MEDICINAL MUSHROOMS IN ITALY AND THEIR *EX SITU* CONSERVATION THROUGH CULTURE COLLECTION

E SAVINO[1]*, C GIROMETTA[1], S CHINAGLIA[1], M GUGLIELMINETTI[1], M RODOLFI[1], A BERNICCHIA[2], C PERINI[3], E SALERNI[3] AND AM PICCO[1]

[1]Department of Earth and Envtl Science (DSTA), University of Pavia, Via S. Epifanio 14, 27100 Pavia, Italy
[2]Department of Agricultural Sciences, University of Bologna, - Via A. Guidotti 39, 40134 Bologna, Italy
[3]Department of Life Sciences (DSV), University of Siena, via P.A.Mattioli 4, 53100 Siena, Italy
elena.savino@unipv.it

ABSTRACT

A wide variety of mushrooms, including the edible, nutraceutical and medicinal ones, are present in Italy, a small country characterized by a great habitat richness. Inevitably, the extensive action of many mycophiles collecting sporophores, mainly for culinary uses, can impact on this important source of biodiversity. In order to protect the rare *taxa*, also collecting their biological material for application purposes, conservation *ex situ* is suggested.

Wood-inhabiting fungi were collected from Italian Alps, Apennines, wood plains and Mediterranean areas. Pure culture isolation was carried out from fruiting bodies in experimental sterile conditions. The culture collection has been registered at the Mycological Laboratory of Pavia University (Italy). In few years about 150 species were isolated and, among them, some rare and precious *taxa* such as *Ganoderma pfeifferi* Bres., *Laricifomes officinalis* (Batsch) Kotl. & Pouzar, *Lenzites warnieri* Durieu & Mont. and *Perenniporia meridionalis* Decock & Staplers deserve to be mentioned. It is well known that medicinal mushroom bioactivities could depend not only on biological characteristics (*i.e.* the genetic profile) but also on the geographical origin and the substrate. Therefore, Italy could really become a new resource of different fungal ecotypes, characterized by different and potentially useful properties worthy to be investigated.

Keywords: medicinal mushrooms, mycelia, sporophores, wood-inhabiting fungi

INTRODUCTION

Italy is located in Southern Europe and comprises the boot-shaped Italian Peninsula and a number of islands including the two largest, Sicily and Sardinia. Most of the land is surrounded by Mediterranean sea. It is crossed by Apennine Mountains, which are like a backbone, while the Alps form most of its northern boundary, where Italy's highest point is located on Mont Blanc (4,810 m/15,782 ft). The longest river is the Po, that flows in the Padan plain, bounding area of the continental climate from the Mediterranean one. In the north a few lakes, due to glacial deposits, lead to Mediterranean microclimates too. The country is situated at the meeting point of the Eurasian Plate and the African Plate, leading to considerable seismic and volcanic activity: there are 14 volcanoes in Italy, four of which are still active.

Thanks to the longitudinal extension of the peninsula and to the mostly mountainous internal conformation, the climate of Italy is highly diverse. In most of the inland northern and central regions, the climate ranges from humid subtropical to humid continental and oceanic. The coastal areas generally fit the Mediterranean climate stereotype (Köppen climate classification Csa). Conditions on peninsular coastal areas can be very different from the interior's higher ground and valleys, particularly during the winter months when the higher altitudes tend to be cold, wet, and often snowy. The coastal regions have mild winters and warm and generally dry summers, although lowland valleys can be quite hot in summer.

Therefore, even if Italy is a relatively small country it is characterized by numerous different habitats where many fungal species are present [1, 2] as well as many mycophiles collecting fruiting bodies mainly for culinary uses. Anyway the use of wild fungi also for other purposes dates back to ancient civilizations, for instance *Amanita caesarea*, Caesar's mushroom [1]. Despite the long tradition the toxicity or edibility of some non-timber forest product is still dubious. Keeping on this topic, it is well known that the genus *Amanita* includes various species edible or not, poisonous and sometimes even deadly: among the group of the white ones, *Amanita ovoidea* is described as both edible and with a good taste, on the

other hand this mushroom seems to have caused several cases of poisoning, at least in Italy, classified as allenic norleucine syndrome. Phytochemical screening shows a very low presence of amino acids and for this the poisoning by allenic norleucine seems to be excluded. In respect to the environment, *A. ovoidea*, like the edible *A. caesarea*, can accumulate high amounts of heavy metals and could therefore be harmful to humans [2]. Finally, as said before, there are still to solve numerous questions even if scientific and technological progress is increased. In spite of the numerous activities of Italian mycologists, belonging to scientific research centres or amateur groups, concerning different aspects such as biodiversity and mapping [3, 4] the importance of fungi in nature conservation is still limited and it has to be vigorously promoted.

Besides, during the last decade, the interest in medicinal mushrooms has been increasing, especially because almost all the better-known species having officinal properties grow in the Italian areas. In order to protect these fungal species from an extensive collection threatening the rare *taxa*, conservation *ex situ* could be suggested [5, 6]. Culture collections play a key role in preservation and maintenance of fungal genetic resources and they are an important tool to get biological material for application purposes [7].

Aims of the present work are: a) to investigate selected areas favorable to the development of wood-inhabiting basidiomata; b) to identify the species by means of the morphological approach; c) to isolate the mycelia in pure culture; d) to confirm the strain identification by molecular analysis.

MATERIALS AND METHODS

Sampling sites

According to the specific research activities of the authors, some Italian environments were investigated more in detail and for a longer time than others. Wood-inhabiting fungi were collected from different habitats of Italian Alps, Apennines, wood plains and Mediterranean areas. The attention has been particularly focused on both "polyporoid" fungi and *Corticiaceae* [8-11].

Pure culture isolation

Isolation of mycelia in pure culture was performed according to the methodology provided by [12] and [13]. Each one of the following steps presupposes high attention in preventing fungal material and culture medium from any external contaminants; decayed and/or infested specimens were avoided as well. 1) The sporophores were opened aseptically to make the context accessible; 2) few mm^3 of context were drawn by means of lancet; 3) drawn material was inoculated by penetration into Petri dishes containing culture medium (e.g. MEA 2% or PDA 4%); 4) most species grew well at 25 °C, during their initial phase. At first, antibiotics were added to the cultural medium. Each strain was registered at the Mycotheque of Pavia University (DSTA) and to the Culture Collection of Miconet Srl (Pavia University-academic spin off). Cultures were maintained at 4 °C on 2% Malt Extract Agar (MEA) plates (Biokar Diagnostics).

Molecular analyses

Cultures were grown in 200-ml Erlenmeyer flasks containing 50 ml of malt extract broth on a rotary shaker (100 rpm) for 10 d at 25 °C. Biomass was collected in microcentrifuge tube and DNA was extracted with CTAB method [14].

CTAB (hexadecyltrimethylammonium bromide) lysis buffer (2%) were added to fresh biomass and mycelium was ground with sterile pestle. Extraction was performed with a phenol-chloroform- isoamyl alcohol (25:24:1) solution. Afterward the phases were separated by centrifugation and DNA was precipitated from the aqueous phase with an equal volume of isopropanol. Total nucleic acids were collected by centrifugation, the pellet was rinsed with 70% ethanol, and the nucleic acids were dissolved in 100 µl sterile deionized water.

Internal transcribed spacer- ITS region was amplified using the primer pair ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 5'–TCCTCCGCTTATTGATATGC-3' [15]. Each PCR reaction contained 1 µl of fungal DNA, 1X DNA polymerase buffer, 0.2 mM dNTP mix (dUTP, dATP, dGTP, dCTP), 0.2 ìM (each) of ITS1 and ITS4, and 0.025 U/îl of *Taq* DNA polymerase (5 PRIME, Germany) in a total volume of 25 µl.

The amplification reactions were run in a BIORAD thermocycler with an initial denaturation of 5 min at 95 °C, followed by 39 cycles of 35 sec at 95 °C, 35 sec at 55 °C, and 50 sec at 72 °C, with a final extension step of 72 °C for 8 min. PCR products were analyzed by electrophoresis in a 1% agarose gel in 1X TAE buffer [(40mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8)], stained with ethidium bromide. Furthermore, the PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and sent to Macrogen (The Netherlands) for sequencing. The obtained sequences were assembled, corrected and subsequently analyzed by BLAST searches using the GenBank (NCBI) and Mycobank (CBS) databases. Taxonomic assignment were based on similarity to reference sequences of these databases; afterward genotypic identification was compared with phenotype identification.

RESULTS AND DISCUSSION

In few years about 150 species were isolated, among them there are some rare and precious *taxa* both with well known medicinal properties, such as *Laricifomes officinalis* (Batsch) Kotl. & Pouzar (1.2.Lo1 & 1.2.Lo2) and *Ganoderma pfeifferi* Bres. (1.2.Gpf1), and others never investigated before, such as *Lenzites warnieri* Durieu & Mont. (1.2.Lw1, 1.2.Lw2, 1.2.Lw3, 1.2.Lw4) and *Perenniporia meridionalis* Decock & Stalpers (1.2.Pm1). To the best of our knowledge, the strains of all these species are maintained in the Mycotheque of Pavia University (DSTA) and in the culture collection of Miconet representing the first Italian isolations.

Molecular analysis were carried out in order to confirm the isolates or to supplement morphological identification of those fungal strains belonging to *taxa* whose identification was critical. The comparison of the obtained ITS sequences obtained with the ones deposited in the available databases has allowed to confirm the taxonomic assignment at the species level, with a similarity higher than 99%. *Ganoderma* resulted the genus with major discrepancies between morphological and molecular analyses; however it is well known that it presents a great variability in macroscopic characters of basidiocarps, thus leading to confusion in the taxonomy of the genus too [16]. The same authors declared that *Ganoderma* is the most difficult genus of all polypores. Almost all the species present in Europe have been collected during this study, for many of them the mycelium was successfully isolated in pure culture. The most remarkable strain resulted *G. pfeifferi*, a species that grows quite exclusively in Europe, till now poorly investigated. Some interesting data published in the last years report the identification of new chemical substances related to it, named ganomycins A, B and K [17, 18]. *G. pfeifferi* has been reported only in few Italian locations, mostly in Tuscany (Central Italy) [3].

Among the macrofungi famous for their medicinal properties, *L. officinalis* was detected, a lignicolous fungus strictly related to the holoartic mountains, on the Alps it grows exclusively on old and thick larch trees (*Larix decidua*), a species endemic for Europe. The fungus has been well known since 1st Century A.D. for its healing properties, being used to treat several diseases, especially pulmonary ones. It is nowadays recognized that *L. officinalis* produces antibiotic substances, particularly efficient against *Mycobacterium tuberculosis* [19, 20]. Due to the past extensive collection, *L. officinalis* became so rare to be supposed almost extinct. Consequently eight European countries (Italy not included) included it into the Red Lists of threatened species. In Switzerland, for example, programs of habitat protection together with a specific census let *L. officinalis* spread again [21]. In Italy, till now, the sporocarps of this species have been registered in only four locations: in the Graian Alps two reports (2000 and 2013)**,** one report in the Retic Alps (2013) and one nearby the "Monte Rosa" (2014). Consequently, its inclusion in the Italian Red Lists as near threatened (NT) has been recently proposed (personal communication). The mycelium was isolated from Graian (Fig. 1) and Retic (Fig. 2) specimens, both collected in late summer 2013. The sporophores did not present all the morphological characters useful to identify to species level. The exact identification was obtained by DNA analysis (Fig. 4) of liquid culture of the mycelia isolated from Graian (1.2.Lo2 – Fig. 3) and Retic (1.2.Lo1) specimens. Sequences are going to be deposited in GenBank as soon as possible.

Among the uncommon or rare and poorly investigated species, *L. warnieri* and *P. meridionalis* deserve more attention. Both prefer warm environments and are considered rare in Europe. However, in recent years the reports of the former in Italy are steadily increasing. The Authors collected *L. warnieri* in different sites of Northern Italy, from humid plains to Apennines. It was registered also in other few Italian areas [3]. The strains of this species are not always are present in the International Culture Collections. In the Mycotheque of the Pavia University four different strains (all collected in Pavia

Figure 1. Specimen from Graian Alps (2013)

Figure 2. Specimen from Retic Alps (2013)

Figure 3. Mycelium in pure culture (1.2.Lo2)

Figure 4. Elettrophoretic running of PCR products: 3a and 3b respectively belong to Retic and Graian strains (1.2.Lo1 & 1.2.Lo2)

province) have been maintained till now and their identification has been confirmed by means of the analysis of ITS sequences, compared with Mycobank and GenBank databases. The genus *Perenniporia* was revised by Decock and Stalpers [22] with the assessment of *P. meridionalis* at species level, previously named *P. medulla-panis*. Despite this taxonomic problem, the species is quite rare in our Country [3, 5]. It was collected only once, in Northern Italy, nearby the Iseo lake. Also in this case, the DNA analysis agrees with the morphological identification. Finally, recent studies [23] revealed that both the detected species possess interesting enzymatic activities, when they grow on a natural substratum.

CONCLUSION

It is well known that medicinal mushroom bioactivities could depend on different characteristics such as the genetic profile, the geographical provenance and the substrate [24]. Therefore Italy, as already happened for other countries, could become a very interesting source of different fungal ecotypes, characterized by new and different properties worthy to be investigated. Furthermore, some species collected by the Authors in Italy (for example *L. officinalis*) are classified as endangered and protected species in other European countries. Their *in situ* conservation would be consequently necessary, avoiding the destruction of the habitat and preserving these fungi from uncontrolled actions of picking. Moreover, as quoted by other authors [25 the *ex situ* conservation by means of culture collections is an indisputable tool to get biological material for application purposes and to improve sustainable agriculture and economy.

ACKNOWLEDGMENTS

This research was supported by "Ministero dell'Ambiente e della Tutela del Territorio e del Mare" within the project "Management of the RNIS e Bosco S. Negri (Zerbolò-Pavia-Italy)". The authors are very grateful to dr. Elisa Altobelli and Mrs. Teresa Mangiapane for their laboratory support.

REFERENCES

[1] Boa E. (2004). Wild edible fungi. A global overview on their use and importance to people. In: Non-food forest products. 17, pp. 147, ISBN 92-5-105157-7.

[2] Biagi M *et al.* (2014). Investigations into *Amanita ovoidea* (Bull.) – Link.: Edible or Poisonous? *Nat. Resour. J*. 5: 225-232.

[3] Onofri S *et al.* (2005). Checklist dei funghi italiani. Basidiomycetes. ISBN 88-7138-387-7.

[4] Siniscalco *et al.* (2013). Storia della micologia italiana e primo contributo alla nomenclatura corretta dei funghi, pp. 512, ISBN 978-88-448-0592-0.

[5] Bernicchia A. (2005). *Fungi Europei - Polyporaceae s.l.*, pp. 808, ISBN 88-901057-5-5.

[6] Donnini D *et al.* (2013). Wild and cultivated mushrooms as a model of sustainable development. *Plant Biosyst.* 147(1): 226-236.

[7] Perini C *et al.* (2011). The important plant areas program from a mycological point of view: the regional experience in an European context. *Fitosociologia*. 48(2) suppl.1: 155-161.

[8] Saitta A *et al.* (2011): Biodiversity of wood-decay fungi in Italy. *Plant Biosyst.* 145(4): 958-968.

[9] Altobelli E *et al.* (2012). Harvesting, isolation and cultivation of polyporoid fungi with medicinal properties. *Micologia Italiana* XLI (1): 3-10.

[10] Mammarella B *et al.* (2014). Macromiceti lignicoli in ambiente mediterraneo: il caso di studio del Parco Regionale della Maremma (Grosseto). *Micol. Veget. Medit.* 29 (1): 65-74.

[11] Bernicchia A. (2010). *Fungi Europei - Corticiaceae s.l.* pp. 1008, ISBN 978-88-901057-9-8.

[12] Stalpers JA. (1978). Identification of wood-inhabiting Aphyllophorales in pure culture. *Studies in Mycology* 16: pp. 248, CBS (NL).

[13] Stamets P. (2000). Culturing Mushroom Mycelium on Agar Media. In: Growing Gourmet and Medicinal Mushrooms, pp. 88-91, ISBN-10: 1-58008-175-4.

[14] Doyle JJ and Doyle JL. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*. 19: 11-15.

[15] White TJ *et al.* (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetic. *PCR protocols: a guide to methods and applications*. 18: 315- 322.

[16] Ryvarden L and Gilbertson RL (1993). *European Polypores.* Part 1 & 2. pp 743, ISSN 0802-8966.

[17] Mothana R *et al.* (2000). Biologically active compounds of *Ganoderma pfeifferi* dms 13239. PCT Int. Appl., WO 2000053207 A1 20000914.

[18] Niedermeyer THJ *et al.* (2013). Isolation of farnesyl hydroquinones from the basidiomycete *Ganoderma pfeifferi*. *Nat. Prod. Bioprospect.* 3(4): 137-140.

[19] Hwang CH *et al.* (2013). Chlorinated coumarins from the Polypore Mushroom *Fomitopsis officinalis* and their activity against *Mycobacterium tuberculosis*. *J. Nat. Prod.* 76: 1916-1922.

[20] Grienke U *et al.* (2014). European medicinal polypores – A modern view on traditional uses *J. Ethnopharmacol*. 154: 564–583.

[21] Senn-Irlet B. (2012). Fiches pratiques sur les champignons: Polypore officinal. *Laricifomes officinalis* (Vill.) Kotl. et Pouz. www.wsl.ch/notice_champignons *Birmensdorf, Institut fédéral de recherches WSL*. 2 p.

[22] Decock C and Stalpers JA. (2006). Proposal to conserve the name Perenniporia against Physisporus with a conserved type (Basidiomycota). *Taxon*. 55(1): 227.

[23] Doria E *et al.* (2014). Evaluation of lignocellulolytic activities of ten fungal species able to degrade poplar wood. *Inter. Biodet. & Biodegr.* 94: 160-166.

[24] Psurtseva N and Ozerskaya S. (2013). Culture collections: Germplasm resources for medicinal mushroom science and production. In: *Abstracts IMMC* 7, 22-24.

[24] Arora DK *et al.* (2005). Current status, strategy and future prospects of microbial resource collections. *Current Science*. 89(3): 488-495.