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IDENTIFICATION OF *TUBER MAGNATUM* PICO DNA MARKERS BY RAPD ANALYSIS

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Summary

Different species of truffle were studied in order to identify species-specific markers. The isolation of two *Tuber magnatum* Pico markers is reported. One of these could be used as a probe in dot blot hybridization, allowing the development of a rapid test able to identify *Tuber magnatum* species.

INTRODUCTION

DNA polymorphisms based on the amplification of arbitrary nucleotide sequences can allow the identification of the species of almost any organism.

Random amplified polymorphic DNA (RAPD) (Williams et al.1990) is a subset of AP-PCR (Arbitrarily Primed - Polymerase Chain Reaction) (Welsh and McClelland 1990, Welsh et al.1991) that uses primers nine or ten bases long. This technique is simpler and faster than RFLP technology (Botstein et al.1990, Donis -Keller et al.1987, Tanksley et al.1989) and can be applied to any species from which DNA can be prepared. Furthermore, it does not require knowledge of the biochemistry or molecular biology of the species being studied. For these reasons we applied the RAPD technique in order to distinguish three different species of truffle: *Tuber magnatum*, *Tuber albidum* and *Tuber maculatum*.

In this paper, we report the identification of anonymous amplified polymorphic DNA markers which were found to be specific for *Tuber magnatum*.

The procedure described here could also be applied in further studies concerning the characterization and identification of ectomycorrhizae in artificially mycorrhized plants.

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MATERIALS AND METHODS

Primers. A total of 12 primers were surveyed, with the following nucleotide sequences: (5'- 3'): 128, GCCTGACAGG; 126, ATTGCCTCC; 124, ATTGCGTCCGAG; 127, GCCTGACAGGTC; 129, GCCTG ACAG; 125, ATTGCGTCCG; 116, TAGACCGGTTC; 100, ATTGCGTCG; 115, TAGACCGGTT; 117, TAG ACCGGTTCCA; TAR A, TGGTCACTGA; TAR B, ACGGTACACT. They were arbitrarily chosen for application in the RAPD technique without DNA template sequence information. Primer composition was kept between 50% and 80% in GC, without palindromic sequences (Beckmann 1988).

Truffle genomic DNA extraction. Truffle fruitbody samples were collected in Central Italy. Total genomic DNA was isolated from fruitbodies of *Tuber magnatum*, *Tuber maculatum* and *Tuber albidum* according to the method of Lee et al. (1990). The *Tuber maculatum* samples used in this study were provided by the "Area Operativa-Agricoltura e Foreste" of the Region of Umbria which classified this species on the basis of morphological characteristics and microscopical analysis of the spores. Microscopical examination of the lysate showed that the spores were intact and the extracted DNA was from the hyphae only.

The DNA was dissolved in Tris-EDTA (TE) and the final concentration was estimated either spectrophotometrically or by agarose gel electrophoresis staining with ethidium bromide.

RAPD conditions. Amplification reactions were carried out in a 25 µl volume containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP and dTTP (Perkin-Elmer/Cetus), 0.2 µM primer, 25 ng of genomic DNA and 0.1 units of SuperTaq polymerase (Sthelin,Basel, Switzerland).

Amplification was performed in a Perkin-Elmer/Cetus DNA Thermal Cycler (Model 480) programmed for an initial denaturation at 94 °C for 5 min, followed by 45 cycles of 30 sec at 90 °C, 1 min at 36 °C, 2 min at 72 °C and a final extension at 72 °C for 7 min.

Reaction products were separated by electrophoresis in 1.4% agarose gel stained with ethidium bromide.

Gel electrophoresis and autoradiography. Electrophoresis of Eco RI digested total truffle DNA was carried out in 0.8% agarose, using Tris-acetate-EDTA 1X (TAE) as running buffer. Southern blotting was carried out by depurination in 0.25 M HCl for 15 min followed by transfer for 12 h to Hybond N$^+$ (Amersham) using 0.4 N NaOH as the transfer solution. RAPD products on agarose gels were blotted to Hybond N$^+$. The specific RAPD fragments were recovered from the gel using low melting agarose and radioactively labelled by the random examer method (Sambroock et al. 1989). Hybridization was carried out at 65 °C in 0.5% sodium dodecyl sulfate (SDS), 0.9 M NaCl, 0.05 M NaH_2PO_4, 0.5 mM EDTA (SSPE 5X) and 5X Denhardt's solution (0.1% w/v BSA, 0.1% w/v Ficoll, 0.1% w/v PVP). Post hybridization washing was in 2X SSPE, 0.1% SDS at room temperature for 10 min, 1X SSPE, 0.1% SDS at 65 °C for 10 min. Autoradiography was carried out with hyperfilm-MP (Amersham) and one intensifying screen for 12 hours.

Dot blot. Dot blots were performed using a dot blot apparatus (BioRad) and a vacuum source. Two micrograms of truffle DNA dissolved in TE 1 mM-0.1 mM were transferred according to the method of Davis et al. (1986). We transferred the fruitbodies both with and without the traditional DNA extraction onto dot blot. A very small amount (0.01 g) of fruitbody was boiled in 50 µl of 0.4 N NaOH for 10 min. The samples were then spun and the surnatant added to the wells. We used N$^+$ membranes and 0.4 N NaOH as transfer buffer. The dot blot hybridization and post hybridization washing were performed as described in *gel electrophoresis and autoradiography.*

RESULTS

Genomic DNA from three different species of truffle was amplified with 12 arbitrary sequence single primers using the standard RAPD protocol. Because of the high sensitivity of the method, sterile conditions and negative PCR controls (no template) were used to exclude the possibility of DNA contaminants.

Six samples each of *Tuber magnatum*, *Tuber maculatum* and *Tuber albidum* were studied and the polymorphisms detectable by fingerprint analysis were investigated. We observed that species can be identified by comparing the polymorphisms in their genomic fingerprints. Our studies allowed us to identify at least two *Tuber magnatum* markers using primers 126 (5'ATTGCGTCC) and 127 (5'GCCTGACAGGTC). These markers were deduced from the high level of homology in the amplification products and were named T1270 and T650 on the basis of their lengths. The genomic fingerprints generated by primer 126 are shown in Fig. 1. The electrophoretic profile showed intraspecific variability among *T. maculatum* and T. *albidum* samples and the presence of a specific polymorphism among *T.magnatum* samples, which we have named T1270 (arrow Fig. 1).

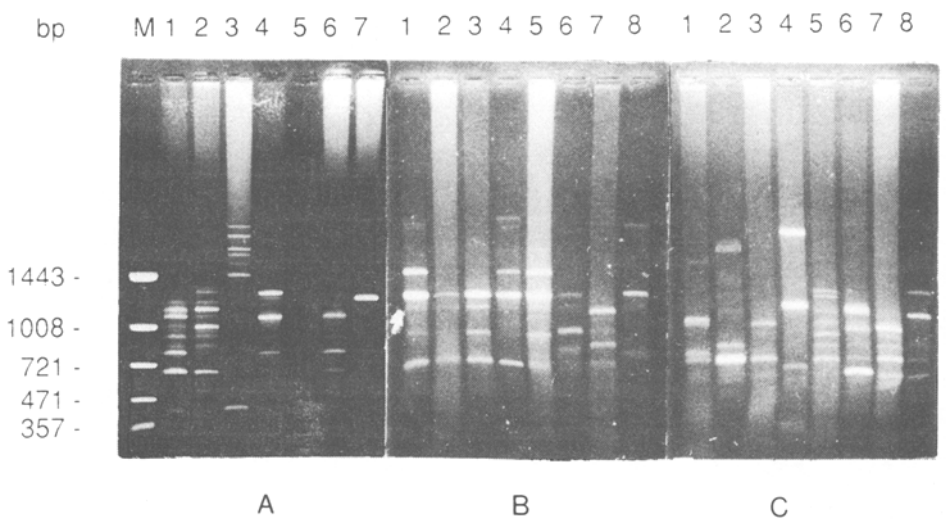

Figure 1. Genomic fingerprints generated by primer 126 from 3 species of Tuber.
A : lanes 1-4, 6, *T. maculatum*; lane 5, control reaction; lane 7, *T.magnatum*.
B : lanes 1-6, *T.magnatum*; lane 7, *T.maculatum*; lane 8, *T.albidum*.
C : lanes 1-6, *T.albidum*; lane7, *T.maculatum*; lane 8, *T.magnatum*.
M : Taq I digested pEMBL8 DNA.

This specific fragment of DNA was selected, then labelled and the RAPD products, blotted to the nylon membrane, were hybridized in order to verify the detection of a *Tuber magnatum* marker.

This experiment highlighted even just a few nanograms of amplified DNA, undetectable on agarose gel, and examined whether or not any bands of *Tuber maculatum* and *Tuber albidum* of the same size as the marker selected also had the same nucleotide information. Autoradiography showed the presence of a radioactive signal in the *Tuber magnatum* samples alone (Fig. 2).

This result confirmed the identification of a specific *Tuber magnatum* DNA marker. The T1270 was also used as probe on the Southern blot of EcoRI digested genomic DNA from *Tuber magnatum*, *Tuber albidum* and *Tuber maculatum*. These results are reported in Fig. 3 and show hybridization of T1270 to highly repetitive DNA in the *Tuber magnatum* sample.

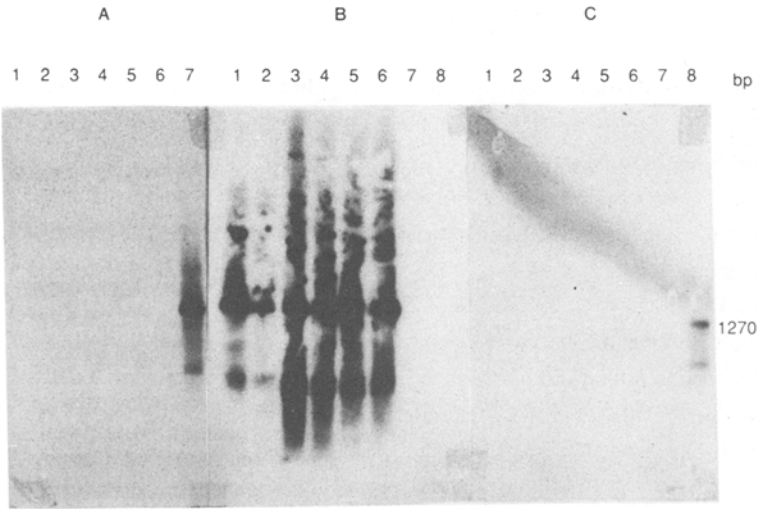

Figure 2. Hybridization of probe T1270 to the RAPD products reported in Fig.1.
A: lanes 1-4, 6, *T. maculatum*, lane 5, control reaction, lane 7, *T. magnatum*
B : lanes 1-6, *T.magnatum*, lane 7, *T. maculatum*, lane 8, *T.albidum*.
C: lanes 1-6, *T.albidum*, lane 7, *T. maculatum*, lane 8, *T.magnatum*.
These results confirm the identification of a specific *T.magnatum* marker.

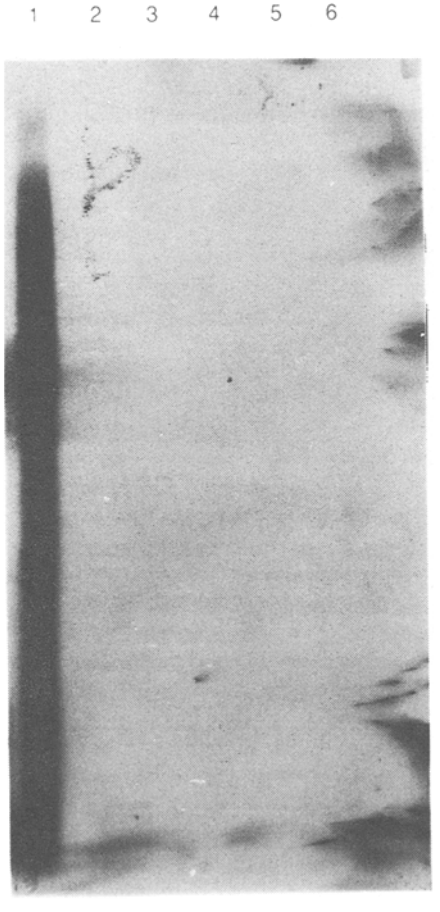

Figure 3. Hybridization of probe T1270 to the Southern blot of EcoRI digested DNA from *T.magnatum* (lane1), *T.maculatum* (lanes 2-3), *T.albidum* (lane 4-5) and from a human being (lane 6). The autoradiogram shows that the marker is specific for *T.magnatum* and probably consists of a repetitive DNA sequence.

Further hybridization experiments using the T1270 probe were performed on a dot blot containing heat-denatured genomic DNA from the three species: 25 samples of *T.magnatum*, 6 samples of *T.albidum* and 6 samples of *T.maculatum*. The results show that the sequence selected is present in the *Tuber magnatum* samples alone (Fig. 4).

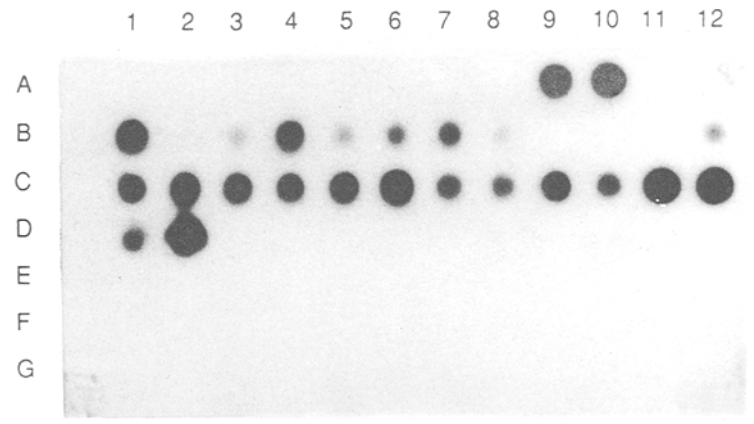

Figure 4. Dot blot hybridization of T1270 to *T. magnatum*, *T. albidum* and *T. maculatum* genomic DNA samples. **A 9-10**,*T. magnatum* . **B 1-8,12**, *T. magnatum*. **B 11**, *T. albidum*. **C 1-12**, *T. magnatum*. **D 1-2**, *T. magnatum*. **E 1-5**, *T.albidum*. **G 1-6**, *T.maculatum*. These results point out that the T1270 sequence is specific for *Tuber magnatum* species.

The same procedure was used for the analysis with primer 127. The genomic fingerprints generated by this primer are shown in Fig. 5. We observed, as in the case of primer 126, a similar banding pattern among *Tuber magnatum* samples, and variability among samples of the other two species. We selected one of these constant bands (T650), labelled it and hybridized RAPD products blotted to nylon membranes. The results reported in Fig.6 confirmed the identification of another specific *Tuber magnatum* DNA marker. The presence of several bands on the autoradiogram suggests that we have probably isolated a repetitive DNA sequence. The autoradiogram of the Southern blot probed with T650 showed hybridization to *T.magnatum* samples alone and bears out the possibility that this marker is a repetitive DNA sequence. The T650 marker used for hybridization in dot blot experiments gave positive signals not only in *T.magnatum* samples, but also in some samples from the other two species.In view of this result , we limited the use of primer 127 to RAPD reactions in fingerprint analyses.

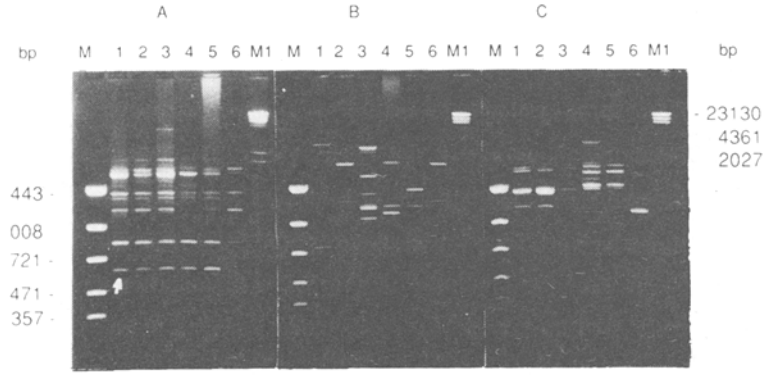

Figure 5. Genomic fingerprints generated by primer 127. **A** : *T.magnatum*; **B** : *T.albidum* ; **C** : *T.maculatum*. **M** : Taq I digested pEMBL8 DNA; **M1** : Hind III digested bacteriophage lambda DNA.The electrophoretic pattern suggests the presence of *Tuber magnatum* specific polymorphisms.

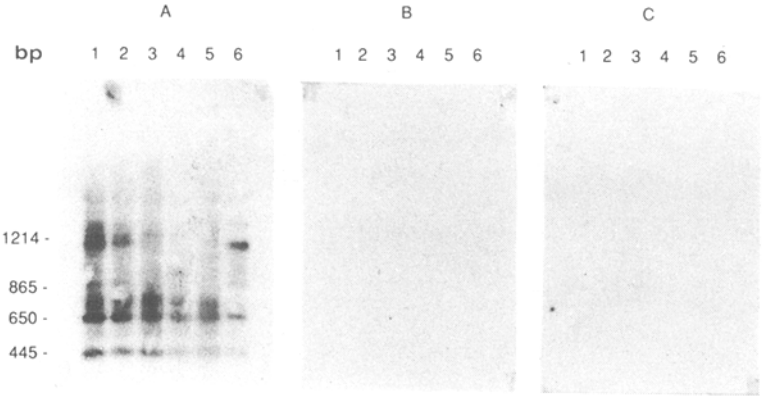

Figure 6. Hybridization of the T650 probe (arrow in Fig.5) to the genomic fingerprints generated by the 127 primer.
A : *T.magnatum.*
B : *T.albidum.*
C :*T.maculatum.*

DISCUSSION

Since we had no information about the truffle genome, we chose to use single random primers and the polymerase chain reaction in order to identify the three Tuber species.

The data reported in this study confirm the applicability of the RAPD method for DNA fingerprinting in species identification. The results obtained from dot blots hybridized with probe T1270 allowed us to develop a fast, simple and inexpensive test able to identify the *Tuber magnatum* species, affording the possibility of future applications of this strategy not only to the fruitbodies but also to the ectomycorrhizae (Harley et al. 1983) related to this species. The application of this procedure could be of interst in the study of mycorrhizae because, at present, there are still no rigorous taxonomic methods to identify the symbiotic phase (Pankow et al. 1991, Zambonelli et al. 1991)). Further studies are in progress in our lab in order to characterize these markers, compare their sequences in data base and obtain specific primers able to amplify a region of the *Tuber magnatum* genome.

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