Peering into the Mediterranean black box: *Lactifluus rugatus* ectomycorrhizas on *Cistus*

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Abstract: We describe the morpho-anatomical features of the ectomycorrhizas (ECMs) formed by *Lactifluus rugatus* on *Cistus*, a genus of about 20 species of woody shrubs typical of the Mediterranean maquis. The description of *L. rugatus* mycorrhizas on *Cistus* is the first ECM description of a species belonging to *Lactifluus* subgen. *Pseudogymnocarpi*. The ECM identity was verified through molecular tools. Anatomically, the characteristic of *L. rugatus* mycorrhiza is the presence of abundant, long “bottle-shaped” cystidia on mantle surface. Indeed, the overwhelming majority of milkcap mycorrhizas are acacidystate. This is the third Lactarius/Lactifluus mycorrhiza to have been described associated with Cistus, the others being Lactarius cistophilus and *L. tesquorum*. The phylogenetic distance between all these taxa is reflected by the diversity of the principal features of their ECMs, which share host-depending ECM features known for *Cistus*, but are otherwise distinguishable on the host roots. Comparison of *Lactifluus rugatus* ECM with those formed by *L. velleureus* and *L. piperatus* on *Fagus* reveals elevated intrageneric diversity of mycorrhizal structures. Such a diversity is supported by analysis of ITS sequences of relevant species within European *Lactifluus* species. Our study extends knowledge of *Cistus* mycorrhizal biology and confirms the informative value of mycorrhizal structures in understanding phylogenetic relationships in ECM fungi.

Key words: confocal laser scanning microscopy  
*Lactarius*  
maquis  
mycorrhizal phylogeny  
*Russulaceae*

INTRODUCTION

The basidiomycete family *Russulaceae* has undergone a dramatic taxonomic revision during the last decade. Studies based on multigene phylogenies of both *Russula* and *Lactarius* have shown that neither of these two classic genera is monophyletic. In the case of *Russula*, a small group of species previously classified as subgen. *Compactae* subset. *Ochricomptae* were found to be a monophyletic entity, for which the new generic name *Multiturca* was introduced; the genus also includes the rare American species *Multiturca furcata* (Buyck et al. 2008). *Lactarius* was found to comprise two clades, with subgenera *Piperites*, *Russularia*, and *Plinthogalus* constituting the newly circumscribed genus *Lactarius*, and subgenera *Lactariopsis*, *Lactarius*, *Lactifluus*, *Russulopsis*, *Gerardii*, and the former *Lactarius* sect. *Edules* constitute the newly recognized genus *Lactifluus* (Buyck et al. 2010, Stubbe et al. 2012, Verbeke et al. 2011, 2012). A more recent multi-gene analysis of *Lactifluus* resulted in a new infrageneric classification, with four supported subgenera: *Lactifluus*, *Lactariopsis*, *Gymnocarpi*, and *Pseudogymnocarpi* (De Crop et al. 2017).

Considered together, *Lactarius* and *Lactifluus* form one of the most prominent groups of ectomycorrhizal (ECM) basidiomycetes (Hutchinson 1999, Rinaldi et al. 2008, Comandini et al. 2012b). With more than 450–500 species described worldwide, these taxa play a significant role as mycobionts of trees and shrubs in a vast range of ecosystems, from boreal coniferous forests to temperate Mediterranean-type maquis, from Mesoamerican Neotropics to the rainforests of South-East Asia, passing through tropical Africa (Comandini et al. 1998, Eberhardt et al. 2000, Comandini et al. 2004, Nuytinck et al. 2004, Montoya & Bandala 2005, 2008, Le et al. 2007, Mueller & Halling 2010, Verbeke & Walley 2010, Comandini et al. 2012a, Flores Arzú et al. 2012). In Europe, some 100–110 *Lactarius* species are recognized, depending how long-standing controversies on synonymies are resolved, and nine of *Lactifluus* (Heilmann-Clausen et al. 1998, Basso 1999, Van de Putte et al. 2016). Several *Russulaceae* are associated as ECM mycobionts of *Cistus*, a genus of flowering plants in the rockrose family *Cistaceae*, containing about 20 species of woody, evergreen or semideciduous shrubs (Comandini et al. 2006). *Cistus* species are found in semi-arid areas from the Canary Islands...
throughout the Mediterranean basin to the Caucasus, where they are significant components of the maquis and garrigue ecosystems, often forming extensive swards (Ellul et al. 2002, Guzmán et al. 2009). Obligate seeders, Cistus species are early-stage colonizers that follow disturbance, particularly the fire operating in Mediterranean ecosystems. Adaptations include physical seed dormancy, high seed longevity, and small and light seeds, allowing the generation of persistent soil seed banks; the sharp rise in temperature generated in top soil layers by fire breaks seed dormancy and leads to germination (Bastida & Talavera 2002). Overall, these ecological characteristics make Cistus mycorrhizal biology particularly interesting.

Here we describe the morpho-anatomical features of the mycorrhizas, collected in Sardinia, Italy, formed by Lactifluus rugatus with Cistus. The identity of the ECMs was also verified through molecular tools. To our knowledge, this is the first ECM description of a species belonging to the newly recognized genus Lactifluus subgen. Pseudogymnocarpi. Furthermore, we compare the ECM anatomical features with those of Lactarius Cistus-specific mycobionts, namely Lactarius cistophilus and L. tesquorum, and also with those formed by other taxa in Lactarius and Lactifluus.

MATERIALS AND METHODS

Study site and fungal collections

Basidiomes of Lactifluus rugatus (Fig. 1A) were harvested in the vast forested area that extends between Capoterra and Santadi (39°8’30" N, 8°53’24" E, 227 m asl), about 20 km south-west of Cagliari (Sardinia, Italy), and in a sandy area close to Gonnesa (39°15’8" N, 8°24’44" E, 94 m asl), about 70 km west of Cagliari; basidiomes were identified in the field on the basis of published descriptions of macroscopic and microscopic characters (Basso 1999). Specimens were collected from under Cistus spp. in a low-density Quercus suber wood and in treeless area covered with classic Mediterranean maquis/garrigue vegetation.

Several Cistus species (C. creticus, C. salvifolius, and C. monspeliensis) were present in the collection sites, so that it was not possible to identify the host(s) of L. rugatus at species level (all attempts to trace mycorrhizas directly to roots of possible hosts failed). Soil cores (about 20 × 20 × 20 cm) were excavated from beneath basidiomes and immersed overnight in water, and ectomycorrhizal roots were carefully separated under a dissecting microscope. Several tips were immediately transferred into 50 % EtOH and stored at −20 °C for subsequent DNA analysis. Reference material for basidiomes (ACR-2010/6, ACR-2014/6, ACR-2015/1) and ectomycorrhizas (ACR-2010/6-E, ACR-2015/1-E) is deposited in the collection of the Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy.

Microscopy

Mantle preparations of fresh ectomycorrhizas were fixed on microscope slides with polyvinyl lactophenol for light microscopy. Observations were made with a Zeiss Axiosplan 2 bright field microscope and a Leica MZ 6 stereomicroscope. Images were acquired with a Leica DFC290 digital camera. For longitudinal sections (2.5 mm thick), ectomycorrhizas were embedded in LR White resin (Multilab Supplies, Surrey, UK), cut with a Leica Ultracut R ultramicrotome and stained with toluidine blue in 1 % sodium borate for 15 s at 60 °C. For confocal laser scanning microscopy, fixed ectomycorrhizas (4 % glutaraldehyde) were mounted in Vectashield Antifade Mounting Medium (Burlingame, CA) and then examined by TCS SP5 Leica confocal microscopy (Leica Microsystems, Mannheim, Germany) equipped with an inverted microscope DMI 6000 CS (Objective HCX PL APO CS 40×1.3 oil) and a VIS Argon laser. The laser excitation wavelength was fixed at 488 nm. The general methodology and terminology used to characterize ectomycorrhizas follows Agerer (1986, 1987–2006, 1991, 1995). Munsell Soil Color Charts (2000) were used as reference for the descriptions of the colours of ectomycorrhizas.

**Fig. 1.** Lactifluus rugatus (ACR-2015/1), from the Mediterranean maquis of Sardinia, Italy. **A.** Basidiomes. **B.** Habit of ectomycorrhiza with Cistus sp.; the abundant short cystidia give a light and hyaline appearance to the mycorrhizal surface.
PCR amplification and sequencing of the ITS rDNA region

Genomic DNAs of the basidiomata were isolated from 20 mg of each dried sample using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Extracts were eluted in 50 µL of sterile water and their DNA concentration estimated using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Madison, WI). The ITS amplifications were performed using ITS1F and ITS4 primers pair (White et al. 1990, Gardes & Bruns 1993) following the protocol reported by Leonardi et al. (2005). A direct PCR approach was applied to identify all ECM morphotypes isolated from soil samples. One to three representative ECM tips per morphotype were selected as PCR targets. A little fragment of ECM mantle was excised from each selected tip as described by Iotti & Zambonelli (2006) and directly amplified in a 50 µL PCR reaction using ITS1F/ITS4. Two microlitre of 20 mg/mL BSA solution (Thermo Fisher Scientific) were added to each reaction tube to prevent PCR inhibition. The amplification conditions were 6 min of the initial denaturation at 95 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension step of 72 °C for 10 min. PCR products were visualized through 1 % agarose gel electrophoresis stained with ethidium bromide. The amplified products were purified using the QIAquick PCR Purification Kit (Qiagen, Milan, Italy) and then directly sequenced using the same primers pair.

Phylogenetic inference

The sequences of the ITS1, 5.8S, and ITS2 regions of the nuclear rDNA obtained were compared with those present in GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and UNITE (http://unite.ut.ee/analysis.php) databases using the BLASTN search (Altschul et al. 1990). Besides sequences from Lactifluus rugatus ECM morphotype and basidiome, ITS sequences from basidiomata of Lactifluus brunneoviolascens and L. cistophilus, both collected in Mediterranean-type maquis in Sardinia, were also obtained. Sequences are deposited in GenBank under accession numbers KU885433–KU885436. After excluding the ambiguous regions at the 5’ and 3’ ends of the chromatograms, sequences were edited using BioEdit v. 7.2.5 (Hall 1999) and aligned by MUSCLE (Edgar 2004). Sequence statistics, nucleotide diversity, and distance based analyses were performed using MEGA v. 6 (Tamura et al. 2013). The best substitution model with the lowest BIC scores (Bayesian Information Criterion) was chosen with the default settings. A phylogenetic tree was obtained by the Maximum Likelihood (ML) method based on the two-parameter distance model of Kimura (1980), selecting “Nearest-Neighbor-Interchange (NNI)” as the ML heuristic method in the tree inference options. Bootstrap tests were performed using 1000 replicates.

RESULTS

Description of ectomycorrhizas

Morphological characters: Mycorrhizal system to 4–5 mm long, monopodial-pyramidal or coralloid, 2–3 orders of ramification. Main axes 0.4–0.5 mm diam. Unramified ends straight to slightly bent, to 1.5 (~1.8) mm long and 0.3–0.4 mm diam. Mycorrhizas pale yellow (2.5y7/3), but the light yellow short cystidia give a lighter and hyaline appearance to the mycorrhizal surface (Fig. 1B); older mycorrhizas pale brown. Surface of unramified ends short spiny, with sporadic longer extramatrical hyphae. Soil particles and hyphal mats often stuck on the mycorrhizal surface; not secreting latex when injured; mantle not transparent. Rhizomorphs and sclerotia lacking.

Anatomical characters of mantle in surface views. Mantle plectenchymatous throughout, hyphal cells hyaline, clamps lacking, abundant cystidia present (Fig. 2A). Outer mantle layers plectenchymatous, net-like, bearing abundant cystidia (type D, Agerer 1995). Hyphae frequently branched, 3.5–5 µm diam, hyphal segments 8–15 µm long, the basal roundish part of the cystidia 4–6 µm diam (Figs 2B, 3A). Middle mantle layers plectenchymatous, very close to the outer mantle layers. Lactifers not observed. Inner mantle layers plectenchymatous, with a gelatinous matrix between the hyphae, hyphae arranged net-like to ring-like, frequently branched (Figs 2C, 3B). Hyphae generally 2.5–4.5 µm thick, not uniform in diam. In some mantle preparations, a few hyphae with content (lactifers?) can be observed. Very tip hyphal arrangement and characteristics as in the other parts of the mantle.

Anatomical characters of emanating elements. Rhizomorphs not observed. Emanating hyphae 3–4 µm thick, hyaline, clamps lacking, Cystidia hyaline, sometimes cylindrical, mainly bottle-shaped, 25–30(–40) µm long and 4–5 µm diam (Fig. 2D). Cystidia tips blunt and straight, but the remaining part sinuous. Hyphal walls less than 1 µm thick in the upper part, becoming thicker toward the base; no contents observable by light microscope, but content inside the cystidia fluorescing and observable by confocal microscopy (Fig. 3C); septa present towards the base, sometimes also in the other parts of the cystidia; cystidia bases generally roundish, 5–7 µm diam (Fig. 3D) but sometimes more elongated (Fig. 2D).

Anatomical characters, longitudinal section. Mantle (30–)35–55(–65) µm thick, different layers discernable: outermost layer, 25–30 µm thick, formed by a loose net of very long cystidia (see above); underlying layer about 15–25 µm thick, formed by the roundish bases of the cystidia, 2–3 µm diam, and by hyphal cells to 30 µm long and 3 µm diam; innermost layer 8–10 µm thick, very compact and formed by hyphal cells, longitudinally orientated, 1–3 µm thick, scarce hyphae with granular content (lactifers?) may be observed; mantle of tip about 20 µm thick, hyphal organization as the remaining part, but structures more compact and single elements difficult to measure. Tannin cells not observed.
Cortical (epidermal) cells of 1–2 rows, radially orientated 20–28(–35) x 8–11 µm. Hartig net paraepidermal, of one row of roundish, 1–4 µm diam hyphal cells, palmetti-type, lobes 1–1.5 µm wide.

Molecular and phylogenetic analyses

The ITS sequences of the basidiomes of Lactifluus rugatus and of the ectomycorrhizas found below them were identical, confirming the identity of the collected ectomycorrhizal morphotype. The amplifications produced a fragment of 565 bp containing the complete ITS1-5.8S-ITS2 sequence. In order to assemble a multiple sequence alignment for phylogenetic analysis, ITS sequences of 21 different European Lactifluus and Lactarius species were imported from the GenBank and UNITE databanks, and the ITS sequences from basidiomes of Lactifluus brunneoviolascens and Lactarius cistophilus, obtained for the first time during this study from samples collected in Mediterranean-type maquis in Sardinia, were also considered. Russula werneri and R. insignis were chosen as outgroup. According to availability, one to four sequences were chosen for each species. The sequence alignment contained 1027 characters of which 402 were variable basepairs and 297 were parsimony informative. K2P distances ranged between 0.002 and 0.28. A tree was constructed using the Maximum Likelihood (ML)
Nodes with bootstrap values lower than 50% were eliminated. The phylogenetic analysis shows that all the European species belonging to Lactifluus are well delineated and the bootstrap values support the segregation of the different taxa in subgenera and sections as delineated by De Crop et al. (2017) (Table 1).

**DISCUSSION**

Lactifluus rugatus is a typically Mediterranean and well-characterized milkcap, with a wrinkled, vividly orange cap (Fig. 1), and known from southern Europe (Portugal, Spain, France, Italy, and Greece) and northern Africa (e.g. Morocco, Algeria, Tunisia) (Kühner & Romagnesi 1954, Malençon 1974, Bertault 1978, Alessio 1979, Lalli & Pacioni 1992, Pierotti 2002, Nounsi et al. 2014, Dimou et al. 2016). The species has long been confused with L. volemus, and some authors have considered L. rugatus a Mediterranean vicariant species of L. volemus (Galli 2006), but molecular studies reveal it as more closely related to the North American L. hygrophoroides and African species such as L. rubiginosus and L. volemoides (Verbeken et al. 2012). The distinctness of L. rugatus and L. volemus is also supported by the results of our analysis of ITS sequence data of European taxa (Fig. 4): overall, our Lactifluus cladogram fits well with the multigene analysis recently presented by Van de Putte et al. (2016), and the analysis of De Crop et al. (2017) showed that the two species actually belong to different subgenera. Hesler & Smith (1979) treated L. rugatus as a variant of L. hygrophoroides occurring in North America, but conspecificity with Mediterranean L. rugatus is most certainly to be excluded. Lactifluus hygrophoroides var. rugatus (Hesler & Smith 1979) probably simply represents an aspect of the natural variability

![Fig. 3. Anatomical characters of Lactifluus rugatus ectomycorrhizas (ACR-2015/1-E). A. Outer mantle surface viewed with confocal laser scanning microscopy (CLSM), the basal roundish parts of the cystidia are visible. B. Plectenchymatous inner mantle layer viewed with a light microscope. C. Mycorrhizal surface as viewed with CLSM; abundant cystidia with fluorescent content visible. D. Outer mantle layer viewed with the light microscope; a single bottle-shaped cystidium with a roundish base is indicated by the arrow. Bars = 5 µm.](image-url)
of L. hygrophoroides (Lalli & Pacioni 1992). Verbeken et al. (2012) treated L. hygrophoroides and L. rugatus as distinct taxa, and the molecular analysis by De Crop et al. (2017) supported this view. Table 1 summarizes recent classification schemes for L. rugatus and related taxa. L. rugatus is by no means a mycobiont linked exclusively to Cistus, although it occurs frequently associated with this shrub. In the field, it is also associated with other members of the Cistaceae, such as Halimium halimifolium (Comandini & Rinaldi, unpubl.). Besides, according to both our field observations and those of several other authors (Basso 1999, Mua & Casula 2012, Nounsi et al. 2014), this species also grows in association with Quercus in the Mediterranean eco-region. Even the original description reads: “Sous Quercus suber, à la Réghaïa, aux environs d’Alger” (Kühner & Romagnesi 1954).

In recent years, we have been carrying out a long-term study on the distribution, ecology, phylogeny and ectomycorrhizal biology of milkcap species occurring in selected ecosystems in Europe and the Neotropics (Comandini et al. 1998, Eberhardt et al. 2000, Comandini et al. 2012a, Flores Arzú et al. 2012, Nuytinck et al. 2004). We explore the milkcap ECM diversity of host trees or shrubs in a given ecologically important ecosystem, and relevant milkcap ECM types are fully characterized from a morpho-anatomical perspective.

Fig. 4. Maximum Likelihood tree obtained from the alignment of ITS rDNA region sequences. Bootstrap values are indicated next to relevant nodes. The scale indicates the number of substitution per site. Sequences of species in boldface were obtained during this study. The different colours in genus Lactifluus correspond to subgenera following the classification proposed by De Crop et al. 2017 (see also Table 1).
and molecular points of view, also comparing ECM anatomical characters with those known from related taxa. Morphological and molecular data obtained for related milkcap taxa, living either in the same habitat or linked to other hosts, are used to clarify the systematic position of the species of interest. Thus, the combination of morphological characters of basidiomes and mycorrhizas and their molecular features contribute to a reliable taxonomy in the genus (Nuytinck et al. 2004, Eberhardt et al. 2000). Such a combined approach could be of general help when dealing with the characterization of given ECM associations, showing the relevance mycorrhiza descriptions still have for the recognition of natural groups, if sufficient and reliable data is available.

The main distinguishing character of *L. rugatus* ECM is the presence of abundant cystidia resembling the terminal cells in the pileipellis of the correspondent fungal symbiont (Lalli & Pacioni 1992, Basso 1999). Not many of the milkcap ECM described so far present cystidia on the mantle surface, which is now known to be the correct name for a North American species (Verbeken et al. 2012, De Crop et al. 2017).

The three species present a plectenchymatous outer mantle layer. Other *Lactarius* ECMs are reported to possess cystidia, such as *Lactarius rubrocinctus* and *L. camphoratus* (Brand 1991), but cystidia here are infrequent and grow from a hyphal net present on the pseudoparenchymatous mantle surface.

Three milkcap mycorrhizas associated with *Cistus* have now been recognized: *L. cistophilus*, a member of *Lactarius* subgen. *Piperites* sect. *Uvidi* subsect. *Uvidini* (Comandini & Rinaldi 2008), *L. tesquorum*, a member of *Lactarius* subgen. *Piperites* sect. *Piperites* (Nuytinck et al. 2004), and *Lactifluus rugatus*. The structure of these three ECM morphotypes is quite diverse (Table 2), which makes it possible to distinguish them on *Cistus* roots. Only *Lactifluus rugatus* possesses numerous and characteristic cystidia that cover mantle surface; *Lactarius cistophilus* presents a pseudoparenchymatous outer mantle layer formed by epidermoid cells; and *L. tesquorum* has a plectenchymatous mantle formed by a loose net of hyphae. The three *Lactarius/ Lactifluus* ectomycorrhizas only share the common host-dependent ectomycorrhizal features described so far for *Cistus*; particularly the small dimensions, small diameter of ectomycorrhizal tips, and a thin mantle. The diversity of the principal features of the ECMs formed by *Lactarius/ Lactifluus* mycobionts with *Cistus* reflects the phylogenetic distance between the three species involved (Fig. 4).

Two other ECMs belonging to *Lactifluus* have been fully described previously: *L. vellereus*, and *L. piperatus*. This offers the possibility of comparing ECMs formed by a species each from subgenera *Lactifluus* (*L. piperatus*), *Lactariopsis* (*L. vellereus*), and *Pseudogymnocarpi* (*L. rugatus*), according to the classification scheme proposed by De Crop et al. (2017) (Table 1). A superficial description

### Table 1. Recent classification schemes of *Lactifluus rugatus* and related species.

| Genus Lactarius | Genus Lactarius | Genus Lactifluus | Genus Lactifluus |
|-----------------|-----------------|-----------------|-----------------|
| Subgenus Lactifluus | Subgenus Lactifluus | Subgenus Lactifluus | Subgenus Lactifluus |
| Section Lactifluus | Section Lactifluus | Section Lactifluus | Section Lactifluus |
| L. volemus | L. volemus | L. volemus | L. volemus |
| *L. rugatus* | *L. rugatus* | *L. rugatus* | |
| *L. luteolus* | *L. luteolus* | |
| Subgenus Lactariopsis | Section Albati | Subgenus Lactariopsis | |
| Section Albati | L. vellereus | |
| L. vellereus | |
| L. bertillonii | |
| Subgenus Lactarius | Section Lactarius | Subgenus Lactarius | |
| Section Lactarius | L. vellereus | |
| L. vellereus | |
| L. bertillonii | |
| Subgenus Lactarius | |
| L. piperatus | |
| L. glaucescens | |
| L. glaucescens | |

1 Neither *L. rugatus* nor *L. luteolus* (i.e. *L. brunneoviolascens*) were included in this treatment which concerned milkcaps from northern Europe.
2 Previously named *Lactifluus luteolus*, which is now known to be the correct name for a North American species (Verbeken et al. 2012, De Crop et al. 2017).
3 A recent study recognized three cryptic species within the morphospecies *Lactifluus volemus*; *L. volemus*, *L. oedematopus*, and *L. subvolemus* (Van de Putte et al. 2016).
of *L. velleurus* mycorrhizas was provided by Ceruti et al. (1988). An ECM from *L. velleurus* was described by Brand & Agerer (1986), but later proved to be formed by *Lactarius biennius*; i.e. it was based on a misidentification (Brand 1987). The only reliable description of *L. velleurus* was provided by *Lactarius blennius* Brand & Agerer (1986), but later proved to be formed by *Russula* (Kumar & Atri 2016). Their description includes pictures and drawings prepared from cross and longitudinal sections, which are per se less informative than direct observations of the mantle surfaces; in addition, no molecular data of either the basidiomes or ECMs were provided to verify the connection. Nevertheless, the outer mantle layer of *L. velleurus* is pseudoparenchymatous, with angular cells and abundant extramatrical thick-walled hyphae, *Russula*-type rhizomorphs are present, and lactifiers have not been observed (Table 2). Finally, while this study was under review, a description of the ECM formed by *L. volemus* var. *volemus* on *Shorea*. (Dipterocarpaceae) from India became available (Kumar & Atri 2016). The description includes pictures and drawings prepared from cross and longitudinal sections, which are per se less informative than direct observations of the mantle surfaces; in addition, no molecular data of either the basidiomes or ECMs were provided to verify the connection. Nevertheless, the outer mantle layer of *L. volemus* var. *volemus* is pseudoparenchymatous, the inner mantle being heterogeneous or more or less pseudoparenchymatous; lactifiers are also present in the inner mantle layer.

Overall, there are no significant features common to the four *Lactifluus* ECM, which is in line with their phylogenetic distance as indicated by molecular analysis. *Lactifluus rugatus* and *L. piperatus* ECMs both lack lactifiers in the mantle, which are also absent in all *Russula* species (Beenken 2004). Interestingly, the absence of lactifiers in ECM of *L. rugatus* contrasts with the presence of abundant, white and mild latex which exudates from basidiomes. *Lactifluus piperatus* and *L. volemus* var. *volemus* ECMs, however, share an important character; the presence of extramatrical hyphae (Table 2).

According to De Crop et al. (2017) these taxa belong to different sections of subgenus *Lactifluus*.

In addition to basidiome/ECM morpho/anatomical and molecular data, comparison of the compounds produced by *Lactarius*Lactifluus* species could have, at least potentially, some value in assessing phylogenetic distances of scrutinized species (Frisvad et al. 1998). For example, *Lactarius*Lactifluus* species are well known to produce a vast array of sesquiterpenes of several kinds (Vidari & Vita-Finzi 1995, Clericuzio et al. 2008). These compounds are usually stored in laticiferous hyphae as inactive precursors, and are converted to biologically active compounds when the fungus is injured. Relevant to our discussion on *Lactifluus*, is the presence of stearyol-velutinal and breakdown products velleral and isovelleral in *L. velleurus* and *L. piperatus* (Camazine & Lupo 1984, Vidari & Vita-Finzi 1995), responsible for the peppery taste of these milkcaps. No velutinal esters or other sesquiterpenes could be detected either in intact or injured basidiomes of *L. rugatus* (Clericuzio et al. 2008), and the same substances are also absent in *L. volemus*, which exudates permanently mild latex and is particularly rich in sugars (Reis et al. 2011). No specific chemical investigations have been carried out so far in *L. brunneoviolascens*, but that species has mild milk and flesh (Basso 1999).

In conclusion, the present study enhances our knowledge of *Cistus* mycorrhizal biology, indicating that *Lactarius/Lactifluus* harbour multiple mycobionts linked to this host, thriving in an ecologically specialized ecosystem. In the future, it would be interesting to test the proposed number of mycobionts-host area relationship and host specificity in the *Lactarius/Lactifluus – Cistus* case, as compared to other host genera pre-eminent in the Mediterranean ecotone, such as *Quercus* and *Pinus* (Newton & Haigh 1998). Moreover, close examination of ECM features revealed differences amongst *Lactifluus* species, a diversity supported by molecular analyses. This confirms, once again, the informative value of mycorrhizal structure, when joined to evidence coming from other approaches, in resolving phylogenetic relationships in ECM fungi (Nuytinck et al. 2004, Mielczko & Ronikier 2007, Benkeen 2011).

Table 2. Comparison of structural features of the described mycorrhizas of *Lactifluus rugatus* and other host- or phylogenetically-related *Lactifluus/Lactarius* species.

| Ectomycorrhizal type                  | System/appearance | SV       | ML    | IV     | RHIZ     | Host      | Reference          |
|--------------------------------------|-------------------|----------|-------|--------|----------|-----------|--------------------|
| *Lactifluus rugatus*                 | m-pyr, short spiny| cys + pl | pl    | pl     | /        | Cistus    | present work       |
| *Lactarius cistophillus*             | simple (pinnate), smooth | ps | dpl + lact | dpl | /        | Cistus    | Comendanti & Rinaldi 2008 |
| *Lactarius tesquorum*               | simple/m-p, smooth | pl | dpl + lact | dpl | hd       | Cistus    | Nuytinck et al. 2004 |
| *Lactifluus piperatus*              | m-p, woolly       | hh + pl  | /     | /      | dif      | Fagus     | Beenken 2004       |
| *Lactifluus volemus var. volemus*    | simple (monopodialpinnate), smooth/cottony | hh + pl | ps | ps + lact | /        | *Shorea*  | Kumar & Atri 2016  |
| *Lactifluus velleurus*              | m-pyr, smooth/mildly grainy | hn + ps | /     | pl + lact | undif    | *Fagus*  | Grebenc et al. 2009 |

SV, surface view; ML, middle layer; IV, inner view; RHIZ, rhizomorphs; cys, cystidia; dpl, densely plectenchymatous; hd, highly differentiated; hh, extramatrical hyphae; hn, hyphal net; lact, lactifiers; m-p, monopodial pinnate; m-pyr, monopodial pyramidal; pl, plectenchymatous; ps, pseudoparenchymatous; undif, undifferentiated.
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