Identification of *Tuber aestivum, T. panniferum* and *T. excavatum* by Restriction Fragment Length Polymorphism

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Abstract: A molecular method for the identification of *Tuber aestivum, T. panniferum* and *T. excavatum* was developed on samples collected in natural truffle-grounds in Sardinia. DNA was analysed by means of PCR using primers ITS1 and ITS4. The amplification gave products of different size for the three species: about 700 bp for *T. aestivum*, 680 bp *T. panniferum* and 650 bp *T. excavatum*. In addition, a 580 bp PCR fragment was obtained from a DNA sample of *T. rufum* fo. *nitidum*. Comparison with the ITS sequences available in GenBank showed differences in the ITS1 region of each species. For this reason, it was possible to choose restriction endonucleases suitable to distinguish the species under study. The enzymes *Apa* I and *Hae* II were selected for this purpose. Only in the ITS amplification product from *T. excavatum* was present a restriction site for *Apa* I at the position 143. The digestion produced two fragments of about 507 and 143 bp. *Hae* II was able to cut the ITS product of *T. aestivum* once (at the position 169) and the *T. panniferum* product twice (positions 150 and 622). *Hae* II digestion produced two fragments of 532 and 169 bp for *T. aestivum* and three fragments of 472, 150 and 58 bp for *T. panniferum*. The ITS amplification product of *T. rufum* fo. *nitidum* was not digested by *Apa* I or *Hae* II as its sequence does not contain restriction sites for these enzymes. RFLP method proved to be very useful for the identification of *T. aestivum, T. panniferum* and *T. excavatum.*

Key words: Restriction Fragment Length Polymorphism (RFLP), *Tuber aestivum, T. panniferum, T. excavatum*, truffles, identification

1 Materials and Methods

1.1 *Tuber* spp. samples

Truffle fruit bodies were collected in Sardinia in natural truffle-ground. The morphological determination revealed they belonged to four different species: *T. rufum* fo. *nitidum* (1 sample), *T. aestivum* (24 samples), *T. panniferum* (9 samples) and *T. excavatum* (4 samples).

1.2 DNA extraction

Total DNA was extracted from 50 mg of fruit body by Dneasy plant mini kit (Qiagen) and analysed by electrophoresis at 5V/cm, in 1.5% agarose gel in TAE running buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8. 0) containing ethidium bromide at a final concentration of 0.5 µg/ml. Gel images were acquired using the Gel Doc 2000 system (Bio-Rad Laboratories, Hercules, CA, USA). The concentration of DNA was determined by comparison with the molecular weight marker 1 kb plus DNA Ladder (Invitrogen), using the Quantity One software (Bio-Rad).

1.3 PCR

Universal primers ITS1 and ITS4[1] were used for the amplification of the ITS1 (Internal Transcribed Spacer) and ITS2 regions, and the rRNA 5.8S encoding gene. Reactions were performed in a volume of 25 µl containing sterile double-distilled water, 2.5 µl of 10X buffer, 200 µM of each nucleotide dATP, dGTP, dCTP, dTTP, 0.5 µM of each primer, 1 Unit of Red Taq DNA polymerase (Sigma), and about 10 ng of template DNA. A thermal cycler (PCR-Express Hybaid Limited, Middlesex, UK) was used with the following program: 1 cycle of 1 min at 94°C; 30 cycles of 1 min at 94°C, 1 min at 45°C and 1.5 min at 72°C; a final step of 7 min at 72°C. Amplification products were analysed by electrophoresis in 1.5% agarose gel. The molecular weight of the fragments was determined by comparison with the 1Kb plus DNA Ladder (Invitrogen).

1.4 RFLP

ITS sequences of *T. aestivum, T. panniferum, T. excavatum* and *T. rufum* found in the GenBank with accession numbers AY226042, AF132507, AJ557545 and AF106862 were compared using BLAST program (www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) and aligned by using CLUSTAL W program (www.ebi.ac.uk/clustalw/). Cut sites for restriction endonucleases were obtained using the software at www.restrictionmapper.org website. In this way, suitable enzymes were chosen in order to distinguish the species under study. Digestions of non-purified PCR products were performed at 37°C for 4 hrs, in a 20 µl total volume containing 5 U of enzyme. The sizes of the fragments were evaluated by electrophoresis in 2.5% agarose gel, using the 1Kb plus DNA Ladder (Invitrogen).

2 Results and Discussion

A total of 38 truffle samples, collected in Sardinia and classified as *T. aestivum, T. panniferum, T. excavatum* and *T. rufum* fo. *nitidum,* were analysed by PCR using primers ITS1 and ITS4. Amplification reactions gave products of different size: 700 bp for *T. aestivum* (22 out of 24 samples), 680 bp for *T. panniferum*, 650 bp for *T. excavatum* and 580 bp for *T. rufum* fo. *nitidum* (Figure 1).
Since these slight differences in size were not easy to observe, a more reliable method, suitable to distinguish the truffle species under study, was set up in this work. Comparison between the ITS sequences of the four species available in the GenBank showed differences mainly in the ITS1 region. On the basis of the restriction

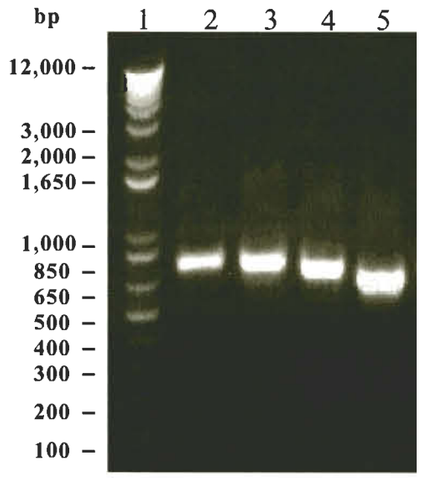

Figure 1. ITS amplification products
Tuber aestivum (lane 2); *T. panniferum* (lane 3); *T. excavatum* (lane 4); and *T. rufum* (lane 5); lane 1: 1 kb plus DNA ladder.

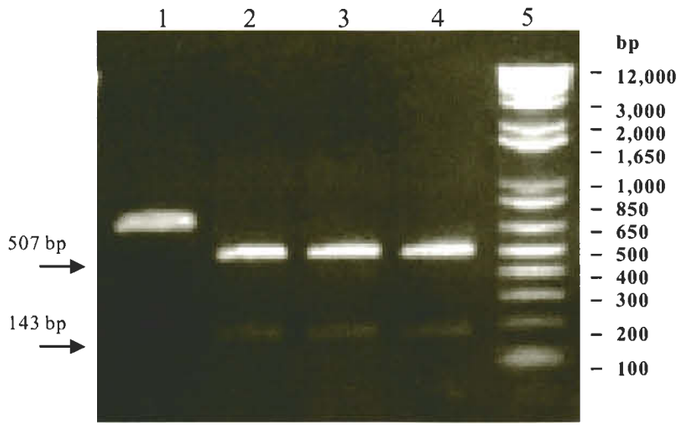

Figure 2. RFLP pattern of *T. excavatum* ITS products
Digested with the restriction enzyme *Apa* I (lanes 2-4); lane 1: uncut product; lane 5: 1 kb plus DNA ladder.

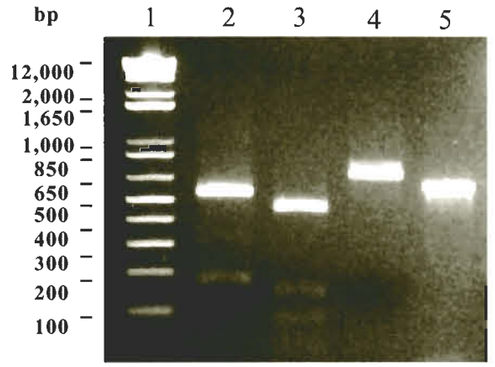

Figure 3. RFLP patterns obtained after digestion of the ITS products with the restriction enzyme *Hae* II
Lane 1: 1 kb plus DNA ladder; lane 2: *T. aestivum* (532 and 169 bp fragments); lane 3: *T. panniferum* (472, 150 and 58 bp fragments). Products of *T. excavatum* (lane 4) and *T. rufum* fo. *nitidum* (lane 5) remained uncut.

maps the endonucleases *Apa* I and *Hae* II were selected. *Apa* I digested only the ITS PCR product obtained from the DNA of *T. excavatum*, producing two fragments of about 507 and 143 bp (Figure 2) as expected. In fact a cut site for *Apa* I was present in the ITS1 sequence of *T. excavatum* at the position 143. *Hae* II was able to digest the ITS product of *T. aestivum* at the position 169, producing two fragments of 532 and 169 bp, whereas *T. panniferum* ITS product was cut twice (at the positions 150 and 622), so that three fragments of 472, 150 and 58 bp were obtained (Figure 3). The ITS amplification product of the *T. rufum* fo. *nitidum* sample was not digested by *Apa* I or *Hae* II as its sequence does not contain restriction sites for these enzymes (Figure 3). All the samples belonging to the same truffle species gave an identical RFLP pattern except for two samples morphologically determined as *T. aestivum*, which gave a PCR product of about 600 bp that besides cannot be cut by *Hae* II. Further analyses are being carried out on both samples. Nevertheless RFLP proved to be an easy and useful method for the identification of *T. aestivum, T. panniferum* and *T. excavatum* and it could be used at any stage of their life cycle.

References

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