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SHORT NOTE

Mycorrhizal morphotyping and molecular characterization of *Chondrogaster angustisporus* Giachini, Castellano, Trappe & Oliveira, an ectomycorrhizal fungus from *Eucalyptus*

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Abstract *Chondrogaster angustisporus* is a hypogeous ectomycorrhizal fungus described from fruiting bodies collected under *Eucalyptus* spp. in Brazil, Uruguay, and Australia. Due to its efficiency in promoting plant growth, we decided to characterize this fungus through mycorrhizal morphotyping and internal transcribed spacer (ITS) (rRNA) sequencing. DNA extracted from mycelium was amplified and sequenced using specific primers. Mycorrhizas were obtained aseptically and analyzed in terms of macroscopic and microscopic characteristics. When compared with other fungal DNA sequences available in the NBCI GenBank, the *C. angustisporus* sequence presented the highest similarity to an uncultured ectomycorrhizal fungus from the Seychelles. It also shows significant similarities to *Gomphus*, *Ramaria*, and *Hysterangium* species supporting the classification of *Chondrogaster* in the subclass Phallomycetidae in the gomphoid–phalloid group. The mycorrhizas were characterized by a narrow mantle with a single tissue layer densely arranged and organized as a net synenchyma with elongated hyphae. Interhyphal spaces were seen only in the external region where hyphae were more loosely organized. Bottle-shaped cystidia with bent necks were observed on the surface of the mantle. Emanating hyphae were larger than those in the mantle and presented a granular content. At regular intervals the hyphae were divided by septa with clamp connections. The Hartig net was of the common type, with typical palmetti and single hyphal rows and limited to the epidermal layer. The mycorrhizal description and the ITS sequence obtained are useful tools to identify this ectomycorrhizal fungus in culture and in association with *Eucalyptus* roots.

Keywords Mycorrhizal morphotyping · Internal transcribed spacer (ITS) · Sequencing · Molecular identification

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Introduction

Eucalypts, the most extensively employed plants in Brazilian reforestation programs, are fast-growing trees able to associate with ectomycorrhizal (ECM) fungi. They are native of Australasia and were introduced to Brazil early in the 20th century to meet the country's demand for timber. High temperatures and abundant water associated with the often-rich soils contributed to improve the establishment and growth of these plants, yielding short rotation cycles

(Lima 1993). Currently, Brazil alone has 4 Mha planted with eucalypt species, of which about 10% are located in Santa Catarina State, Southern Brazil.

In Australasia, many of the ectomycorrhizal fungi associated with eucalypts have been catalogued and described (Bougher and Castellano 1993; Castellano and Bougher 1994; Bougher and Syme 1998; Lu et al. 1999; Bougher and Lebel 2001; Midgley et al. 2007). In Brazil, however, very little is known concerning ectomycorrhizal fungi associated with eucalypts.

The most intensive survey of ectomycorrhizal fungi in eucalypt plantations in Brazil was performed by Giachini and co-workers (Giachini et al. 2000, 2004). In that study, 49 presumed ECM taxa representing nine orders and 12 families were identified, including epigeous and sequestrate species. Many were first reports for South America, and three were new species.

Amongst the new species was found *Chondrogaster angustisporus* Giachini, Castellano, Trappe & Oliveira, a hypogeous fungus found in soil, either solitary or more frequently in clusters of several specimens embedded in a profuse, white, mycelial colony under *Eucalyptus dunnii* Maiden, in Santa Catarina State, Southern Brazil (Giachini et al. 2000). This fungus was also collected in Australia under *Eucalyptus* spp. (J. M. Trappe, personal communication) and in Montevideo, Uruguay under *E. grandis* (Giachini et al. 2000). More recently, *C. angustisporus* was found in eucalypt plantations in Spain by Alvarez and Cerceda (2005).

Souza et al. (2004) studied an isolate of *C. angustisporus* which significantly improved P uptake and growth of *E. dunnii* seedlings under greenhouse conditions. This raised the possibility of using the fungus in seedling inoculation programs (Rossi 2006). The objective of this study was to describe the mycorrhizas formed by *C. angustisporus* and *E. dunnii* seedlings and to compare the internal transcribed spacer sequence of the isolate UFSC-Ch163 with those from other fungi in order to obtain tools to recognize this fungus in pure culture or when growing in association with *Eucalyptus* roots.

Materials and methods

Chondrogaster angustisporus isolate

A *C. angustisporus* isolate (UFSC-Ch163) from the culture collection of ectomycorrhizal fungi stored at the 'Laboratório de Ectomicorrizas', Universidade Federal de Santa Catarina (UFSC), in Florianópolis, Santa Catarina, Brazil was used in this study. This fungus was isolated by A. J. Giachini and V.L. Oliveira in 1997 from fruiting bodies harvested from *E. dunnii* plantations in Santa Catarina State, and was described for the first time by Giachini et al. (2000).

Fungal culture and DNA extraction, amplification, and sequencing

Mycelium used in this study was obtained from 3-week-old liquid cultures in Modified Melin Norkrans (MMN) medium (Marx 1969). After decanting the culture medium, approximately 300 mg of fresh mycelium was used for DNA extraction by the techniques described by Gardes and Bruns (1993).

After DNA extraction, samples were treated with a 2% *v*/*v* solution of RNAse A (10 mg ml^{-1}) at 37°C in a water bath for 30 min. The presence of DNA was verified by electrophoresis in agarose gel 1.2% (*w*/*v*) in Tris–borate–EDTA (TBE buffer) (Sambrook et al. 1989), stained with 0.5 µl of an ethidium bromide solution (10 mg ml^{-1}) followed by observation under UV light (360 nm).

Amplification of the ITS region by polymerase chain reaction (PCR) was achieved with fungi-specific primers that amplify ITS region from asco and basidiomycetes. Primers used were: ITS1 (5′ TTC CGT AGG TGA ACC TGC GG 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (White et al. 1990). PCR reaction was performed as following: 1.0 µl DNA; 2.5 µl PCR buffer 10×; 3.0 µl dNTPs (1.5 mM); 2.0 µl $MgCl_2$ (20 mM); 3.0 µl of each primer (25 pmol); 0.5 U *Taq* polymerase (5 U μl^{-1}); and 10.5 µl ultrapure water.

Amplification was run in a MJ Research, Inc. machine (Model PTC-100) under the following conditions: first extension at 94°C for 30 s; denaturation at 94°C for 45 s; annealing temperature of 55°C (30 s) for 35 cycles; and a final extension at 72°C for 10 min. The PCR product was fractionated by electrophoresis on an agarose gel 1.2% (*w*/*v*) in TBE buffer, then stained with ethidium bromide under UV light (360 nm).

The product received 1 vol of 13% polyethylene glycol (PEG 8000) (10 ml ultrapure water, 1.3 g PEG, and 0.94 g NaCl) (Dunn and Blattner 1987) and was left in the refrigerator overnight. After that, samples were centrifuged at 18,894 ×*g* for 15 min. The PEG solution was then removed and 200 µl of 70% alcohol was added. The mixture was then centrifuged again at 18,894 ×*g* for 10 min. The alcohol was removed and the DNA was re-suspended in ultrapure water (Sambrook et al. 1989).

DNA was sequenced using a double-stranded DNA template of PCR product following the protocol supplied by Amersham Bioscience with their MegaBACE™ 500. The ITS sequence obtained for the isolate was compared to those available in the GenBank of the National Center for Biotechnology Information (NCBI) (Altschul et al. 1997). Those presenting the highest scores were selected and aligned using the algorithm ClustalW. A phylogenetic tree was built using the neighbor-joining method by the MEGA software, version 4, with 5,000 replicates (Kumar et al. 2004). The

similarities between the nucleotide sequences were calculated using the DNASTAR software (LASERGENE 1994).

Mycorrhizal synthesis

C. angustisporus mycorrhizas on *E. dunnii* seedlings were produced in vitro using the technique described by Yang and Wilcox (1983). The fungus was cultivated on MMN–agar medium in Petri dishes at 25±1°C for 20 days. From these cultures, mycelial plugs, 8 mm in diameter, were cut and tested for viability on fresh MMN–agar. After 2 days, when new hyphae were observed from the edges of the plugs and no contamination was detected, the plugs were used to inoculate *E. dunnii* seedlings.

E. dunnii seeds were germinated on agar medium containing boric acid (3 μM), glucose (2 g l^{-1}) and calcium sulfate (500 μM), pH 5.7, agar (7 g l^{-1}), in Petri dishes. Before sowing, the seed surfaces were disinfected by immersion in 70% ethanol solution for 30 s followed by a 20-min immersion in sodium hypochlorite 1%. Afterwards, seeds were rinsed three times in sterilized distilled water and placed on the germination medium. Dishes were then incubated in a growth chamber at 25±1°C, with a 24-h photoperiod with 30 μmol s^{-1} m^{-2} of light intensity.

Two weeks later, seedlings were transferred to ten glass tubes (20×2.5 cm) containing 30 ml of a mixture of vermiculite and peat (1:20, *v*/*v*). A sheet of filter paper (11× 11 cm) was placed between the vermiculite–peat mixture and the wall of the tube. The substrate was humidified with 10 ml of distilled water. Tubes were closed with a cellophane sheet and sterilized at 121°C for 20 min. Ten milliliters of MMN solution was dispensed to each tube followed by a second sterilization cycle under the same temperature and time conditions.

One seedling of *E. dunnii* was placed in each tube with its roots carefully arranged between the filter paper and the tube wall in order to allow the observation of the root system through the course of the experiment. A mycelial plus was placed on each side of the root system. The tubes were returned to the growth chamber and maintained at 25±2°C with a 12-h photoperiod.

After 5 weeks, seedlings were removed from the tubes and mycorrhizas were separated from the rest of the roots and studied using a dissecting microscope (10–30×) for their morphological characteristics including shape, branching, color, and texture. They were then photographed and placed in a NaCl solution (0.85%) and kept in the refrigerator overnight, before sections were cut using a cryo-microtome (20–30 μm) and mounted on glass slides with a drop of PVLG (16.6 g polyvinyl alcohol+100 ml lactic acid+10 ml glycerol+100 ml distilled water) and stained with a drop of cotton blue–lactoglycerol (0.04 g cotton blue+10 ml lactic acid+20 ml glycerol+10 ml distilled water). They were examined under an optical microscope (100–1,000×) in terms of external and internal microscopic features including the presence of extramatricial hyphae and rhizomorphs, mantle organization, Hartig net morphology, and details of external elements such as cystidia (Agerer 1987–1993; Ingleby et al. 1990; Goodman et al. 1996–1998). In all cases, mycorrhizal structures were noted and photographed.

Results

Amplification of the rRNA gene of *C. angustisporus* (isolate UFSC-Ch163), with primers ITS1 and ITS4 produced a 610-bp fragment containing ITS1, ITS2, and 5.8S regions. These regions presented a total of 237, 210, and 163 bp, respectively. The sequence falls in the same range length as those of the ITS1 and ITS2 regions of other ectomycorrhizal fungi available in the National Center for Biotechnology Information (NCBI) GenBank, even though their lengths varied among species and isolates.

Table 1 List of the ten closest relative fungi of *Chondrogaster angustisporus* isolate UFSC-Ch163 (accession EF989122[a]) as determined by the comparison of their ITS sequences

Organism	Accession[a]	Similarity (%)	References
Uncultured ectomycorrhizal	AM412270	58.5	Tedersoo et al. (2007)
Hysterangium sp.	DQ974736	45.8	Smith et al. (2007)
Gloeocantharellus purpurascens	AY872281	44.1	Hughes et al. (2004)
Gomphus clavatus	DQ365637	42.8	Dunham et al. (2006)
Ramaria sp.	DQ365606	42.6	Dunham et al. (2006)
Uncultured basidiomycete	DQ672289	42.2	Midgley et al. (2007)
Gautieria sp.	AF377077	39.2	Bidartondo and Bruns (2001)
Ramaria cedretorum	AJ408353	39	Daniels et al. (2001)
Ramaria botrytis	AJ292294	38.8	Daniels et al. (2001)
Sphaerobolus sp.	DQ979014	37.5	Tlapa and Dela Torre (2006)

[a] Accession number in the GenBank database, National Center of Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov)

When the ten highest scores obtained with the sequence of UFSC-Ch163 (accession EF989122) were compared with those of other fungi registered in the NCBI GenBank, it formed a separate cluster with an uncultured ectomycorrhizal fungus from the Seychelles (Tedersoo et al. 2007), accession AM412270 (Table 1).

The similarity of the 5.8S region of *C. angustisporus* and those of the ten other fungi was also elevated. In this case, *C. angustisporus* and the uncultured ectomycorrhizal fungus from the Seychelles, studied by Tedersoo et al. (2007), also formed a separated cluster. The results indicate a similarity between *C. angustisporus* and species of *Gloeocantharellus*, *Ramaria*, *Gautieria*, *Gomphus*, *Hysterangium*, and *Sphaerobolus*.

Colonized root tips (0.1 to 0.6 mm) were mostly unbranched and presented a light brown mantle with a slightly grainy texture when observed under a dissecting microscope (Fig. 1a). Rhizomorphs were not seen, though white hyphae emanating from the mycorrhiza surface were giving a felty aspect to the mantle. The general aspect of the colonized roots was similar to that of mycorrhizas formed on eucalypts by *Hysterangium* spp. described by Brundrett et al. (1996).

Transversal sections of these mycorrhizas observed under an optical microscope revealed the mantle to be formed by a single tissue layer, which was narrow but densely arranged, with ca. 8 µm width at the tip base. Mantle organization was of the net synenchyma type, with compact elongated hyphae, but without any obvious interhyphal spaces, except in the more external regions where hyphae were more loosely organized (Fig. 1b). Emanating hyphae were larger than the hyphae in the mantle, with a diameter of 2.5 µm and a granular content. At regular intervals, they were divided by septa with clamp connections (Fig. 1d). Bottle-shaped cystidia, with bent necks, were seen on the surface of the mantle (Fig. 1e).

The Hartig net, although easy to detect, was difficult to describe perhaps due to the quality of the sections. The

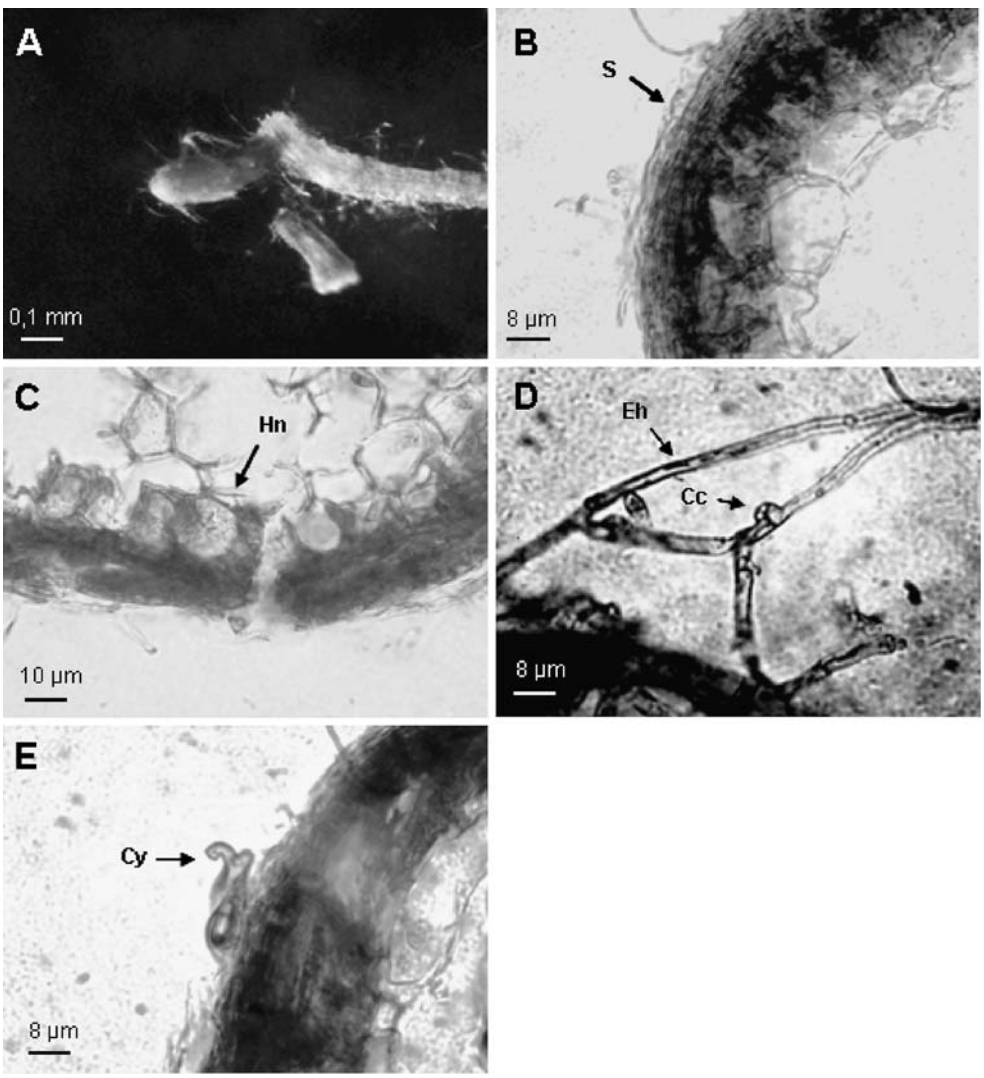

Fig. 1 Ectomycorrhizas of *Eucalyptus dunnii* seedlings formed with *Chondrogaster angustisporus*, UFSC-Ch163. **a** External view; **b–c** transversal sections showing the mantle (*S*) and the Hartig net (*Hn*); **d** extramatricial hyphae (*Eh*) showing clamp connections (*Cc*); **e** detail of the mantle showing bottle neck cystidia (*Cy*)

mycorrhizas were of a very delicate nature and sections were hard to obtain. Nevertheless, the Hartig net was of the common and widely distributed type according to Goodman et al. (1996–1998), presenting typical palmetti and single hyphal rows (Fig. 1c). The Hartig net penetration was limited to the epidermal layer which is typical of eucalypt ectomycorrhizas and other angiosperms (Brundrett et al. 1996; Peterson et al. 2004). At most of the points, there was only a partial encircling of the epidermal cells by the hyphae producing the so-called para-epidermal Hartig net but occasionally Hartig net encircled the whole epidermal cell resulting in a peri-epidermal type (Fig. 1c).

Discussion

In this study, we verified that the ITS sequence of *C. angustisporus* is similarly related to the ITS sequences of some ectomycorrhizal fungi available in the database. This is the first ITS sequencing from this ectomycorrhizal fungus. Its 5.8S gene sequence presents a high similarity with those obtained from species of other putative ectomycorrhizal fungi of the genera *Gloeocantharellus*, *Ramaria*, *Gautieria*, *Gomphus*, *Hysterangium*, and *Sphaerobolus* and supports the present view that places the genus *Chondrogaster* in the subclass Phallomycetidae formed by the gomphoid–phalloid group, as proposed by Hosaka et al. (2006).

The high similarity between *C. angustisporus*, isolate UFSC-Ch163, in relation to the uncultured ectomycorrhizal fungus reported by Tedersoo et al. (2007) on *Eucalyptus robusta* mycorrhizas in Seychelles, suggests that this latter fungus has been introduced in the Seychelles from Australasia perhaps with eucalypt seedlings, seeds, or soil. This may also be the case in Brazil, Uruguay, and in Spain where *C. angustisporus* and other related fungi such as *Hysterangium* spp. and *Ramaria* spp. have been commonly observed with eucalypt plants. Strong support of this hypothesis is demonstrated by the results of Tedersoo et al. (2007) where the fungus was observed on *E. robusta* root tips but not on the native plants examined, although *E. robusta* and native plants shared other ectomycorrhizal fungi.

The similarity between UFSC-Ch163 and the uncultured ectomycorrhizal fungus (AM412270) studied by Tedersoo et al. (2007) was particularly high in relation to the ITS2 and the 5.8S regions. However, the differences in terms of the ITS1 regions were such that the two fungi could not be considered as belonging to the same species or potentially the same genus. Another species of this genus, *Chondrogaster pachysporus* Maire, was described by Maire in 1924, and has been found in other regions of the world (Castellano et al. 1989).

The morphological characters of the fruiting bodies have been used to distinguish *Chondrogaster angustisporus* (Giachini et al. 2000, 2004; Alvarez and Cerceda 2005) from other ectomycorrhizal fungi. But this is the first description of the mycorrhizas formed by this fungus on eucalypt roots.

This present isolate is part of an ectomycorrhizal fungi culture collection available at the Microbiology Department of the Federal University of Santa Catarina, Florianópolis, Brazil. These cultures are maintained on MMN–agar medium, where sucrose, present in the original medium (MN) at 2.5 g l^{-1} (Norkrans 1949), is replaced by glucose at 10 g l^{-1} (Marx 1969). Activated charcoal is added to this culture medium at a rate of 2 g l^{-1} in order to improve fungal viability. As with most ectomycorrhizal fungi, this isolate grows slowly in comparison with free-living microorganisms. In liquid culture, this isolate produces a specific growth rate (μ_X) of 0.19 day^{-1} and a productivity of 0.26 g l^{-1} day^{-1} (Rossi 2006).

The ITS sequences from this work could facilitate the development of further specific PCR primers for use in a rapid and reliable identification of *Chondrogaster angustisporus* regardless of environmental or host conditions, allowing study of the host range of C. *angustisporus*. Future studies are needed in order to compare the present isolate with other *Chondrogaster* isolates/species using their ITS regions, in order to elucidate the level of inter- and intra-specific polymorphisms within the rRNA gene ITS region of these ectomycorrhizal fungi.

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