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# Rapid typing of truffle mycorrhizal roots by PCR amplification of the ribosomal DNA spacers

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**Abstract** DNA analyses were developed to type mycorrhizas of two *Tuber* species of commercial value (*T. melanosporum, T. borchii*) and a competitive fungus (*Sphaerosporella brunnea*) which forms ectomycorrhizas with plants usually considered hosts for truffles. Polymerase chain reaction (PCR) amplification of DNA isolated from fruitbodies, mycelia, mycorrhizas and leaves of host plants, was performed with a primer pair for an internal transcribed spacer ITS1-4. ITS amplification followed by restriction fragment length polymorphism (RFLP) analysis of the amplified products clearly distinguished the two *Tuber* species at the fruitbody, mycorrhiza and mycelium levels.

**Key words** Polymerase chain reaction · Ectomycorrhiza · *Tuber* · Ribosomal genes

## Introduction

Truffles are ascomycetous fungi which form ectomycorrhizas with the roots of trees such as oak, poplar, willow and hazel (Ceruti 1989; Harley and Smith 1983), and shrubs such as *Cistus* (Fontana and Giovannetti 1978–79). Their biology and life cycles are still obscure (Pegler et al. 1993), although they have been the subject of intense applied studies since the 1970s due to the remarkable commercial value of some species.

Programmes for large-scale mycorrhizal production have been elaborated in southern European countries to increase truffle production (Chevalier 1994). The main steps are production of mycorrhizal roots in controlled conditions using spore or root inocula, planting out of the mycorrhizal seedlings, checking for the presence of the introduced *Tuber* species among the ectomycorrhizal symbionts, and harvest of fruitbodies.

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Identification of truffles during their symbiotic phase is one of the major topics in truffle research. Truffle fruitbodies are usually identified from the size and shape of their spores and asci, wall ornamentation, structure of the peridium and gleba. These features, however, are lost when the hyphae of the mantle and Hartig net are developed at the area of contact with the plant root. For these reasons, discrimination between truffle mycorrhizas is often a difficult task. Italian and French projects designed to formulate reliable, simple and inexpensive protocols for the identification of truffle species have already successfully applied molecular methods to fruitbodies. Random amplified polymorphic DNA (RAPD) markers and primers designed for the amplification of the internal transcribed spacer regions (ITS) of ribosomal genes have led to the rapid identification of many fruitbodies (Henrion et al. 1994; Lanfranco et al. 1993, 1995; Paolocci et al. 1995a, b). However, many problems remain for reliable, easy identification of the fungal symbiont when mycorrhizal roots are considered.

This paper reports the investigation of two truffle species during their symbiotic phase by examination of their ITS regions. Fingerprints were produced from 15–20 mycorrhizal tips associated with three host plants grown in vitro, in a semi-sterile system, and in the field. Mycorrhizas produced by *Tuber borchii* were distinguished from those produced by an antagonistic mycorrhizal fungus, *Sphaerosporella brunnea.*

## Materials and methods

Fruitbodies of *T. melanosporum, T. borchii* and *S. brunnea* were collected from the following Italian sites: *T. melanosporum* Vitt. from Val Curone (Piedmont) and Ceva (Cuneo), *T. borchii* Vitt. from Urbino (Pesaro), *S. brunnea* (A. & S.) Svrcek & Kubicka from the greenhouses of the Centro di Studio sulla Micologia del Terreno (Torino).

Hazelnut and oak seedlings of different origin (Table 1) were gently washed and mycorrhizal tips were carefully selected under a stereomicroscope. Mycelium of *T. borchii* isolated from the fruitbody was grown on modified MMN medium (Mischiati and

**Table 1** Mycorrhizal roots examined and the sites from which they were collected

| Species | Site |
|---|---|
| *Tuber melanosporum*/*Quercus pubescens* | Bruno Robin, Breeder, St Laurent-du-Cros (France)* |
| *Tuber melanosporum*/*Quercus cerris* | Bruno Robin, Breeder, St Laurent-du-Cros (France)* |
| *Sphaerosporella brunnea*/*Corylus avellana* | Centro di Studio sulla Micologia del Terreno (Torino)* |
| *Tuber albidum*/*Quercus robur* | Forestry Service at Padova and Rovigo (Veneto)° |

* Greenhouse
° Field

Fontana 1993). In vitro mycorrhizas were obtained starting from an aseptic meristem culture of poplar (*Populus alba*) and the *T. borchii* mycelium. The mycorrhizas were produced according to Piagnani (1988), and were kindly provided by Dr. M. Marchetti (CSMT-CNR). Leaves or nonmycorrhizal roots of poplar, oak and hazelnut seedlings were used as control material in polymerase chain reaction (PCR) experiments.

### DNA extraction

Total DNA was extracted from mycelia, fruitbodies, leaves, roots and from ectomycorrhizal root tips according to Henrion et al. (1994) with slight modifications. DNA was extracted from fresh material as follows: 15–20 mycorrhizal tips for each mycorrhizal plant were suspended in lysis buffer (1.4 M NaCl, 100 mM Tris-HCl pH 8, 20 mM EDTA, 2% CTAB, 0.2% $\beta$-mercaptoethanol), then crushed with a pestle in a 1.5-ml tube. Fruitbodies, leaves and mycelia were crushed in the presence of liquid nitrogen and then suspended in lysis buffer. All samples were incubated for 1 h at 65 °C, centrifuged for 5 min at 14000 *g*, extracted twice with 1 volume of phenol/chloroform/isoamyl alcohol (25/24/1/v/v/v) and 1 volume of chloroform (Sambrook et al. 1989), and centrifuged for 15 min at 14000 *g* for each extraction. Nucleic acids in the supernatant were precipitated with 1.5 volumes of isopropanol, incubated for 30 min at 4 °C, centrifuged for 30 min and the pellet washed with 200 µl of 70% ethanol, well dried, and resuspended in 50–100 µl of 1× TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). DNA from 100–200 mg of fruitbody, mycelium or leaf was measured by reading its optical density at 260 nm. DNA from mycorrhizal tips was not quantified because of its low amount. The DNA was used in PCR analysis at the dilutions of 1/10, 1/50, 1/100 and was stored at −20 °C.

### PCR and restriction fragment length polymorphism analysis

The primers used to amplify the ITS region (ITS1/ITS4) have been described by White et al. (1990). They were supplied by Primm (Milan, Italy). The cycling parameters were: an initial denaturation at 95 °C for 3 min, followed by 40 cycles at 95 °C for 90 s, 50 °C for 45 s, 72 °C for 90 s and a final extension at 72 °C for 5 min. The components for 50-µl PCR reactions were: 0.1–10 ng of total DNA, 50 pmol of each primer, 0.2 µM dNTPs, 0.5 U SuperTaq-Polymerase (Stehelin, Basel, Switzerland) and 1× buffer (10 mM Tris-HCl at pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/ml BSA). Negative controls (no DNA template) were included in every experiment. The amplification reaction was performed in a Perkin Elmer thermal cycler and the products fractionated in 1.7% agarose gel in 1× TBE. One-fifth of the amplified DNA was digested for 1 h with 4–6 units of restriction enzymes (*Sau*3A I, *Hinf* I). The restriction fragments were separated in a 2% agarose gel and stained by soaking in a solution of 0.5 µg/ml ethidium bromide.

## Results

PCR amplification from mycorrhizal roots with the primer pair for ITS distinguished symbionts with different degrees of resolution. The banding pattern of amplified DNA from mycorrhizas of *T. melanosporum* on oak (*Quercus pubescens*) roots depended on the sample dilution (Fig. 1a). The undiluted sample, as extracted, showed two bands: the upper weak one matched that of the leaves of *Q. pubescens,* the other, of about 600 bp, corresponded to that shown by the fruitbody of *T. melanosporum.* The diluted sample (1/10) showed only a band corresponding to that of the fruitbody. The same results were obtained from mycorrhizal roots of *T. melanosporum* on *Q. cerris* (Fig. 1a).

*Hinf* I digestion of the PCR products of *T. melanosporum* mycorrhizas on *Q. pubescens* and on *Q. cerris* roots confirmed the previous experiments: ITS products from *T. melanosporum* fruitbody and mycorrhizas had one band of the same size (300 bp), whereas *Q. pubescens* and *Q. cerris* leaves showed a different pattern (Fig. 1b). Amplification of DNA from in vitro ectomycorrhizas gave two close bands; the upper band had a molecular weight corresponding to that obtained from non-inoculated *Populus alba* roots (about 620 bp), the other corresponded to that shown by *T. borchii* fruitbody and mycelium (550 bp) (Fig. 2).

Mycorrhizas of *S. brunnea* on hazelnut (*Corylus avellana*) were distinguished by amplification of the ITS region followed by restriction fragment length polymorphism (RFLP) (Fig. 3). The amplification products gave two bands for the mycorrhizas corresponding to the fungal and host plant products. Digestion with *Hinf* I and *Sau*3A I showed a banding pattern which included both *S. brunnea* and *C. avellana* digestion patterns (Fig. 3).

ITS amplified fragments from mycorrhizas of *S. brunnea* on *C. avellana* and mycorrhizas of *T. borchii* on *Q. robur* had similar sizes (Fig. 4a). Two bands of about 550 and 700 bp were displayed after PCR amplification from both the mycorrhizas. The lower one matched that of the corresponding fruitbodies (Fig. 4a), while the upper one corresponded to that shown by the two host plants. However, after *Sau*3A I digestion, the mycorrhizas of *S. brunnea* showed a banding pattern completely different from that presented by the mycorrhizas of *T. borchii* (Fig. 4b). The plant ITS bands remained undigested (lanes 3, 6), while the fungal fragments were differently cut: *T. borchii* gave two bands of 350 and 150 bp (lanes 1, 2) and *S. brunnea* two bands of 290 and 210 bp (lanes 4, 5).

## Discussion

Identification of ectomycorrhizal truffles has been mainly based on morphological and biochemical features. In some cases, however carefully performed, these analyses are not enough for species typing. More-

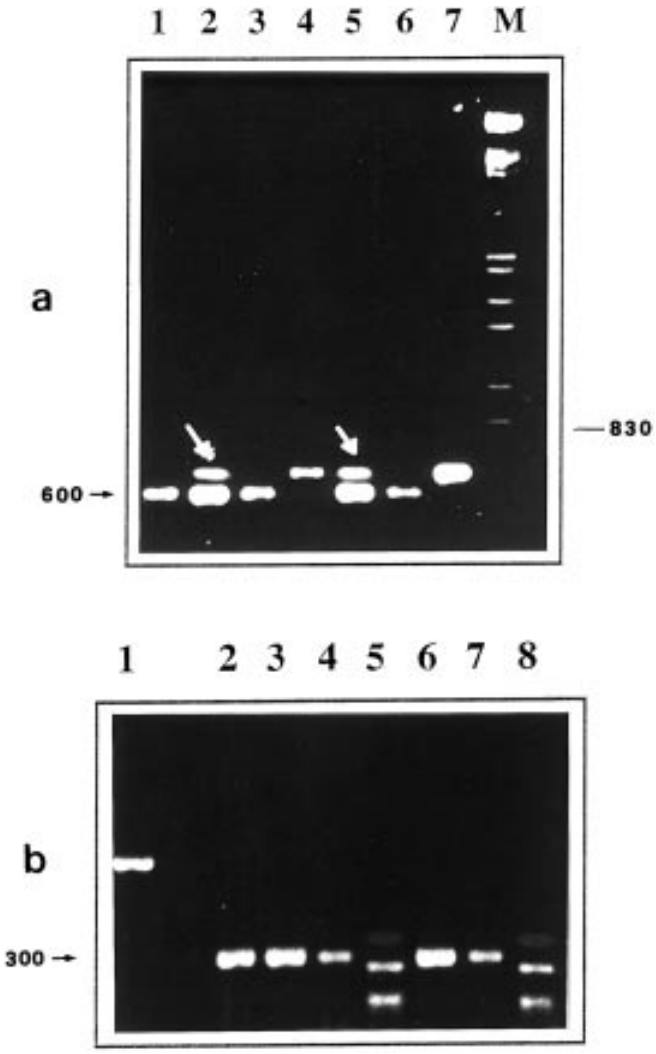


**Fig. 1a, b** PCR-amplified ITS regions: **a** fruitbody of *Tuber melanosporum* (lane 1); mycorrhiza of *T. melanosporum* on *Quercus pubescens* (undiluted-diluted samples, lanes 2–3); leaf of *Q. pubescens* (lane 4); mycorrhiza of *T. melanosporum* on *Q. cerris* (undiluted-diluted samples, lane 5–6); leaf of *Q. cerris* (lane 7); Lambda DNA digested with *Eco*R I-*Hind* III (lane M). The *white arrows* point to the host plant bands in the undiluted samples. **b** undigested (lane 1) and digested with *Hinf* I, lanes 2–8: fruitbody of *T. melanosporum* (lane 1); fruitbody of *T. melanosporum* (lane 2); mycorrhiza of *T. melanosporum* on *Q. pubescens* (undiluted-diluted samples, lane 3–4); leaf of *Q. pubescens* (lane 5); mycorrhiza of *T. melanosporum* on *Q. cerris* (undiluted-diluted sample, lane 6–7); leaf of *Q. cerris* (lane 8)

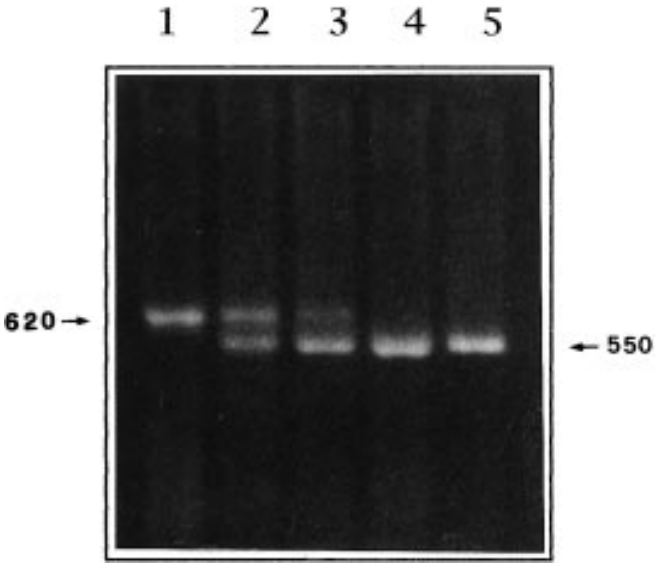


**Fig. 2** Amplification products with primers ITS1 and ITS4 of DNA extracted from noninoculated roots (lane 1), two different isolates of in vitro mycorrhizas of *Tuber borchii* on *Populus alba* (lanes 2, 3), fruitbody of *T. borchii* (lane 4), mycelium of *T. borchii* (lane 5)

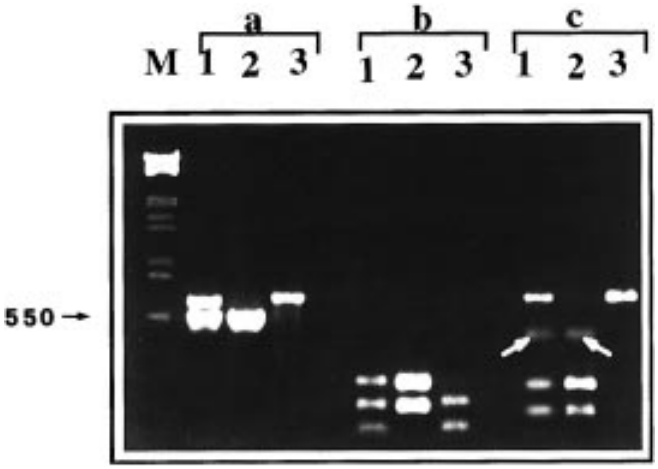


**Fig. 3** Amplified products with primers ITS1 and ITS4 (*a*), amplified products cut by *Hinf* I (*b*) or *Sau*3A I (*c*) of DNA extracted from mycorrhiza of *Sphaerosporella brunnea* on *Corylus avellana* (lane 1), fruitbody of *S. brunnea* (lane 2), *C. avellana* leaf (lane 3). Lambda DNA digested with *Eco*R I-*Hind* III (lane M). The *white arrows* point to a weak band shared by mycorrhizas and fruitbody

over, many of the structures important for taxonomic identification are absent during filamentous growth and establishment of mycorrhizas. The increasing market for seedlings inoculated with selected edible truffles has stimulated the search for unequivocable techniques for typing fruitbodies, mycelia and mycorrhizas.

Amplification of the ITS1-4 region combined with RFLP revealed a distinct pattern in each fungus-host plant interaction. Our analyses of material from an in vitro, a semi-sterile system, and the field led to firm identification of the ectomycorrhizal symbiont, irrespective of the host species, namely *Q. pubescens, Q. cerris, Q. robur, C. avellana* and *P. alba.* Even if in the present study we had not faced the problem of fungal intraspecific variation, it is known that ITS1-4 primers do not allow distinction of fungal isolates (Gardes et al. 1991). Similar results have been obtained for isolates of *Tylospora fibrillosa* (Erland et al. 1994), *Tuber melanosporum* (Henrion et al. 1994), and many isolates of *Tuber borchii* (Longato and Bonfante in press). Some

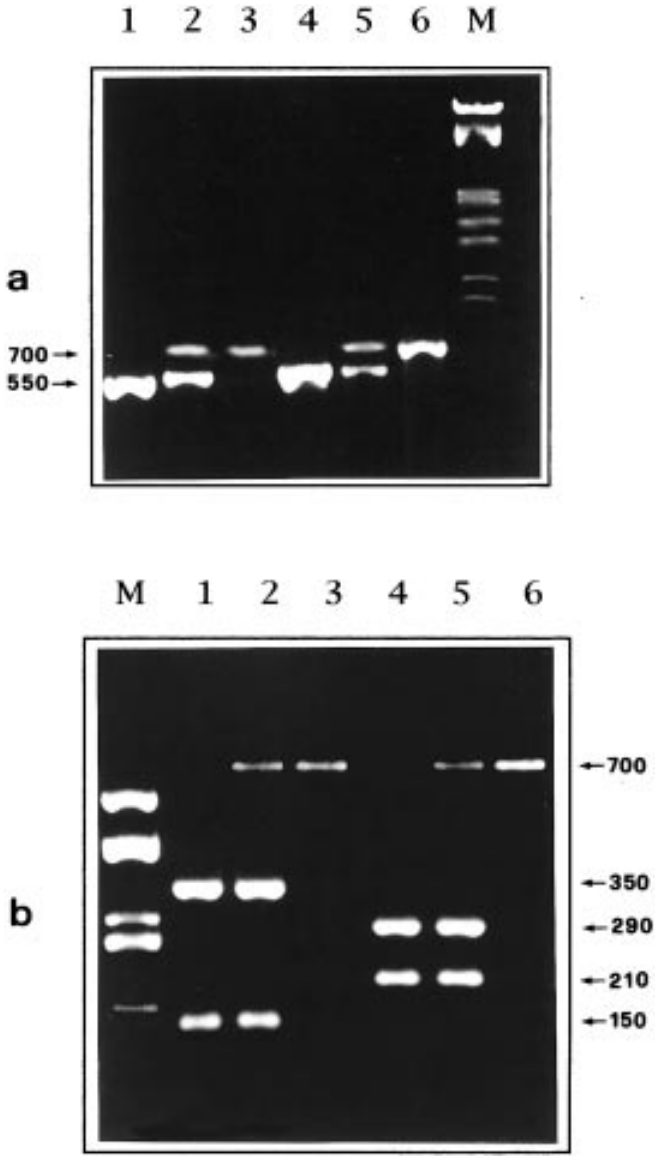


**Fig. 4a, b** PCR-amplified ITS regions: **a** fruitbody of *T. borchii* (lane 1); mycorrhiza of *T. borchii* on *Quercus robur* (lane 2); *Q. robur* leaf (lane 3); fruitbody of *S. brunnea* (lane 4); mycorrhiza of *S. brunnea* on *C. avellana* (lane 5); *C. avellana* leaf (lane 6); Lambda DNA digested with *Eco*R I-*Hind* III (lane M). **b** digested with *Sau*3A I: fruitbody of *T. borchii* (lane 1); mycorrhiza of *T. borchii* on *Q. robur* (lane 2); *Q. robur* leaf (lane 3); fruitbody of *S. brunnea* (lane 4); mycorrhiza of *S. brunnea* on *C. avellana* (lane 5); *C. avellana* leaf (lane 6); pUC18 DNA *Hae* III digest (lane M)

of these results indicate a low level of polymorphism within truffles, in their ITS region, as already suggested by RAPD experiments (Lanfranco et al. 1993).

The results with ITS-RFLP are of particular interest for *S. brunnea*; this agressive fungus forms its mycorrhiza in continuous high humidity (Meotto and Carraturo 1992) with many plants, including *Castanea, Quercus* and *Fagus,* usually considered specific hosts for truffles and *Boletus edulis* (Meotto and Carraturo 1987–88). It strongly competes with truffles, mostly in greenhouse conditions, where it produces mycorrhizas morphologically similar to those produced by white truffles, like *T. magnatum* and *T. borchii.* Morphological analyses of the root system have demonstrated the simultaneous presence of both *Sphaerosporella* and *Tuber* in pots containing seedlings previously inoculated with *Tuber* for large-scale mycorrhizal production. In our experiments, *S. brunnea* mycorrhizas were distinguished from those of *T. borchii.*

ITS region primers designed by White et al. (1990) for fungal ribosomal RNA genes have been demonstrated to amplify the ITS region in various plant species (Gardes and Bruns 1993). Even the primers ITS4-B, intended to be specific for basidiomycetes, gave faint amplification of the ITS region in many plants (Gardes and Bruns 1993). In our experiments on truffle mycorrhizas, DNA of host plants was amplified by ITS1-4 fungal primers, in agreement with Gardes and Bruns (1993) and Paolocci et al. (1995a). The presence of the host band in ITS amplification from mycorrhizas does not raise any serious problem, despite the remarks of Paolocci et al. (1995a, b). Typing of the three fungal species from ectomycorrhizal DNA was easy with RFLP analysis of PCR-amplified products. The same two-step analysis must also be made on DNA from leaves, or noninoculated roots of host plants, to compare with the fingerprint pattern of fruitbodies. However, the band of host origin is not always present in PCR products, depending on the template dilution and, therefore, on the relative quantity of fungal and plant DNA, as already suggested by Gardes and Bruns (1993).

The protocol used in these experiments is useful when only a small quantity of DNA is available. Only 15–20 mycorrhizal tips are required to type the fungal species, in comparison with the higher amount of DNA (about 2 g of ectomycorrhiza) required to identify the fungus in the mycorrhizal root by Southern analysis (Paolocci et al. 1995a). A further advantage is the rapidity of the DNA extraction method compared with CsCl gradient purification. The latter produces DNA of better quality but makes the DNA extraction procedure longer and more complex. An easy, quick DNA extraction protocol is certainly crucial in programmes for the routine analysis of mycorrhizas.

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