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Single step molecular characterization of morphologically similar black truffle species

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Abstract

Species-specific internal ITS primers that amplify polymerase chain reaction (PCR) products of different lengths were selected to distinguish the morphologically similar ectomycorrhizal fungi *T. melanosporum*, *T. brumale* and *T. indicum* by aligning their internal transcribed spacer sequences and taking into account any incidence of intraspecific variability. In multiplex PCR experiments, the species-specific primers yielded the expected amplicons on template DNA isolated from the above mentioned species, while there was no amplification in PCR reactions carried out on fungal DNA from competing truffle species and host plants.

Keywords: Black truffle species; ITS; Species-specific primer; Multiplex polymerase chain reaction

1. Introduction

Truffles are ascomycetous fungi belonging mainly to the genus *Tuber* that establish symbiotic associations, ectomycorrhizae, with roots of trees and shrubs [1,2]. The ascocarps of some *Tuber* spp. are edible and marketed worldwide. Because certain truffle species, the European black *Tuber melanosporum* Vitt., and white, *Tuber magnatum* Pico, have a very distinctive flavour and command high prices, the market for them is particularly active and represents a valuable source of agricultural income in inland areas of some European countries. Although programmes of large scale truffle-inoculated plant production have been developed in these countries for reforestation and expanding the truffle market [3], the biology, ecology and identification of these fungal species remain insufficiently understood [4,5]. *Tuber* species characterization is based on morphological analysis, but reliable ascocarp, and above all, ectomycorrhizal identification may be hindered by the environmental and physiological dependent expression of the anatomical traits used [6]. Among black truffle species, *T. melanosporum*, *T. indicum* and *T. brumale* share similar morphological characteristics throughout their entire life cycle. Moreover, because the fruit bodies of *T. melanosporum* and the less prestigious Asiatic *T. indicum* species are very similar, differentiation between them is not easy on the basis of sporal microscope studies alone [7]. This

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has led to the witting, or unwitting, massive and increasing importation of *T. indicum* into Europe and their worldwide recommercialization as *T. melanosporum*. The economic consequences of uncontrolled importation of Asiatic black truffle ascocarps are obvious and its potential environmental impact worrying [8]. We report a single step technique that uses highly species-specific ITS primers to discriminate between *T. indicum*, *T. melanosporum* and *T. brumale* fruit bodies overcoming any intraspecific variability.

The use of these primers in multiplex polymerase chain reaction (PCR) analysis for identification of ectomycorrhizae is also proposed.

2. Materials and methods

2.1. Sample sources and DNA isolation

Ascocarps of the *Tuber* species listed in Table 1 and 110 black *T. indicum* truffles imported in France and Italy in 1995, 1996 and 1997 were used in this study. All truffles were first examined macro- and microscopically [9,10]. Fungal and host plant genomic DNA from *Quercus* spp. , *Corylus* spp. , *Populus* spp. and *Pinus pinea* were isolated as described by Henrion et al. [11].

2.2. Molecular analyses

The ITS region of *T. brumale* was amplified as below using the primers ITS1/ITS4 [12] from DNA isolated from a fruit body collected in Central Italy (Perugia) and the resulting product (about 900 bp) was cloned in pGM T-Easy vector (Promega). Double strand sequencing was carried out with Thermosequenase (Amersham, Cleveland, OH, USA) according to the supplier's instructions. The primers used for sequencing were: vector primers T7 and Sp6, the ITS1 and ITS4 and two nested primers 5.8SF (5′-TCGATGAAGAACGCAGC-3′) and 5.8SB (5′-GCTGCGTTCTTCATCGA-3′) deduced from the alignment of the 5.8S region of *Tuber* spp. and other fungi using the Clustal program [13]. The forward primer ITSML (5′-TGGCCATGTGTCAGATTTAGTA-3′), specific to *T. melanosporum*, and the backward primer ITS4LNG (5′-TGATATGCTTAAGTTCAGCGGG-3′) were designed previously by aligning the ITS sequence

Table 1
List of ascocarps of *Tuber* species and their collection sites[a]

T. melanosporum			**T. magnatum**		
1 Abruzzo (L'Aquila)	(19)	Italy	1 Molise	(4)	Italy
2 Umbria (Perugia)	(11)	Italy	2 Marche	(3)	Italy
3 Lazio (Campoli Appennino)	(5)	Italy	3 Piemonte (Val Curone)	(1)	Italy
4 Lazio (Latina)	(4)	Italy	4 Piemonte (Asti)	(1)	Italy
5 Toscana (Arezzo)	(3)	Italy	5 Lazio (Frosinone)	(4)	Italy
6 Marche	(2)	Italy	6 Umbria (Città di Castello)	(1)	Italy
7 Meuse	(2)	France	**T. borchii**		
8 Dordogne	(10)	France	1 Umbria	(9)	Italy
T. brumale			2 Basilicata	(6)	Italy
1 Abruzzo (L'Aquila)	(2)	Italy	**T. excavatum**		
2 Umbria (Valtopina)	(4)	Italy	1 Abruzzo	(1)	Italy
3 Marche (Acqualagna)	(3)	Italy	**T. macrosporum**		
4 Umbria (M. Tezio)	(7)	Italy	1 Marche (Acqualagna)	(2)	Italy
5 Lazio	(7)	Italy	2 Toscana (Arezzo)	(3)	Italy
6 Umbria (Città di Castello)	(3)	Italy	3 Umbria (Pietralunga)	(5)	Italy
6 Umbria (Monte S.M. Tiberina)	(4)	Italy	**T. aestivum**		
7 Vaucluse	(4)	France	1 Molise (Quadri)	(5)	Italy
8 Meuse	(5)	France	2 Emilia-Romagna (Piacenza)	(3)	Italy
T. rufum			3 Abruzzo	(11)	Italy
1 Meuse	(1)	France	**T. mesentericum**		
2 Umbria (Perugia)	(2)	Italy	1 Abruzzo	(4)	Italy

[a]The numbers in brackets are the truffles processed for each collection site.

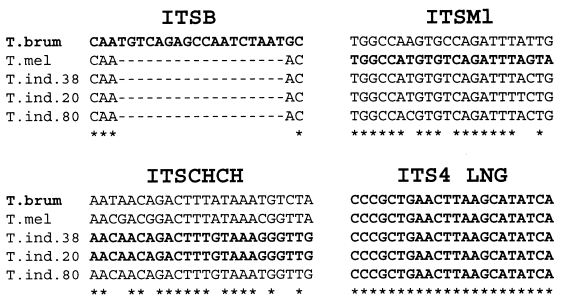

Fig. 1. Multiple sequence alignment of ITS subregions of black truffle species where the ITSB, ITSML, ITSCHCH and ITS4LNG primers span. *T.brum*: *T. brumale*; *T.mel*: *T. melanosporum* (U89359); *T.ind.*38: *T. indicum* ascocarp 38 (U89361); *T.ind.*20: *T. indicum* ascocarp 20 (U89362); *T.ind.*80: *T. indicum* ascocarp 80 (U89360). In brackets are the accession numbers.

of *T. melanosporum* with that of *T. indicum* [14]; a primer specific to *T. brumale*, ITSB (5′-CAATGTCAGAGCCAATCTAATGC-3′) and one specific to *T. indicum*, ITSCHCH (5′-AACAACAGACTTTGTAAAGGGTTG-3′), by aligning the ITS sequences of all black truffle species considered (Fig. 1). All primers were supplied by Life Technology (Glasgow, Scotland, UK).

The ITS sequence of *T. brumale* is deposited in GenBank under the accession number AF001010.

2.3. PCR amplification

PCR amplification with species-specific primer pairs was carried out in an OMNI-E (Hybaid, UK) thermal cycler using 0.3–5 ng of target DNA isolated from control plants and ascocarps of truffles species by: a first denaturation step at 95°C for 3 min; 23 cycles consisting of 30 s at 94°C, 30 s at 63°C and 45 s at 72°C; a final extension step for 7 min at 72°C. The amplifications were carried out in a final aqueous volume of 50 μl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 4 mM $MgCl_2$, 20 pmol of each specific primer, 200 mM of each dNTP, in the presence of 2.5 units of Taq Polymerase (Pharmacia Biotech, Uppsala, Sweden). Twenty μl of PCR products were run on 2% agarose gels.

PCR amplification with ITS1 and ITS4 primers on host plants and ectomicorrhizal fungi was performed as above, but the annealing temperature was decreased to 55°C. Positive and negative controls (no DNA template) were included in all experiments.

*Rsa*I/RFLPs analysis of ITS fragments amplified from all the *T. indicum* and *T. melanosporum* isolates were carried out and gel fractionated as previously described [14].

3. Results

3.1. Amplification and analysis of ITS

All *T. brumale* ascocarps displayed an amplification product of about 900 bp with the primer pair ITS1/ITS4 and sequence analysis and alignment of *T. brumale*, *T. melanosporum* and *T. indicum* ITS revealed length polymorphism in the ITS1 region of the first species, which was about 300 bp longer than the other two species. No length polymorphism was detected between the *T. indicum* and *T. melanosporum* ITS fragments and ITS/RFLPs analysis with the restriction enzyme *Rsa*I revealed the three molecular classes of *T. indicum* already described [14], but no ITS intraspecific variability among the *T. melanosporum* ascocarps under investigation (data not shown).

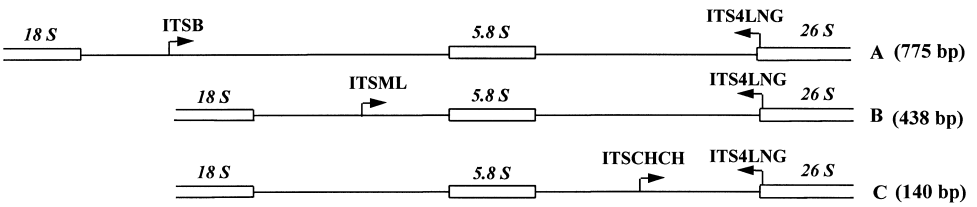

Fig. 2. Schematic representation of annealing sites of ITS species-specific primers on black truffle ITS sequences. a: *T. brumale*; b: *T. melanosporum*; c: *T. indicum*. In brackets are the approximate lengths of the expected amplicons.

3.2. Species-specific ITS primers selection and multiplex PCR amplification

The primer pairs ITSCHCH/ITS4LNG, ITSML/ITS4LNG and ITSBR/ITS4LNG gave, respectively, PCR fragments: about 140 bp on all the genotypes of *T. indicum* irrespective of their *Rsa*I ITS restriction patterns; 438 bp on *T. melanosporum* and 775 bp on *T. brumale* (Fig. 2), but none yielded amplification of ITS on any DNA from host plants or the other *Tuber* species listed in Table 1. Moreover, ITSCHCH/ITS4LNG never amplified ITS of *T. brumale* or *T. melanosporum*, which were highly and clearly amplified only by their specific forward primers ITSB and ITSML, respectively (data not shown). Pooled DNA isolated from the three fungus species studied and amplified in a multiplex PCR in the presence of all four primers showed the three specific bands, whereas no amplicons were obtained when the target DNA were isolated from other truffle species or from host plants (Fig. 3). As a control the same target DNAs were amplified with the primer pair ITS1/ITS4 (Fig. 4).

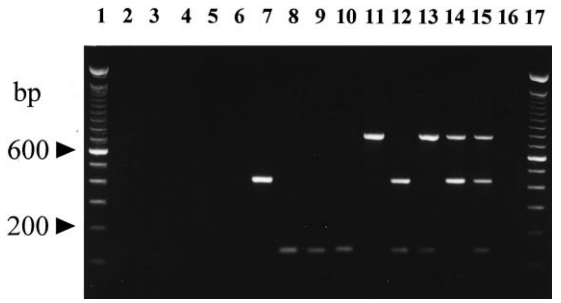

Fig. 3. Multiplex PCR amplification of DNA isolated from *Tuber* species ascocarps and host plants with black truffle species-specific ITS primers ITSB, ITSML, ITSCHCH and ITS4LNG. Lanes 1 and 17: 100-bp Ladder (Gibco-BRL); lane 2: *T. aestivum*; lane 3: *Corylus avellana*; lane 4: *T. borchii*; lane 5: *T. magnatum*; lane 6: *T. rufum*; lane 7: *T. melanosporum*; lane 8: *T. indicum* ascocarp 20; lane 9: *T. indicum* ascocarp 38; lane 10: *T. indicum* ascocarp 80; lane 11: *T. brumale*; lane 12: mixture of *T. melanosporum* and *T. indicum* DNA; lane 13: mixture of *T. brumale* and *T. indicum* DNA; lane 14: mixture of *T. brumale* and *T. melanosporum* DNA; lane 15: mixture of *T. brumale*, *T. indicum* and *T. melanosporum* DNA; lane 16: negative control (no DNA template).

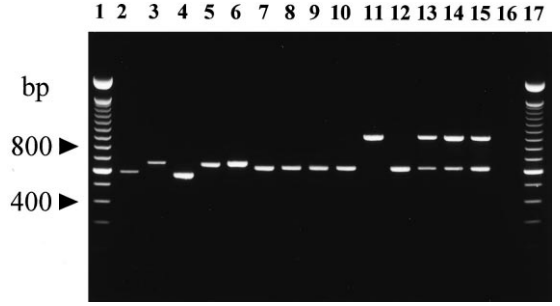

Fig. 4. PCR amplification of truffle ascocarps and host plants by universal primers ITS1/ITS4. Lanes are as in Fig. 3.

4. Discussion

The ITS1 length polymorphism of *T. brumale* vs. those of *T. indicum* and *T. melanosporum* allowed a *T. brumale* specific primer to be designed. The specificity was later confirmed as all *T. brumale* genotypes displayed the expected amplicon of about 775 bp with ITSB/ITS4LNG primers, whereas *T. melanosporum*, *T. indicum* and host plant species did not (data not shown). The ITSML and ITSCHCH primers were designed, respectively, for *T. melanosporum* and *T. indicum*, whose ITS exhibit no length polymorphism (Fig. 4), to: (a) allow efficient amplification by highly specific primers that perfectly match the target DNA at the 3′ end [15,16]; (b) overcome the low and high genetic variability present, respectively, within *T. melanosporum* and *T. indicum* [14,17]; (c) yield amplicons of different lengths to distinguish *T. brumale*, *T. indicum* and *T. melanosporum* when they are present in a single reaction.

Despite the high intraspecific variability previously reported in *T. indicum* ITS sequences [14,17], the ITSCHCH primer spans a ribosomal subregion which is well conserved in all genotypes so far considered and so overcomes this problem. Moreover, all the *T. melanosporum* and *T. indicum* samples tested in this study were clearly amplified only by their own specific forward primers ITSML and ITSCHCH, respectively, whereas none of the competing truffle species did.

Extensive morphological analyses have been carried out on Asiatic truffles and new Asiatic-origin truffle species have been recently described on the basis of sporal morphology [18]. The molecular anal-

ysis we adopted on all Chinese truffles examined confirmed the presence of three *Rsa*I ITS/RFLPs classes among these samples, despite the high heterogeneity for the epispore structure among and within each ascocarp. As the greatest progress in understanding taxonomic and phylogenetic relationships of these mycorrhizal fungi will be achieved by combining classical morphological data with information obtained by using molecular tools, we are presently carrying out extensive morphological and molecular studies on highly polymorphic Asiatic fungi.

The multiplex PCR we propose for dealing with the massive importation of cheaper *T. indicum* ascocarps into Europe and their commercialization as the more prestigious *T. melanosporum*, offers a more powerful and rapid diagnostic method than the use of ITS/RFLPs, SCAR and PCR amplification with species-specific primers that exhibit amplicons of the same length on different templates [14,17].

With our method, the three black truffle species can unambiguously be differentiated by performing a single amplification reaction, and comparing the length of amplicons obtained. It should also be possible to apply this molecular approach to accurately monitor the ectomycorrhizae of the species investigated since none of the ITS species-specific primers revealed PCR products on the most common host plants. These findings should have not only considerable economic, but more importantly, ecological impact, seeing that Asiatic black truffles may be knowingly or unknowingly introduced as spore donor for the production of artificially inoculated plants to be planted out in Europe.

The PCR based approach reported should also be applicable to other truffle species whose fruit bodies and/or ectomycorrhizae cannot be clearly typed by morphological analysis, as is the case with *T. magnatum* vs. *T. borchii* species.

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