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Characterization and identification of field ectomycorrhizae of *Boletus edulis* and *Cistus ladanifer*

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Abstract: Field ectomycorrhizae sampled under *Boletus edulis* and *Cistus ladanifer* have been characterized and described in detail based on standard morphological and anatomical characters. The described ectomycorrhiza has traits typical of Boletales: whitish with three differentiated plectenchymatous layers in the mantle in plan view forming ring-like structures and rhizomorphs with highly differentiated hyphae. The inflated, smooth cystidia-like clavate end cells on the surface of the rhizomorphs and their slightly twisted external hyphae are additional characterizing features. The Hartig net occupies 1½ rows of cortical cells, partly reaching the endodermis. Not all hyphae have clamps. The identification of the fungal symbiont as *B. edulis* was confirmed by ITS rDNA sequence comparison between mycorrhizas and sporocarps. The singularity of this symbiotic association, as well as its ecological and practical implications, are discussed.

Key words: anatomy, description, ITS rDNA, morphology

INTRODUCTION

Boletus L., especially the *Boletus edulis* species complex, is a cosmopolitan genus of ectomycorrhizal fungi widely represented in the warmer parts of the Northern Hemisphere. These species have a great economic importance for their edibility (Singer 1986, Hall et al 1998). The genus comprises more than 1000 species with epigeous fructification, inhabiting forests in tropical and midlatitudes, forming ectomycorrhizae mainly with trees and shrubs of Pinaceae, Fagaceae and Betulaceae (Singer 1986).

The *B. edulis* species complex includes four species: *B. aereus* Bull., *B. aestivalis* (Paulet) Fr., *B. edulis* Bull. and *B. pinophilus* Pilát & Dermek. The identification of the fruiting bodies of these four species traditionally has been difficult because it is based exclusively on a few, highly variable morphological characters. Recent studies showed that these four species can be successfully discriminated by an extensive analysis of the internal transcribed spacer of the nuclear rDNA region (Leonardi et al 2005).

The plants of the Cistaceae family are fairly abundant in the Northern Hemisphere and South America. The family has eight genera with almost 200 species (Muñoz and Navarro 1993). The *Cistus* genus is represented in the Iberian Peninsula by 12 shrub species, all belonging to primary succession stages of tree stands, growing readily in degraded areas. The Cistaceae species in general are pyrophytic. Their germination is related to high temperatures, and they are adapted to fires in Mediterranean forests (Alonso et al 1992). *Cistus ladanifer* L. lives in the western Mediterranean, from Portugal and Morocco to the French Riviera and Algeria, in zones with hot, dry summers, 0–1500 m a.s.l., on silicon soil in the southern half of the Iberian Peninsula and on slate and granite in the western part (Demoly and Monserrat 1993).

All Cistaceae are ectomycorrhizal plants (Brundrett 2002, Smith and Read 1997), but available descriptions for *Cistus* ectomycorrhizal types are scarce. Only four morphotypes of ectomycorrhizae described in association with *Cistus* sp. have been found; they are *B. rhodoxanthus* (Krombh.) Kallenb. with *Cistus* cf. *ladanifer* (Hanh 2001), *Laccaria laccata* (Scop.) Fr. with *C. ladanifer* (Torres et al 1995), *Lactarius tesquorum* Malençon with *Cistus* sp. (Nuytinck et al 2004) and *Tuber nigrum* Allioni with *C. incanus* L. (Fontana and Giovanetti 1978–1979, Fusconi 1983, Wenkart et al 2001). Rockroses (*Cistus* and *Helianthemum*) are ecologically important species because they may act as a reservoir of mycorrhizal fungi inoculum after a forest disturbance (Torres et al 1995, Díez 1998).

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TABLE I. Literature references of harvesting of Boletales carpophores in stands with Cistaceae species in Spain

Species	Host	Reference
Boletus aemilii Barbier	*Cistus* sp.	Llamas and Terrón 2003
Boletus aereus Bull.	*Cistus* sp.	Sánchez Rodríguez et al. 2004
Bolatus aestivalis (Paulet) Fr.	*Cistus* sp.	Sánchez Rodríguez et al. 2004
Boletus corsicus Rolland	*Cistus ladanifer* L.	Oria de Rueda and Díez 2002
Boletus impolitus Fr.	*Cistus monspeliensis* L.	Pando 2000
Boletus queletii Schulz. var. *zugazae* Moreno	*Cistus ladanifer* L.	Moreno 1977
Boletus rhodoxanthus Kallenb.	*Cistus* cf. *ladanifer* L.	Hahn 2001
Chalciporus piperatus (Bull. ex Fr.) Bataille.	*Cistus ladanifer* L.	Pando 2000
Leccinum corsicum (Roll.) Sing.	*Cistus* sp.	Llamas and Terrón 2003
	Cistus sp.	Sánchez Rodríguez et al. 2004
	Cistus albidus L.	Moreno Arroyo et al. 1996
	Cistus ladanifer L.	Moreno Arroyo et al. 1996
		Pando 2000
	Cistus laurifolius L.	Oria de Rueda and Díez 2002
	Cistus monspeliensis L.	Moreno Arroyo et al. 1996
Leccinum hispanicum Moreno	*Cistus ladanifer* L.	Moreno 1977
Leccinum lepidum (Bouchet ex Essette) Quadr.	*Cistus ladanifer* L.	Pando 2000
Leccinum quercinum (Pilát) E.E. Green & Watling	*Cistus* sp.	Pando 2000
Paxillus rubicundulus Orton	*Cistus populifolius* L.	Pando 2000
Xerocomus chrysenteron (Bull.) Quél	*Cistus* sp.	Pando 2000
Xerocomus ichnusanus Alessio, Galli et Littini	*Cistus ladanifer* L.	Pando 2000

Previous references to Cistaceae associations with Boletales in Spain have been compiled (TABLE I). No previous worldwide literature references have been found about the harvest of *Boletus edulis* sporocarps in pure stands of *Cistus* sp. The aim of this paper is to provide a first description and characterization of the ectomycorrhizae of *B. edulis* on *C. ladanifer* collected in their natural habitat, as well as the molecular analyses of the fungal symbiont.

MATERIAL AND METHODS

The *Boletus edulis* sporocarps and ectomycorrhizae were collected in Nov 2004 in a single area of the province of Zamora, in the municipality of Riofrío (Castilla y León, España), UTM co-ord.: 29T0 735590, 4633695, about 872 m a.s.l., in loamy soil composed of slate and sandstone, pH 5.0. The harvested fungal specimens were collected in pure stands of 8 y old *Cistus ladanifer* shrubs. No trees were present. Soil cores were collected from beneath the sporocarps and stored at 4 C for analysis in the laboratory. The roots with ectomycorrhizae and the soil rhizomorphs were extracted carefully with the aid of a stereomicroscope. The use of water was avoided because of the clay soil. To complete the cleaning, the excised roots and ectomycorrhizae were placed in an ultrasonic bath with deionized water and some drops of Tween 20® detergent at 20 C for 15 min. Samples of the sporocarps, ectomycorrhizae and rhizomorphs were frozen immediately at −20 C for further molecular analyses. Dried sporocarps and ectomycorrhizae fixed in FAA (Verlhac et al 1990) were stored as voucher specimens in the Dpto. Inv. Exp. For. Valonsadero with the codes: VALONSADERO—FUNGI 2081 and VALONSADERO—MYCORRHIZA 019 respectively.

The general methodology and terminology for characterizing the ectomycorrhizae follows Agerer (1987–2002, 1991) and Agerer and Rambold (2004–2005). For observation of the mantle ectomycorrhizae were grated with the peeling technique (Agerer 1991). Mantle and rhizomorph preparations of fresh ectomycorrhizae were fixed on slides with lacto-glycerine for microscope observation. For longitudinal and cross sections (5–7 µm thick) ectomycorrhizae and rhizomorphs were embedded in liquid parafine, cut with a Microm HM 340E microtome and stained with hematoxilin-eosin.

Molecular characterization was carried out by sequencing fragments of the nuclear ribosomal DNA region of sporocarps, ectomycorrhizae and rhizomorphs. DNA extraction from fungal tissue, ectomycorrhizae and rhizomorphs was performed with the QIAGEN® DNeasy Plant Mini Kit. Amplifications of ITS rDNA sequences were carried out with an Applied Biosystems® 9700 PCR machine using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al 1990) as well as the fungal specific ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns 1993) and the *Boletus* specific BED-4 (5′-GTTTGTATACATTCTGGACATGCG-3′) (Moor et al 2002). Sequence alignments were performed with the BioEdit program version 5.0.9 (Hall 1999). Identification was carried out by comparing our sequences with the existing ones in the GenBank database.

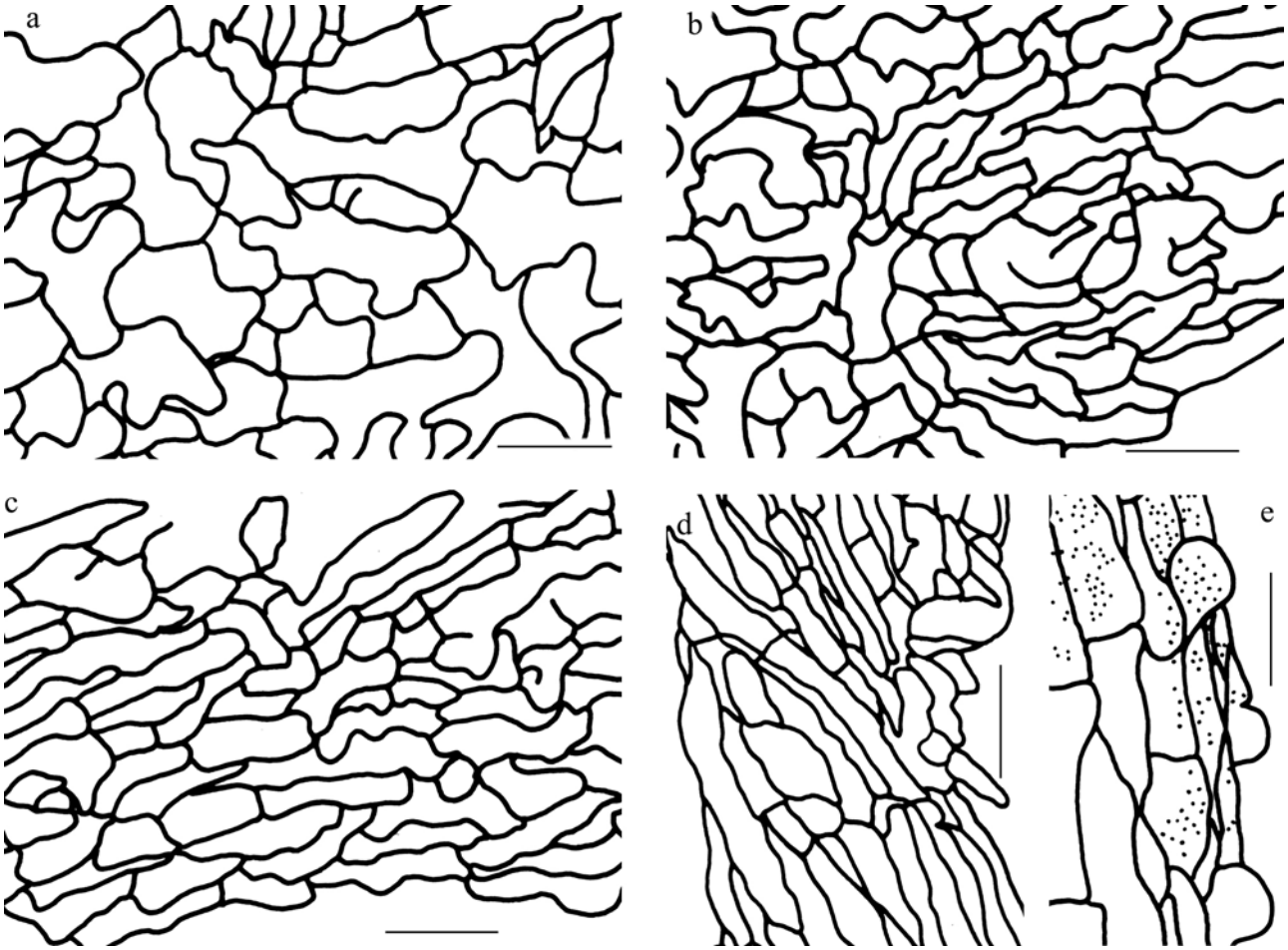

FIG. 1. Anatomical characters of *B. edulis* ectomycorrhizae. (a) Outer mantle layer with a plectenchymatous structure formed by a loose net of hyphae. (b) Middle mantle layer with a plectenchymatous structure forming ring-like structures. (c) Inner mantle layer with a densely plectenchymatous structure. (d) Surface of rhizomorph, showing slightly twisted hyphae. (e) Vesicles on the margin of rhizomorph. Bars = 10 μm.

RESULTS

Morphological characters.—Ectomycorrhizal system infrequently found, 1.5–1.8 mm long, monopodial-pinnate, ramifications of second order lacking or less developed, with four tips per 10 mm (FIG. 2a). Main axes 0.2–0.3 mm diam. Unramified ends 0.4–0.6 mm long, 0.2–0.3 mm diam, sinuous, yellow whitish getting more yellow with age, whitish tip, not contrasting, inflated, club-shaped. Surface of unramified ends smooth and shiny, distinct, mantle not transparent and cortical cells not visible, rhizomorphs infrequent, emanating hyphae absent. Rhizomorphs, thin (0.25–0.1 mm) when originating directly from ectomycorrhizae, at a greater distance thick, up to 2 mm diam, white near the mycorrhiza and at the base of the fruitbody and nearby, frequently ramified at restricted points, round in cross-section, surface smooth, connection to mantle kind distinct, origin location distal and proximal, joint angle to the mantle 30°, ramification common with an angle of 60°. Sclerotia not observed.

Anatomical characters of mantle in plan views.—Mantle plectenchymatous in all layers; all hyphae colorless, clamps lacking. Outer mantle layer (FIG. 1a) with a net of branching hyphae in a regular ring-like arrangement, hyphae 4–5(8) μm diam, cells 22–23 μm long, colorless, matrix not observed, hyphae junctions angle 120°, septa as thick as walls, cells slightly inflated in middle portions, cell wall surface smooth. Middle mantle layer (FIG. 1b) densely plectenchymatous, with distinct hyphal bundles forming ring-like patterns like the outer layers, hyphae colorless, 4–5 μm thick, cells 20 μm long, cell walls smooth, anastomoses not observed, matrix lack-

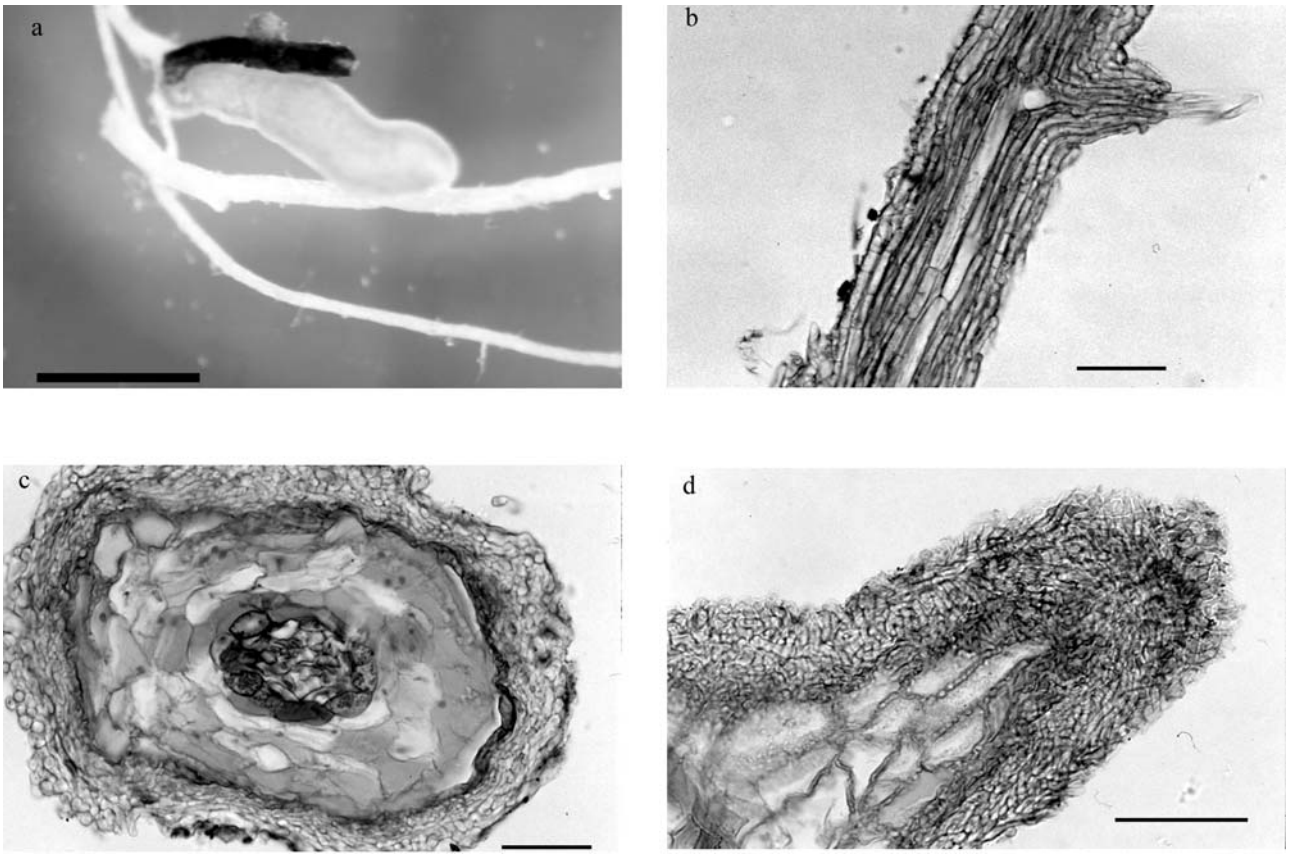

FIG. 2. Anatomical characters of *B. edulis* ectomycorrhizae. (a) Morphological aspect of an ectomycorrhizae and rhizomorphs. Bar = 1 mm. (b) Longitudinal section of a rhizomorph. Bar = 25 µm. (c) Cross section of a mycorrhiza. Bar = 25 µm. (d) Longitudinal section of a mycorrhiza. Bar = 25 µm.

ing. Inner mantle layer (FIG. 1c) densely plectenchymatous, with broad streaks of parallel hyphae, colorless, 3–5 µm diam, cells 20–22 µm long. Tip with the same structural characteristics as in the older parts of mantle.

Anatomical characters of emanating elements.—Rhizomorphs highly differentiated (type E according to Agerer 1987–2002), forming internal nodia and nodia at branching points, with hyphae empullate and conical young side-branches, presence of trumpet-like inflated hyphae, 10–11 µm thick; central vessel-like hyphae present (FIG. 2b), 7–8 µm diam, cells 70–85 µm length, 1–1,5 µm thick wall, ramification with one side-branch at septum; central nonvessel-like hyphae with septa as thick as the cell walls, 7 µm diam, cells 27 µm long; peripheral hyphae not specialized, 3–4 µm diam, cells 34 µm long, colorless, mostly smooth, but sometimes little granules on the cell wall present, slightly dotted surface, with the hyphae slightly twisted (FIG. 1d) and some vesicles appear in the margin (FIG. 1e) formed by empullated septa of the peripheral hyphae. Clamps lacking. Emanating hyphae lacking. Cystidia lacking. Chlamydospores not observed.

Anatomy of the mantle in longitudinal section.—Mantle 18–24 µm thick, 20–22 µm at ectomycorrhizal tip, three different layer discernable (FIG. 2d), all of them plectenchymatous, outer mantle with few calyptra cell remains, hyphae 5–7 µm tangential length, 3–4 µm radial diam; middle mantle plectenchymatous, hyphae 7–8 µm tangential length, 2–3 µm radial diam; inner mantle plectenchymatous, hyphae 6–7 µm tangential length, 3–4 µm radial diam. Tannin cells absent. Cortical cells tangentially oval to elliptic or cylindrical and obliquely oriented, 24–36 µm tangential length, 12–24 µm radial diam, CCt = 30 µm, CCq = 18 µm. Hartig net present in one or in one-half row of cortical cells, adjoining endodermis free of this, hyphal cells around cortical cells beaded, 2–3 µm thick, two hyphal

rows around cortical cells. Hartig net structure (in plan view) infrequently lobed, lobes without septa, 1.5–2 µm width.

Anatomy of mantle in cross-section.—Different layers discernible in the mantle (FIG. 2c). Outer mantle layer plectenchymatous, without calyptra cell remains, hyphae 12–14 µm tangential length, 7–9 µm radial diam. Middle mantle layer plectenchymatous, hyphae 13 µm tangential length, 4 µm radial diam. Inner mantle layer plectenchymatous, hyphae 7 µm tangential diam, 4 µm radial diam. Cortical cells rectangular, 17–18 µm tangential length, 12–17 µm radial diam, CCt = 18 µm, CCq = 15 µm. Hartig net apparently 1½ rows deep, hyphal cells around cortical cells beaded, 2 µm thick filling two rows around cortical cells.

Chemical reactions.—Brilliant cresyl blue, dense blue; formol 40%, only the mantle turns gray-greenish; Melzer's reagent, dextrinoid; ruthenium red, pink reddish; toluidin blue, dense blue. The rest (acid fuchsin, anilin, etanol 70%, $FeSO_4$, guaiac, KOH 10%, lactic acid, phenole, phenole-anilin, sudan III, sulpho-vanillin and water) absent.

DNA analysis.—Sequences of the nuclear ribosomal DNA fragments were registered in the NCBI GenBank database with these accesion numbers: DQ002921 for the sporocarp sequence, DQ002922 for the mycorrhiza sequence and DQ002923 for the rhizomorph sequence. ITS1/ITS4 amplifications were successful for the sporocarp samples but failed with mycorrhizas and rhizomorphs, which were amplified successfully using the specific ITS1F/BED-4 primers pair. Alignments of the three structures had a 100% coincidence in the ITS1 region. A search for highly similar sequences by the MegaBLAST procedure was performed to compare our complete sporocarp ITS1, 5.8S and ITS2 sequence with the GenBank ones. A 99–100% identity with 13 *B. edulis* entries, 2 *B. aestivalis*, 2 *B. persoonii* Bon and 1 *B. venturii* Bon was found.

DISCUSSION

Ectomycorrhiza description and characterization.—Characterization of rhizomorph structures seems to be important for distinguishing ectomycorrhizae in the Boletales (Brand 1989). After the revision of the previously published *Boletus* genus ectomycorrhizae descriptions by Ceruti et al (1983–1984), Ceruti et al (1987–1988), Garrido (1988), Gronbach (1988), Agerer and Gronbach (1990), Franz and Acker (1995), Hahn (2001), Palfner (2001) and Agerer and Rambold (2004–2005) it can be concluded that the ectomycorrhizae of this genus are characterized by the lack of hyphal clamps, the plectenchymatous mantle and rhizomorphs with differentiated hyphae. The mantles of all of the *Boletus* ectomycorrhizae described are formed by three plectenchymatous layers of colorless hyphae forming ring-like structures (type A, Agerer 1991).

Hahn (2001) described vesicles in the margin of the rhizomorphs formed by *B. rodoxanthus* similar to those described in this study for *B. edulis*; however, whereas the vesicles of the latter species are smooth, the vesicles of the former species are covered with a dense layer of smooth crystals. Rhizomorphs of *B. loyo* Phillippi and *B. putidus* E. Horak (Palfner 2001) are similar to the *B. edulis* described here, but both present cystidia. The two descriptions of *B. aestivalis* (Ceruti et al 1983–1984, Garrido 1988) described the characters of the mantle exclusively. All descriptions of *B. edulis* ectomycorrhizae (Ceruti et al 1987–1988, Gronbach 1988, Garrido 1988, Agerer and Gronbach 1990, Franz and Acker 1995, Palfner 2001, Agerer and Rambold 2004–2005) report smooth hyphae and differentiated rhizomorphs according to Agerer (1999). Although some of the hyphae of the ectomycorrhizae described in this paper are slightly dotted and the external hyphae of the rhizomorphs are slightly twisted, those characteristics could not be considered definitive.

Molecular characterization allowed the identification of the fungal symbiont present in mycorrhizas and rhizomorphs as *B. edulis*. GenBank sequence comparisons were based mainly on the data provided by Leonardi et al (2005). Although the similarity between the sequence obtained in this work and a few other *Boletus* species or varieties was also high, all of them belong to the *B. edulis* species complex. Because average nucleotide diversity inside the *B. edulis* species is low compared to other species of the complex (Leonardi 2005), the full coincidence of the fragment amplified from mycorrhizas and rhizomorphs of the sporocarp turned out to be informative for confirming the identity of the fungal partner. On the other hand the lack of success in the PCR amplification from ectomycorrhizas and rhizomorphs when using the universal primers ITS1 and ITS4 indicates that specific primers for PCR amplification can be necessary when working with field, nonaseptic material.

Ecological and practical implications.—*B. edulis* species complex is associated with a wide range of host trees. *B. edulis* and *B. pinophilus* sporocarps

are found in temperate conifer and broadleaf forests, whereas *B. aereus* and *B. aestivalis* sporocarps are more termophilic and usually are found in broadleaf and conifer forests (Alessio 1985).

There are few references of mycorrhizal associations of *B. edulis* with shrubs. Manavella (2004) harvested sporocarps of this species in the Italian Alps, at 2500 m a.s.l., with presence of *Juniperus communis* L. subsp. a*lpina* (Suter) Čelak. and *Arctostaphylos uva-ursi* (L.) Spreng. Both shrubs can form ericoid and vesiculo-arbuscular mycorrhizae, whereas the former also forms ectomycorrhizae (Harley and Harley 1987). Molina and Trappe (1982a) reported members of the Boletales forming arbutoid mycorrhizae with ericaceous shrubs and ectomycorrhizae with coniferous trees.

The extent by which plants benefit from a symbiosis with mycorrhizal fungi varies depending on identity of the plant and the fungus, the physiological state of the plant and environmental conditions (van der Heijden and Sanders 2002). Allen (1991) stated that some plants may form symbiosis with certain fungi depending on the ecological conditions. Molina et al (1992) proposed the concept of ecological specificity that is the influence of biotic and abiotic factors on the ability of plants to form functional mycorrhizae with particular fungi in natural soils. Also Brundrett (2002) suggested that mycorrhizal fungi have a limited capacity for distinguishing the roots of different plant species, so plants primarily would regulate specificity.

Boletus edulis is one of the species that seems to follow this pattern, being able to produce sporocarps in association with unusual host plants such as *C. ladanifer*, a pioneer early stage shrub, when species of Fagales or Pinaceae are absent. This situation would favor the maintenance of soil inoculum reservoria for successional stages. Also the fact that *B. edulis* is able to fruit when associated with 8 y old rockroses may be seen as a dispersion strategy to assure genetic variation (Horton and Bruns 2001).

Studies on wild sporocarp production of edible *Boletus* have been carried out in different environmental situations (Rondet and Leprince 2001, Martínez 2003, Salerni and Perini 2004). Controlled cultivation and mycorrhizal synthesis studies with *Boletus* are relatively abundant (Pantidou 1961, 1962, 1964; Tozzi et al 1980, 1981; Molina and Trappe 1982b; Poitou et al 1982; Ceruti et al 1983, 1985; Poitou and Mamoun 1984; Zucherelli 1988, Meotto and Pellegrino 1989). The association with *C. ladanifer* reported in this study, together with the early sporocarp production, offer an alternative economic resource for developing countries and for marginal and inland areas with low incomes. The only attempts to produce edible sporocarps have been done with *Helianthemum* inoculated with *Terfezia* (Morte et al 2004). Nursery-controlled inoculations designed to establish short-term production plots could be seen as a feasible and promising way to exploit this peculiar symbiosis.

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