Multiplex PCR for the identification of white Tuber species

Antonella Amicucci a, Chiara Guidi a, Alessandra Zambonelli b, Lucia Potenza a, Vilberto Stocchi a,*

a Istituto di Chimica Biologica 'Giorgio Fornaini', Università degli Studi di Urbino, Via Saflì 2, 61029 Urbino (PS), Italy
b Dipartimento di Protezione e Valorizzazione Agroalimentare, Università di Bologna, Via Filippo Re 8, 40126 Bologna, Italy

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Abstract

Species-specific primers selected from the internal transcribed spacer region sequence were used to set up a multiplex polymerase chain reaction (PCR) able to simultaneously identify the white truffle species Tuber magnatum, Tuber borchii, Tuber maculatum and Tuber puberulum. Furthermore, a primer specific for the competitive fungus Sphaerospora brunnea was designed and added to the multiplex PCR set, allowing the detection of the Tuber species and the contaminant fungus in a one-step reaction. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Truffles are soil fungi that live in intimate association with many higher plants. Together they form ectomycorrhizae, mutualistic symbioses with a central role in the capturing of soil nutrients. The advantages provided by the mycorrhizae for the host plant and the flourishing world-wide market for edible truffle ascocarps have led to an increase in the production and marketing of seedlings inoculated with different Tuber species. However, quality control of these products requires reliable and sensitive identification tools able to verify the success of the mycorrhization experiments and to avoid commercial frauds.

The characterisation of Tuber species has traditionally been based on morphological analysis of the macroscopic and microscopic features of their fruitbodies [1]. However, during some developmental stages these anatomical traits are scarce and at times not distinctive enough to allow their identification. As a consequence, several molecular tools, mainly based on polymerase chain reaction (PCR), have been developed to support these methods [2–7]. In particular, the intragenic internal transcribed spacer (ITS) of the ribosomal genes has been successfully used as the target region.

In this study we set up a rapid and sensitive molecular tool able to identify four Tuber species, as well as an invasive contaminant fungus, Sphaerospora brunnea, that is often present in truffle orchards. In particular we developed a specific multiplex PCR able to simultaneously identify white Tuber species (Tuber magnatum Pico, Tuber borchii Vittad., Tuber maculatum Vittad., Tuber puberulum Berk. & Br.) and the contaminant Sphaerospora brunnea (A. & S. ex Fr.) Svcek & Kubicka, in a single reaction.

2. Materials and methods

2.1. Sample source and DNA isolation

The truffle fruitbodies analysed were collected from different locations in central Italy (Marche, Umbria and Emilia Romagna regions) and were identified by anatomical-morphological methods, according to Pegler et al. [1]. Mycorrhizal root tips were collected from greenhouses (T. borchii/Tilia platyphyllos Scop., S. brunnea/Ostrya carpinifolia Scop., T. puberulum/Pinus pinea L.) and harvested in the field (T. maculatum/O. carpinifolia Scop., T. borchii P. pinea L.). The substrate was carefully washed and each...
The root system was examined under a dissection microscope for sampling. The mycorrhizae were then frozen in liquid nitrogen and the ectomycorrhizal DNA was extracted using the method of Henrion et al. [2]. Total genomic fruitbody DNA was isolated according to Lee and Taylor [8].

2.2. Selection of primers

The primer–primer interactions were analysed using the program 'Primer!' (downloaded from http://www.william-stone.com/primers/calculator/). The primers TmagI [4], TpuI [4], rTmacII (5'-CGGGGCTATCAGTGCTGCTG-3') and rTboII (5'-GAAGTTGACCGTGGTAATAG-3'), respectively specific for T. magnatum, T. borchii, T. maculatum, T. puberulum, were used as forward primers and the universal primer ITS4 [9] as reverse primer. The ITS region of the contaminant species S. brunnea was sequenced using the ABI Prism 310 Genetic Analyzer (PE Applied Biosystem). The sequence obtained was compared to the ITS sequences of T. magnatum, T. borchii, T. maculatum and T. puberulum, using the Pileup program available as part of the GCGpackage, and the primer SB2 (5'-GGAGGTCTACATCAAAACCATTGC-3') was selected for coupling with the universal primer ITS4. The new primer was added to those used in the multiplex PCR set up for the Tuber species.

2.3. PCR amplification

The amplification conditions (buffer, MgCl2 and dNTP concentrations) tested are listed in Table 1. The best yields were obtained by using the following conditions: a 25-μl reaction mix, composed of 0.8 × of buffer B, 6.7 mM MgCl2, 1 mM dNTPs. The use of the adjuvants was not recommended because they affected differently the amplification efficiency and specificity of the DNA targets.

### Table 1

| 10× Buffer composition | MgCl2 | dNTPs | Adjuvants
|------------------------|-------|-------|-----------
| A                      |       |       | formamide, DMSO, (NH₄)₂SO₄ |
| Tris-HCl pH 8.8        | 670 mM| 1.5–10.3 mM | 200 μM–1.3 mM | 1–5% | 0.5–10% | 100–500 mM |
| NA₂EDTA                | 68 μM |       |           |       |       |           |
| B                      |       |       |           |       |       |           |
| Tris-HCl pH 8.0        | 100 mM|       |           |       |       |           |
| KCl                    | 500 mM|       |           |       |       |           |
| Triton X-100           | 1%    |       |           |       |       |           |
| C                      |       |       |           |       |       |           |
| Tris–HCl pH 8.3        | 100 mM|       |           |       |       |           |
| KCl                    | 500 mM|       |           |       |       |           |

*aThe PCR buffers were used in concentrations ranging from 0.8–2×. The optimal amplification conditions were obtained at 0.8× of buffer B, 6.7 mM MgCl2, 1 mM dNTPs. The use of the adjuvants was not recommended because they affected differently the amplification efficiency and specificity of the DNA targets.

*bDMSO = dimethylsulfoxide, (NH₄)₂SO₄ = ammonium sulfate.

3. Results and discussion

The availability of the previously selected pairs of primers specific for the species T. magnatum, T. borchii, T. maculatum and T. puberulum [4], led us to set up a multiplex PCR, in which a set of primers simultaneously amplify different target DNAs [10].

The choice of the primers must assure that all the DNA targets are satisfactorily amplified, in that a pair of primers that works well individually can, however, lead to poor yields in multiplex reactions. The predicted sizes of the amplification products and the high primer–primer interactions highlighted induced us to study several combina-

![Fig. 1. Organisation of the rDNA region. The black arrowheads represent the 3' end of the primers selected for the multiplex PCR, that give rise to amplification products easily distinguishable in size.](https://academic.oup.com/femsl/divisions/189/2/265/article-abstract/524834/524834)
tions of primers, with the aim of setting up a multiplex PCR able to give rise to differently sized amplification products for separation by gel electrophoresis. In order to reach this aim we used the reverse sequence of some of the specific primers (rTmacII and rTboII) (Fig. 1) and the specificity of the new combinations of primers was confirmed by testing several truffle species (Fig. 2). The technique was optimised by testing different reaction buff-


Fig. 3. Multiplex PCR amplification obtained with the set of primers ITS4+rTmacII/TpuI/rTboII/TmagI. M: Marker VIII (Boehringer). M': specific amplification fragments of *T. maculatum*, *T. puberulum*, *T. borchii* and *T. magnatum*. (A) Amplification fragments obtained starting from fruitbody DNA. Lane 1, DNA mix of the four *Tuber* species; lane 2, *T. magnatum*; lane 3, *T. puberulum*; lane 4, *T. maculatum*; lane 5, *T. borchii*. (B) Amplification fragments obtained starting from ectomycorrhizal DNA. Lane 1, *O. carpinifolia* roots inoculated with *T. maculatum*; lane 2, *P. pinea* roots inoculated with *T. puberulum*; lane 3, *P. pinea* roots inoculated with *T. borchii*; lane 4, *T. maculatum* and *T. puberulum* ectomycorrhizal DNA mix; lane 5, *T. maculatum*, *T. puberulum* and *T. borchii* ectomycorrhizal DNA mix. (The quality of the images was improved using the program Adobe Photoshop version 5.)
ers and taking into account the concentration ratio between the MgCl₂ and the deoxyribonucleotides [10–11] (Table 1).

First of all, fruitbody target DNA was used to optimise the technique (Fig. 3A) and the species was unequivocally identified both when the DNA of a single species was added to the reaction mix and when a mixture of DNA from all four species was used. This multiplex PCR has potential for application mainly in the typing of mycorrhized roots, and in particular for those harvested in the field, in which case several mycorrhizal species are frequently found in the same root system [12]. In fact, using the described set of primers, multiplex PCR was performed on 1–3 ng DNA extracted from roots of T. platypyllum inoculated with T. borchii grown in nurseries, from mycorrhizae of T. maculatum on roots of O. carpinifolia and from mycorrhizae of T. borchii on P. pinea harvested in the field, where amplification inhibitors can often lead to false negative results (Fig. 3B). Also in these cases the ectomycorrhizae were clearly identified, even when the mycorrhizal tips from the different species were mixed before DNA isolation (Fig. 3B, lanes 4 and 5).

Subsequently, we considered the contaminant species S. brunnea, an ectomycorrhizal fungus that can interfere severely with the production of seedlings infected with specific symbionts, especially for highly prized truffles, such as T. magnatum and Tuber melanosporum Vittad. S. brunnea grows rapidly and its ascospores germinate easily on ordinary media; it is characterised by a prolific production of fruitbodies in the greenhouse [13]. Furthermore, it produces mycorrhizae morphologically similar to those produced by prized truffles, such as T. magnatum, T. borchii and T. melanosporum, with which it can be easily confused [14].

In order to develop a sensitive molecular tool more rapid than the restriction fragment-length polymorphism (RFLP) analyses [15] for application in the screening of seedlings inoculated with selected truffle species, in greenhouses or truffle orchards, we sequenced the ITS region of this species and selected specific primers for use in specific and multiplex PCR experiments. Taking in to consideration the locations and predicted amplification product size of the several different oligonucleotides found, primer SB2 was selected for coupling with the universal primer ITS4. The specificity of this primer was tested on several truffle species and its applicability to the screening of seedlings mycorrhized with both white and black truffles was confirmed (Fig. 4A). The new primer was added to those used in the multiplex PCR set up for the Tuber species, obtaining successful results even in ectomycorrhizal typing starting from 1 to 3 ng of DNA (Fig. 4B). The method set up thus permits reliable molecular identification and detection of the invasive contaminant fungus S. brunnea in plant roots mycorrhized in vitro and transplanted in nurseries.

In conclusion, the multiplex PCR set up in this study represents a powerful, sensitive and inexpensive molecular tool for rapid typing of mycorrhized plant roots, in that it is able to reveal different species simultaneously present in the root system. This strategy may be of great importance in applications involving the typing of mycorrhized root tips harvested in greenhouses and in the field. A further advantage is the small amount of DNA required for identification: only 1–5 root tips, in comparison with the larger amount (15–20 mycorrhized tips) required for RFLP analyses [15] and for Southern blot analysis [16]. Finally, the availability of multiplex PCR to simultaneously detect both different truffle species and one of the most aggressive and common contaminant fungi represents a considerable practical advantage in evaluating the success of mycorrhizal synthesis in inoculated seedlings, in monitoring the persistence of a selected truffle (such as T. magnatum or T. melanosporum), in out-planting of seedlings mycorrhizae of: lanes 1 and 2, S. brunnea; lane 3, T. magnatum; lane 4, T. melanosporum; lane 5, T. borchii; lane 6, T. maculatum; lane 7, T. dryophilum; lane 8, T. puberulum; lane 9, T. uncinnatum; lane 10, T. brumale; lane 11, T. aestivum; lane 12, T. macrosporum; lane 13, T. excavatum; lane 14, T. mesentericum. (B) Multiplex PCR amplification obtained with the set of primers ITS4+TmacII/TpubII/TbtoII/TmagI/SB2. M: Marker VIII (Boehringer). Amplification fragments obtained starting from DNA of: lane 1, T. magnatum; lane 2, T. puberulum; lane 3, T. maculatum; lane 4, T. borchii; lane 5, S. brunnea fruitbody; lane 6, S. brunnea ectomycorrhizae; lane 7, T. magnatum, T. borchii, T. maculatum, T. puberulum and S. brunnea DNA mix. (The quality of the images was improved using the program Adobe Photoshop version 5.)
corrhized in vitro and in studying truffle orchards and natural truffle grounds.

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