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Molecular and taxonomic characterization of a endophytic fungus isolated from *Helleborus bocconei* subsp. *intermedius* (*Ranunculaceae*)

Abstract

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A non-sporulating fungus was isolated from different organs of *Helleborus bocconei* subsp. *intermedius* (*Ranunculaceae*) endemic to southern Italy and Sicily, known for the traditional use of dried roots in the treatment of lung diseases of cattle and horses.
Molecular characterization of endophytic fungus based on the internal transcribed spacer (ITS) region of the rRNA gene sequences was done. The DNA sequence of full length ITS region of the studied fungus was a 100% match to that of *Chaetomium strumarium* strain dH 21642 (GenBank accession number JX280851.1). The morphological characters of colony and mycelium of this microfungus are reported here.

Key words: *Chaetomium strumarium*, *Chaetomiaceae*, endophyte, endemic plant, molecular analysis, folk veterinary medicine.

Introduction

On the basis of the elements of biological and phytochemical importance arose during the biological and ethno-pharmaco-botanical study of *Helleborus bocconei* subsp. *intermedius* (Guss.) Greuter & Burdet, (Spadaro 2006), further investigations were carried out in order to explain the pharmacological action trough which the traditional use of the dried roots of the plant in the veterinary treatment of lung diseases of cattle and horses (Raimondo & Lentini 1990) is based. As we know, *H. bocconei* subsp. *intermedius* [*H. bocconei* subsp. *siculus* (Schiffner) Merxm. & Podl.], *Ranunculaceae* family, endemic to Sicily and southern Italy, locally known as “radicchia”. Its study has shown particular endophytic relationships between a microfungus and the host plant, that had been found during morpho-anatomical observations also in other taxa of the same genus as *Helleborus lividus* subsp. *corsicus* (Willd.) Tutin, *H. viridis* L., *H. orientalis* Lam., *H. foetidus* L. and *H.* × *hybridus* Hort. ex Vilmorin (Spadaro & al. 2006; Spadaro & al. 2007). Phytochemical investigations, however, allowed the isolation and characterization of new biologically active molecules (Rosselli & al. 2006) revealing, some antibacterial properties in the

extracts of the roots and aerial parts of the plant investigated (Rosselli & al. 2007) and a cytotoxic activity of some compounds isolated from methanolic extract of the roots (Rosselli & al. 2009). According to these studies, and considering the importance of endophytic microorganisms as sources of new biologically active secondary metabolites (Schulz & al. 2002); further mycological analyses of *H. bocconei* subsp. *intermedius* from Sicily were considered advisable. Compared to the preliminary data for the first isolation of an endophyte from the same plant and referred to *Botrytis byssoidea* Walker (Spadaro & al. 2007), after a deeper study several endophytic and commensal microfungi were identified. Among them, an endophyte was constantly found - by preliminary antibiosis assays – showing an antibacterial activity (Spadaro & al. 2011), which is still under investigation. What has above summarized the shows the interest of characterizing and identifying the micromicete in question. Its endosymbiosis with Sicilian Hellebore becomes very important in view of a larger and more comprehensive study aimed to prove scientifically the reliability of the empirical use of the plant in the veterinary treatment of lung diseases of cattle and horses.

Materials and methods

The *Helleborus bocconei* subsp. *intermedius* materials were collected in different seasons, between April and September 2010, in the locality Grotta del Garrone, near Monte Pizzuta (Piana degli Albanesi, Palermo). In each season, five plants were collected and transported to the lab in sterile polythene bags. The samples were stored at 4°C and processed within 24 hours from collection.

A voucher specimen (No. 3/10) was besides deposited in the *Herbarium Mediterraneum Panormitanum* (PAL) of the University of Palermo.

Isolation of endophyte

The endophytic fungi were isolated from different organs (root, rhizome, stem and leaf) with surface-sterilizing method (Bayman & al. 1997). A preliminary analysis was carried to determine the parameters of surface sterilization by H_2O_2, owing to the sterilization last varies from plant to plant (Hallmann & al. 2006).

Plants were washed under running water, then washed again in sterile distilled water to remove adhering soil particles and the majority of microbial surface epiphytes and other occasional colonizers. The samples were cut under sterile conditions to separate root, rhizome, stem and leaf, and surfaces sterilized with 3.5% hydrogen peroxide for 8' and, in the case of the root, 10'; these were successively re-immersed in sterile distilled water to remove residues of sterilizing. The plant organs so sterilized were cut into small pieces of 1cm and plated on Mycological agar (Difco) with 200 mg/l of streptomycin sulphate (sterilized by filtration -0.2µm pores) to prevent bacterial growth. The plates were incubated at 25° C for three weeks. Hyphal tips of fungi emerging out of the plant tissues were picked and grown on Mycological agar in pure cultures.

The morphological identification was carried on microscopic slides under light microscope.

To obtain sporulation of non–sporulanting strains several different media were utilized: Potato Dextrose Agar (PDA-Difco), Czapek Yeast Agar (saccharose 30 g, sodium nitrate 2 g, dipotassium phosphate 1g, magnesium sulphate 0.5 g, potassium chloride 0.5 g, ferrous sulphate 0.01 g, yeast extract 5 g, agar 15 g dissolved in 1000 ml of distilled H_20) and Malt Extract Agar 2% (20g malt extract, peptone 1g, glucose 20g, 15g agar dissolved in 1000 ml of distilled H_20).

DNA isolation, amplification and sequencing

Genomic DNA was extracted using Automated DNA Purification on the Maxwell® 16 Instrument. The protocol was performed using the cartridges "Cell LEV (Low Elution Volume) total RNA purification kit" in a final volume of 50 µl of Nuclease-Free Water (provided in the kit). For the molecular analysis the Internal Transcribed Spacer region (ITS1-5.8S-ITS2) of the nuclear ribosomal DNA (rDNA), was amplified by PCR. The amplification was carried out in a final volume of 25 µl, using 2 µl of extracted DNA and the reaction mixture containing 0.5 µl of each primer 10 mM, 2.5 µl of MgCl2 25mM (Promega), 1.5 µl of 5x buffer (Promega), 0.5 µl of dNTPs 10 mM, and 5 U of Go-Taq Polymerase (Promega); the final volume was reached adding ultrapure water. After amplification, the species identification was carried out by direct DNA sequencing of the PCR products. For the ITS region (ITS1-5.8S-ITS2) rDNA, was amplified a 520 bp fragment using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), (White et al., 1990). The PCR amplification was performed as follow: 3 min at 95 °C, followed by 30 cycles of 30 sec at 95 °C, 30 sec at 54 °C, 45 sec at 72 °C, and a final extension of 10 min at 72 °C (White et al., 1990). ITS sequences were checked for similarity using a basic local alignment search tool (BLAST), (http://www.ncbi,nml.nih.gov/BLAST/). Taxonomic assignments were based on phylogenetic position and similarity to reference sequences of the GenBank database.

ITS sequences showing the highest score and other ITS sequences representative of the genus *Chaetomium* (*Chaetomiaceae*) were retrieved from a GenBank and aligned using ClustalX software version 1.8 (Thompson & al. 1997). A neighbour-joining tree was obtained using the software MEGA version 4 (Tamura & al. 2007) based on two-parameter distance model of Kimura (1980).

Results and discussion

In the present study some endophytic and commensal microfungi were identified. On the whole for each taxon found no significant variations were observed within the same communities with respect to different organs as well as to material sampled in different seasons; only the strain RR1 resulted recurrently present in all organs studied. Preliminary study on this fungus also showed an interesting antibacterial activity (Spadaro & al. 2011).

In Mycological agar (Fig 1 a), the development of RR1 strain is rapid, tumultuous, perfectly centrifugal, in 24 hours reaches a diameter of 1.2 mm; in 48 hours 8 mm; in 72 hours 14 mm. The colonies, after 15 days of development, are woolly, light brown in the middle,

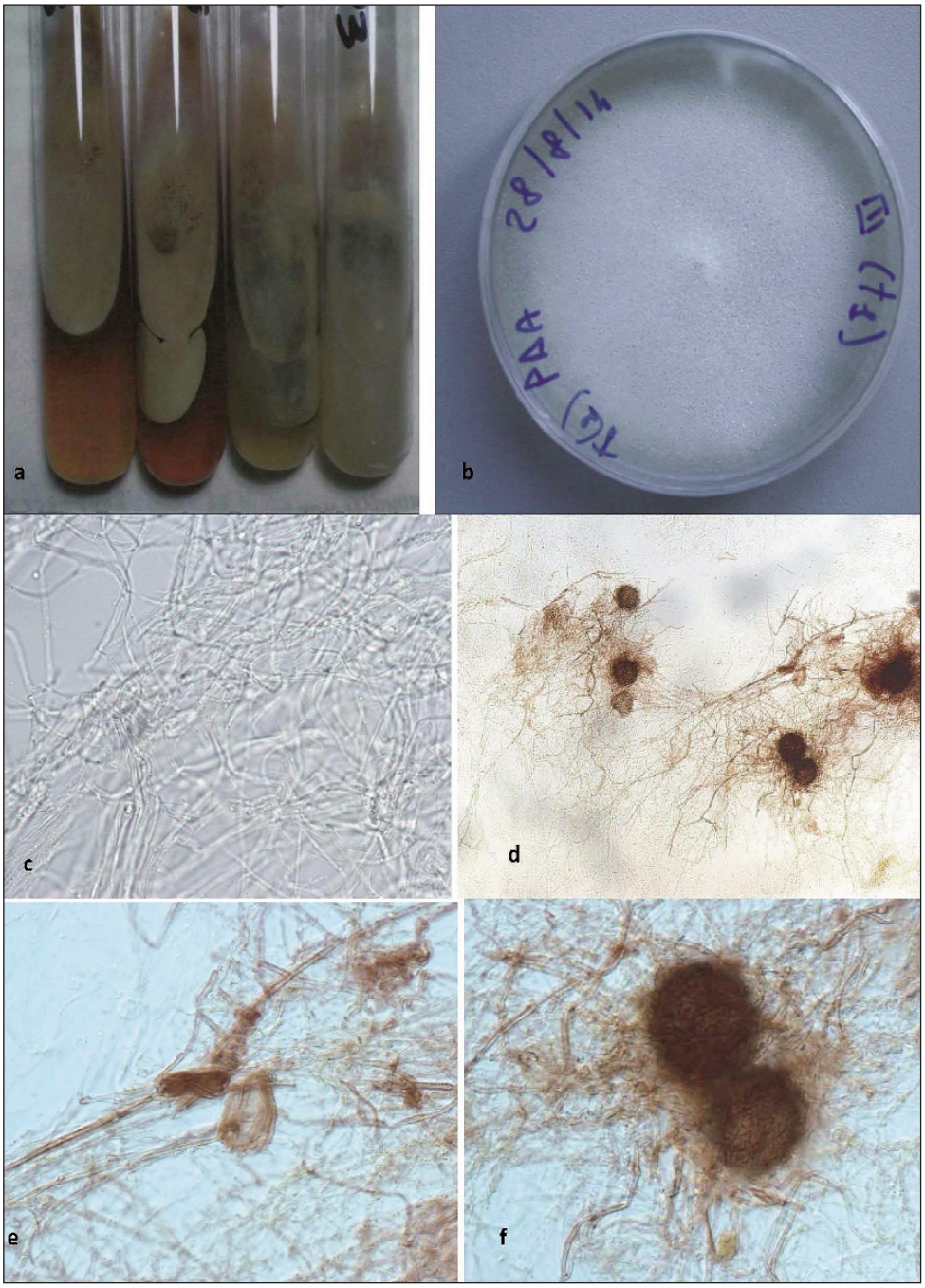

Fig. 1. *Chaetomium strumarium* strain RR1 : **a.** colony on Mycological agar; **b.** colony on PDA; **c.** mycelium with hyaline hyphae (×400); **d.** cleistotecial-like structures on MEA 2% (×200); **e.** mycelium with enlarged coiled hyphae (×400); **f.** cleistotecial-like structures on MEA 2% (×400).

with colours that fade to white towards the periphery, the reverse has richer colours: reddish brown in the middle, with colours that blend into the surrounding areas. They are composed of abundant mycelium with hyphae – observed at 750 magnifications – smooth-walled, frequently branched, septate, hyaline or slightly chlorine (Fig. 1 c), with straight or slightly sinuous trends, generally with homogeneous protoplasmic content, 4 to 5 μm in width. Sometimes, they are greatly enlarged and spiralate (Fig. 1e), folding back on itself repeatedly. No reproductive structures were found (Fig. 1b).

To obtain sporulation different media were utilized PDA, CYA and MEA 2%. No conidial structure was observed; in MEA 2% cultures up to 90 days old, some sterile cleistothecial-like structures were observed (Fig. 1 d, f).

RR1 endophyte produced only sterile mycelium and was not taxonomically identifiable by morphological study; for taxonomic attribution the strain was subjected to the molecu-

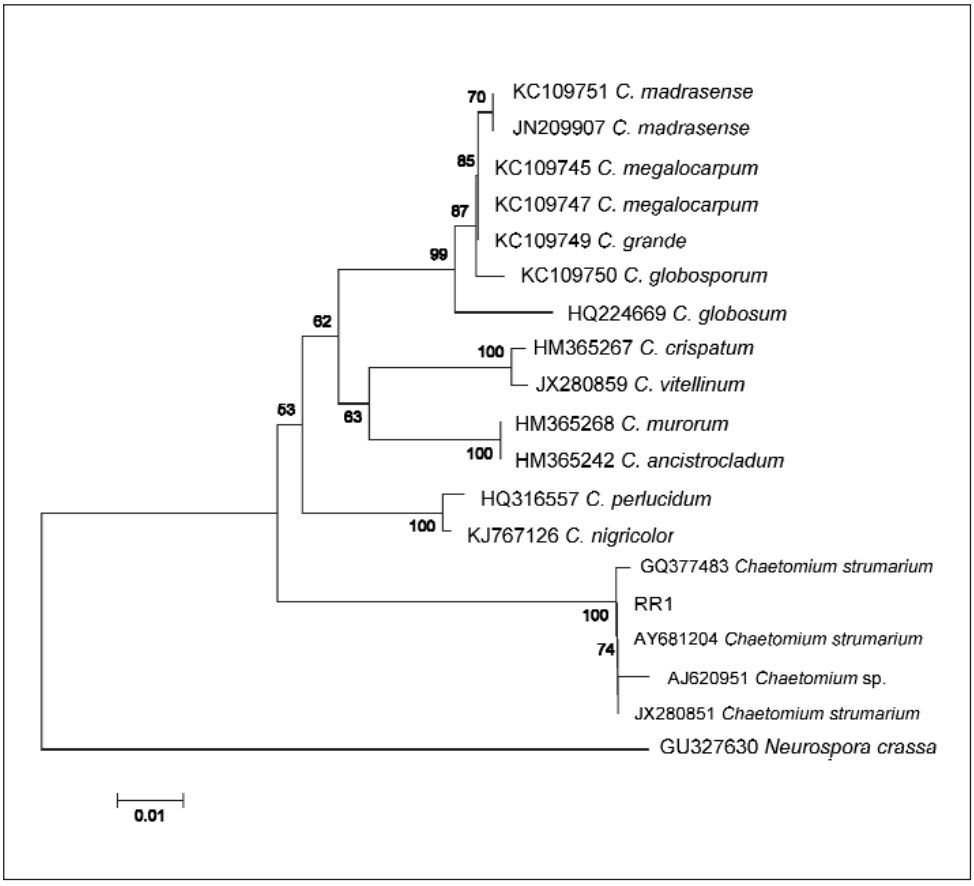

Fig. 2. ITS neighbor-joining tree showing the relationship of RR1 sample with other *Chaetomium* species. Numbers near the branches indicate bootstrap values (percentage over 1000 replicates). *Neurospora crassa* Shear & B.O. Dodge is outgroup.

lar analysis of ITS region (Aggarwal & al. 2013; Quadri & al. 2013). PCR amplification with IT5/ITS4 primers pairs produced an amplicon of about 550pb. Similarity search against GenBank database using blast showed that the obtained sequence is similar to ITS sequences of *Chaetomium strumarium* (J.N. Rai, J.P. Tewari & Mukerji) P.F. Cannon [*Achaetomium strumarium* J.N. Rai, J.P. Tewari & Mukerji; *Achaetomium cristalliferum* Faurel & Locq.-Lin.].

On the basis of molecular analysis the strain RR1 identified as *Chaetomium strumarium* (identity 98 to 100%) quite identical to *Chaetomium strumarium* strain dH 21642 (de Hoog et al. 2013).

In order to evaluate more in detail the taxonomic group to which our strain RR1 can be ascribed, ITS of references species were retrieved from GenBank and used to perform a phylogenetic analysis. The sequences considered in the phylogenetic analysis were selected on the basis of the blast results and sequences referenced in published works (Asgari & Zare 2011; Aggarwal & al. 2013; de Hoog & al. 2013). The phylogenetic analysis (Fig. 2) confirms the blast results. The sample RR1 forms a highly supported cluster (100% of bootstrap replicates) with *Chaetomium strumarium* (syn. *Achaetomium strumarium*).

Conclusions

The above described mycological and molecular analyses have allowed to characterize and identify as *Chaetomium strumarium* (*Chaetomiaceae*) the RR1 fungal strain isolated from different organs of *Helleborus bocconei* subsp. *intermedius*. Other authors have used the sequences of ITS to determine non-sporulating species of *Chaetomium* (Mootha & al. 2012; Quadri & al. 2013). The identification of this endophyte of the Sicilian Hellebore, further enriches the research undertaken by new elements and, therefore, interesting. In recent literature (Ranadive & al. 2013), *C. strumarium* is reported among the fungi with antimicrobial activity. This would, of course, support the antibacterial activity found in strain examined in the preliminary antibiosis assays. The results encourage the authors who propose to make further isolations not only of Sicilian Hellebore, in order to consider additional fungal endophytic component, but also from other congener taxa on which they were made, as already mentioned, morpho-anatomical observations. In addition, attention will be focused even more on antibacterial activity of the endophyte isolated and here analyzed, in order to be able to contribute to explain on the one hand the traditional use of dried roots of *Helleborus bocconei* subsp. *intermedius* in the veterinary treatment of lung diseases of cattle and horses, on the other side the possible applications in the field of therapeutic of potential pharmaceutical products obtainable.

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