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SHORT NOTE

Membranomyces species are common ectomycorrhizal symbionts in Northern Hemisphere forests

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Abstract *Membranomyces* (Clavulinaceae, Cantharellales) Jülich consists of two described species of resupinate (crust-like) basidiomycetes. Previous studies indicated that *Membranomyces* falls within the Clavulinaceae, but the phylogenetic position of the genus has not been fully resolved. *Membranomyces* species were thought to be saprotrophic until 2003 when Tedersoo et al. detected *Membranomyces delectabilis* on ectomycorrhizal roots of *Populus* and *Picea*. *Membranomyces* was previously known only from collections made in eastern Canada and Europe. We recently sequenced the ITS rDNA barcode region from Scandinavian herbarium specimens identified as *M. delectabilis* and *Membranomyces spurius*. Phylogenetic analyses of these sporocarp sequences and similar environmental sequences indicated that *Membranomyces* is more diverse than previously thought and forms ectomycorrhizas with hosts from a diverse range of plant families in many north temperate ecosystems.

Keywords Basidiomycota · Cantharellales · *Clavulina* · Clavulinaceae · Resupinate fungi

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Introduction

Membranomyces Jülich was erected in 1975 to accommodate resupinate (crust-like) basidiomycetes with a smooth hymenophore, inamyloid, smooth basidiospores, basidia with curved sterigmata, and monomitic hyphae lacking clamp connections (Larsson et al. 2004). Jülich (1975) erected the genus based on the type species *Membranomyces spurius* (Bourd.) Jülich which was originally described as *Corticium spurium* Bourdot. Some authorities recognize a second species, *Membranomyces delectabilis* (H.S. Jacks.) Kotiranta and Saarenoksa (=*Corticium delectabile* H.S. Jacks.), that differs from *M. spurius* in basidiospore length and the absence of cystidioid hymenial hyphae (Eriksson et al. 1981; Kotiranta and Saarenoksa 1993). *C. delectabile* was once included in *Clavulicium* (Eriksson and Ryvarden 1973; Hjortstam 1973), but morphological and molecular evidence indicates no close relationship with the type of *Clavulicium*, hence supporting recognition of *Membranomyces* as an independent genus (Larsson et al. 2004).

A recent shift has occurred in our understanding of the systematics and ecological habit of *Membranomyces*. Several studies indicated that *Membranomyces* is related to the coral fungus genus *Clavulina* (Clavulinaceae, Cantharellales, Fig. 1b) although the exact phylogenetic relationship was unclear (Tedersoo et al. 2003; Larsson et al. 2004). Moncalvo et al. (2006) resolved *M. delectabilis* as sister to *Clavulina* whereas Uehling et al. (2012a) found *M. delectabilis* nested within *Clavulina*. Larsson et al. (2004) and others noted a remarkable similarity in basidium morphology between *Membranomyces* and *Clavulina*. *Membranomyces* species were once thought to be saprotrophs due to their fruiting habit on woody substrata, but are now known

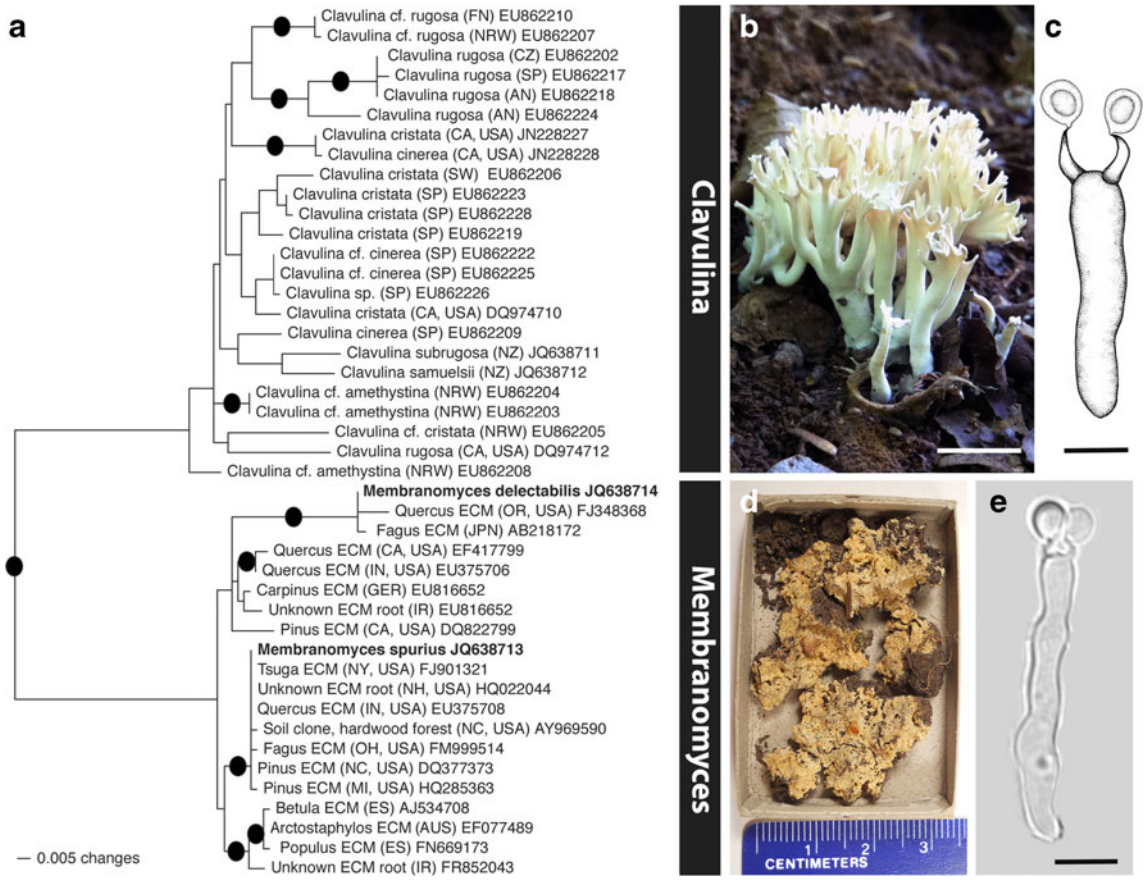

Fig. 1 **a** Midpoint-rooted maximum likelihood (ML) phylogeny of *Membranomyces* and *Clavulina* species based on ITS rDNA (likelihood value=−ln 2,272.125). Sequences from *Membranomyces* fruiting bodies are shown in *bold text*. Sequences from root tips or soil cores indicate host plant (when known), location, and GenBank accession number. Country codes for sample locations are: Andorra (*AN*), Finland (*FN*), Germany (*GER*), Iran (*IR*), Japan (*JPN*), New Zealand (*NZ*), Norway (*NRW*), Spain (*SP*), Sweden (*SW*), and United States (*USA*). *Filled black circles* indicate ML and Maximum Parsimony bootstrap support greater than 85 %. **b** Basidiomata of *Clavulina* cf. *cristata* specimen MES461 showing coralloid fruiting habit, *scale bar*=10 mm. **c** Bisterigmate basidium with developing basidiospores of *Clavulina cerebriformis* showing classical diagnostic morphology, *scale bar*=10 mm. **d** Basidioma of *Membranomyces* cf. *spurius* specimen Saarenoksa 39091, showing resupinate fruiting habit. **e** Bisterigmate basidium with developing basidiospores of *M. delectabilis* specimen K. H. Larsson 13499, *scale bar*=10 μm

to form ectomycorrhizas that are morphologically similar to those formed by *Clavulina* (Tedersoo et al. 2003; Larsson et al. 2004). Tedersoo et al. (2003) found *M. delectabilis* in association with both spruce and aspen in a mixed Estonian forest, suggesting a generalist status on gymnosperm and angiosperm hosts, consistent with findings for some other ectomycorrhizal (ECM) fungi (Bonito et al. 2010).

Relative to known sporocarp species diversity of Clavulinaceae, belowground diversity can be high in a variety of ecosystems when root-based molecular surveys are performed. Numerous temperate ECM community studies have found Clavulinaceae root tip sequences that were unresolved at the species level (Tedersoo et al. 2003; O'Brien et al. 2005; Krpata et al. 2007; Parrent et al. 2007; Peay et al. 2007; Avis et al. 2008; Morris et al. 2008; Southworth et al. 2009; Burke et al. 2009; Bahram et al. 2011, 2012; Lang et al. 2011). In the neotropical forests of Guyana, molecular studies of ECM fungal communities documented a high diversity of *Clavulina* sequences, including many that were not conspecific with known regional *Clavulina* sporocarps (Smith et al. 2011). At the same site in Guyana, new species of *Clavulina* with small resupinate sporocarps were recently discovered, which led us to hypothesize that at least some of the "missing" *Clavulina* belowground diversity may be composed of additional taxa with cryptic sporocarps (Uehling et al. 2012). Given the large number of unresolved Clavulinaceae sequences from many parts of the world, we suspected that a similar "missing diversity" situation may also apply to *Membranomyces*, providing the stimulus for the current study.

We initially sequenced the internal transcribed spacer region (ITS) of rDNA from herbarium specimens identified as *M. delectabilis* and *M. spurius* to provide a database from which to search for potentially congeneric or conspecific

root or soil sequences already deposited in GenBank. Here, we present our analyses of self-generated and GenBank environmental sequences of *Membranomyces* and other Clavulinaceae and show that *Membranomyces* species are widespread, generalist, ECM fungi distributed across the Northern Hemisphere.

Methods

Specimens identified as species of *Membranomyces* and *Clavulina* were obtained from herbaria at the University of Gothenburg, Sweden (GB), University of Helsinki, Finland (H), and University of Tennessee, USA (TENN). Rehydrated fungal tissue from these specimens was mounted in water and 3 % KOH, and micromorphological features were photographed on a Qimaging Micropublisher 3.3 camera system. At least 20 individual basidiospores were measured for each species to confirm consistency with reported spore dimensions. Sporocarp tissues were homogenized with a micropestle and DNA was extracted with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA USA). PCR protocols for the amplification of ITS1-5.8s-ITS2 rDNA followed those of Uehling et al. (2012). We generated full-length ITS sequences for *M. delectabilis* (KH Larsson 13499, GenBank JQ638714), *M. spurius* (R Saarenoksa 39091, GenBank JQ638713, Fig. 1d), *Clavulina samuelsii* (TENN 065723, JQ638712), and *Clavulina subrugosa* (TENN 43395, JQ638711). Sequences were viewed and edited in Sequencher v.4.1.4 (Gene Codes Corp., Ann Arbor, MI). We subjected our newly generated *Membranomyces* ITS sequences to BLAST analysis using the blastn algorithm in GenBank to identify potentially congeneric or conspecific environmental sequences to include in our analysis (www.ncbi.nlm.nih.gov/). Taxa with greater than 97 % sequence homology were considered conspecific for our purposes (Smith et al. 2007; Hughes et al. 2009). Along with the *Membranomyces* sporocarp and environmental sequences, we included a limited number of temperate *Clavulina* sporocarp sequences from Genbank. We excluded tropical *Clavulina* sequences from our analysis because their ITS regions are so variable that alignment is challenging and requires exclusion of many more nucleotides from the data set (Smith et al. 2011). A more comprehensive, multi-gene dataset, and a detailed taxonomic assessment will be needed to address the higher-level placement of these *Membranomyces* species within the *Clavulina* lineage. Since the main aim of this study was to examine geographic distribution of and phylogenetic diversity within *Membranomyces*, we limited the dataset and focused on this group. We subsequently assembled an alignment of 44 ITS sequences derived from *Membranomyces* and *Clavulina* sporocarps and Clavulinaceae ECM root tips and soil cores.

DNA sequences were compiled for phylogenetic analysis in Mesquite 1.1 and aligned with the aid of MUSCLE (Edgar 2004; Maddison and Maddison 2010). We excluded 144 ambiguously aligned characters mostly from the ITS1 one region, leaving 617 characters in the final alignment. Parsimony analysis was performed with the default settings and parsimony bootstrapping was conducted with 500 replicates in PAUP 4.0a112 (Swofford 2002). Maximum likelihood analysis and bootstrapping with 500 replicates were performed using the default settings in Garli 0.951 (Zwickl 2006).

Results

We examined the *Membranomyces* herbarium specimens and confirmed that *Membranomyces* and *Clavulina* species are similar in basidium and basidiospore micromorphology (e.g. Fig. 1c, e; Larsson et al. 2004). The two *Membranomyces* specimens that were sequenced were generally consistent with descriptions in the literature, with basidiospores measuring 7–10×7–8(9)μm in *M. spurius* and 9–12×(7)8–10 μm in *M. delectabilis* (Kotiranta and Saarenoksa 1993; Bernicchia and Gorjón 2010). The ITS sequences generated from these sporocarps were significantly different using a 97 % similarity definition of species-level taxa. BLAST searches revealed strong matches between our *Membranomyces* sporocarp sequences and environmental Clavulinaceae ECM root tip and soil sequences on GenBank. Subsequent phylogenetic analysis indicated that *Membranomyces* sporocarp sequences grouped closely at the generic level with numerous root and soil sequences from both angiosperms (*Arctostaphylos*, *Betula*, *Carpinus*, *Fagus*, *Populus*, and *Quercus*) and gymnosperms (*Pinus* and *Tsuga*).

The maximum parsimony (MP) analysis utilized 102 parsimony informative characters and yielded four equally parsimonious trees of 271 steps. Each of the four trees showed a strong separation between *Membranomyces* and *Clavulina* lineages and their topologies differed only in minor rearrangements of taxa within the *Clavulina* lineage. The maximum likelihood (ML) phylogeny (−ln 2,272.125, Fig. 1a) also had a similar topology to the four MP trees. Each of the 18 unresolved environmental Clavulinaceae ITS sequences included in our analyses fell within the *Membranomyces* lineage. Both MP and ML analyses recovered several different groups of *Membranomyces* sequences that roughly correspond to the ITS sequence groups that share greater than 97 % similarity (Fig. 1a).

Discussion

The original range of *Membranomyces* was considered to include eastern Canada and Europe (Kotiranta and Saarenoksa

1993; Yurchenko and Kotiranta 2007; Bernicchia and Gorjón 2010). Our results indicate that the genus is much more widespread, spanning much of the Northern Hemisphere. The new inferred distribution includes Andorra, Belarus, eastern Canada, Denmark, Finland, France, Germany, Italy, Iran, Japan, Norway, Russia, Spain, Switzerland, Turkey, United Kingdom, and various regions of the USA. Additionally, the presence of multiple well-supported and widespread species groups within *Membranomyces* indicates that the currently described species are more broadly distributed and that additional species exist. For example, the Finnish specimen of *M. spurius* grouped closely with ECM root tip sequences from the eastern USA in our analyses, while the Swedish *M. delectabilis* grouped with ECM root tip sequences from the western USA and Japan. A third well-supported cluster within *Membranomyces* that is dissimilar at the species level to the two known species includes ECM sequences from Estonia, Austria, and Iran. Several other ECM sequences from Iran, Germany, and various parts of the USA are nested at alternative positions in *Membranomyces*, but are not statistically supported with the other species-level clusters.

Prior to this study, there were three *Membranomyces* sequences from two specimens deposited on GenBank, composed of a full length ITS sequence from *M. spurius* (KM 131627), a short 5.8S sequence, and a longer 28 S sequence from *M. delectabilis* (KH Larsson 11147) (GenBank numbers GQ981509, AY463442, AY586688 respectively). When analyzed using blastn, the ITS sequence from KM 131627 (GenBank GQ981509) was approximately 90 % similar to other *Clavulina* and *Membranomyces* sequences, but was not closely related to any ECM root tip sequences, including those in our analysis. Given the uniqueness of its ITS sequence, this specimen may represent another undocumented generic level lineage within Clavulinaceae. The ITS sequence from *M. delectabilis* specimen KH Larsson 11147 was approximately 200-bp long and mostly derived from the conserved 5.8S region. Thus, blastn analysis with full-length ITS sequences from *Membranomyces* species on ECM roots or soil do not match closely with this reference sequence due to length differences. Such poor blastn matching with pre-existing *Membranomyces* sporocarp sequences on GenBank is the likely reason that the numerous *Membranomyces* environmental sequences have previously gone undetected.

The ubiquity of resupinate ECM fungi in groups such as the Sebacinales and Thelephorales became clear when sporocarp sequences from herbarium specimens were matched to environmental sequences (Kõljalg et al. 2000; Larsson et al. 2004; Weiss 2004). Here, we have shown that species of *Membranomyces* are similarly widespread ECM symbionts that may have been repeatedly overlooked in the field due to their cryptic sporocarp morphology. It is estimated that nearly a third of fungal rDNA sequences recently added to GenBank are environmental sequences with the majority being inadequately identified (Brock et al. 2009; Ryberg et al. 2009; Hibbett et al. 2011). As demonstrated here with *Membranomyces*, ITS sequencing from herbarium specimens can be a valuable approach to identifying fungi in this ever-growing pool of unresolved environmental sequences.

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