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Morchella tomentosa: a unique belowground structure and a new clade of morels

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Abstract: Mechanisms involved in post-fire morel fructification remain unclear. A new undescribed belowground vegetative structure of *Morchella tomentosa* in a burned boreal forest was investigated north of Fairbanks, Alaska. The name ''radisciscleroti um'' is proposed to define this peculiar and elaborate belowground vegetative structure of *M. tomentosa*. Bayesian and maximum parsimony analyses based on ITS rRNA regions and nLSU gene strongly supported a new clade composed of *M. tomentosa* within the genus *Morchella*.

Key words: belowground structure, burned boreal forest, connective mycelium, post-fire morels, radisciscleroti a, rRNA phylogeny, sclerotia

INTRODUCTION

Morel biology and ecology are complex and poorly understood. Morel fructification occurs either in healthy or stressed forests, with different mycelial dynamics, alternating between saprotrophic and symbiotic behaviours (Buscot and Roux 1987, Buscot 1989, Buscot and Bernillon 1991, Dalhstrom et al. 2000). In healthy stands, morel thalli grow quite regularly every spring in hardwood forests, particularly under poplars or dead elms (Pegler 2003). Morel ascomata appear also as a response during the spring following some disturbances such as insect ravages, forestry practices, droughts or wildfires (Pilz et al. 2004). Wildfire is the most common disturbance occurring at a large scale and prompting the most impressive fructification episodes: Pegler (2003) reported fructification of 20 tons in a single season on a 0.5 ha burned site in Austria.

Despite the clear relationship between disturbances and fructification initiation, one can wonder about the inoculum's origin. Besides ascomata formation, morels develop belowground vegetative structures such as pseudosclerotia or sclerotia (Ower 1982; Wipf et al. 1997; Mayer 1982 quoted by Buscot 1987; Buscot and Roux 1987; Buscot and Kottke 1990; Buscot 1992a, 1992b; Dahlstrom et al. 2000). The occurrence of these structures and their lifetime in forest soil, preceding or following aboveground disturbances, has not yet been established. The nature of the signals from disturbances and the subsequent mechanisms leading to morel fructification such as massive post-fire fructification remains unknown (Wurtz et al. 2005). Sclerotia may form structures linking the belowground mycelium to the ascomata. In the present study, the presence of belowground structures of morel ascomata from burned forests was investigated north of Fairbanks, Alaska. Peculiar belowground structures attached to three ascomata of the recently described *M. tomentosa* M. Kuo (Kuo 2008) were observed and characterized. As these structures were apparently new for morels, we analyzed the phylogenetic position of *M. tomentosa* within the Morchellaceae based on rRNA sequences.

MATERIALS AND METHODS

Field sampling and herbarium specimen.—In Jun 2006, 51 post-fire morels were collected north of Fairbanks, Alaska, USA. At the collecting site, the soil was carefully removed around the base of the stipe to investigate the presence of belowground structures (BGS) using a soft brush and a needle. The BGS of three morel ascomata from a one-year-old burned black spruce stand were collected. Ascocarps and BGS were dried 24 h after collecting while one BGS subsample from each ascocarp was kept in a cacodylate solution for further microscopic observations. Herbarium

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TABLE I. List of herbarium specimens examined and GenBank accession numbers. Specimen IDs beginning with QFB were deposited in the René Pomerleau Herbarium at the Laurentian Forestry Centre, specimen IDs beginning with 061 are from the Mycology Collection of the Field Museum of Natural History in Chicago and the specimen ID beginning with DAOM is from the National Mycological Herbarium in Ottawa

Herbarium specimen	Herbarium ID	Origin	GenBank accession number	
			ITS rRNA regions	nLSU rRNA gene
Gyromitra infula	QFB 9249	Québec	GQ304944	GQ305046
Gyromitra sphaerospora (Peck) Sacc.	QFB 3131	Québec	GQ304943	GQ305045
Morchella conica Pers.	QFB 3231	Québec	GQ304962	GQ305027
Morchella conica Pers.	QFB 3209	Québec	GQ304963	GQ305031
Morchella conica Pers.	QFB 3211	Québec	GQ304964	GQ305032
Morchella esculenta (Vent.) Pers.	QFB 17140	Québec	—	GQ305042
Morchella tomentosa	DAOM 240079	Alaska	GQ304951 to 53	GQ305016 to 18
Morchella tomentosa	QFB 8582	Alaska	GQ304947 to 50	GQ305012 to 15
Morchella sp.	QFB 8581	Alaska	GQ304982 to 86	GQ305033 to 37
Morchella tomentosa Kuo (holotype)	06150405	Montana	GQ325232	GQ325238
Morchella tomentosa Kuo	06150402	Montana	GQ325233	GQ325239
Morchella tomentosa Kuo	06150403	Montana	GQ325234	GQ3252340
Morchella tomentosa Kuo	06150406	Montana	GQ325235	GQ3252341
Morchella tomentosa Kuo	06150407	Montana	GQ325236	GQ3252342
Morchella tomentosa Kuo	06150409	Montana	GQ325237	GQ3252343
Verpa conica (Mull.) Swartz	QFB 3221	Québec	—	GQ305044
Verpa bohemica (Krombh.) Schröt	QFB 16542	Québec	GQ304945	GQ305043

samples of *Gyromitra*, *Verpa* and *Morchella* from the QFB Herbarium (Laurentian Forestry Centre, Québec, Canada) and the Mycology Collection of the Field Museum of Natural History in Chicago were included in the phylogenetic analyses (TABLE I).

Microscopy.—BGS of about 1 cm^3 were fixed with 4% paraformaldehyde in 0.1 M cacodylate, washed, progressively dehydrated with ethanol and embedded in paraffin. Thin (5 μm) slices were stained with hematoxilin-eosine and examined and photographed under a light microscope (Olympus BX-51).

Molecular analyses.—For genomic DNA (gDNA) extraction, small pieces of the following ascomata were excised: 51 dried ascocarps collected in Alaska, eight subsamples from two dried BGS, two *Gyromitra* specimens [*G. sphaerospora* (Peck) Sacc. and *G. infula* (Schaeff.) Quél.], one *Verpa bohemica* (Krombh.) Schröt and 10 *Morchella* specimens [one *M. esculenta* (Vent.) Pers., three *M. conica* Pers. and six *M. tomentosa* Kuo]. Samples were crushed in liquid nitrogen with micropestles and incubated at 65 C for 1 h in 400 μL Carlson lysis buffer and 2 μL β-mercaptoethanol (Carlson et al. 1991). Samples were mixed every 15 min. Four hundred μL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) were added and the aqueous phase was collected after centrifugation (5000 g for 10 min). Samples were incubated 1 h at −20 C in 70 μL of 7.5 M ammonium acetate and 600 μL isopropanol for nucleic acids precipitation. After centrifugation (9000 g for 10 min), pellets were washed with 800 μL 75% ethanol, dried on a thermoblock for 10 min at 55 C and re-suspended in 50 μL of TE-8 (Tris EDTA buffer, pH 8).

Genomic DNA was diluted 1 : 50 before amplification. The ITS1-F (Gardes and Bruns 1993) / LR5 (Vilgalys and Hester 1990) primer pair was used in a single PCR to amplify the internal transcribed spacers (ITS) and nuclear large subunit (nLSU) of the ribosomal RNA regions. The PCR mixture included 0.5 μM of each primer, 25 μg of bovine serum albumin (Sigma), 1.6 mM of $MgCl_2$, 10× PCR buffer (Roche Diagnostics, Mannheim, Germany), 1.25 mM of each deoxynucleotide triphosphate, and 1 unit of *Taq* DNA polymerase (Roche Diagnostics, Mannheim, Germany). The PCR program was as follows: initial denaturation at 95 C for 2 min, 38 cycles of 94 C for 45 s, 58 C for 1 min, 72 C for 1 min, and a final elongation at 72 C for 10 min. PCR reactions were run on a MJ Research PTC-200 (MJ Research Inc., Waltham, Massachusetts). PCR products were sequenced on a 96-capillary 3730 × l DNA analyzer at the Genomic Sequencing and Genotyping Platform, Centre de recherche du Centre hospitalier de l'Université Laval (CRCHUL, Québec, Canada).

Bioinformatic analyses.—Sequences were corrected and contigs were assembled with Sequencher v4.6 (GeneCodes, Ann Arbor, Michigan). The similarity threshold for sequences belonging to the same operational taxonomic unit (OTU) was set to 99% to serve as a proxy for 'species', which corresponds to values used in other studies using these rDNA regions (Lynch and Thorn 2006, Arnold et al. 2007, Porter et al. 2008). We used BLASTn (Altschul et al. 1990) to find similar sequences in the NCBI GenBank database of voucher specimens. Sequences were aligned with Muscle software v3.5 (Edgar 2004) with default parameters. Alignments were edited in eBioX version 1.5.1 (<www.ebioinformatics.org>) and manually refined. The NCBI GenBank database was parsed for *Gyromitra*, *Morchella* and *Verpa* voucher ITS and nLSU sequences. Sequences retrieved from GenBank were added to the alignments.

Because large insertions/deletions occur within *Gyromitra*, *Morchella* and *Verpa* ITS sequences, phylogenetic analyses were based on the last part of the ITS1 section (86 bp), the complete 5.8S (168 bp), and the first and last parts of the ITS2 section (201 bp). The nLSU phylogeny was based on the first 494 base pairs (bp) of the nLSU rRNA gene. Maximum-parsimony analyses were performed using PAUP v4.b10 (Swofford 2002), with 1000 trees saved. A heuristic search was executed with 1000 random stepwise additions, the branch swapping was conducted using the nearest-neighbor interchange (NNI) algorithm and multiple parsimonious trees were saved (MULTREES in effect). Significant support of the clusters was assessed with 5000 bootstrap resamplings through a heuristic search with random addition sequence.

The DNA substitution model was determined for the ITS rRNA regions and the nLSU rRNA gene using the hierarchical likelihood ratio test implemented in MODELTEST 3.06 (Posada and Crandall 1998) and results were included in the subsequent phylogenetic analyses. Bayesian phylogenetic analyses were performed on each dataset using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003), running four Markov Chains Monte Carlo (MCMC) for 10 million generations. The number of trees saved was set to 100 000 and the first 10% of the trees were excluded for computing the consensus tree (BURNIN period set to 10 000). Phylogenetic trees were edited in FigTree v1.3.1 (Rambaut 2009).

RESULTS

Morphology.—A peculiar belowground structure associated with the newly described *M. tomentosa* M. Kuo was observed under three ascocarps collected in two different plots (FIG. 1). Such a structure has never been reported or described. We propose the name ''radisciscleroti um'' for this singular and complex underground vegetative structure of *M. tomentosa*. Looking at the radisciscleroti um from top to bottom, it appears to be an extension of the *Morchella tomentosa* stipe below the soil surface (FIG. 1A, C). It rapidly branches out into two or three root-like structures (FIG. 1B, D). Radisciscleroti a were 5–15 mm in diameter, rigid, and whitish after the dirt has been removed. Thin plant roots were embedded within the radisciscleroti a (FIG. 1B). Microscope observations of radisciscleroti a transversal sections (FIG. 2) show straight, oversized hyphae, 8.7 (± 2.9 SD) × 90 (± 60 SD) µm, embedded in a tight matrix. Long hairlike paraphyses typical of *M. tomentosa* were observed (160–360 µm) on sterile ridges. Specimens were deposited in the QFB Herbarium (QFB 8582) and DAOM240079.

Ecology.—*Morchella tomentosa* is a post-fire morel in black spruce forests found in the spring of the year following a fire and probably a few years after. This species can be distinguished from other black morels by long hairs on sterile ridges (Kuo 2008) using a hand lens. *Morchella tomentosa* ascocarps represented about 14% (7/51) of the morel ascocarps collected in our sampling. Radisciscleroti a were observed under three ascocarps randomly excavated in two different plots the year following forest fire during the second half of June. Sequence data showed they all belong to *M. tomentosa*. Therefore, radisciscleroti a can potentially be used for *M. tomentosa* diagnostics.

Molecular analyses and phylogeny.—The contig analysis based on ITS sequences showed one dominant group clustering 44 black morel ascomata sequences from Alaska, followed by a second cluster composed of seven sequences of ascomata from Alaska, eight sequences from radisciscleroti a subsamples, the holotype and five isotypes of *M. tomentosa*. All sequences were phylogenetically analyzed except for the dominant contig for which we only considered 5 sequences out of 44 to avoid redundancy.

Similarity between ITS sequences of *M. tomentosa* sampled in Alaska and *M. tomentosa* from Montana (Kuo 2008) ranged from 99.3% (873/877 bp for isotypes 06150402, 06150403 and holotype 06150405) to 99.5% (872/878 bp for isotypes 06150406, 06150407, 06150409). Contig analysis based on nLSU sequences provided similar clusters and singletons as in the ITS rRNA analysis.

The closest ITS sequence to *M. tomentosa* found in GenBank was *M. costata* with 95% similarity for a 50% coverage, matching the end of the ITS1, the 5.8S and the beginning of the ITS2. The following closest sequences found in GenBank were sequences of *M. angusticeps* and *M. conica* with 92% similarity and covering 51% of the same region as *M. costata*.

The two Bayesian phylogenetic reconstructions (FIG. 3A, B) clustered *M. tomentosa* sequences into a single clade, statistically supported by Bayesian and bootstrap values. The phylogenetic relationships of *M. tomentosa* with the other *Morchella* clades were not resolved. The phylogenetic analysis based on the ITS data set considered *M. tomentosa* as a new clade within the black morel, and the blond morel basal to the black morel including the *M. tomentosa* clade. The phylogenetic analysis of the nLSU gene showed that the *Morchella* was a polytomy of three well-supported clades: *M. tomentosa*, the blond and the black morels. Sequences of *M. tomentosa* ascomata sampled in Alaska were closely related to *M. tomentosa* collected in Montana and they clustered with strong support in analyses of both data sets. The other black morels sampled in Alaska clustered into a single branch within the clade composed of *M. conica* – *M. angusticeps* – *M. costata* – *M. elata* with strong Bayesian and bootstrap values. Similarity between ITS sequences of black morels sampled in Alaska and *M. costata* and *M. angusticeps* was 93% and

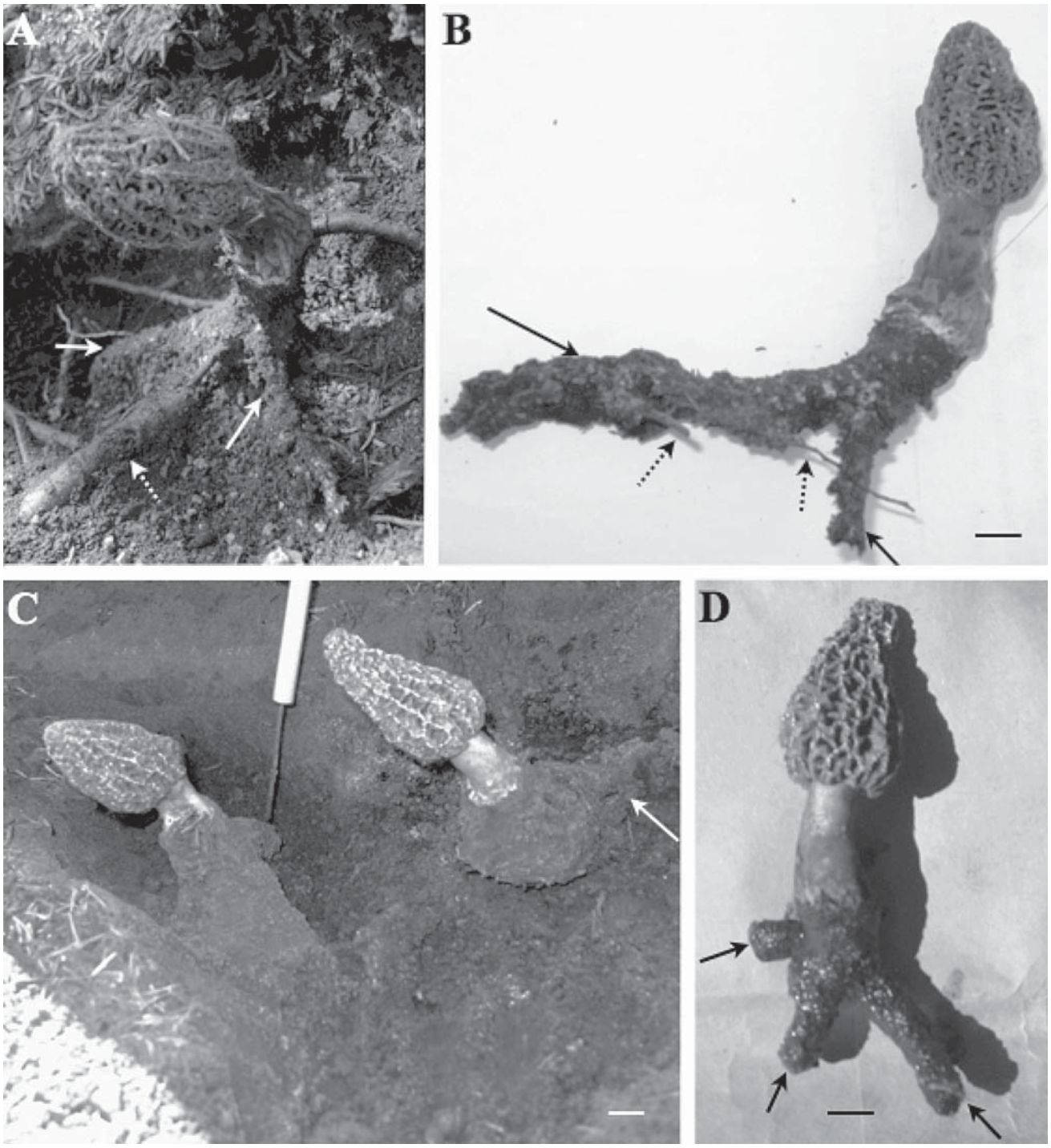

FIG. 1. *Morchella tomentosa* in the field (A, C). Radisciscleroti thrusting deep into the ground (full line arrows) and plant roots (dotted line arrows). *Morchella tomentosa* (B) and radisciscleroti (D). Bar scale is 1 cm.

94%. Similarity between nLSU sequences of black morels sampled in Alaska and the *M. conica* – *M. elata* cluster was 98%. Maximum parsimony analyses showed similar trees to the Bayesian analysis (data not shown). Phylogenetic trees and the data sets were deposited in TreeBASE as accessions T26724, T26752 and M4849, M4730.

DISCUSSION

To our knowledge, this is the first report of this type of belowground structure, the radisciscleroti, in post-fire morels in a boreal forest. However, other types of underground structures have been described as connective mycelia, linking *M. rotunda* (Fr.) Boudier

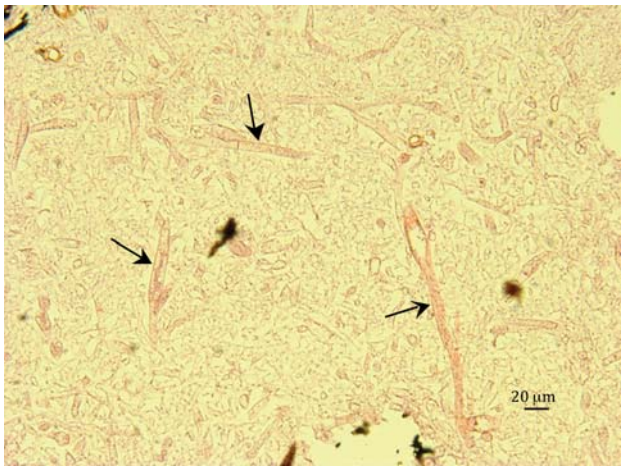

FIG. 2. Transverse section of a radiscisclerotium. Arrows show oversized hyphae (magnification 20 ×).

ascocarps to a sclerotia-like mycelial muff, enveloping the roots of ligneous or herbaceous plants (Buscot and Roux 1987). The underground structures connected to *M. tomentosa* stipes are similar to the connective mycelium of *M. rotunda* and *Mitrophora semilibera* (DC.) Lév. associated with *Fraxinus excelsior* L. and *Cornus sanguinea* L. roots (Buscot 1987) but they are far better defined macroscopically as root-like structures. Goldway et al. (2000) mentioned some sort of an underground structure of *M. conica* associated with the roots of *Fraxinus syriacus* [syn. *Fraxinus angustifolia* subsp. *syriaca* (Boiss.)Yalt.]. Buscot (1987) suggested that the connective mycelium grows only during the initial development of the morel ascocarps and degenerates when the ascocarps begin to sporulate. This could explain why it is so difficult to observe these structures in *Morchella*. Buscot and Roux (1987) and Buscot (1987) pointed out the ephemeral nature of *M. rotunda* and *M. semilibera* loose connective mycelia, which makes it impossible to observe their morphogenesis. By contrast, radiscisclerotia of *M. tomentosa* are strongly attached to the mature ascocarps and are solid and compact hypogeous structures, suggesting that they represent a distinct structure.

The observation that radiscisclerotia remained a compact and well-defined structure after *M. tomentosa*

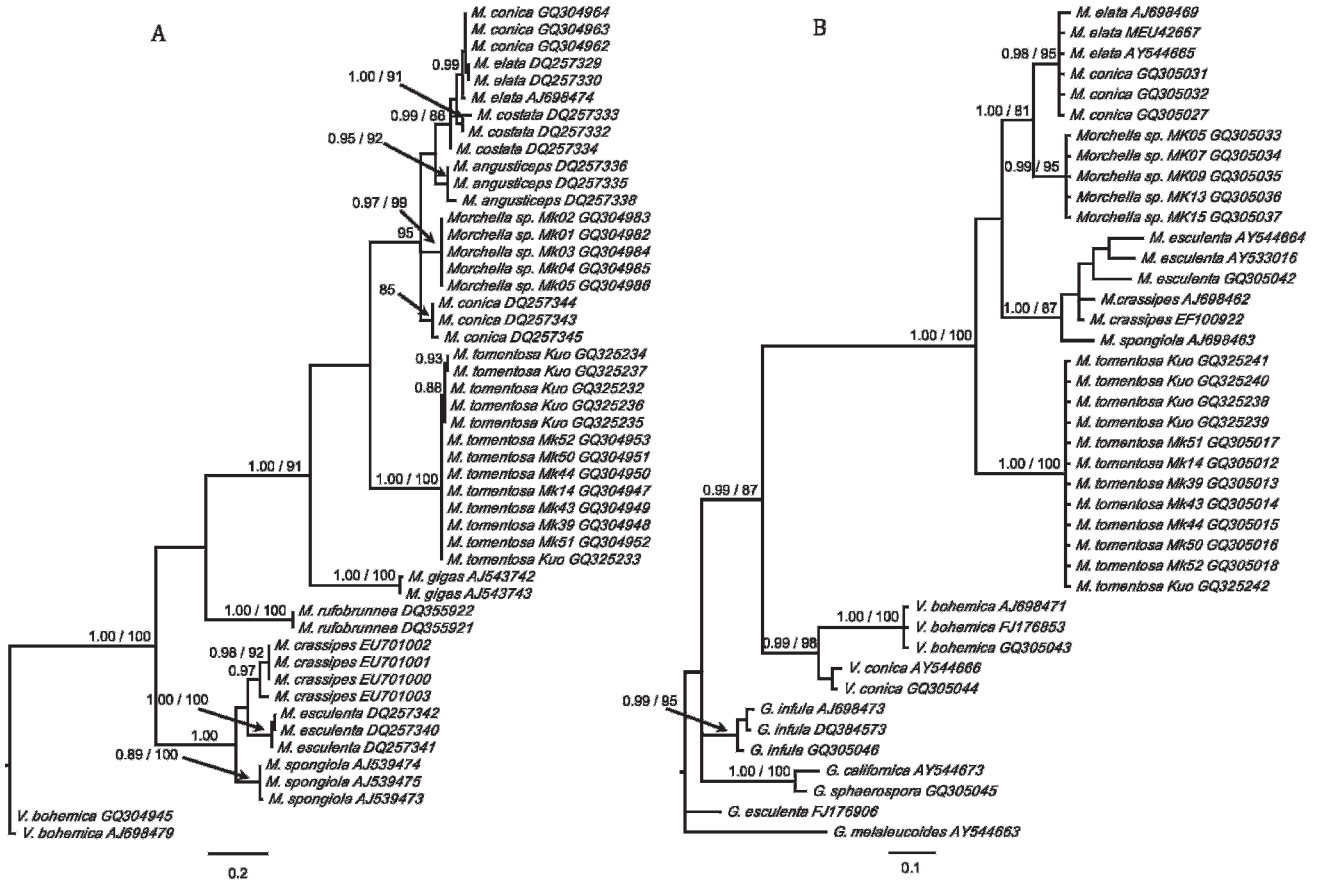

FIG. 3. Bayesian 50% majority consensus trees based on (A) the analysis of the ITS rRNA regions and (B) the analysis of the LSU rRNA gene. Values above nodes reflect Bayesian posterior probabilities (left) and bootstrap support (right). Only Bayesian posterior probabilities and bootstrap values greater than 0.85 and 80, respectively, are shown. The scale gives the substitution rate.

ascocarp formation implies it may not play the same role as the temporary resource reservoir observed in *M. rotunda*. It may be a time-resistant resource reservoir that could last a few years, allowing second- and third-year ascocarp production. We doubt that this structure could remain throughout the complete fire rotation period awaiting the next fire episode. However, morphogenesis of radiscisclerotia remains to be understood. Wurtz et al. (2005) suggested that sclerotia/pseudosclerotia provide nutrients in order to allow a massive fructification in response to the lack of nutrients transferred from the host via the root tips in case of host injury or death.

All collections of this new clade of black morel were only done in burned conifer forests, fruiting during the year following a forest fire. This ecological niche is also shared with some members of the black morel *M. conica-elata* group, but the latter group can also be found in undisturbed sites (Pilz et al. 2004, Pilz et al. 2007). The ITS and nLSU phylogenies did not resolve the phylogenetic relationships of *M. tomentosa* with other morel clades. Phylogenetic analyses of well-identified morels based on coding genes are required to better resolve the relationships of the different morel clades and to increase understanding of their ecology. The strong genetic divergence observed between *M. tomentosa* and the other morels might suggest very different biological behavior. Up until now, *M. tomentosa* has only been reported from burned forest sites (Kuo 2008), which emphasizes its saprophytic behavior.

North America is the apparent centre of diversity of the genus *Morchella* (Pilz et al. 2004) and O'Donnell et al. (2003) estimated that 22 morel species may be endemic. Phylogenetic analyses based on DNA sequences could help in sorting out morel taxonomy, an essential step to better define morel diversity and to increase the understanding of their biology. *Morchella tomentosa* is a recently described species that represents a new clade within *Morchella*. Fortunately, this morel can easily be identified in the field, a rare feature in the morel world.

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