Intraspecific Polymorphisms Among *Tuber borchii* Vittad. Mycelial Strains

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Abstract

Four mycelial strains of *Tuber borchii* Vittad. were studied in order to find DNA polymorphisms. Polymerase chain reaction-based techniques for assaying differences in the genome were used. Although a very low level of genetic variability was observed, the few polymorphisms found were sufficient to elaborate strain-specific markers suitable for use in biotechnological applications.

Keywords: *Tuber borchii* mycelium, intergenic spacer, random amplified polymorphic DNA, microsatellite region, ectomycorrhizal fungi

1. Introduction

Truffles are ascomycetous fungi belonging to the genus *Tuber*, which form ectomycorrhizal symbiosis with the roots of some trees as well as shrub species (Pegler et al., 1993). The life cycle of a truffle requires the succession of many steps: spore dispersion and germination, production of mycelia, contact with the host root, formation of the fungal mantle and the Hartig net, development of extraradical mycelia and fruitbodies.

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These ectomycorrhizal fungi are of great interest for forestry and are also in great demand due to the organoleptic properties of some species such as *Tuber magnatum* Pico and *Tuber melanosporum* Vittad. Several studies were recently carried out on the molecular characterization of these fungi, including the typing of different *Tuber* species (Lanfranco et al., 1993; Henrion et al., 1994; Potenza et al., 1994; Amicucci et al., 1996; Amicucci et al., 1997; Gandeboeuf et al., 1997; Longato and Bonfante, 1997; Paolocci et al., 1997; Amicucci et al., 1998; Bertini et al., 1998a, 1998b) and the development of experimental models representative of the three distinct phases of the life cycle: mycelium, mycorrhiza and fruitbody (Sisti et al., 1998). Little is known instead about truffle mycelium (Saltarelli et al., 1998), the biomolecular characterization of which is important in perfecting mycorrhization procedures and understanding the mechanisms involved in mycorrhiza formation. The aim of the present study was the analysis of genetic variability among four mycelial strains of *Tuber borchii*, a white truffle species, in order to identify, at the isolate level, specific markers suitable to investigate the structure and dynamics of populations in nurseries and plantations. DNA polymorphisms were investigated using PCR-based techniques involving analyses of random amplified polymorphic DNA (RAPD), intermicrosatellite regions and intergenic spacers of nuclear ribosomal DNA.

2. Materials and Methods

Mycelial strains

Four different mycelial strains designated 1BO (ATCC 96540), 10RA, 17BO and Z43 were isolated from fresh *Tuber borchii* fruitbodies. Strain 1BO was isolated from a fruitbody harvested in *Pinus pinea* and *Pinus pinaster* wood located in Cervia (Ravenna, Central Italy). The other three strains were isolated from fruitbodies harvested in experimental truffle-grounds of *Pinus pinea*, large about 1,000 m^2, located in Marina di Ravenna (Ravenna, Central Italy). Dried voucher specimens and mycelia are preserved in the "Dipartimento di Protezione e Valorizzazione Agroalimentare" of the University of Bologna, Italy. The isolates were grown in the dark at 24°C, with no agitation, in modified Melin-Norkrans nutrient solution (MMN), pH 6.6, according to the method of Molina (1979) in 100 ml flasks, each containing 70 ml of medium inoculated with fungus cultured in potato dextrose agar plugs.

DNA extraction

Genomic DNA of mycelia was isolated from 1-month-old cultures of *T.*

borchii (1BO, 10RA, 17BO, Z43) following the protocol described by Erland et al. (1993). Mycorrhizae were *in vitro* obtained using the method set up by Sisti et al. (1998). The ectomycorrhizal DNA was extracted using the method of Henrion et al. (1994). A purification step using Gene Clean II (Bio 101 Inc.) was performed on ectomycorrhizal DNA to avoid the PCR failure which can result from the presence of inhibitors such as phenols, polysaccharides and salts. The DNA was dissolved in Tris-EDTA (T.E.) and the final concentration was estimated either spectrophotometrically or by agarose gel electrophoresis staining with ethidium bromide.

PCR conditions

Amplification reactions were performed in a final volume of 25 µl using 100–200 ng of genomic DNA from fruitbody and mycelium or 25 ng from ectomycorrhiza.

Amplification with the universal primers ITS1-ITS4 and with the primers TBI-TBII

PCR amplifications of the Internal Transcribed Spacers were performed as described in Henrion et al. (1992, 1994). Primers TBI and TBII were used in amplification reaction as in Bertini et al. (1998 b).

RAPD analysis

Primers of arbitrary sequences, were obtained from Operon Technologies, Inc. (Alameda, CA, USA). They were OPL01, OPL02, OPL05, OPL06, OPL09, OPL13, OPAI10, OPC03, OPC18. RAPD amplification reactions were carried out according to Potenza et al. (1994).

Microsatellite analysis

The amplification reactions were performed in a 25 µl mixture volume containing 1X reaction buffer, 1.5 mM $MgCl_2$, 50 pmol of microsatellite primers and 1.5 U of Taq polymerase enzyme (Promega, Madison, WI, USA). Primers $(GTG)_5$, $(GAC)_5$, $(CAG)_5$, $(CA)_8$, $(CAGA)_4$ and $(TGTC)_4$ (Genenco, Florence, Italy) were used in accordance with a "touchdown" PCR protocol (Don et al., 1991). The amplification reaction with $(GTG)_5$, $(GAC)_5$, $(CAG)_5$ primers was performed in a 2400 Perkin Elmer Thermal Cycler programmed as follows: starting at an annealing temperature of 65°C, reduction by 1°C at each subsequent cycle to 55°C, 20 cycles at 55°C. Reactions with $(CAGA)_4$, $(TGTC)_4$ and $(CA)_8$ primers were run with an annealing temperature of 48°C for the final 20 cycles.

rDNA IGS analysis

The primers used to amplify the nuclear rDNA intergenic spacer 1 (IGS1), between the 25S and 5S rDNAs, were CNL12 and 5SA, originally designed by Anderson and Stasovski (1992). PCR amplifications of this IGS region were performed as described in Henrion et al. (1992, 1994).

Cloning and sequencing PCR products

Amplification products were purified using GenElute Agarose Spin Columns (Supelco). The purified DNA was re-amplified and ligated into the pCR2.1 Vector (TA Cloning kit, Invitrogen). Double-stranded plasmid DNA was sequenced using the dideoxy-chain termination method with universal reverse and forward M13 primers. The IGS1 sequences were deposited in GenBank under the following accession numbers: AF141611 (1BO), AF141020 (10RA1), AF141608 (10RA2), AF141609 (17BO), AF141610 (Z43).

Sequence analysis and selection of specific pairs of primer

The Pileup programe available in the Genetics Computer Group (GCG) Sequence Analysis Software Package version 7.1 (Devereux et al., 1984) was used for sequence alignment.

The pairs of primers specific for the strains of the species under study were selected from the IGS sequences. The choice of these pairs of primers was checked using the "Oligo" software, version 5 (National Biosciences Inc., Plymouth, MN, USA). Their sequences are the following:
10RAI 5'AGGGGTGGGTTGGGTTAGTT3';
10RAII 5'CTTTTGGTGGGCGAGATGT3';
1BOI 5'AAAGGGGAGGTTGGTGGAG3';
1BOII 5'CAATTCCTCTAAACACCTGTA3'.

Restriction enzyme analysis of the amplified products

One μg of 17 BO and Z43 amplified DNA was digested for 5 h with 2.5 units of various restriction enzymes (*Hinf*I, *Taq*I, *Bsr*I, *Alu*I). The restriction fragments were size-fractionated using 2.5% agarose gel electrophoresis (Sambrook et al., 1989). Agarose gels were stained with ethidium bromide and photographed under u.v. light.

3. Results

Before starting our study we confirmed that the four strains belonged to the

species *Tuber borchii* by using PCR strategies set up in previous work concerning species-specific identification (Bertini et al., 1998a, 1998b). The pair of primers TBI-TBII, specific for *Tuber borchii*, and restrictions with *Taq*I and *Hinf*I enzymes on ITS1-ITS4 amplification products, which give rise to electrophoretic patterns specific for this species, were used. Thus ten random primers 10 mer in length were used in RAPD (Williams et al., 1990) in order to identify strain-specific markers. A low level of polymorphism was observed among RAPD patterns of the four strains and for this reason no strain-specific marker was found (Fig. 1).

Primers corresponding to microsatellite sequences were also used to identify genetic polymorphisms. Using 6 microsatellite primers a low level of variability was observed. However, one polymorphic band obtained with primer $(GTG)_5$ was selected from strain 1BO, excised from the gel and used as probe on $(GTG)_5$ amplifications of the four strains blotted on a nylon membrane (Fig. 2). The specificity of this probe was not confirmed by autoradiography which also showed a signal from a bigger band common to the four strains. These fragments were sequenced. The common band revealed the same sequence, which was then aligned with that specific for 1BO. The alignment showed a similarity of 87.88%. Sequence analysis of inter-primers region did not reveal GTG repetitions. The high percentage of similarity did not allow us to select specific pairs of primers from the two sequences. The $(CA)_8$ primer gave no amplification products at an annealing temperature of 48°C.

More interesting results were obtained from the analysis of the intergenic ribosomal region amplified with primers CNL12 and 5SA (IGS1), which were complementary to sequences in the 3' region of 25S and 5' region of 5S, respectively. From the electrophoretic gel, no length polymorphism of the amplification products was observed in any of the four strains except for strain 10RA which showed an additional larger band. The common band was about 400 bp in length (Fig. 3). The absence of size variation of the IGS1 among strains led us to clone and sequence these products. From the alignment we obtained a detailed survey of nucleotide differences among strains: occurrence of deletion and/or insertion and base mutations were detected (Fig. 4). Two pairs of primers were selected from 10RA and 1BO sequences, which gave specific amplification products of 279 bp and 203 bp in length, respectively, only from these two strains (Fig. 5). These pair of primers were also applied in the characterization of the mycelium in the ectomycorrhizal phase. Ectomycorrhiza obtained *in vitro* after the infection of *Tilia platyphillos* Scop. with the four *T. borchii* strains (Sisti et al., 1998) were analysed. Specific amplification products were obtained from the ectomycorrhiza induced by 10RA and 1BO (Fig. 6). No PCR amplification was found for the other ectomycorrhizae and for the host plant, the DNA quality of which was checked by rDNA amplification using the NS1-NS2 primers (Dams et al., 1988). These results confirmed the specificity of the

Fig. 1   Fig. 2   Fig. 3
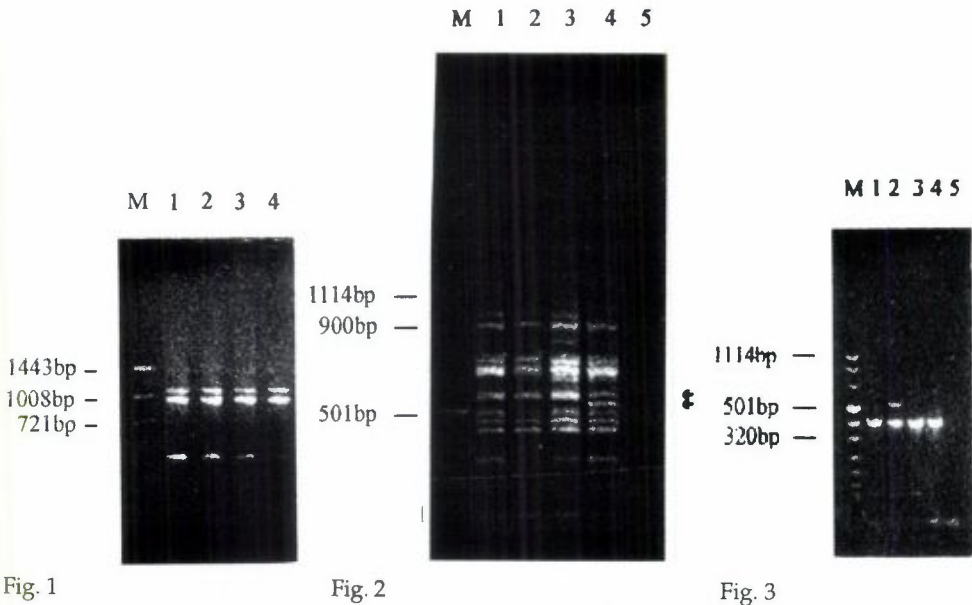
Figure 1.   Random amplification of total DNA from 4 isolates of *Tuber borchii* mycelium
using primer OPL 01. Lane 1: 1BO; lane 2: 10RA; lane 3: 17BO; lane 4: Z43. M:
DNA molecular weight marker pEmbl 8/*Taq*I.

Figure 2.   Amplification of the 4 strains of *Tuber borchii* with the (GTG)5 microsatellite
primer. Lane 1: Z43; lane 2: 17BO; lane 3: 10RA; lane 4: 1BO; lane 5: control
reaction. M: DNA molecular weight marker VIII (Boehringer). Upper arrow
indicates the common band; lower arrow indicates the band specific for 1BO
strain.

Figure 3.   Amplification of IGS1 using primers 5SA and CNL12 on DNA extracted from the
4 strains under study. Lane 1: 1BO; lane 2: 10RA; lane 3: 17BO; lane 4: Z43; lane
5: control reaction. M: DNA molecular weight marker VIII (Boehringer).

selected markers for 10RA and 1BO strains. Pairs of primers were not available
for the remaining strains (17BO and Z43) in that they show the same sequence,
except for five nucleotides. In order to discriminate between these strains we
identified restriction enzymes, which revealed these differences in nucleotide
sequences and provided strain specific restriction patterns. In Fig. 7 digestions of
Z43 and 17BO strains with *Hinf*I and *Taq*I are reported. The restriction
patterns with these enzymes allowed us to distinguish these strains.

4. Discussion

The four mycelial strains of *Tuber borchii* (1BO, 10RA, 17BO and Z43)

```
        1                                                          50
17bo    ..........  ..CAGAGTCC  TATGGCCGTG  GAT..CTCAT  AGTTGCGTTC
z43     ..........  ..CAGAGTCC  TATGGCCGTG  GAT..CTCAT  AGTTGCGTTC
10ra2   ..........  ..CAGAGTCC  TATGGCCGTG  GAT..CTCAT  AGTTTCGTTC
1bo     ..........  ..CAGAGTCC  TATGGCCGTG  GATATCTGAG  A.CGAAGTCC
10ra1   CACAGTACAA  GGTATGGCCA  TAAAAAAGTG  GTACGAAGCA  GGGGTGGGTT

        51                                                         100
17bo    GGCATCGTCG  AGCTTTCG..  ..........  .....GGAAT  GGAACGCTAC
z43     GGCATCGTTG  AGCTTTCG..  ..........  .....GGAAT  GGAACGCTAC
10ra2   GGCATCGTTC  AGCTTTTG..  ..........  .....GGAGT  GGAACGCCAC
1bo     GTTCAAGAGC  TTTCTGCGAC  GGATGAAAGG  GGAGGTTGGT  GGAGTGTAGT
10ra1   GGGTTAGTTT  CGGACAAAAT  TGTGGCACAC  GGCAACCAAA  CCGCCCTCTC

        101                                                        150
17bo    TGGGTGTAGT  CGTTTTCCAA  GGTATTGTG.  .ATAATACAC  ACCCTAGGGC
z43     TGGGTGTAGT  CGTTTTCCAA  GGTATTGTG.  .ATAATACAC  ACCCTAGGGC
10ra2   TGGGTGTAAC  CGCTTCCCAA  GGTATTGTG.  .ACAATACGT  AGCCTAGGGC
1bo     CTGTCATGGC  TCTTGTGAAG  TCCAAGGTGT  TCAAGAACGC  GTGGGAAACA
10ra1   ACAGAAAATA  TGTCACAATG  GTGATTTTTG  ATTTTTTCTT  TTTTCTGTTT

        151                                                        200
17bo    GAAGTTACTG  GCGTCGGTTT  CTACCACCGT  TTTTCTATCT  GGG...TAGA
z43     GAAGTTACTG  GCGTCGGTTT  CTACCACCGT  TTTTCTATCT  GGG...TAGA
10ra2   AACCTCACTG  GCGTCGGTTT  CTTTCCACTGT  CTTTCTATCT  GGG...TAGA
1bo     AATCTTTCGA  TAGCGGGAGG  GAGCTAGGAT  AGGACTGTAG  CTTGAGTTAT
10ra1   TTTTTATATA  TCAGTTTTGC  ATTTTATTCG  TACAACTACC  AGGAAAAACC

        201                                                        250
17bo    AATGTGCTAG  GATTGGTGCG  G.TCGTAAAT  TTCCTTTTCA  GCTCTTCGAA
z43     AATGTGCTAG  GATTGGTGCG  G.TCGTAAAT  TTCCTTTTCA  GCTCTTCGAA
10ra2   AATGTGCTAG  GATTGGCGCG  GATTGTCAAC  TTTCTTTTTC  ATTCCTCAAA
1bo     TAGAAGAGAT  ATGCCCACAG  ATTTGTAAGA  GGTGTTTACA  GGTGTTTAGA
10ra1   AGACATGCAG  CGTTAGCTAT  GGCCAAGAAA  AACCTACGCA  TCTCTCCCGT

        251                                                        300
17bo    TGCTCTGTCC  TGCTCTTGCC  CCCAGTAAAA  GGGTTTTTTC  ACCCCTTTCT
z43     CGCTCTGTCC  TGCTCTTGCC  CTCAGTAAAA  GGGTTTTTTC  ACCCCTTTCT
10ra2   GGTTTTTTCT  TGCTCCTGTC  CCCAGTAAAA  AGGGGTTTTC  ACCCCTTTCT
1bo     GGAATTGACC  AAGCTTANGG  GCCCCGCCGT  CAGATTTTAT  GCNAGACANT
10ra1   CATGGGATCA  AAGCCACGCT  CCCAAGACCC  TGTGGTATGG  GCCCAACCGC

        301                                                        350
17bo    CCTGATTCTT  TAGGGTATC.  GGTTATTGGT  TTACATATTC  CAGAGAAGTT
z43     CCTGTTTCTT  TAGGGTATC.  GGTTATTGGT  TTACATATTC  CAGAGAAGTT
10ra2   CTTAGTTTTT  TAGGGTATC.  GGTTGTTGGT  TTACATATTT  CAGGTAGGTT
1bo     GCTGATTAAT  TCATNGAAG.  AANAGATGTG  GGTGAAATTC  ACNACATCNA
10ra1   ACATCTCGCC  CACCAAAAGT  AGCAATACTC  CTAGCGAGCA  AGCAGGAAGA

        351                                                        400
17bo    TTTTATGGAT  CTTCGGTAGA  AATTTGCAAA  CCCAATAAAG  ATTTGTACCT
z43     TTTTATGAAT  CTTCGGTAGA  AATTTGCAAA  CCCAATAAAG  ATTTGTACCT
10ra2   TTTTAT.AAC  CTTCGGTAGA  AATTTGCACA  CCCAATGAAG  ATTTGTACCT
1bo     CGTCAAANAN  GGCNNGGGCT  CAATTCAATT  CCNTCAAAAC  CTTTGTACCT
10ra1   CCCAGAAAAC  CCTTCCGAAA  GCTCCCCTGA  ACAAACGAAA  AGTTTACAAA

        401                                                        450
17bo    CTTTTACTGA  CTTAGAGGCG  TTCAG.....  ..........  ..........
z43     CTTTTACTGT  CTTAGAGGCG  TTCAG.....  ..........  ...:......
10ra2   CTTTTACTGA  CTTAGAGGCG  TTCAG.....  ..........  ..........
1bo     CTTTTACTGA  CTTAGAGGCG  TTCAG.....  ..........  ..........
10ra1   AGAAAGCCCG  CACAAACGGG  AAAAGAAAGA  AACTCCACTA  ATCGAAAACC

        451                                                        500
17rbo   ..........  ..........  ..........  ..........  ..........
z43     ..........  ..........  ..........  ..........  ..........
10ra2   ..........  ..........  ..........  ..........  ..........
1bo     ..........  ..........  ..........  ..........  ..........
10ra1   TAACAAAGAA  TGAACACATC  TGACTTAGAG  GCGTTCAG..  ..........
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Figure 4. Alignment of the IGS1 nucleotide sequence of the four strains. The underlined sequences indicate strain-specific primers 10RA I-II; the sequences in bold type indicate strain-specific primers 1BO I-II. Strain 10RA presents a common band (10RA2) and an additional larger band (10RA1).
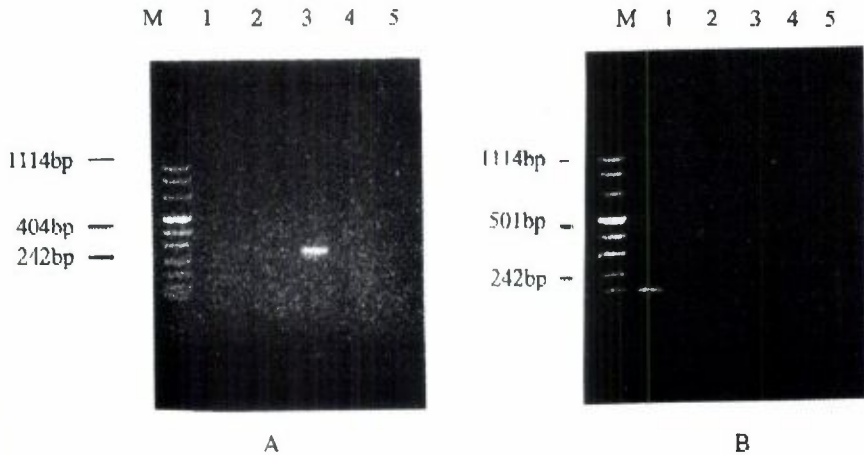
Figure 5. Amplifications of 4 strains of *Tuber borchii* mycelium with strain-specific primers 10 RAI-II (A) and 1BOI-II (B). Lane 1: 1BO; lane 2: 17BO; lane 3: 10RA; lane 4: Z43; lane 5: control reaction. M: DNA molecular weight marker VIII (Boehringer).
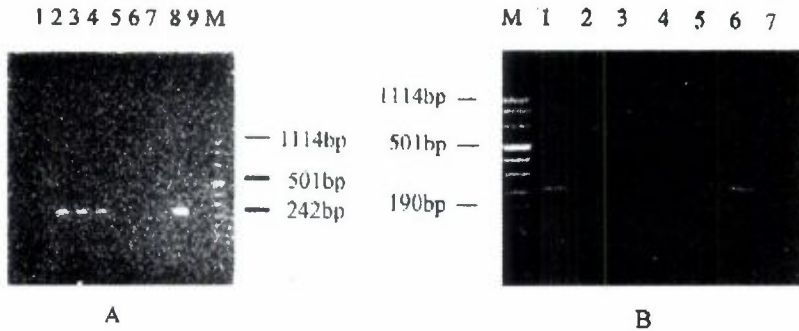

Figure 6. Use of strain-specific primers 10RAI-II (A) and 1BOI-II (B) on mycelium, ectomycorrhiza and host plant leaves. (A) Lane 1: ectomycorrhiza of 1BO; lanes 2-4: ectomycorrhiza of 10RA; lane 5: ectomycorrhiza of 17BO; lane 6: ectomycorrhiza of Z43; lane 7: leaf from *Tilia platyphyllos*; lane 8: 10 RA mycelium; lane 9: control reaction. (B) Lane 1: ectomycorrhiza of 1BO; lane 2: ectomycorrhiza of 10RA; lane 3: ectomycorhiza of 17BO; lane 4: ectomycorrhiza of Z43; lane 5: leaf from *Tilia platyphyllos*; lane 6: 1BO mycelium; lane 7: control reaction. M: DNA molecular weight marker VIII (Boehringer).

showed different morphological characteristics in liquid cultures and developed mycorrhiza with different anatomical-morphological features (Giomaro et al., 1998). In this study, we investigated if these strains, isolated from fruitbodies, grown *in vitro* in the same conditions, show genetic
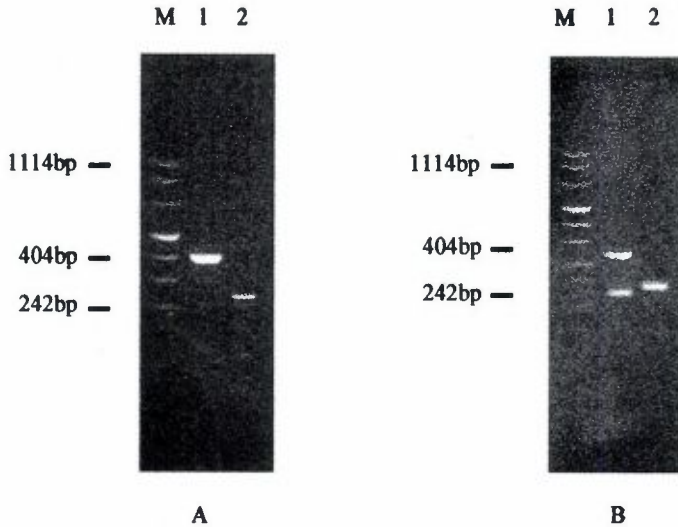
Figure 7.   Restriction analysis of amplified IGS1 region. Lane 1: Z43; lane 2: 17BO. M: DNA molecular weight marker VIII (Boehringer). A *Hinf*I; B *Taq*I. Strain restriction patterns are provided.

variability which could explain the different mycelial morphology and mycorrhizal competence observed.

Tuber borchii was selected because at present it is one of a few *Tuber* species that have been grown successfully *in vitro* as competent mycelium for plant mycorrhization. Several PCR applications have been used in the identification of intraspecific DNA polymorphisms. RAPD technique was selected in that the use of a single random primer allows the identification of inter and intraspecific polymorphism. Microsatellites were also used to reach this goal. Microsatellites in fact are a special class of tandem repeats that involve a motif of 1–10 bp repeated up to 100 times (Tautz, 1993). These sequences were first observed by Hamada et al. (1982), to have a different number of repeats and to be distributed along the genome in vertebrates and yeast. Their high mutation rate makes them very informative for screening genome differences (Bowcock et al., 1994; Meyer et al., 1991; 1993; Meyer and Mitchell, 1995; Weising et al., 1995; Longato and Bonfante, 1997; Martin et al., 1997). Despite the characteristics of these two strategies a low variability was revealed among the four strains. These results are in agreement with those observed for other *Tuber* species (Lanfranco et al., 1993; Amicucci et al., 1997; Bertault et al., 1998). In previous work we found a low degree of polymorphism among fruitbodies of *Tuber magnatum* species collected from different locations in

Central Italy (Amicucci et al., 1997). (GTG)$_5$ primers were also used by Longato and Bonfante (1997) in the detection of intraspecific polymorphisms. Low variability (S=90%) was revealed when isolates of *T. borchii* collected from the same geographical area were analysed (Urbino, Central Italy). Likewise, Bertault et al. (1998) analysed fruitbodies of *T. melanosporum* from French and Italian regions by RAPD and microsatellite-primed PCR. They also found an extremely low level of polymorphism over the whole study area for both types of marker.

In higher fungi, it is known that intergenic spacers present length and sequence variations within a species (Selosse et al., 1996; Hwang et al., 1998) and given this characteristic we selected this region for further analysis. The sequence analysis of the IGS region allowed us to select genetic markers and strain restriction patterns. These results demonstrated that the IGS1 region represents a suitable and attractive target in the determination of pure truffle cultures. The availability of strain-specific molecular markers may have biotechnological applications. In fact they can be used to confirm the persistence of the strain used for the infection or to reveal overwhelming by other competitive mycobionts. Furthermore, they provide a tool able to identify the strains in competition tests designed to evaluate mycorrhizal competence. It is in fact known that some individual fungi may be more competitive than others (Dahlberg and Stenström, 1991). This strategy allows an unequivocal strain identification, thus avoiding the time-consuming study of morphological features which can sometimes be ambiguous. This research undertaken using a small sample does not permit us to make general conclusions on genetic intraspecific variability, but it could have applications in optimizing large-scale inoculation programs of *Tuber borchii* species. Furthermore, the present typing approach could be easily extended to a wide range of species and also applied in ecological studies to estimate the distribution of isolates of ectomycorrhizal fungi in natural ecosystems.

Finally, the successful amplification of IGS1 obtained with primers CNL12 and 5SA, confirmed information about the localization of 5S rRNA in *Tuber*, already shown in Henrion et al. (1994).

In eukaryotes the 18-5.8-28S genes are transcribed by RNA polymerase I as a large transcript that is subsequently cleaved to yield the individual rRNAs, and the 5S rRNA is transcribed by RNA polymerase III (Van Heerikuhuizen et al., 1985; Clark, 1987). In fungi, 5S rRNA can be found either in the rDNA repeat or elsewhere in the genome (Selker et al., 1987). Our results showed that 5S is in the same strand as the 17-5.8-25S transcription unit. Further studies will be carried out to find genomic interspersed 5S.

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