Molecular Tools for the Identification of *Tuber melanosporum* in Agroindustry

Nathalie Séjalon-Delmas,* Christophe Roux, Monique Martins, Michel Kulifaj, Guillaume Bécard, and Robert Dargent

Mycologie Végétale, UMR 5546 CNRS/Université P. Sabatier, Pôle de Biotechnologie végétale, 24 Chemin de Borde-Rouge, BP 17, 31326 Castanet-Tolosan, France

Tuber melanosporum Vitt., *Tuber magnatum* Pico, and *Tuber uncinatum* Chat. can be differentiated by their morphological characters. Fraud problems have arisen recently with the importation to Europe of truffles from China. *T. melanosporum* is morphologically very close, but distinct from the Chinese species [*Tuber indicum* (Cooke and Massee) and *T. himalayense* BC (Zhang and Winter)]. We have optimized molecular tools to unequivocally identify *T. melanosporum*. DNA extraction from ascocarps of black truffles is not straightforward. Problems to obtain pure DNA are due to high contents of phenolic compounds, melanine, and various polymers (proteins, polysaccharides, etc). These compounds coprecipitate with the DNA during extraction and strongly inhibit the PCR reaction. We have developed an efficient and reliable protocol for DNA extraction from truffle ascocarps. It was used successfully for DNA extraction from mycorrhizal root tips as well as from canned preparations of *T. melanosporum*. Several approaches to identify *T. melanosporum* by PCR were developed. Two specific primers for *T. melanosporum* were designed after comparison of the ITS region of this species with those of three Chinese fungi. They proved to be efficient to specifically detect the presence of *T. melanosporum* by PCR. The mycorrhizal status of trees inoculated with *T. melanosporum* but unable to produce truffles was confirmed in a single-step PCR reaction. A multiplex PCR approach was also developed with three sets of primers (including a specific one for Chinese truffles) to detect, in one PCR reaction, the presence of any other *Tuber* species mixed with *T. melanosporum* ascocarps. This optimized protocol, in association with the specific primers we designed, is applicable to quality control in the truffle industry from the production stages to final commercial products.

Keywords: *Alcalase; Black truffles; Chinese truffles; BLOTTO (Bovine Lacto Transfer Technique Optimizer); DNA extraction; ITS (internal transcribed spacer) sequence; PCR specific detection*

INTRODUCTION

Several truffle species are among the most valuable mushrooms on the market because of their unique organoleptic properties. *Tuber melanosporum* among the black truffles and *Tuber magnatum* (Pico) among the white truffles are by far the two species with the highest economic value. The market for them is particularly active and represents a valuable source of agricultural income in some areas of southern Europe. French production has been decreasing since the beginning of the century (Kulifaj, 1994). Programs for large-scale production have been set up to increase truffle production and expand the market. The main steps are the production of mycorrhizal seedlings (using spores or mycorrhizal root inocula), planting and cultivating the mycorrhizal trees, monitoring the presence of inoculated fungal species, and fruitbody harvesting. The identification of *Tuber* at the ectomycorrhizal stage is critical to control inoculation and production of mycorrhizal seedlings. The difficult identification of the fungal symbiont at this stage and the increasing demand for mycorrhizal plants could easily lead to propagation of mistaken mycorrhizal fungal species. Fruitbodies can be subject to fraud. For example, *Tuber indicum*, recently imported from China, is very similar to *T. melanosporum* ascocarps and sometimes mixed with them on European markets. The correct identification of truffle fruitbodies is not always easy. Among black truffles, *T. melanosporum*, *T. indicum*, but also *Tuber brumale* Vitt. share similar characteristics throughout their entire life cycle. Their fruitbodies can only be differentiated after close examination of the peridium and microscopic investigations. In the case of *T. melanosporum* and *T. indicum*, differentiation is possible only on mature ascocarps (Janex-Favre et al., 1996). Tools are then needed for a rapid control of production quality and identification of truffle fruitbodies (fresh and canned).

Several analytical approaches have been developed to identify and characterize *Tuber* species: biophysical (Papa et al., 1987), biochemical (Mouches et al., 1981; Dupré and Chevalier, 1991; Palenzona et al., 1990; Cameleyre and Olivier, 1993; Gandeboeuf et al., 1994), olfactory (Pacioni and Pomponi, 1989), and immunological (Corocher et al., 1992, Zambonelli et al., 1993, Neuner-Plattner et al., 1999). These approaches can be species-specific, but their use to specifically identify mycorrhiza is limited by the amount of biological material required (around 100 mycorrhizal root tips per analysis). In contrast, PCR-based DNA detection can be performed on minute amounts of tissue. It can be used

* To whom correspondence should be addressed. Phone: 33 5 62 19 35 04 Fax: 33 5 32 19 35 02. E mail: ndelmas@cict.fr.

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Table 1. Origin of the Fungal Material and Sequences Used for the Alignment and the Design of Specific Primers

species	isolate	origin	GenBank accession no.
Tuber melanosporum (Vitt.)	Tm13, TmM2, TmA1	? L'Aquila (Italy)	AF132501,[a] (EMBL) Y09790, U89359
Tuber indicum (Cooke & Massee)	Ti17, Ti80 , Ti20	Huidong (China) ? ?	AF132502,[a] U89360, U89362
Tuber himalayense (Zhang and Minter)	Th20	Huidong (China)	AF132503[a]
Tuber pseudo-himalayense ?	Ti38		U89361
Tuber mesentericum Vitt.			AF132508[a]
Tuber panniferum Tul.			AF132507[a]
Tuber borchii Vitt.			AF132505[a]
Tuber ferrugineum Vitt.			AF132506[a]
Tuber uncinatum Ch.			AF132509[a]
Tuber brumale Vitt.			AF132504[a]
Tuber brumale f *moschatum*			AF001010
Tuber magnatum			AF003913
Tuber puberulum			AF003918
Tuber dryophilum			AF003917
Tuber maculatum			AF003919
Tuber aestivum			U95175
Tuber excavatum			AF073509

[a] Sequences obtained in this study. The others were obtained from GenBank or EMBL.

like the above methods for specific detection of fruitbodies, but is ideally suited to the identification of small samples such as mycelium and ectomycorrhizae. The internal transcribed spacers (ITS) of rDNA are commonly used in mycorrhizal diagnostic (Gardes et al., 1993). ITS regions are present in multiple copies and tend to be similar within and variable between fungal species. Primers designed for the amplification of species-specific ITS regions have been used to develop sensitive techniques for the detection of *Tuber* species (Amicucci et al., 1998; Henrion et al., 1994; Longato and Bonfante, 1995; Paolocci et al., 1995, 1997; Rubini et al., 1998). Other PCR-based methods have also been used: RAPD (Gandeboeuf et al., 1997a,b), microsatellites (Lanfranco et al., 1993), and the corresponding primers specifically designed. However, many problems remain for the easy and reliable identification of fungal species in mycorrhizal roots. PCR reactions are easily inhibited by phenolic substances, polysaccharides, and humic acids, which are often mixed with the DNA extracted from plant roots (Tsai et al., 1992).

In the present study, we optimized DNA extraction and improved the PCR conditions for the identification of truffles. We illustrate the performance of our optimized methods with difficult samples such as ectomycorrhizae and canned truffles. This method, along with multiplex PCR, may be used to detect any fungal species mixed with *T. melanosporum*.

EXPERIMENTAL PROCEDURES

Fungal Materials. The origins of the *T. melanosporum* and Chinese ascocarps used for multiplex PCR are listed in Table 1. Canned truffles were provided by Pebeyre S.A (Cahors 46000, France). Briefly, the truffles had been autoclaved twice for 2 h at 120 °C and then for 1 h more. Mycorrhizal root tips from truffle-producing and nonproducing trees were collected in an experimental field at Le Bugue, 46000 France. All truffle materials were kept at −20 °C prior to analysis.

Optimization of DNA Extraction. DNA from 50 mg of truffle ascocarps (frozen or canned) or from a single mycorrhiza (from *Quercus pubescens* Wild.) was extracted by grinding the tissues in 200 μL CTAB extraction buffer (Henrion et al., 1994). After centrifugation, the supernatant was mixed with 10 μL of Alcalase 2.4.L (Novo Nordic, 92017 Nanterre, France), for 5 min at 65 °C. Then, 10 μL of RNAse A (10 mg/mL) was added for 10 min at 37 °C. Potassium acetate (5 M, pH 6) was added to the previous mix (0.5 v/v) and incubated for 10 min at 4 °C. After centrifugation, 15 min at 10 000 rpm, the supernatant was mixed with chloroform (v/v) and centrifuged for 5 min at 8000 rpm. This last step was repeated twice. 2-Propanol (0.6 vol) was added to the aqueous phase and DNA was precipitated for 10 min to 1h at −20 °C. After centrifugation, 10 min at 10 000 rpm, the DNA pellet was washed twice with 70% cold ethanol. It was then air-dried and resuspended in 50 μL of 10 mM Tris- HCl and 1 mM EDTA buffer, pH 8. The quality and quantity of DNA were estimated after measurement of the optical density at 210, 260, 280, and 320 nm. DNA was diluted to 2.5 μg/mL for PCR reactions.

The juice surrounding the canned truffle was also used for DNA extraction. A volume of 2.5 mL of the juice was added to 5 M potassium acetate pH 6 (1v/2v) and left for 10 min at 4 °C. DNA extraction was carried out as described above.

Specific Primers for *T. melanosporum*. To specifically detect *T. melanosporum* by PCR, primers were designed after comparison of ITS sequences from several *Tuber* species (GenBank accession number is in Table 1). Alignment was performed with Seaview software, using the Clustal W algorithm. We designed the primers for the ITS region where no variation had been observed in a previous PCR-RFLP analysis (Roux et al., 1999). The first set of primers was called TM1/TM2. A second set was designed for use with "universal fungal primers" (ITS1F, ITS4) in multiplex reactions: TM1b associated with ITS4 was specific for *T. melanosporum*. Reverse Ti3 primer was mixed with ITS1F to specifically detect *T. indicum* or *T. himalayense* (Table 2; Figure 1).

PCR Reaction Protocol. The amplifications were conducted using 25 μL of DNA template solution in 50 μL of reaction mixture. The final solution contained 25 μM each of dATP, dCTP, dGTP, and dTTP (Euromedex, Belgium), 0.5 μM of each primer and 1 unit of thermostable polymerase (Red Hot, Fischer Scientific, France). The cycling conditions consisted of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles at 52 °C (1 min), 72 °C (1 min), and 94 °C (1 min). Six microliters of the amplified products was visualized by 2% agarose gel electrophoresis. For multiplex PCR reaction, 30 ng of each DNA template (from *T. indicum*, *T. brumale*, and *T. melanosporum*, or *T. melanosporum* and *T. indicum*) were mixed in the tube with 25 μL of reaction mixture containing 1.25 μM ITS1F, Ti3, and ITS4 with 1 μM TM1b primer. The PCR cycling procedure consisted of 30 cycles with 1 min 30 at 52 °C for the annealing step. A positive control (DNA sample from an ascocarp of *T. melanosporum*) which was always amplified and a negative control (with no DNA template) were included in each experiment. The PCR reaction was optimized by adding 2% (v/v) of a 10% (w/v)

Table 2. Primers for the Amplification of ITS Sequences of *T. melanosporum* and of Chinese Truffles

primers	forward	reverse
TM 1	CCTGTGGGAGATCTCTAT	
TM 2		GATGGGGGTCCTTAAGGTAA
TM 1b	GCACCTGTGGGAGATCTCTAT	
Ti3		CCTACAACCATTTACAAAGTCTGTT
ITS 1F[a]	CTTGGTCATTTAGAGGAAGTAA	
ITS4[b]		TCCTCCGCTTATTGATATGC

[a] Gardes and Bruns, 1993. [b] White et al., 1990.

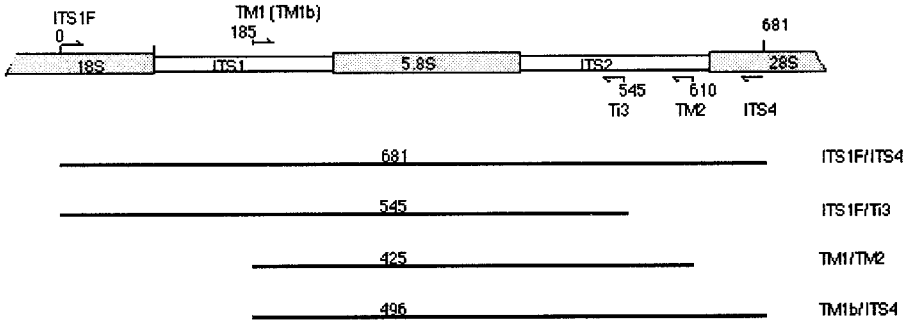

Figure 1. Position on the ITS sequences of the different primers used in this study, with the length of the amplified products. Ti3 specific primer of *Tuber indicum* is drawn on the same figure as the *T. melanosporum* specific primers, but hybridizes only on *Tuber indicum* ITS sequence.

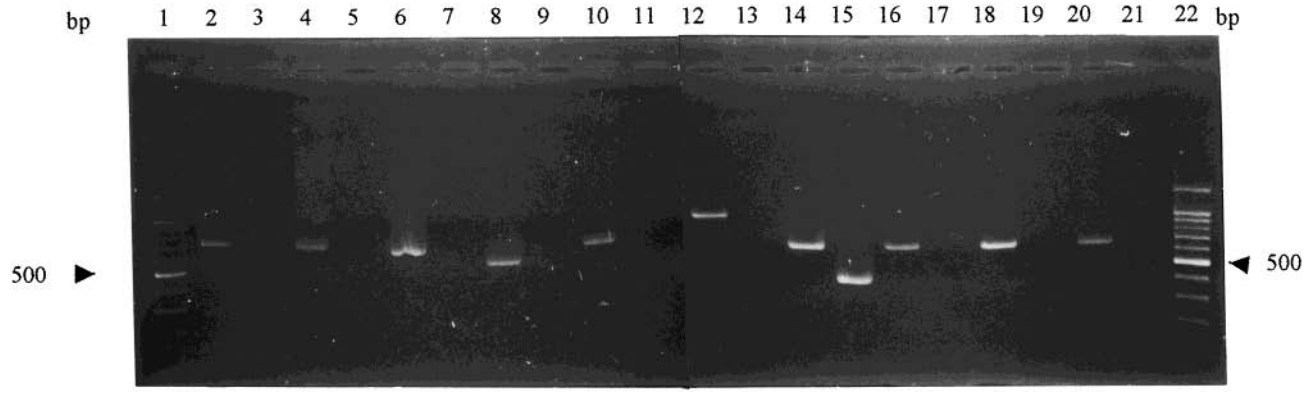

Figure 2. PCR amplification of the rDNA ITS1-5.8S ITS2 sequences of various species of *Tuber* with ITS1F/ITS4 (even lanes) and TM1/TM2 (odd lanes) primers. Lanes 1 and 22: DNA ladder, 100 bp. Lanes 2 and 3, *T. panniferum*. Lanes 4 and 5: *T uncinatum*. Lanes 6 and 7: *T. ferrugineum*. Lanes 8 and 9: *T. borchii*. Lanes 10 and 11: *T. mesentericum*. Lanes 12 and 13: *T. brumale*. Lanes 14 and 15: *T. melanosporum*. Lanes 16 and 17: *T. indicum*. Lanes 18 and 19: *T. himalayense* sample 15. Lanes 20 and 21*: T. himalayense*, sample 20.

skimmed milk solution [BLOTTO (Bovine Lacto Transfer Technique Optimizer)] to the mix (De Boer et al., 1995).

RESULTS

Alignment of ITS sequences from different species of truffle allowed the design of primers for the specific detection of *T. melanosporum*. The specificity of these primers was assessed on different *Tuber* species (Figure 2). To exclude false negatives, DNA quality was checked using ITS1F/ITS4 universal primers. Except for *T. melanosporum* and *T. indicum*, every truffle species tested was discriminated on the basis of its ITS size (Figure 2). TM1/TM2 primers did not allow amplification of *Tuber* species other than *T. melanosporum*. This set of primers was used for the identification of ectomycorrhizae. DNA from a single mycorrhizal tip was extracted using a protocol optimized by addition of Alcalase. This step was important to improve the quality of the extracted DNA (data not shown). DNA amplification on mycorrhizae was also optimized by addition of BLOTTO to the PCR mix. The addition of BLOTTO allowed a better amplification of DNA from root samples, reducing the number of false negatives. TM1/TM2 primers amplified the ITS sequence of *T. melanosporum* from roots of a productive tree (Figure 3, lane 2), but also from roots of a tree not producing truffles (lane 4). Oak seedlings growing under both trees were also used for *T. melanosporum* detection. A positive reaction was obtained from roots of all seedlings, indicating the presence in the soil of *T. melanosporum* mycelium under both the productive and the nonproductive tree. No amplification products were ever obtained with DNA from the host tree.

This optimized protocol was used for extraction and amplification of DNA from canned ascocarps. Amplification products of the expected size were obtained with DNA extracted from the cooked ascocarps but also from the canning juice (Figure 4). PCR reactions carried out without BLOTTO were negative in both cases.

Two additional primers Ti3 and TM1b were designed after alignment of ITS sequences of *T. melanosporum*, *T. indicum*, and *T. himalayense*. These primers were designed to work in multiplex reaction with ITS1F/ITS4 universal primers. Ti3/ITS1F specifically amplified *T.*

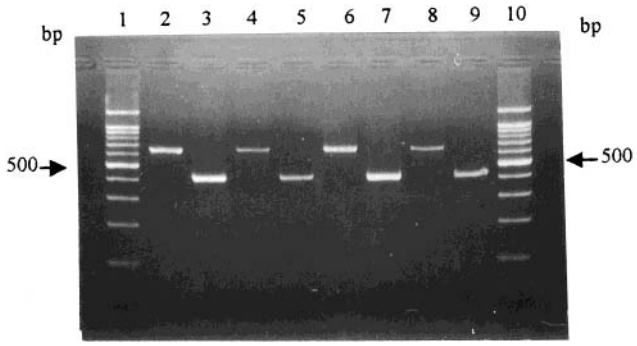

Figure 3. PCR amplification, optimized with the use of BLOTTO, of DNA extracted from mycorrhizae sampled from trees producing truffles and not, using ITSF/ITS4 (even lanes) and TM1/TM2 (odd lanes) primers. Lanes 1 and 10: DNA ladder, 100 bp. Lanes 2 and 3: mycorrhizae from a productive tree. Lanes 4 and 5: mycorrhizae from seedlings growing under the productive tree. Lanes 6 and 7: mycorrhizae from a nonproductive tree. Lanes 8 and 9: mycorrhizae from seedlings growing under the nonproductive tree.

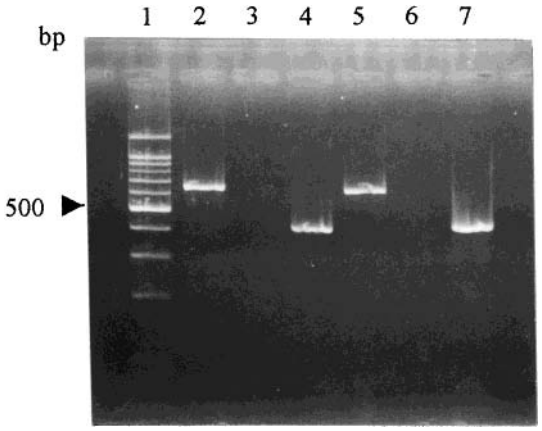

Figure 4. PCR amplification of ITS sequence of DNA extracted from canned *T. melanosporum* and from the canning juice. Lane 1: DNA ladder, 100 bp. Lanes 2 and 3: cooked ascocarp with ITS 1F/ ITS4 primers in the presence or absence of BLOTTO, respectively. Lane 4: cooked ascocarp with TM1/ TM2, in the presence of BLOTTO. Lanes 5 and 7: canning juice with ITS1F/ITS4 primers, in the presence or absence of BLOTTO, respectively. Lane 6: canning juice with TM1/TM2 primers, in the presence of BLOTTO.

indicum and *T. himalayense* whereas TM1b/ITS4 amplified only *T. melanosporum*. A multiplex reaction carried out with a mixture of DNA from *T. indicum*, *T. melanosporum*, and *T. brumale* showed an amplification of *T. indicum* with Ti3/ITS1F and *T. melanosporum* with TM1b/ITS4. Amplification of *T. melanosporum* and *T. indicum* with ITS1F/ITS4 combination gave two amplification products of the same size (680 bp) confounded in a single band, whereas *T. brumale* was identified on the basis of its ITS size after amplification with ITS1F/ITS4 (Figure 5).

DISCUSSION

Tuber species are determined on the basis of ascocarp and/or ascospore morphology. This can pose problems to differentiate between the various white or black truffles. The recent occurrence, on the European market, of Asian truffles, morphologically very close to *T. melanosporum* (Janex-Favre et al., 1996), raises two kinds of problems: (i) fraud, since Asian truffles are cheaper and their organoleptic qualities are not certified

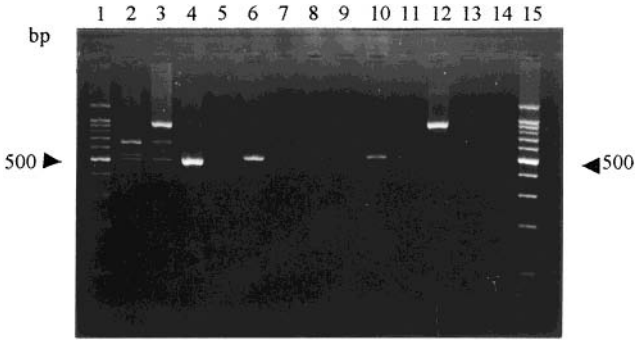

Figure 5. Multiplex PCR amplifications of DNA isolated from black truffles with the set of primers specific for *T. melanosporum* and Chinese truffles. Lanes 1 and 15: DNA ladder 100 bp. Lane 2: multiplex reaction with ITS1F/ITS4 and TM1b/Ti3 primers on mixed DNA from *T. melanosporum* and *T. indicum*. Lane 3: multiplex PCR on a DNA mixture of *T. melanosporum*, *T. indicum*, and *T. brumale*. Lanes 4 and 5: amplification of *T. melanosporum* with the sets of primers TM1b/ITS4 and Ti3/ITS1F, respectively. Lanes 6 and 7: amplification of *T. indicum* with Ti3/ITS1F and TM1b/ITS4, respectively. Lanes 8 and 9: amplification with Ti3/ITS1F and TM1b/ITS4 of *T. himalayense*, sample Th20. Lanes 10 and 11: amplification with Ti3/ITS1F and TM1b/ITS4 of *T. himalayense* sample Th15, Lanes 12, 13, and 14: amplification of *T. brumale* with ITS1F/ITS4, TM1b/ITS4, and Ti3/ITS1F, respectively.

as reaching those of *T. melanosporum*; (ii) ecological, since Asian black truffles may be used (knowingly or not) as inoculum for the production of ectomycorrhizal seedlings to be planted in Europe.

Fungal identification from morphological analyses of ectomycorrhizae is even more problematic than fruit body identification. Several approaches such as enzymatic polymorphism analyses (Dupré et al., 1993; Gandeboeuf et al., 1994; Urbanelli et al., 1998) and immunological techniques (Neuner-Platter et al., 1998) have been developed through the years. For field studies, it is important to be able to work on single root tips. It has been shown by Urbanelli et al. (1998) that a given host plant can be mycorrhized by several taxa on very small and precise root segments. The above techniques are not sufficiently sensitive to make reliable analyses of small samples. Molecular approaches are considered as more suitable for this purpose but, as underlined by Gandeboeuf et al. (1997), the molecular markers have to be adequately chosen to avoid false negatives. Henrion et al. (1994) demonstrated the feasibility of typing several *Tuber* species using the length of amplified ITS. On this basis, we showed that *T. borchii* Vitt., *T. mesentericum* Vitt., *T. panniferum* Tul., and *T. uncinatum* Ch. could be differentiated from *T. melanosporum*. There are two restrictions to using ITS lengths as determination criteria: (i) on ectomycorrhizae, mistaken identification of the fungal symbiont may occur due to amplification of contaminant DNA of other origins; (ii) among the black truffles, *T. indicum*, *T. himalayense*, and *T. melanosporum* exhibit the same ITS length of about 680 bp. In this case, it is however possible to differentiate *T. indicum* from *T. himalayense* and from *T. melanosporum* using RFLP analysis (Gandeboeuf et al., 1997; Roux et al., 1999). The use of species-specific primers, designed to amplify the ITS region allows a fungal identification by a single step PCR amplification. The high performance of the method is partly due to the repetitiveness of ITS sequences along the genome. Fungal contaminants are no longer a problem since the primers used specifically target ITS amplification of a

single fungus. One of the limits of this approach in truffles was false-negatives due to inhibition of PCR reaction by the low purity of the DNA obtained with the standard extraction procedure. High levels of phenolic compounds, polysaccharides, and proteins are present in truffle ascocarps and mycorrhiza. Contamination of the crude DNA extract by these molecules strongly inhibits Taq polymerase activity. The decrease of the amplification yield leads to poor reproducibility of the PCR method resulting in a low significance of the diagnostic procedure. One way to solve the problem of DNA purity is to add a purification step with commercial kits, as successfully tested for mycorrhizae (Bertini et al., 1998). However, this procedure is expensive and time consuming for routine use, and it gave no results with canned truffles (data not shown). Sterilization of truffles in cans causes extensive chemical modifications, like protein coagulation and DNA degradation. Under these conditions, we found that Proteinase K, usually used in DNA extraction protocols, was not efficient enough. Alcalase2.4L is a protease already reported to be efficient by Duffy et al. (1991) and appeared to be particularly suitable for DNA extraction of our degraded canned samples. The standard procedure of protein precipitation during DNA extraction was also modified as we observed that the phenol-chloroform step induced a decrease of DNA quality and recovery (data not shown). In our protocol, proteins were precipitated by a chloroform step in the presence of chaotropic salts (potassium acetate, 5 M). Most of the pigments, assumed to be melanin and polyphenolic intermediates (Harki et al., 1997), are transferred to the chloroform phase. PCR reproducibility was greatly enhanced by the addition of BLOTTO (Bovine Lacto Transfer Technique Optimizer) to the PCR mix. BLOTTO was shown to optimize PCR reactions with plant or soil samples (bacterial DNA extracted from soil, De Boer et al., 1995). The low cost of nonfat milk cocktails makes them particularly suitable for large-scale PCR experiments, such as those required for instance in population studies. Paolocci (1999) obtained a higher amplification of ectomycorrhiza and fresh ascocarp DNA by adding bovine serum albumin to the PCR mix. However, our study shows, for the first time, the successful extraction and amplification of DNA from canned ascocarps.

Our optimized protocol combining the use of Alcalase2.4L instead of proteinase K, potassium acetate-chloroform instead of phenol-chloroform, and addition of BLOTTO to the PCR mix was used for analysis of mycorhizae and fresh and canned ascocarps. *T. melanosporum* specific primers TM1/TM2 were designed to hybridize with an ITS region found to be specific and perfectly conserved among various isolates of *T. melanosporum* (Roux et al., 1999). Using these primers, we were able to specifically amplify *T. melanosporum* ITS of fresh and cooked ascocarps, as well as from DNA directly extracted from canning juice. The method proved to be sensitive enough for use on a single root tip. We demonstrated the presence of *T. melanosporum* in roots of a 20-year-old tree, exhibiting a "brûlé", but producing no truffles. Seedlings growing under trees, productive or not, were used in this molecular study and were revealed to be also mycorrhized with *T. melanosporum*. To detect the presence of Asian truffles (*T. indicum* or *T. himalayense*) or of any other species among *T. melanosporum* samples, we designed species-specific primers to be used in a multiplex reaction in a single-step experiment. Primers TM1b (paired with ITS4) and Ti3 (paired with ITS1F) were designed for the specific detection of *T. melanosporum* and *T. indicum* (and *T. himalayense*), respectively. These primers were designed such that they give amplicons of different lengths. Two isolates of *T. himalayense*, Th20 and Th15, from two different geographical origins were used, to be sure that a variation in the isolate will not prevent amplification with Ti3 specific primer. The presence of "universal primers" in the multiplex reaction allows the detection of any additional fungal contaminants, and avoids the use of other species-specific primers to detect fungi potentially mixed with *T. melanosporum*, for instance *T. brumale*.

CONCLUSION

The identification of *Tuber* taxa before fruitbody development is of great interest to control truffle production. It allows the early verification that the expected truffle species is developing correctly and the early detection of the presence of intruders which could compromise truffle production. The optimized protocol for DNA extraction and PCR reaction which we proposed, combined with species-specific primers, proved to be efficient for fungal analysis of ectomycorrhizae. It can be used to assess the quality of commercialised inoculated seedlings, but also, in the field, to check mycorrhizal status and consequently truffle production.

Our protocol also proved to be potentially useful to identify, in one PCR step, the presence of Chinese truffles or of any other fungal species, either in a fresh batch of truffles or in canned truffles.

It can therefore be applied to quality control in the truffle industry from production stages to final commercial products.

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