes*-4) and *M. rufobrunnea* (*Mruf*) were selected to root the Elata Clade phylogeny (Fig. 3). MP and ML analyses of the four-locus data matrix provided a highly resolved phylogeny of the Elata Clade and strong support (MP-BS and ML-BS = 100%) for the reciprocal monophyly of nine of the 11 species represented by two or more collections. Additional sampling of three genetically divergent species (*Mel*-8, *Mel*-11 and *Mel*-17), represented by single collections, is needed to accurately assess their species boundaries. The remaining nine species, including the *Mel*-12-to-24 subclade, were strongly supported by bootstrap analyses of at least three of the four individual data partitions (BS = 77–100%) (S6). Most of the phylogenetic diversity represented in the reduced Elata Clade dataset was represented by eight species restricted to western North America (Fig. 3), including two of the earliest diverging lineages (*Mel*-1 = *Morchella tomentosa*, Kuo, 2008; and *Mel*-2). The *Morchella semilibera* or half-free morel *Mel*-3-to-*Mel*-5 subclade was deeply nested within the Elata Clade and includes allopatric species within Europe (*Mel*-3 = *M. semilibera*), eastern North America (*Mel*-4 = *Morchella punctipes*) and western North America (*Mel*-5).

Phylogenetic analyses of four individual data partitions and the combined 4.6-kb 51-taxon dataset were conducted to develop hypotheses of species limits within the Elata *Mel*-12-to-24 subclade (Fig. 4, Table 4). MP and ML analyses of the combined data partitions provided strong support for all but four nodes along the backbone of the phylogeny. The genealogical exclusivity of

Table 4
Mel-12-to-24 subclade 51-taxon tree statistics and summary sequence (see Fig. 4).

Locus	# Characters[a]	# MPTs[b]	MPT length	CI[c]	RI[d]	Aut[e]	Syn[f]	PIC/bp[g] (%)
ITS-LSU rDNA	1315	>2000	131	0.878	0.965	51	48	3.7
RPB1	794	125	67	0.91	0.96	35	25	3.1
RPB2	906	>2000	72	0.931	0.965	41	24	2.6
EF-1α	1598	22	200	0.875	0.96	77	87	5.4
Combined	4613	502	513	0.815	0.93	204	184	4

[a] No nucleotide characters were excluded as ambiguously aligned.
[b] MPTs, most-parsimonious trees.
[c] CI, consistency index.
[d] RI, retention index.
[e] Aut, autapomorphic or parsimony uninformative character.
[f] Syn, synapomorphic or parsimony informative character.
[g] PIC/bp, parsimony-informative characters/base pair.

10 species represented by two or more collections was strongly supported by bootstrap analysis of one or more of the single-locus data partitions (MP-BS 91–100%). Additional sampling is needed to assess species boundaries of *Mel*-13 (from India and China). *Mel*-13 only received 71% MP-BS and 70% ML-BS support in analyses of the combined data partitions, compared with 92% MP-BS and 94% ML-BS for the nine other species analyzed. Because *Mel*-17 (from China) was represented by a single collection, additional sampling is needed to assess its species boundaries. Even though a single collection of *Mel*-18 (from Dominican Republic) and *Mel*-24 (from MI-USA) was included in the present study, analyses of one additional collection of *Mel*-18 from the Dominican Republic (M375) and five additional collections of *Mel*-24 from eastern North America (MDP 0506062 from NY-US, and MDP 05110306, 05110405, 05120401 and 05150302 from MI-US) establish that each species fulfills the requirements of GCPSR (K. O'Donnell, unpublished). Based on the available sampling data, the 21 species represented by multiple collections within the Elata Clade appear to exhibit continental endemism, and with the exception of two Eurasian species (*Mel*-19 and *Mel*-20), they exhibited provincialism.

3.2. Divergence time estimates and historical biogeography of *Morchella*

Sequences of five non-Morchellaceae outgroup taxa (*see Materials and Methods*) were used to calibrate the Morchellaceae chronogram against the geological time scale (Walker et al., 2009), using published diversification time estimates (Heckman et al., 2001; Blair, 2009). Initially, chronograms were obtained for the 49-taxon Morchellaceae data matrix. When the data were evaluated under the unconstrained model versus biologically realistic models, the former had a significantly higher likelihood value than the latter (Supporting information Table S7). This odd result was likely obtained due to the fact that four taxa (from the Dominican Republic, Hawaii, Canary Islands, and Venezuela) represent probable anthropogenic introductions, as all but one of these four species was collected near vascular plants that were knowingly introduced for use in horticulture or silviculture. When we repeated the analysis excluding these four taxa, the likelihood scores were substantially better under the biologically realistic models than under the unconstrained model (see below). We interpret this result as further evidence that these species have a high probability of human-mediated dispersal into their current ranges rather than transoceanic long distance dispersal (LDD).

Analysis of the 45-taxon dataset resulted in a divergence time estimate of the Morchellaceae at 243.63 (95% highest posterior density [HPD] interval: 169.35–319.89) Mya in the middle Triassic and the divergence of *Morchella* from its epigeous sisters, *Verpa* and *Disciotis*, at 129.61 (95% HPD interval: 90.26–173.16) Mya in the early Cretaceous, both within western North America. The basal most divergence within *Morchella* was represented by the monotypic *M. rufobrunnea* lineage based on collections from Mexico and California (Figs. 1 and 5; Table 1). The early divergence of the remaining ingroup taxa comprising the Elata and Esculenta Clades was dated at 101.78 (95% HPD interval: 71.91–133.80) Mya in the early Cretaceous, followed by radiation of the Elata Clade in the late Cretaceous 73.48 (95% HPD interval: 51.92–97.87) Mya and the Esculenta Clade in the mid-to-late Paleocene 59.18 (95% HPD interval: 38.40–82.14) Mya. Our results indicate that approximately three-quarters of lineage diversification within *Morchella* took place between the middle Miocene and present, including the rapid diversification of the *Mel*-12-to-24 subclade in the late Miocene 8.55 (95% HPD interval: 5.70–11.85) Mya.

Ancestral area reconstructions (AARs) with the highest likelihood value for the 45-taxon Morchellaceae dataset were obtained using the 70 Mya dispersal-extinction cladogenesis (DEC) model (Supporting information Table S7). The optimal 70 Mya LAGRANGE AAR required 20 dispersals that were equally divided between intra- and intercontinental range expansions (Fig. 5; three of the dispersals involved the outgroup species which were pruned to highlight *Morchella*). Intracontinental dispersals were asymmetric, with three of four involving western North America-to-eastern North America range expansions in the Nearctic, compared with five of six Asia-to-Europe range expansions in the Palaearctic. Of the eight intercontinental dispersal events within *Morchella*, five involved adjacent areas. Except for the root node of the Esculenta Clade, which suggests a Laurasian migration across the Thulean North Atlantic Land Bridge into Europe by the mid-to-late Paleocene 59.18 (95% HPD interval: 38.40–82.14) Mya, the remaining seven range expansions are hypothesized migrations across Beringian land bridges at different times from the Nearctic into Asia ($N = 4$) or the Palaearctic into the New World ($N = 3$) starting as early as the Eocene. The AARs also revealed that range expansions involving dispersals within *Morchella* were predominately from ancestral ranges comprising a single area, or rarely from two areas, consistent with widespread allopatric speciation. All 33 Holarctic species represented by multiple collections exhibited continental endemism. Moreover, 16/18 North American and 13/15 Eurasian species appeared to exhibit provincialism (Fig. 6).

4. Discussion

4.1. Phylogenetic species recognition within and molecular phylogeny of *Morchella*

One of the primary objectives of the present study was to clarify species limits within the economically important true morel genus *Morchella* using GCPSR (Taylor et al., 2000). To test hypothesis of species boundaries within *Morchella*, we constructed a four-gene dataset comprising 3.7-to-4.6 kb of aligned nucleotide sequence

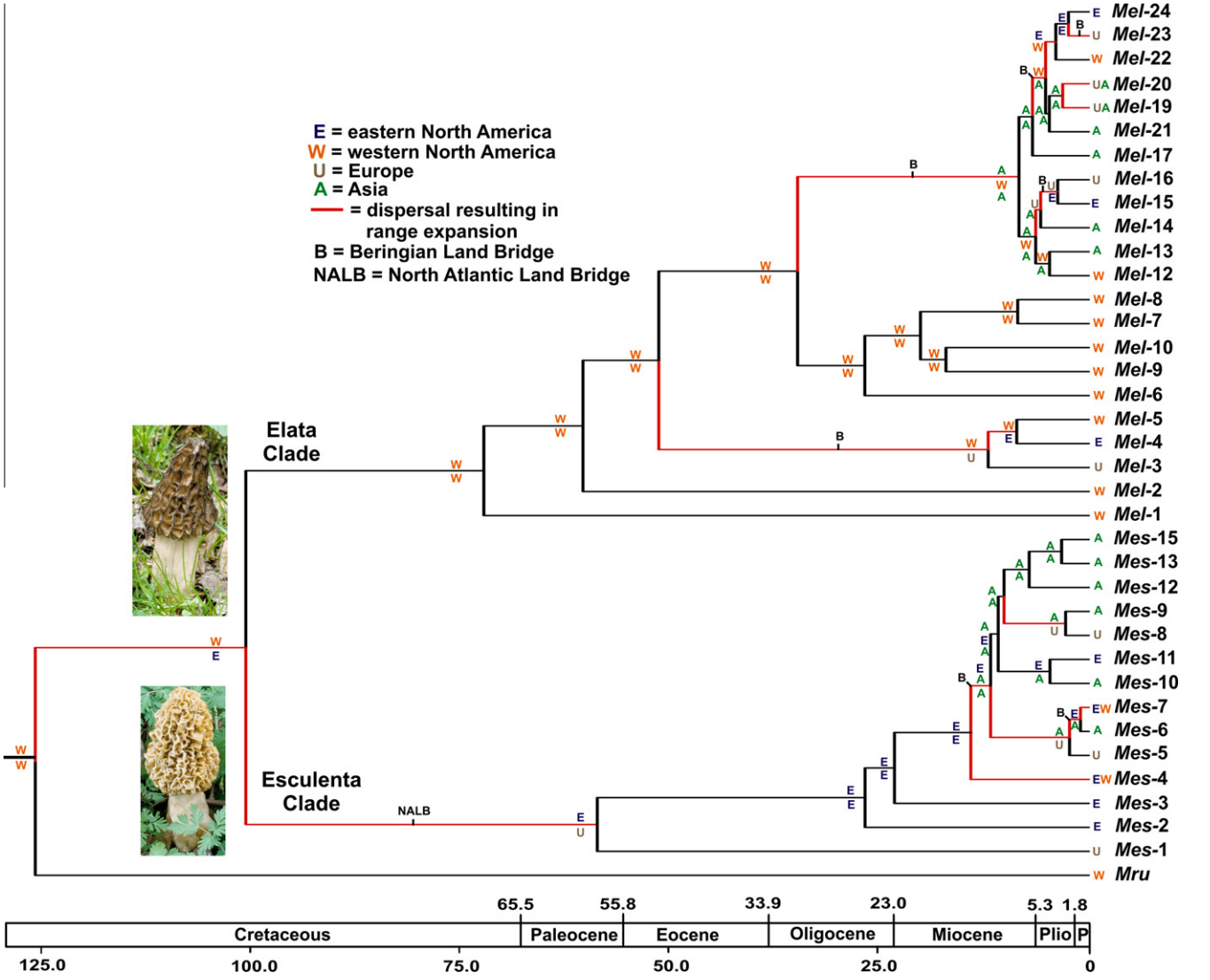

Fig. 5. Ancestral area reconstruction of the true morel genus *Morchella* using the maximum likelihood-based program LAGRANGE (Ree and Smith, 2008), with four ancestral areas in the Northern Hemisphere. The outgroup taxa *Verpa* and *Disciotis* were pruned from the chronogram to focus on the geographic range evolution of *Morchella*. Divergence times were estimated using sequences of five calibration taxa (*see Materials and Methods*) and published divergence times for these taxa (Heckman et al., 2001; Blair, 2009). With the exception of two nodes within the Esculenta Clade, all ancestral ranges are represented by a single area. Red branches identify dispersals resulting in range expansions. Dispersals theorized to have involved the North Atlantic (NALB) and Beringian (B) land bridges between the Old and New Worlds are indicated. The geological time scale is in millions of years before present (Walker et al., 2009). *Mru* = *M. rufobrunnea*, Plio = Pliocene, P = Pleistocene. See Supporting information dataset S5 for the NEXUS file.

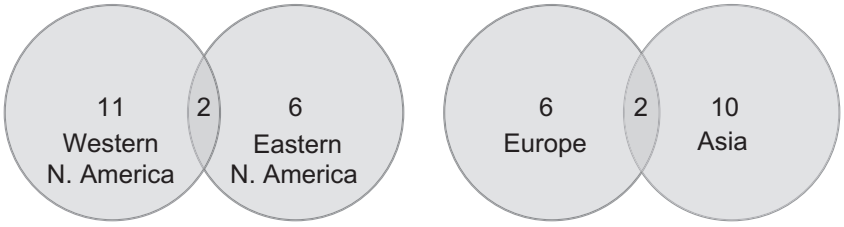

Fig. 6. Venn diagrams depicting the high level of continental endemism and provincialism of *Morchella* in the Northern Hemisphere.

data from a global collection of 177 members of the Morchellaceae. In addition to the traditionally used domains D1 and D2 of the LSU 28S rDNA (O'Donnell et al., 1997), the loci sampled included portions of three protein coding genes (*RPB1*, *RPB2*, and *EF-1α*) whose utility at the species level has been documented recently in diverse Ascomycota (Hansen et al., 2005; Rehner and Buckley, 2005) and Basidiomycota (Matheny et al., 2002; Frøslev et al., 2005; Matheny, 2005). Consistent with prior studies, *RPB1*, *RPB2* and *EF-1α* contributed over 90% of the phylogenetic signal in our analyses of the Morchellaceae (Fig. 1, Table 1), and the Esculenta (Fig. 2, Table 2) and Elata (Fig. 3, Table 3) Clades. Collectively, these results indicate that the LSU 28S rDNA is too conserved for phylogeny reconstruction within the Morchellaceae, as evidenced by its failure to resolve *Morchella* as monophyletic (Bunyard et al., 1995). In addition, due

to significant length mutations within the ITS rDNA region within the Elata (616–858 bp) and Esculenta (821–1132 bp) Clades, as determined DNA sequence analysis (K. O'Donnell, unpublished), use of this locus for molecular phylogenetics within *Morchella* has been limited to only three closely related species within the Esculenta Clade (Kellner et al., 2005), and analyses of the highly conserved, and phylogenetically uninformative, 5.8S rDNA gene within the ITS region (Wipf et al., 1999). Although the nuclear small subunit (SSU) rDNA has been used widely for phylogenetic inference within diverse taxonomic groups, analyses of a 38-taxon dataset of nearly complete sequences (1710 bp), representing the phylogenetic diversity of the Morchellaceae, revealed that it was too conserved for species level studies within this group (K. O'Donnell, unpublished).

Our multilocus analysis of *Morchella* contributes to a growing number of GCPSR-based studies directed at clarifying species boundaries within medically (Koufapanou et al., 1997; Kasuga et al., 2003; Pringle et al., 2009) and agriculturally important fungi (Geiser et al., 1998; O'Donnell et al., 2000; Rehner and Buckley, 2005), the model system fungus *Neurospora crassa* (Dettman et al., 2003), and our preliminary assessment of *Morchella* species diversity in Turkey (Taskin et al., 2010). Following the criteria proposed by Dettman et al. (2003), we recognized phylogenetic species if their genealogical exclusivity was supported by bootstrap analysis of at least one of the single-locus genealogies and by the combined data partition, and their monophyly was not contradicted by analyses of any of the four loci tested. Of the 41 phylogenetic species recognized within *Morchella*, all 36 lineages represented by two or more collections fulfilled the GCPSR criteria of Dettman et al. (2003). Our rationale for provisionally recognizing the remaining five lineages represented by single collections as cryptic species was based on the fact that they were highly divergent genetically from their sisters who were, with only two exceptions (*Mel*-7 and *Mel*-13), found on separate continents. A similar approach was adopted for recognizing lone lineages as phylogenetically distinct species within the human pathogen *Histoplasma capsulatum* (Kasuga et al., 2003). Knowledge of the geographic origin of the lone lineages within *Morchella* provides a means by which these under sampled species, especially in Asia, can be targeted for additional sampling to fully assess their species status.

Results of the present study and those of our recent GCPSR-based investigation of *Morchella* diversity in Turkey suggests species may occupy allopatric latitudinal ranges within a continent, as evidenced by the discovery that only three of the 15 species collected in Turkey (i.e., *Mel*-3, *Mel*-20 and *Mes*-8) were also represented in Europe (Taskin et al., 2010). The high species endemism detected within Turkey resulted from the discovery of seven novel species within the *Mel*-12-to-24 subclade (i.e., *Mel*-20-to-31) and *Mes*-17. It is also worth mentioning that four of the 15 species identified (i.e., *Mel*-2, *Mel*-7, *Mel*-9 and *Mel*-10), representing 35.6% (88/247) collections genotyped, appear to have been introduced to Turkey from western North America (Taskin et al., 2010). Our working hypothesis is these species were introduced in association with horticulture or silviculture.

The molecular phylogenetic results clearly negate the hypothesis that morel species are cosmopolitan in their distribution. To the contrary, species distributions within *Morchella*, which are quintessentially Laurasian, show extraordinarily high levels of continental endemism and provincialism in the Northern Hemisphere. Given that morel fruit bodies produce thousands of wind-dispersed ascospores with an ellipsodial drag-minimizing shape (Roper et al., 2008), why are their ranges so restricted? Two aspects of the morel life cycle may help explain why range expansion via LDD appears to be rare in *Morchella*. Morel ascospores germinate directly to give rise to haploid colonies that appear to be predominately, if not exclusively, self-sterile (heterothallic). In the absence of a chance encounter with a colony of the opposite mating type, they would be unable to form fruit bodies, complete the sexual cycle and form ascospores, the only highly dispersive propagules formed during the morel life cycle. As is typical of most ascomycetous fungi, morels produce mitotic spores (asexual conidia), but they are thin-walled and hyaline, suggesting that they are poorly adapted to LDD.

Surprisingly, the restricted distributions of iconic basidiomycetous macrofungi such as the Chinese black mushroom shiitake (*Lentinula edodes*; Hibbett, 2001) and the psychotropic fly agaric (*Amanita muscaria*; Geml et al., 2008) indicate these heterothallic aconidial species also do not disperse readily by LDD. In addition to the aforementioned studies of macrofungi, a growing number of GCPSR-based studies have shown that broadly defined medically and agriculturally important microfungi harbor multiple cryptic species with significant biogeographic structure (reviewed in Taylor et al. (2006) and Giraud et al. (2008)). Collectively, these studies highlight the importance of allopatric cladogenesis in the evolutionary diversification of diverse macro- and microfungi. As reported recently for the rubiaceous angiosperm *Psychotria* (Ree and Smith, 2008), and according to our DEC model based analyses, range expansions within *Morchella* were accompanied by allopatric speciation following dispersals from ancestral ranges, which typically occupied a single area, or at most two areas (Fig. 5).

One of the most important findings to emerge from the present study is that *Morchella* comprises three lineages: a monotypic basal lineage represented by the commercially cultivated *M. rufobrunnea* and two sister lineages comprising the early diverging Esculenta and Elata Clades. In addition, our multilocus analyses of *Morchella* have provided the first robust phylogenetic framework for elucidating their genotypic diversity, a prerequisite for developing a classification that accurately reflects their evolutionary history. The divergence time estimates suggest that the *Morchella* body plan evolved at least 129.61 (95% HPD interval: 90.26–173.16) Mya in the early Cretaceous and has remained remarkably static during its long evolutionary history. Not surprisingly, their extreme morphological stasis has contributed to the enormous taxonomic confusion within this genus. Morels, for example, can easily be identified as members of the Esculenta (yellow morels) or Elata (black morels) Clades, but only one species within each clade (*Mes*-1 = *M. steppicola* and *Mel*-1 = *M. tomentosa*) can be distinguished currently using phenotypic data. Furthermore, due to the morphological plasticity of the commercially cultivated morel *M. rufobrunnea*, it is sometimes confused with *Morchella esculenta* (Ower et al., 1986). The most conspicuous evolutionary transition in ascocarp body plan within the genus is found in the *M. semilibera* or half-free morel subclade (*Mel*-3-to-5; Figs. 1 and 3) in which the base of the cap in its three allopatric species has become detached from the stalk. Some European authors classify *M. semilibera* in the separate genus *Mitrophora* (Breitenbach and Kränzlin, 1984), but our phylogenetic results clearly show that it is deeply nested within the Elata Clade of *Morchella*. As proposed for the basal enigmatic ascomycete *Neolecta* (Landvik et al., 2001), which has been characterized as a living fossil, *Morchella* also exhibits extreme bradytelic morphological evolution as evidenced by the retention of the ancestral ascocarp body plan. Results of the present study have important implications for elucidating the systematic of *Morchella*. Our results indicate that *Morchella's* phylogenetic diversity within North America and Asia far exceeds the number of published taxa for these areas. We attribute this in part to their homoplastic evolution and the widespread misapplication of European names to predominately undescribed North American and Asian taxa. By contrast, our phylogenetic results indicate that there are approximately 10 times more validly published names as there are European species (see Index Fungorum http://www.indexfungorum.org/Names/Names.asp). In the absence of morphological

apomorphies, the aligned four-locus DNA sequence dataset for the Morchellaceae represents an indispensible resource for advancing their systematics and for elucidating fundamental questions such as their mycorrhizal-like associations with plants (Buscot, 1994; Hobbie et al., 2002).

4.2. Divergence time estimates and historical biogeography of *Morchella*

A potential bias in our analysis of divergence times is with respect to the choice of outgroup taxa. Recently, it has been suggested that certain life history traits (e.g., generation time) are correlated with the rate of molecular evolution in angiosperm plants (Smith and Donoghue, 2008; Smith, 2009). This correlation has never been found for fungi. But if it does hold true, the fact that we used an uncorrelated lognormal model in our divergence time estimation should account for any discrepancies in lineage-specific rate differences that might occur (Drummond et al., 2006; Smith, 2009).

Because the historical biogeography of too few fungi has been studied in detail to identify general patterns in their movements within the Holarctic (Hibbett, 2001; Mueller et al., 2001; Jeandroz et al., 2008; Sung et al., 2008), we compared range evolution in *Morchella* with those documented for plants and animals within the Northern Hemisphere (Sanmartín et al., 2001; Donoghue and Smith, 2004). In contrast to many Gondwanic taxa, clades in all three groups were not restricted to a single area, reflecting the complex history of biotic exchanges within and between the Nearctic and Palaearctic. Whereas many plant groups originated within and dispersed out of Asia (Donoghue and Smith, 2004), this pattern was not observed in *Morchella* and animals (Sanmartín et al., 2001). Results of the AAR suggest that *Morchella* evolved in western North America initially and then migrated into eastern North America during the early Cretaceous. We hypothesize that the *Mes*-1 (*M. steppicola*) lineage subsequently dispersed into Europe across the Thulean North Atlantic Land Bridge between the mid-Cretaceous and late Paleocene prior to the disruption of this dispersal corroder. However, phylogeographic analyses of *M. steppicola* in Eastern Europe and Central Asia are needed to clarify the geographic range evolution of this lineage. In all subsequent range expansions of *Morchella* over the past 30 million years, as in the majority of plants (Donoghue and Smith, 2004), shiitake mushrooms (Hibbett, 2001) and true truffles (Jeandroz et al., 2008), Beringian land bridges appear to have served as the dispersal corridor between the Old and New World. Although eastern Asia–eastern North America disjunctions accounted for half of the intercontinental disjunctions in plants (Donoghue and Smith, 2004), this category was comparatively small in *Morchella* and animals (Sanmartín et al., 2001). Disjunctions in this category were typically less than 30 million years old in plants and *Morchella*, compared with those in animals that were much older (Sanmartín et al., 2001). Aridification-related extinctions during the Miocene in western North America have been proposed to explain why eastern Asia–eastern North America is the largest disjunction category in plants (Donoghue and Smith, 2004). Morels and shiitake mushrooms (Hibbett, 2001), as well as diverse animals in both hemispheres (Sanmartín et al., 2001), also appear to have been impacted by the late Miocene desertification. In addition to plant extinctions resulting from late Miocene climate changes and aridification, morels may have been negatively impacted because species were missing from western North America or Asia in 5 of 10 nonadjacent Old World–New World disjunctions, suggesting widespread extinctions within *Morchella*. Moreover, fragmentation of ancestral ranges due to late Miocene aridification is hypothesized to have contributed to the relatively recent and rapid allopatric speciation of the Elata *Mel*-12-to-24 subclade.

The origin and diversification of *Morchella* in western North America may best explain their marked intracontinental dispersal asymmetry. Given their hypothetical ancestral area, it is not surprising that three of four dispersals within the Nearctic were from western into eastern North America. Similarly, four separate Beringian dispersals of *Morchella* out of western North America into Asia may have contributed to the pronounced (5:1) Asia-to-Europe dispersal asymmetry observed in the Palaearctic. We theorize that the Mid-Continental Seaway and subsequent orogenic events that led to the desertification of regions east of the Rocky Mountains in the Nearctic functioned as a vicariant agent that helped partition the Esculenta and Elata Clades, respectively, in eastern and western North America until limited biotic exchanges became possible during the Miocene (Sanmartín et al., 2001). The marked geographic partitioning of the majority of the Esculenta and Elata Clade species in eastern temperate deciduous and western boreal forests, respectively, in North America suggests that niche conservation has limited their geographic ranges (Donoghue, 2008). Much like the presence of the Mid-Continental Seaway in the Nearctic, the presence of the Turgai Sea and subsequent aridification of central Asia may have played a vicariant role partitioning European and Asian lineages in the Palaeartic up until the start of the Miocene (Sanmartín et al., 2001). Multiple factors, including the aforementioned vicariant agents, niche conservation, low vagility and heterothallism appear to provide the best explanation for the extraordinarily high level of continental endemism and provincialism observed within *Morchella*. In addition, adaption to fire may have restricted range expansion of several Elata Clade species whose fruiting (i.e., sexual reproductive cycle) appears to be dependent on the natural cycle of lightning-induced forest fires in western North America, and whose incidence is predicted to increase dramatically over the next 50 years due to global warming (Spracklen et al., 2009). As noted for fire-adapted plants (Simon et al., 2009), this adaptation appears to have evolved convergently in *Morchella*.

Given that it is highly unlikely that a fossil morel will ever be found to use as an internal calibration point, the only way our divergence time estimates for *Morchella* can be tested will be by obtaining better external calibration points. In addition, as more fungal genomes become available, it should also be possible to develop more robust data sets and include additional collections from under sampled areas to reevaluate our hypothesized historical biogeography of *Morchella*.

4.3. Implications for morel conservation

Results reported here add to the nascent field of mushroom conservation genetics initiated by studies of shiitake, the Chinese black mushroom *L. edodes* (Hibbett and Donoghue, 1996). As advocated by these authors for shiitake, by employing phylogenetic species recognition (Taylor et al., 2000), we identified a large number of morphologically cryptic species within the true morel genus *Morchella* whose distributions are restricted geographically. This phylogenetic knowledge should be highly instrumental in formulating sound conservation policies directed at protecting and maximally preserving morel genetic diversity in the areas sampled, and promoting informed management practices that help insure the sustainability of commercial harvests (Pilz et al., 2007). Moreover, given their marked provincialism, targeting poorly sampled areas for future studies should greatly expand our knowledge of their genetic diversity and geographic distribution.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.09.006.

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