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ARTICLE

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 acystidiate. 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. vellereus* 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
molecular phylogeny
Russulaceae

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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* subsect. *Ochricompactae* were found to be a monophyletic entity, for which the new generic name *Multifurca* was introduced; the genus also includes the rare American species *Multifurca furcata* (Buyck *et al.* 2008). *Lactarius* was found to comprise two clades, with subgenera *Piperites*, *Russularia*, and *Plinthogalus* constituting the larger 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, Verbeken *et al.* 2011, 2012). A more recent multigene 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, Verbeken & Walleyn 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

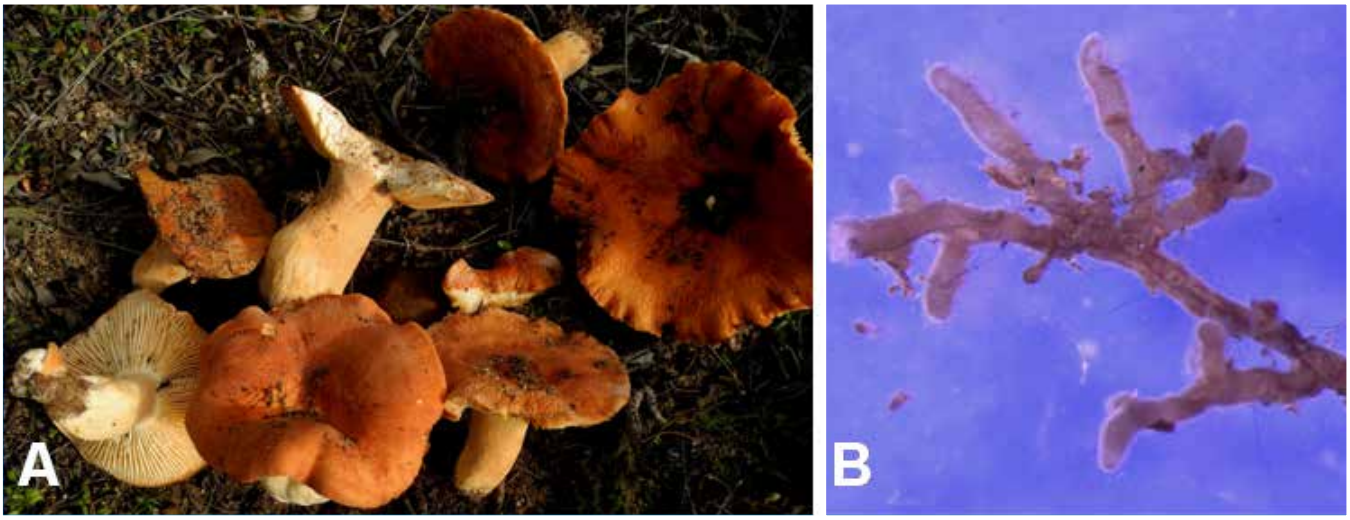

Fig. 1. *Lactifluus rugatus* (ACR-2015/1), from the Mediterranean maquis of Sardinia, Italy. **A.** Basidiomes. **B.** Habit of ectomycorrhiza with *Cistus* sp.; the abundant short cystidia give a light and hyaline appearance to the mycorrhizal surface.

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 Axioplan 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.

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 basidiomes 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.

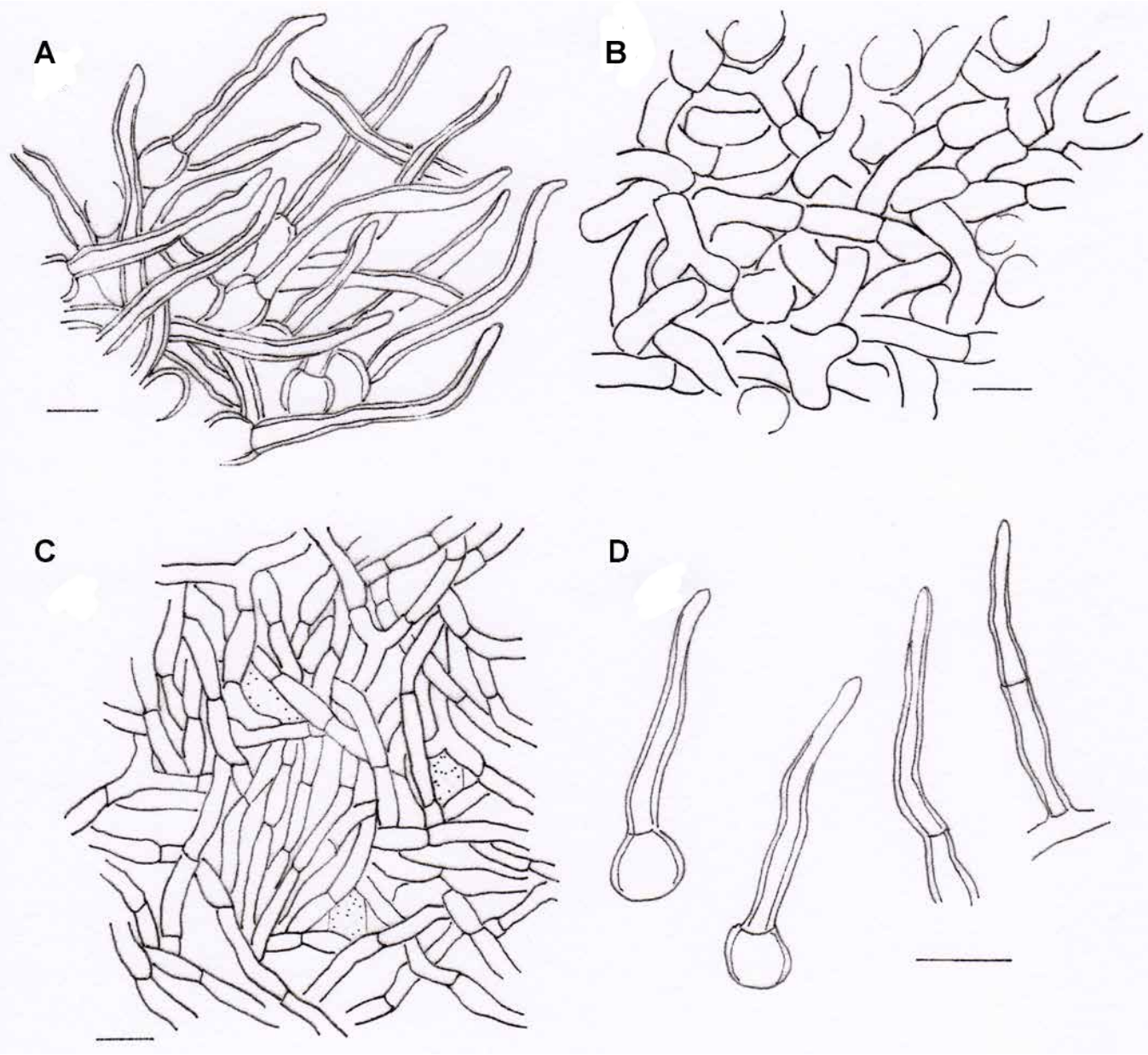

Fig. 2. Anatomical characters of *Lactifluus rugatus* ectomycorrhizas (ACR-2015/1-E). **A.** Outer mantle surface, characterized by abundant cystidia. **B.** Plectenchymatous outer mantle mantle layer formed by frequently branched hyphae, hyphal segments and the basal roundish part of cystidia. **C.** Inner mantle layer with a densely plectenchymatous structure; a few hyphae with content (lactifers?) can be observed in some mantle preparations. **D.** Cystidia, mainly bottle-shaped, sinuous, with blunt tips; cell walls become thicker toward the base which is generally roundish, but sometimes more elongated. Bars = 5 µm.

Cortical (epidermal) cells of 1–2 rows, radially orientated 20–28(–35) × 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)

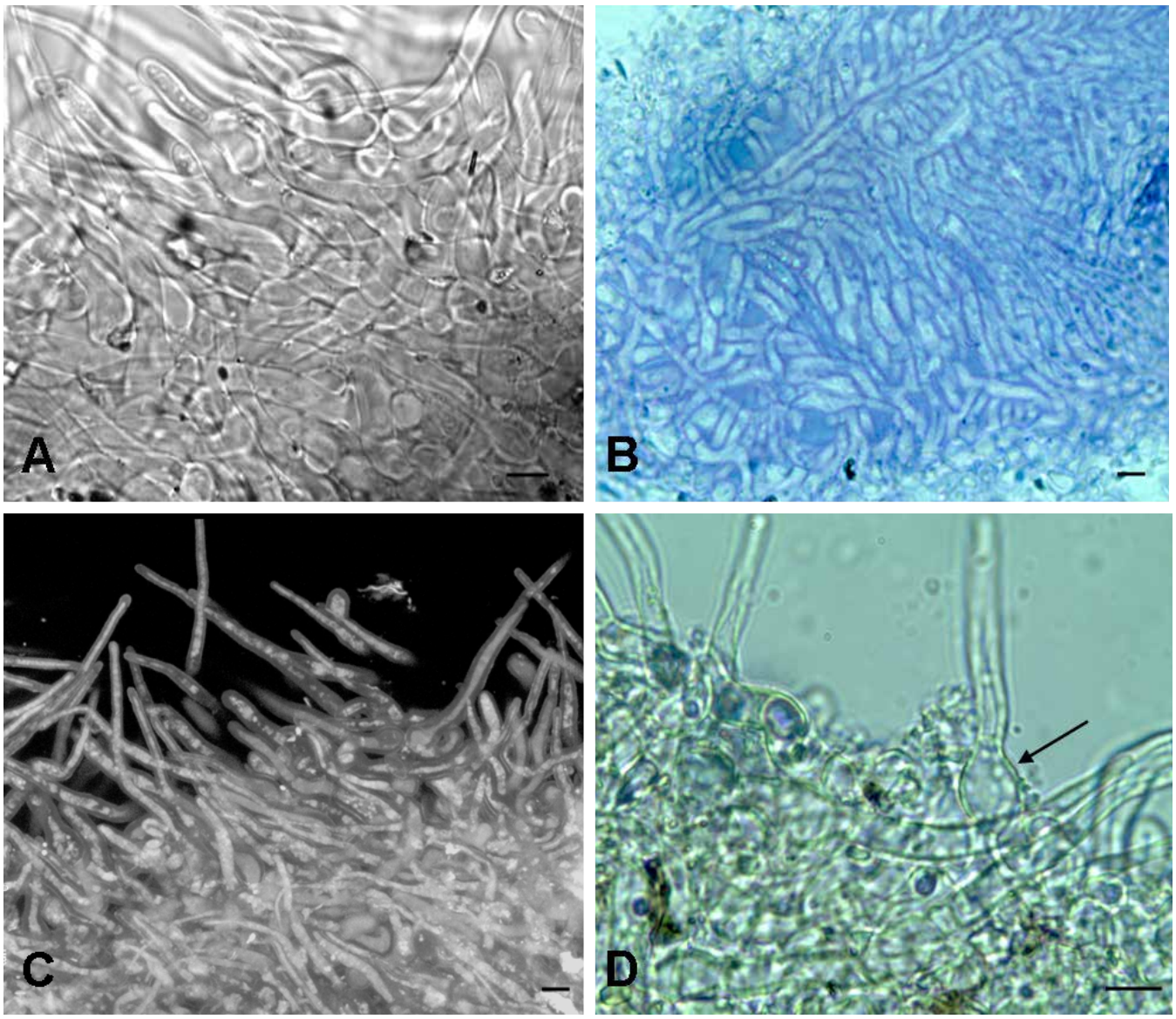

Fig. 3. Anatomical characters of *Lactifluus rugatus* ectomycorrhizas (ACR-2015/1-E), **A.** Outer mantle surface viewed with confocal laser scanning microscopy (CLSM), the basal roundish parts of the cystidia are visible. **B.** Plectenchymatous inner mantle layer viewed with a light microscope. **C.** 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.

(Fig. 4). 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

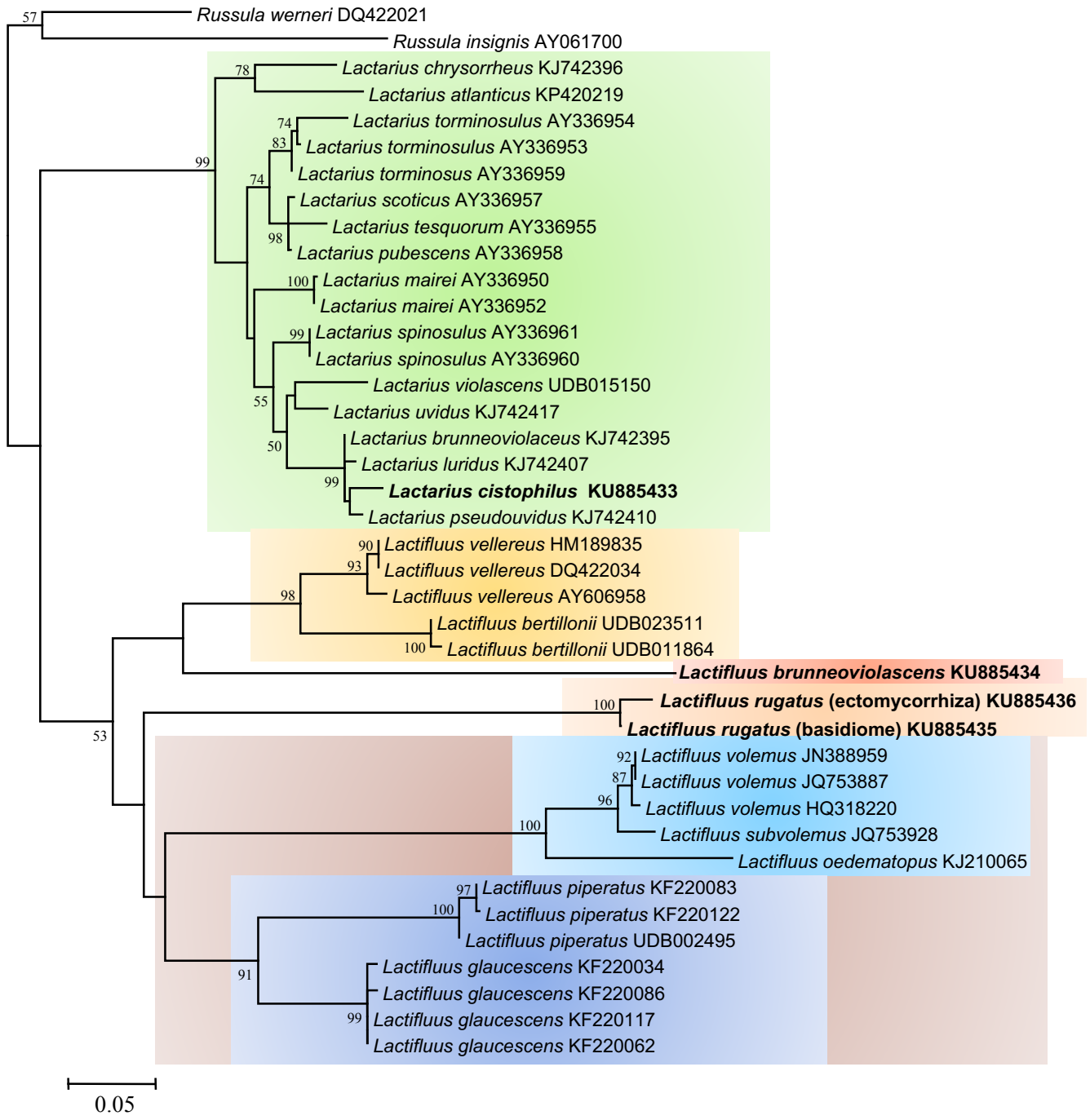

Fig. 4. Maximum Likelihood tree obtained from the alignment of ITS rDNA region sequences. Bootstrap values are indicated next to relevant nodes. The scale indicates the number of substitution per site. Sequences of species in boldface were obtained during this study. The different colours in genus *Lactifluus* correspond to subgenera following the classification proposed by De Crop *et al*. 2017 (see also Table 1).

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*, á 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

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

Heilmann-Clausen *et al.* (1998)	Basso (1999)	Verbeken *et al.* (2011, 2012)	De Crop *et al.* (2017)
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*** [1])	***L. rugatus***	Section *Tomentosi*	*L. subvolemus* [3]
(*L. luteolus* [1])	*L. luteolus*	***L. rugatus***	*L. oedematopus* [3]
Subgenus *Lactariopsis*	Section *Albati*	Section *Phlebonemi*	Section *Piperati*
Section *Albati*	*L. vellereus*	*L. brunneoviolascens* [2]	*L. piperatus*
L. vellereus	*L. bertillonii*	Subgenus *Lactariopsis*	*L. glaucescens*
L. bertillonii	Subgenus *Lactarius*	Section *Albati*	Subgenus *Pseudogymnocarpi*
Subgenus *Lactarius*	Section *Lactarius*	*L. vellereus*	Section *Pseudogymnocarpi*
L. piperatus	*L. piperatus*	*L. bertillonii*	***L. rugatus***
L. glaucescens	*L. glaucescens*	Subgenus *Piperati*	Subgenus *Gymnocarpi*
		Section *Piperati*	Section *Luteoli*
		L. piperatus	*L. brunneoviolascens*
		L. glaucescens	Subgenus *Lactariopsis*
			Section *Albati*
			L. vellereus
			L. bertillonii

[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).

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, and, when present, the characteristics and abundance of these elements are variable. Besides *L. rugatus*, only mycorrhizas of *Lactarius acris* (Brand 1991) and *L. lignyotus* (Kraigher *et al.* 1995), both belonging to *Lactarius* subgen. *Plinthogalus,* present a mantle surface completely covered by “latex-containing hyphal ends resembling cystidia” or “short cystidia-like hyphal ends”, respectively. Moreover, all 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* subsect. *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 2. Comparison of structural features of the described mycorrhizas of *Lactifluus rugatus* and either host- or phylogenetically-related *Lactifluus*/*Lactarius* species.

Ectomycorrhizhal type	System/appearance	SV	ML	IV	RHIZ	Host	Reference
Lactifluus rugatus	m-pyr, short spiny	cys + pl	pl	pl	/	*Cistus*	present work
Lactarius cistophilus	simple (pinnate), smooth	ps	dpl + lact	dpl	/	*Cistus*	Comandini & Rinaldi 2008
Lactarius tesquorum	simple/m-p, smooth	pl	dpl + lact	dpl	hd	*Cistus*	Nuytinck *et al*. 2004
Lactifluus piperatus	m-p, woolly	hh + ps	/	/	dif	*Fagus*	Beenken 2004
Lactifluus volemus var. *volemus*	simple (monopodialpinnate), smooth/cottony	hh + pl	ps	ps + lact	/	*Shorea*	Kumar & Atri 2016
Lactifluus vellereus	m-pyr, smooth/mildly grainy	hn + ps	/	pl + lact	undif	*Fagus*	Grebenc 2005, 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, lactifers; m-p, monopodial pinnate; m-pyr, monopodial pyramidal; pl, plectenchymatous; ps, pseudoparenchymatous; undif, undifferentiated.

of *L. vellereus* mycorrhizas was provided by Ceruti *et al*. (1988). An ECM from *L. vellereus* was described by Brand & Agerer (1986), but later proved to be formed by *Lactarius blennius*; i.e. it was based on a misidentification (Brand 1987). The only reliable description of *L. vellereus* ECM became available more recently (Grebenc 2005, Grebenc *et al*. 2009). The main character of this ECM type is a pseudoparenchymatous outer mantle layer formed by epidermoid cells and with a hyphal net lying on it; abundant lactifers are present in the inner plectenchymatous mantle, and infrequent type B rhizomorphs have been observed (Table 2). A partial description, with comments and pictures, of *L. piperatus* ECM has been provided by Beenken (2004). An earlier description by Luppi & Gautero (1967) did not include enough information for a critical comparison. The outer mantle layer of this *Lactifluus* ECM is pseudoparenchymatous, with angular cells and abundant extramatrical thick-walled hyphae, *Russula*-type rhizomorphs are present, and lactifers 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 robusta* (*Dipterocarpaceae*) from India became available (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. volemus* var. *volemus* ECM is plectenchymatous with extramatical hyphae. The middle and inner mantle layers are pseudoparenchymatous, the inner being heterogeneous or more or less pseudoparenchymatous; lactifers 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 lactifers in the mantle, which are also absent in all *Russula* species (Beenken 2004). Interestingly, the absence of lactifers 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 stearoyl-velutinal and break-down products velleral and isovelleral in *L. vellereus* 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 milk 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, Mleczko & Ronikier 2007, Benkeen 2011).

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REFERENCES

Agerer R (1986) Studies on ectomycorrhizas II. Introducing remarks on characterization and identification. *Mycotaxon* **26**: 473–492.

Agerer R ed (1987–2012) *Colour Atlas of Ectomycorrhizae*. 15 parts. Schwäbisch Gmünd: Einhorn-Verlag.

Agerer R (1991) Characterization of ectomycorrhiza. In: *Methods in Microbiology.* Vol. 23. *Techniques for the Study of Mycorrhiza* (Norris JR, Read DJ, Varma AK eds): 25–73. San Diego: Academic Press.

Agerer R (1995) Anatomical characteristics of identified ectomycorrhizas: an attempt towards a natural classification. In: *Mycorrhiza: structure, function, molecular biology and biotechnology* (Varma A, Hock B eds): 685–734. Berlin: Springer-Verlag.

Alessio CL (1979) *Lactarius rugatus* Kühner et Romagnesi. *Bollettino Gruppo Micologico Bresadola* **22**: 10–14

Altschul SF, Gish W, Miller W, Myers EW, Lipman D (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403–410.

Basso MT (1999) *Lactarius Pers.* [Fungi Europaei vol. 7.] Alassio: Mykoflora.

Bastida F, Talavera S (2002) Temporal and spatial patterns of seed dispersal in two *Cistus* species (*Cistaceae*). *Annals of Botany* **89**: 427–434.

Benkeen L (2004) *Die Gattung Russula Untersuchungen zu ihrer Systematik anhand von Ektomykorrhizen*. PhD thesis, University of Munich; https://edoc.ub.uni-muenchen.de/3175/1/Beenken_Ludwig.pdf

Benkeen L (2011) *Russula densifolia* Secr. ex Gill. + *Fagus sylvatica*. *Descriptions of Ectomycorrhizae* **5**: 147–155.

Bertault R (1978) Lactaires du Maroc. *Bulletin de la Société Mycologique de France* **94**: 273–288.

Brand F (1987) *Lactarius vellereus*. In: *Colour Atlas of Ectomycorrhizae* (Agerer R, ed.): pl. 6. Schwäbisch Gmünd: Einhorn-Verlag.

Brand F (1991) Ektomykorrhizen an *Fagus sylvatica*. Charakterisierung und Identifizierung, ökologische Kennzeichnung und unsterile Kultivierung. *Libri Botanici* **2**: 1–229.

Brand F, Agerer R (1986) Studien an Ektomykorrhizen VIII. Die Mykorrhizen von *Lactarius subdulcis*, *Lactarius vellereus* und *Laccaria amethystina* an Buche. *Zeitschrift für Mykologie* **52**: 287–320.

Buyck B, Hofstetter V, Eberhardt U, Verbeken A, Kauff F (2008) Walking the thin line between *Russula & Lactarius*: the dilemma of *Russula* subsect. *Ochricompactae*. *Fungal Diversity* **28**: 15–40.

Buyck B, Hofstetter V, Verbeken A, Walleyn R (2010) Proposal to conserve *Lactarius* nom. cons. (*Basidiomycota*) with a conserved type. *Taxon* **59**: 295–296.

Camazine S, Lupo AT jr (1984) Labile toxic compounds of the lactarii: the role of the laticiferous hyphae as a storage depot for precursors of pungent dialdehydes. *Mycologia* **76**: 355–358.

Ceruti A, Benvenuti R, Luppi Mosca AM (1988) Micorrize di *Fagus sylvatica* con specie di *Lactarius*, *Russula*, *Laccaria* e *Cortinarius*. *Allionia* **28**: 125–134.

Clericuzio M, Gilardoni G, Malagòn O, Vidari G, Vita-Finzi P (2008) Sesquiterpenes of *Lactarius* and *Russula* (mushrooms): an update. *Natural Product Communications* **3**: 951–974.

Comandini O, Pacioni G, Rinaldi AC (1998) Fungi in ectomycorrhizal associations of silver fir (*Abies alba* Miller) in central Italy. *Mycorrhiza* **7**: 323–328.

Comandini O, Haug I, Rinaldi AC, Kuyper TW (2004) Uniting *Tricholoma sulphureum* and *T. bufonium*. *Mycological Research* **108**: 1162–1171.

Comandini O, Contu M, Rinaldi AC (2006) An overview of *Cistus* ectomycorrhizal fungi. *Mycorrhiza* **16**: 381–395.

Comandini O, Rinaldi AC (2008) *Lactarius cistophilus* Bon & Trimbach + *Cistus* sp. *Descriptions of Ectomycorrhizae* **11/12**: 83–88.

Comandini O, Erős-Honti Z, Jakucs E, Flores Arzú R, Leonardi M, Rinaldi AC (2012a) Molecular and morpho-anatomical description of mycorrhizas of *Lactarius rimosellus* on *Quercus* sp., with ethnomycological notes on *Lactarius* in Guatemala. *Mycorrhiza* **22**: 279–287.

Comandini O, Rinaldi AC, Kuyper TW (2012b) Measuring and estimating ectomycorrhizal fungal diversity: a continuous challenge. In: *Mycorrhiza: occurrence in natural and restored environments* (Pagano M, ed.): 165–200. New York: Nova Science Publishers.

De Crop E, Nuytinck J, Van de Putte K, Wisitrassameewong K, Hackel J, Stubbe D, *et al.* (2017) A multi-gene phylogeny of *Lactifluus* (*Basidiomycota*, *Russulales*) translated into a new infrageneric classification of the genus. *Persoonia* **38**: 58–80.

Dimou DM, Polemis E, Konstantinidis G, Kaounas V, Zervakis GI (2016) Diversity of macrofungi in the Greek islands of Lesvos and Agios Efstratios, NE Aegean Sea. *Nova Hedwigia* **102**: 439–475

Eberhardt U, Oberwinkler F, Verbeken A, Pacioni G, Rinaldi AC, Comandini O (2000) *Lactarius* ectomycorrhizae on *Abies alba*: morphological description, molecular characterization, and taxonomic remarks. *Mycologia* **92**: 860–873.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**: 1792–1797.

Ellul P, Boscaiu M, Vicente O, Moreno V, Rossello JA (2002) Intra- and interspecific variation in DNA content in *Cistus* (*Cistaceae*). *Annals of Botany* **90**: 345–351.

Flores Arzú R, Comandini O, Rinaldi AC (2012) A preliminary checklist of macrofungi of Guatemala, with notes on edibility and traditional knowledge. *Mycosphere* **3**: 1–21.

Frisvad JC, Bridge PD, Arora DK (eds) (1998) *Chemical Fungal Taxonomy*. New York: Marcel Dekker.

Galli R (2006) *I Lattari dalla Natura*. Milan: Libreria della Natura

Gardes M, Bruns TD (1993) ITS primers with specificity for basidiomycetes: application to the identification of mycorrhizae and rust. *Molecular Ecology* **2**: 113–118.

Grebenc T (2005) *Types of ectomycorrhizae on beech (Fagus sylvatica L.) in natural and managed forest*. PhD thesis, University of Ljubljana; http://www.dlib.si/details/URN:NBN:SI:doc-8KMFOI0B.

Grebenc T, Christensen M, Vilhar U, Čater M, Martín MP, *et al.* (2009) Response of ectomycorrhizal community structure to gap opening in natural and managed temperate beech-dominated forests. *Canadian Journal of Forest Research* **39**: 1375–1386.

Guzmán B, Lledó MD, Vargas P (2009) Adaptive radiation in Mediterranean *Cistus* (*Cistaceae*). *PLoS ONE* **4**: e6362.

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis13 program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Heilmann-Clausen J, Verbeken A, Vesterholt J (1998) The genus *Lactarius*. In: *Fungi of Northern Europe* (Læssøe T, Petersen JH, Elborne SA, eds) **2**: 1-287. Copenhagen: Svampetryk.

Hesler LR, Smith AH (1979) *North American Species of Lactarius*. Ann Arbor, MI: University of Michigan Press.

Hutchinson LJ (1999) *Lactarius*. In: *Ectomycorrhizal Fungi: key genera in profile* (Cairney JWG, Chambers SM, eds): 269–285. Berlin: Springer-Verlag.

Iotti M, Zambonelli A (2006) A quick and precise technique for identifying ectomycorrhizas by PCR. *Mycological Research* **110**: 60–65.

Kimura M (1980) A simple method for estimating evolutionary rates of nucleotide substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111–120.

Kraigher H, Agerer R, Javornik B (1995) Ectomycorrhizae of *Lactarius lignyotus* on Norway spruce, characterized by anatomical and molecular tools. *Mycorrhiza* **5**: 175–180.

Kühner R, Romagnesi H (1954) [“1953”] Compléments à la “Flore Analytique”. II. Espèces nouvelles ou critiques de *Lactarius*. *Bulletin de la Société Mycologique de France* **69**: 361–388.

Kumar J, Atri NS (2016) Characterization of ectomycorrhizae of *Russula* and *Lactifluus* (*Russulaceae*) associated with *Shorea* from Indian Shiwaliks. *Nova Hedwigia* **103**: 501–513.

Lalli G, Pacioni G (1992) *Lactarius* sect. *Lactifluus* and allied species. *Mycotaxon* **44**: 155–195.

Le HT, Stubbe D, Verbeken A, Nuytinck J, Lumyong S, Desjardin DE (2007) *Lactarius* in Northern Thailand: 2. *Lactarius* subgenus *Plinthogali*. *Fungal Diversity* **27**: 61–94.

Leonardi M, Paolocci F, Rubini A, Simonini G, Pacioni G (2005) Assessment of inter- and intra-specific variability in the main species of *Boletus edulis* complex by ITS analysis. *FEMS Microbiology Letters* **243**: 411–416.

Luppi AM, Gautero C (1967) Ricerche sulle micorrize di *Quercus robur*, *Q. petraea* e *Q. pubescens* in Piemonte. *Allionia* **13**: 129–148.

Malençon G (1974) Un lactaire méditerranéen nouveau: *Lactarius kuehnerianus* n. sp. *Bulletin Mensuel de la Société Linnéenne et des Sociétés Botanique de Lyon* **43**: 245–254.

Mleczko P, Ronikier M (2007) Features of ectomycorrhizae confirm molecular phylogenetics of *Suillus* (*Boletales*) rather than carpophore-based systematics: insights from studies on *Suillus variegatus, S. plorans* and related species. *Nova Hedwigia* **84**: 1–20.

Montoya L, Bandala VM (2005) Revision of *Lactarius* from Mexico. *Persoonia* **18**: 471–483.

Montoya L, Bandala VM (2008) A new species and new records of *Lactarius* (subgenus *Russularia*) in a subtropical cloud forest from eastern Mexico. *Fungal Diversity* **29**: 61–72.

Mua A, Casula M (2012) *Atlante dei Funghi della Sardegna*. Dolianova: Edizioni Grafica del Parteolla.

Mueller GM, Halling RE (2010) *Macrofungi of Costa Bica*; http://www.nybg.org/bsci/res/hall/.

Munsell (2000) *Soil Color Charts*. Grand Rapids, MI: Munsell Color Company.

Newton AC, Haigh JM (1998) Diversity of ectomycorrhizal fungi in Britain: a test of the species–area relationship, and the role of host specificity. *New Phytologist* **138**: 619–627.

Nounsi A, Outcoumit A, Selmaoui K, Ouazzani Touhami A, *et al.* (2014) Inventaire des champignons ectomycorhiziens du Maroc. *Journal of Applied Biosciences* **79**: 6826–6854.

Nuytinck J, Verbeken A, Leonardi M, Pacioni G, Rinaldi AC, Comandini O (2004) Characterization of *Lactarius tesquorum* ectomycorrhizae on *Cistus* sp., and molecular phylogeny of related European *Lactarius* taxa. *Mycologia* **96**: 272–282.

Pierotti A (2002) Il sottogenere *Lactifluus* (Burl.) Hesler & A.H. Smith emend. Verb. *Pagine di Micologia* **18**: 27–50.

Reis FS, Heleno SA, Barros L, Sousa MJ, Martins A, *et al.* (2011) Toward the antioxidant and chemical characterization of mycorrhizal mushrooms from northeast Portugal. *Journal of Food Science* **76**: 824–830.

Rinaldi AC, Comandini O, Kuyper TW (2008) Ectomycorrhizal fungal diversity: separating the wheat from the chaff. *Fungal Diversity* **33**: 1–45.

Stubbe D, Wang X-H, Verbeken A (2012) New combinations in *Lactifluus*. 2. *L.* subgen. *Gerardii*. *Mycotaxon* **119**: 483–485.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* **30**: 2725–2729.

Van de Putte K, Nuytinck J, De Crop E, Verbeken A (2016) *Lactifluus volemus* in Europe: three species in one revealed by a multilocus genealogical approach, Bayesian species delimitation and morphology. *Fungal Biology* **120**: 1–25.

Verbeken A, Walleyn R (2010) *Monograph of Lactarius in Tropical Africa*. Meise: National Botanic Garden.

Verbeken A, Nuytink J, Buyck B (2011) New combinations in *Lactifluus*. 1. *L.* subgenera *Edules*, *Lactariopsis* and *Russulopsis*. *Mycotaxon* **118**: 447–453.

Verbeken A, Van de Putte K, De Crop E (2012) New combinations in *Lactifluus*. 3. *L.* subgenera *Lactifluus* and *Piperati*. *Mycotaxon* **120**: 443–450.

Vidari G, Vita-Finzi P (1995) Sesquiterpenes and other secondary metabolites of genus *Lactarius* (basidiomycetes): chemistry and biological activity. *Studies in Natural Products Chemistry* **17**: 153–206. Amsterdam: Elsevier.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a guide to methods and applications* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds): 315–322. San Diego: Academic Press.