Mycol Progress (2013) 12:151–155
DOI 10.1007/s11557-012-0841-y

SHORT COMMUNICATION

Phylogenetic analysis of rDNA sequences indicates that the sequestrate *Amogaster viridiglebus* is derived from within the agaricoid genus *Lepiota* (*Agaricaceae*)

Zai-Wei Ge · Matthew E. Smith

Received: 9 May 2012 / Revised: 19 June 2012 / Accepted: 21 June 2012 / Published online: 20 July 2012

Abstract The rare sequestrate fungus *Amogaster viridiglebus* is known from only one collection in California where it was discovered among *Populus* roots. Based on sporocarp coloration and spore morphology, this sequestrate taxon was putatively considered to be an ectomycorrhizal member of the *Boletales*. However, no molecular data were previously available to definitively determine the closest relatives of this fungus. Here we revisit the morphology of *Amogaster viridiglebus* and present a phylogenetic analysis based on ITS and 28S ribosomal DNA. Our phylogeny indicates that *Amogaster viridiglebus* is nested in the genus *Lepiota*, suggesting that this rare species has a saprobic trophic mode and does not form ectomycorrhizae with plants. A new combination, *L. viridigleba,* is made based on these phylogenetic results.

Keywords *Agaricales* · Fungal systematics · Phylogeny · *Lepiota* · Sequestrate fungi

Introduction

Many fungi have limited morphological characters, so it has been difficult to infer their evolutionary history based on morphology alone. The sequestrate fungi (those with enclosed fruiting bodies where the spores are not forcibly discharged) are particularly difficult in this regard because they often have compact fruiting bodies that are morphologically similar to one another, yet distinct from the fruiting bodies of their closest relatives. In recent years, molecular phylogenetic approaches have shown that sequestrate fungi are the result of convergent evolution; they have evolved separately in many different groups of fleshy *Ascomycota* and *Basidiomycota* as well as a few *Zygomycota* and *Glomeromycota* (Trappe et al. 2009). The evolutionary relationships of many groups of sequestrate fungi have been determined in recent years, but for some genera we still do not know where they belong on the fungal tree of life.

The majority of sequestrate fungi obtain their nutrients through ECM associations with plants but some of them have also evolved within many different lineages of saprotrophic fungi (e.g. *Geastrum*, *Lycoperdon*, *Sclerogaster, Weraroa*). Recent molecular studies of the saprotrophic family *Agaricaceae* Chevall. have revealed that several sequestrate genera are derived from epigeous, mushroom-forming relatives. For example, the secotioid genus *Endoptychum* Czern. was shown to be a heterogeneous assemblage of taxa that fall into several different clades of *Agaricaceae.* Based on their phylogenetic affiliations, *Endoptychum depressum* Singer & A.H. Sm. was transferred to *Agaricus* L. as *A. inapertus* Vellinga (Vellinga et al. 2003) and *Endoptychum agaricoides* Czern. was transferred to *Chlorophyllum* as *C. agaricoides* (Czern.) Vellinga (Vellinga 2002). Australian species of sequestrate *Agaricaceae* are nested within the genera *Agaricus* and *Macrolepiota* Singer (Lebel and Syme 2012). Similarly, Kropp et al. (2012) recently erected a new sequestrate genus *Cryptolepiota* Kropp & Trappe to accommodate a lineage from the western USA that was derived from the agaricoid genus *Lepiota* (Pers.) Gray (Kropp et al. 2012). *Lepiota sardoa* (Padovan & Contu) Vila & Castellón, a secotioid species originally described from Italy, is considered to be a member

Electronic supplementary material The online version of this article (doi:10.1007/s11557-012-0841-y) contains supplementary material, which is available to authorized users.

Z.-W. Ge · M. E. Smith (✉)
Department of Plant Pathology, University of Florida, Gainesville, FL, USA
e-mail: trufflesmith@ufl.edu

Z.-W. Ge
Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

of the genus *Lepiota* based on morphology. However, no molecular data are available to determine the closest relatives of this fungus (Padovan and Contu 2001; Vila and Castellón 2003).

The monotypic genus *Amogaster* Castellano was erected to accommodate a small sequestrate fungus with a greenish gleba and a thin, whitish, tomentose peridium that bruises orange or red (Castellano 1995). The single described species, *Amogaster viridiglebus* Castellano (as "*viridigleba*"), is extremely rare and has only been collected once in association with *Populus* species in California (Castellano 1995). Trappe et al. (2009) later reported collections of *Amogaster* from Oregon but subsequent examination of those specimens indicate that they are a different, and as yet undescribed, truffle species (M. Castellano, personal communication). Based on the green gleba color, the reddish staining response, and the potential symbiotic association with the tree genus *Populus*, Castellano (1995) considered *A. viridiglebus* as a member of the *Boletales*, and suggested that it might be related to *Gyroporus* Quel. or *Paragyrodon* (Singer) Singer. However, nothing is known about the phylogenetic position of *A. viridiglebus*. Until now, no *Amogaster* sequence data have been available to determine the closest relatives of this genus or to determine whether this fungus is saprotrophic or ectomycorrhizal (ECM) (Tedersoo et al. 2010). The aim of this study was to determine the phylogenetic position of the genus *Amogaster* and to gain insight into the trophic mode of this rare fungus.

Materials and methods

Materials examined and morphological observations The isotype of *A. viridiglebus*, which is deposited in the Mycological Herbarium of the University of Florida (FLAS), was examined. This specimen was collected by M. Amaranthus (Trappe 9493) on 13 June 1987, at the SFSU field campus on Highway 49 in the Plumas National Forest, Sierra County, California, USA. The holotype specimen is deposited at OSC with additional isotype material deposited at FLAS and SFSU.

In the description, macromorphology is based on a portion of the dried specimen. For descriptions of fresh material see Castellano (1995). Micromorphology is based on observations under a light microscope at 1,000× magnification. Spores and basidia were observed after the tissue was sectioned by hand and mounted in 3 % KOH to rehydrate. Melzer's reagent was used to test the amyloidy of spores. Spore wall reaction to Cresyl Blue and Congo Red were also checked. Dimensions for basidiospores are given using the notation form (a) b–c (d), with the range b–c containing a minimum of 90 % of the 30 measured values. Extreme values (a and d) are given in parentheses. Q indicates the "length/width ratio" of a spore in side view; avQ is the average Q of all basidiospores±standard deviation.

DNA isolation and amplification A small piece of dried basidiome tissue was ground in a 1.5 ml Eppendorf tube using a plastic pestle and genomic DNA was extracted with a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Gardes and Bruns 1993). ITS ribosomal DNA was amplified using primers ITS1F and ITS4 (White et al. 1990; Gardes and Bruns 1993), and the LSU rDNA was amplified using primer pairs LR0R and LR3 (http://www.biology.duke.edu/fungi/mycolab/primers.htm). The sequence produced in this study has been deposited in Genbank with accession number JX014271.

Phylogenetic analyses Sequences obtained from this study were subjected to BLASTn analysis against GenBank (Altschul et al. 1990). Several species of *Lepiota* (e.g. *Lepiota subgracilis* Kühner, *Lepiota maculans* Peck, and *Lepiota ignivolvata* Bousset & Joss. ex Joss.) showed high sequence similarity with the ITS sequence from *Amogaster* and were retrieved directly from GenBank. After further examination of recent studies of *Lepiota* and related *Agaricaceae* (Vellinga et al. 2003; Kropp et al. 2012), we compiled an ITS-LSU sequence dataset that included several species of *Lepiota* and *Cryptolepiota* as well as related genera of *Agaricaeae* (Table 1). The dataset was aligned using the MAFFT software package (http://align.bmr.kyushu-u-ac.jp/mafft/software), followed by manual inspection and correction in MEGA (Tamura et al. 2011). The final alignment was deposited in TreeBASE (Accession http://purl.org/phylo/treebase/phylows/study/TB2:S12706).

The resulting combined ITS-LSU dataset was evaluated using the Maximum Likelihood (ML) strategy performed in RAxML version 7.2.3 (Stamatakis et al. 2008), with GTRGAMMAI as the model of evolution. Branch support was assessed through 1,000 bootstrap partitions (BP) with the rapid bootstrap option. To test alternative phylogenetic relationships, maximum parsimony (MP) analysis was performed using PAUP* 4.0b10 (Swofford 2003). One hundred heuristic searches were conducted with random sequence addition of sequences, and the tree bisection-reconnection (TBR) branch-swapping algorithms, and MaxTrees were set to 2,000. Bootstrap values (BS) were obtained from 200 replicates from the maximum parsimony analysis (Felsenstein 1985). The analyses were performed under the heuristic search option with TBR and Multrees option on, and 100 replicates of random addition sequence were conducted. Gaps were treated as missing data in all analyses. Bayesian analysis was also conducted to test the alternative phylogenetic relationships using MrBayes3.1.2 (Ronquist and Huelsenbeck 2003) under the GTR + I + G model as

selected by MrModeltest (Nylander 2004). Bayesian posterior probabilities were determined twice by running one cold and tree heated chains for five million generations, saving trees every 500th generation. We discarded the first 276 trees as burn-in, as the Markov Chain converges when the SDSF fell below 0.01 after 516,000 generations. A 50 % majority rule consensus tree was used to calculate posterior probabilities.

Results

Mophology

The isotype collection contains approximately one half of a basidiome that measures approximately 8.0×3.5 mm. The specimen is yellowish-brown with a thin, evanescent peridium that does not rehydrate well in any of the solutions used for microscopy in this study. Gleba yellowish, with irregular, empty locules. Basidiospores 11.2–13.2 (15.2)×5.2–6.4 (7.6) μm, Q=1.88–2.21 (2.33), avQ=2.07±0.12 (Fig. 1), amygdaliform to subfusiform, asymmetrical in side view, smooth, slightly thick-walled, pale yellowish-brown in 3 % KOH, with a tiny sterigmal attachment at the base. Basidiopores pale yellow brown in Melzer's reagent, unreactive in Congo Red, becoming light blue to blue in Cresyl blue stain (1 g Cresyl blue dissolved in 100 ml 0.8 % NaCl). Basidia four-spored, 22.4–25.6×6.4–8.0 μm, subcylindric to narrowly clavate. Basidia hyaline, thin-walled, sometimes constricted in the middle. Clamp connections not observed.

Phylogenetic results

The aligned ITS-LSU dataset was 1,704 base pairs long but 268 ambiguously aligned base pairs were excluded from the analyses, yielding a total of 1,436 analyzed nucleotides. The three different analyses (MP, ML, BI) produced phylogenies with similar overall topologies. The RAxML analyses resulted in the ML phylogeny shown in Fig. 1 (Final ML Optimization Likelihood: −10831.090912). The MP analyses resulted in seven equally parsimonious trees based on 331 parsimony-informative characters, with tree length of 1,841 steps (consistency index=0.3911, retention index=0.5493, rescaled consistency index=0.2148). The best Bayesian tree was also similar to the ML tree and the MP consensus tree (data not shown). Bootstrap and posterior probability values were also mostly congruent in their support for major relationships. Because of these strong similarities in the results from the three different analyses, we discuss the results together.

In all three analyses, the genus *Lepiota* was resolved as paraphyletic with several other genera nested within it. The genera *Chamaemyces*, *Cryptolepiota*, *Cystolepiota* and *Melanophyllum* are all nested in *Lepiota.* These results are also congruent with previously published phylogenetic studies of the genus *Lepiota* and *Cryptolepiota* (Vellinga 2003; Kropp et al. 2012). All three of the analyses recovered a strongly supported clade that corresponds to *Lepiota* section *Lepiota* and was referred to by Vellinga (2003) as *Lepiota* clade 1 (Fig. 1). Most of the nodes within *Lepiota* clade 1 were also supported by all three of the phylogenetic analyses. *Amogaster viridiglebus* was consistently resolved as a member of *Lepiota* clade 1 and all three analyses placed this sequestrate species as sister taxon to *L. subgracilis*. These two taxa form a sister relationship with a clade comprised of *L. maculans* and *L. ignivolvata*, and the four taxa jointly form a strongly supported lineage. Other notable taxa that were resolved within *Lepiota* clade 1 are the type species of the genus *Lepiota*, *L. clypeolaria,* as well as three sequestrate species that were recently named in the genus *Cryptolepiota* (*C. americana, C. mengei,* and *C. microspora*). It was notable that the two sequestrate groups (*Amogaster viridiglebus* and the *Cryptolepiota* species) are more closely related to agaricoid taxa than to one another.

The phylogenetic analysis above confirms that *A. viridiglebus* is nested within the genus *Lepiota* section *Lepiota*. Based on this phylogenetic placement we have decided to formally transfer *A. viridiglebus* to the genus *Lepiota*.

Taxonomy

Lepiota viridigleba (Castellano) Z. W. Ge & M. E. Sm., comb. nov.

α *Amogaster viridiglebus* Castellano (as '*viridigleba*'), Mycotaxon 55: 186. 1995

Mycobank number: MB 800493

Discussion

Lepiota viridigleba was originally collected among roots of *Populus* and was putatively considered an ECM member of the *Boletales* (Castellano 1995). However, our phylogenetic analysis based on ITS and LSU ribosomal DNA unequivocally shows that *L. viridigleba* is nested within the genus *Lepiota. Lepiota* belongs to *Agaricaceae* and there are no known ECM species in this fungal lineage. Although sequestrate fungi are often associated with particular plants, and thus are assumed to form mycorrhizae, the simple presence of a fungus with plant roots is not sufficient to prove an ECM relationship. Our phylogenetic results indicate that *L. viridigleba* is a saprotrophic rather than an ECM fungus.

All three of the different phylogenetic methods we used in this study indicate that *L. viridigleba* is nested within

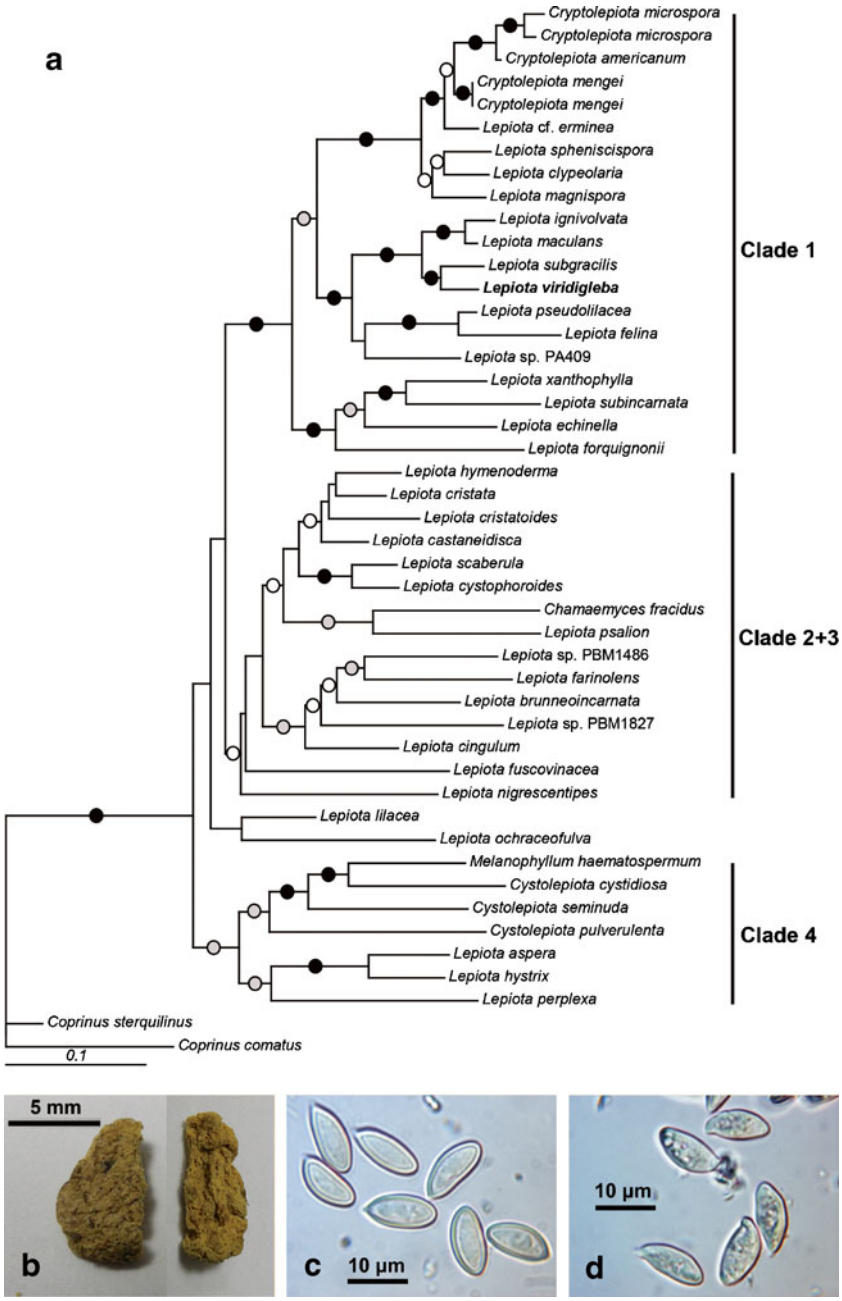

Fig. 1 Phylogeny and morphological features of *Lepiota viridigleba*. Maximum Likelihood tree (**a**) based on combined ITS-LSU rDNA sequences. Statistical support is indicated by filled *black circles* (supported by Maximum Parsimony bootstrap value >75, Maximum Likelihood bootstrap value >75 and Bayesian posterior probability value >0.95), filled *grey circles* (supported by two of three phylogenetic methods), or *white circles* (supported by only one phylogenetic method). Clade names correspond to those used by Vellinga (2003). Basidiome of *Lepiota viridigleba* isotype specimen Trappe 9493 (**b**) with a thin evanescent peridium (*left*) and the gleba comprised of irregular, empty locules (*right*). Subfusiform basidiospores of *L. viridigleba* (**c**) are morphologically similar to those of *Lepiota clypeolaria* specimen FLAS-FF9647 (**d**)

section *Lepiota* (i.e. *Lepiota* clade 1 sensu Vellinga 2003). This clade also contains the type species of *Lepiota*, *L. clypeolaria,* as well as members of the sequestrate genus *Cryptolepiota*. Morphologically, species within *Lepiota* section *Lepiota* are characterized as having fusiform to amygdaliform spores, clamp connections, and a trichodermal pileus covering comprised of cylindrical to narrowly clavate elements.

Lepiota viridigleba has some important morphological similarities to other species in *Lepiota* section *Lepiota*. For example, this sequestrate taxon has asymmetrical, amygdaliform to subfusiform basidiospores that are typical of agaricoid species in the section. Its spores generally resemble those of its sister species, *L. subgracilis,* and the type species of *Lepiota*, *L. clypeolaria* (Fig. 1). *Lepiota viridigleba* also bruises orange or pale red when handled and this feature is similar to the related species *Lepiota ignivolvata* and *L. maculans* (Fig. 1) (Birkebak et al. 2011). Unlike most species within section *Lepiota*, where clamp connections are commonly observed, *L. viridigleba* apparently lacks clamp connections in all tissues.

Lepiota viridigleba also has several unique features when compared with related agaricoid species. This sequestrate taxon has a fine, evanescent peridium that lacks cystidia and does not rehydrate well after drying. Furthermore, the sequestrate fruiting habit of *L. viridigleba* means that its

spores are not dispersed by wind. The exact dispersal mechanism is unknown for this rare species, but many sequestrate species are dispersed by animals and this may be the case for *A. viridiglebus* (Trappe et al. 2009).

Macroscopically, *Lepiota viridigleba* is more similar to species of *Cryptolepiota* than to other species of *Lepiota*, despite the fact that *L. viridigleba* and the *Cryptolepiota* lineage clearly represent two distinct evolutionary events leading to the sequestrate lifestyle (Fig. 1). *Lepiota viridigleba* is more closely related to *L. subgracilis, L. machulans,* and *L. ignivolvata* whereas the *Cryptolepiota* species are more closely related to *L.* cf. *erminea, L. clypeolaria,* and *L. magnispora*. The secotioid species *Lepiota sardoa* may represent a third evolutionary event leading to the sequestrate form within section *Lepiota*, although this has yet to be confirmed by molecular data. Morphologically, *L. viridigleba* is most similar to *Cryptolepiota mengei* because that species also has greenish tones in its gleba and reddish tones on its peridium. However, *L. viridigleba* can be easily distinguished from *C. mengei* because *C. mengei* has subglobose to ovoid spores and has only been found in southern California and Utah whereas *L. viridigleba* has asymmetrical, amygdaliform to subfusiform spores and is only known from northern California.

The case of the sequestrate *Lepiota viridigleba* highlights the fact that many rare sequestrate species (and genera) still remain poorly studied and have not been phylogenetically characterized. For example, in a recent review on ECM fungi Tedersoo et al. (2010) named 12 sequestrate fungal genera for which the trophic mode was unknown and no DNA sequence data were available. Since the unique morphology of sequestrate fungi can obscure their true relationships and sequestrate taxa have evolved multiple times (even within individual genera such as *Lepiota*) molecular analyses are needed for more of these taxa to resolve their placement in the fungal tree of life.

Acknowledgments We appreciate the discussion and comments from Dr. M.A. Castellano who declined co-authorship because he was uncomfortable assigning this species to *Lepiota*. Financial support for this work came from the University of Florida's Institute for Food and Agricultural Sciences (IFAS). The authors would like to thank two anonymous reviewers for their helpful comments on the manuscript and thank Dr. Bradley Kropp for sending information on DNA sequences of *Cryptolepiota*. Morphological examination of W.A. Murrill's specimen FLAS F9647 of *Lepiota clypeolaria* was made possible through support from the Florida Museum of Natural History and the Fungal Herbarium of the University of Florida (FLAS).

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410. doi:10.1006/jmbi.1990.9999

Birkebak JM, Vellinga EC, Franco-Molano AE, Wood MG, Matheny PB (2011) *Lepiota maculans*, an unusual mushroom rediscovered after 105 years. Southeast Nat 10(2):267–274. doi:10.1656/058.010.0207

Castellano MA (1995) NATS truffle and truffle-like fungi.4: *Amogaster viridigleba* gen. et sp. nov., a new truffle-like fungus from the Sierra Nevada. Mycotaxon 55:185–188

Felsenstein J (1985) Confidence-limits on phylogenies—an approach using the bootstrap. Evolution 39(4):783–791. doi:10.2307/2408678

Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol Ecol 2(2):113–118. doi:10.1111/j.1365-294X.1993.tb00005.x

Kropp BR, Albee-Scott S, Castellano MA, Trappe JM (2012) *Cryptolepiota*, a new sequestrate genus in the *Agaricaceae* with evidence for adaptive radiation in western North America. Mycologia 104(1):164–174. doi:10.3852/11-046

Lebel T, Syme A (2012) Sequestrate species of *Agaricus* and *Macrolepiota* from Australia: new species and combinations and their position in a calibrated phylogeny. Mycologia 104(2):496–520. doi:10.3852/11-092

Nylander JAA (2004) MrModeltest v2. Program distributed by the author. Evolutionary Biology Centre, Uppsala University

Padovan F, Contu M (2001) *Notholepiota sardoa* sp. nov., un nuovo gasteromicete filogeneticamente correlato alle *Lepiotaceae* scoperto in Sardegna. Boll del Gruppo Micologico 'G Bresadola', n Ser 44(2):31–36

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19(12):1572–1574. doi:10.1093/bioinformatics/btg180

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web servers. Syst Biol 57(5):758–771. doi:10.1080/10635150802429642

Swofford DL (2003) PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739. doi:10.1093/molbev/msr121

Tedersoo L, May TW, Smith ME (2010) Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. Mycorrhiza 20(4):217–263. doi:10.1007/s00572-009-0274-x

Trappe JM, Molina R, Luoma DL, Cazares E, Pilz D, Smith JE, Castellano MA, Miller SL, Trappe MJ (2009) Diversity, ecology, and conservation of truffle fungi in forests of the Pacific Northwest. U S Forest Service General Technical Report PNW-GTR-772. FS United States Department of Agriculture, Pacific Northwest Research Station (eds) OR: USA

Vellinga EC (2002) New combinations in *Chlorophyllum*. Mycotaxon 83:415–417

Vellinga EC (2003) Phylogeny of *Lepiota* (*Agaricaceae*) – Evidence from nrITS and nrLSU sequences. Mycol Prog 2(4):305–322

Vellinga EC, de Kok RPJ, Bruns TD (2003) Phylogeny and taxonomy of *Macrolepiota* (*Agaricaceae*). Mycologia 95(3):442–456. doi:10.2307/3761886

Vila J, Castellón E (2003) Some remarks on *Notholepiota sardoa* Padovan et Contu. Cryptogam Mycol 24(4):303–307

White TJ, Bruns TD, Lee S, Taylor J (1990) Analysis of phylogenetic relationships by amplification and direct sequencing of ribosomal RNA genes. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic, San Diego, pp 315–322