Identification of Internal Transcribed Spacer Sequence Motifs in Truffles: a First Step toward Their DNA Bar Coding^{▽†}

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This work presents DNA sequence motifs from the internal transcribed spacer (ITS) of the nuclear rRNA repeat unit which are useful for the identification of five European and Asiatic truffles (*Tuber magnatum*, *T. melanosporum*, *T. indicum*, *T. aestivum*, and *T. mesentericum*). Truffles are edible mycorrhizal ascomycetes that show similar morphological characteristics but that have distinct organoleptic and economic values. A total of 36 out of 46 ITS1 or ITS2 sequence motifs have allowed an accurate in silico distinction of the five truffles to be made (i.e., by pattern matching and/or BLAST analysis on downloaded GenBank sequences and directly against GenBank databases). The motifs considered the intraspecific genetic variability of each species, including rare haplotypes, and assigned their respective species from either the ascocarps or ectomycorrhizas. The data indicate that short ITS1 or ITS2 motifs (≤50 bp in size) can be considered promising tools for truffle species identification. A dot blot hybridization analysis of *T. magnatum* and *T. melanosporum* compared with other close relatives or distant lineages allowed at least one highly specific motif to be identified for each species. These results were confirmed in a blind test which included new field isolates. The current work has provided a reliable new tool for a truffle oligonucleotide bar code and identification in ecological and evolutionary studies.

The internal transcribed spacers (ITS) of nuclear rRNA repeat units have frequently been used to identify fungal species in fields (9, 11, 14, 20). The emergence of sequence-based identification with a BLAST similarity search connected to public databases (1, 25) has resolved several experimental and taxonomic constraints. However, generalized databases can contain either misidentified or misannotated sequences (7, 17, 23, 24). In addition, BLAST outcomes give no information about species delimitation for closely related species. The identification of unknown ITS sequences based on these approaches therefore needs to be supported by phylogenetic analysis (19, 20).

At the same time, the ITS offers sequence motifs that are useful for the development of fungal DNA bar coding. This is a technique that uses short DNA sequences from a standardized region of the genome as a diagnostic “biomarker” for species, and it is becoming a must in species identification (8, 33, 42, 43, 47), as testified to by the creation of the Consortium for the Barcode of Life (<http://www.barcoding.si.edu/>). Different species have different DNA bar codes, which allow them to be used to (i) identify specimens, (ii) discover possible new species, and (iii) make taxonomy more effective for science and society (<http://www.barcoding.si.edu/>). According to the previously quoted authors, all the approaches that use short DNA sequence motifs or bar codes are called DNA oligonucleotide bar coding or microcoding. The sequence motif approach is

also popular for the assignment of protein functions to the family and/or subfamily levels in public databases such as MIPDB and PROSITE (10, 12).

DNA bar coding has already been applied to the fungal kingdom. Druzhinina et al. (7) recently designed ITS oligonucleotide bar codes for rapid identification of saprotrophic or pathogenic *Trichoderma/Hypocrea* species (*Ascomycota*) using the public Web application *TrichOKEY* v1.0. Specific ITS oligonucleotides, fixed to a flexible membrane-based array, were also used to identify and detect pathogenic or saprotrophic *Pythium* species or groups of species (*Oomycota*) (48).

To our knowledge, the ITS sequence motif approach has not been used yet, as oligonucleotide bar codes, to identify edible mycorrhizal fungi, such as truffles. Truffles are associated with the roots of shrubs and trees and form symbiotic organs called ectomycorrhizas, as well as filamentous mycelia and hypogeous fructifications. More than 70 truffle species have been recorded in the world, and 32 species have been listed in Europe (5). Some truffle species have ecologic, organoleptic, and socioeconomic values but at the same time share some morphological traits with other less-appreciated truffle species. The white truffle *T. magnatum* (sold at around 3,000 to 4,000 Euros/kg) can be morphologically mistaken for other white truffles, such as *T. maculatum*, which does not have the same organoleptic qualities. On the other hand, the black truffle, *T. melanosporum* (sold at around 300 to 400 Euros/kg), is morphologically similar to the Chinese truffle, *T. indicum*. This has led to the development of controls for commercial fraud in the truffle market as well as inoculated host seedlings. In recent years, several studies have focused on truffle phylogeny using several loci, and these have allowed the main species to be clearly defined (15, 31, 36, 51–53, 56). As an example, Wang et al. (51, 52) investigated the European and Asian truffle phylogeny by

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sequencing several genomic loci. These authors confirmed that *T. melanosporum* is phylogenetically related to *T. indicum*, while *T. magnatum* is related to *T. mesentericum* and *T. aestivum*. The intraspecific ITS variability in *T. magnatum*, *T. melanosporum*, *T. mesentericum*, *T. aestivum*, and *T. indicum* has been widely investigated (31, 35, 36, 51–53, 56), and it has been revealed that the most expensive species (*T. magnatum* and *T. melanosporum*) have a low level of ITS variability. On the other hand, *T. mesentericum*, *T. aestivum*, and *T. indicum* showed a higher diversity level.

Because of the availability of this information, we focused our investigation on the design and testing of ITS sequence motifs for oligonucleotide bar codes to reliably and rapidly identify five truffle species, selected from the best-characterized truffle taxa from Europe and Asia (*T. magnatum*, *T. melanosporum*, *T. indicum*, *T. aestivum*, and *T. mesentericum*) (2, 3, 6, 15, 28, 30–32, 34, 35, 36, 51–53, 55, 56). We first designed ITS sequence motifs and tested their specificity in silico against downloaded *Tuber* data sets, as well as directly against generalized and specialized databases (GenBank/EMBL and UNITE), using pattern matching and BLAST analyses. With the use of dot blot hybridization analyses, *T. melanosporum* and *T. magnatum* motif specificities were successfully evaluated against a collection of fungi as being phylogenetically related or unrelated to *Tuber* species.

MATERIALS AND METHODS

Source of the truffle ITS entries and sequence selection. In order to retrieve truffle ITS entries, we downloaded entries as flat and sequence files by querying the EMBL database of the European Bioinformatics Institute (25) (release 82; <http://www.ebi.ac.uk/Databases/>) via the Sequence Retrieval System server (<http://srs.ebi.ac.uk/>). The data were subjected to two main selection processes to select the truffle ITS sequences. First, the data were examined to retrieve entries that corresponded to the *Tuber* genus based on taxonomic and loci annotations, i.e., “*Tuber*” and “ITS” in the “Organism” and “Description” fields, respectively. The *Tuber* data were then examined in detail on the basis of a combination of three criteria, i.e., annotations, knowledge background, and sequence analysis. We examined six main sources of data for each species: (i) the reference sequences and identified specimens; (ii) the number of specimens studied; (iii) the geographical origin in Europe and Asia; (iv) the presence or absence of underlined nucleic acid bases, the size, and the strand (forward or reverse) of the ITS sequences; (v) the methods and the number of loci used for the identification; and (vi) the congruency of the identifications obtained with these methods in literature and in annotations.

Before the design of the sequence motifs, the analyses and comparisons of the annotations, knowledge background, and the sequences of the *Tuber* data led us to construct two main data sets, “training” and “testing” sets which differ in “quality” and data features. The first high-quality training set gathered the five frequently and well-studied *Tuber* species, *T. aestivum*, *T. indicum*, *T. magnatum*, *T. melanosporum*, and *T. mesentericum*. Each species has several entries, including its respective reference and/or identified sequences. The isolates were collected from different countries in Europe and Asia and have comprehensive, full-length ITS sequences (i.e., >460 bp). They were clearly identified from the ascocarps by morphology as well as by molecular fingerprinting and phylogenetic inference, including single nucleotide polymorphism and haplotype genotyping of several loci, such as ITS, β -tubulin, translation elongation factor 1- α , and protein kinase C (29, 31, 34, 36, 38, 40, 51–53, 56). The isolates also showed high sequence homology with their corresponding reference and well-identified sequences in our groups as well as in other groups: accession no. AJ459541 for *T. melanosporum* (35), accession no. AJ586269 for *T. magnatum* (31), accession no. DQ329363 for *T. indicum* (51, 52), accession no. AF516786 for *T. aestivum* (36), and accession no. AF516798 for *T. mesentericum* (36). A BLASTn similarity search (1) (<http://www.ncbi.nlm.nih.gov/BLAST/BLAST.cgi>) was then performed with the *Tuber* data set using ITS of reference specimen sequences. The training set was also analyzed through phylogenetic inference (see below). All entries and sequences that did not match these criteria were included in the

second so-called “testing set” together with some of the five *Tuber* species that matched these criteria.

Motif identification, testing, and validation. The ITS sequence motifs were identified from aligned sequences of the training data set for each *Tuber* species using PRATT software (<http://www.ebi.ac.uk/pratt/>). The minimum percentage of sequences to match (C%) parameter was adjusted to report pattern matching at 100% of the sequence input. The motifs were expressed using the DNA alphabet (A, T, C, G) in PROSITE language (4). The validation of the motifs was performed for each *Tuber* species against the training set using a “PATTERN MATCHING” Web application (http://genoweb.univ-rennes1.fr/Serveur-GPO/outils_acces.php3?id_syndic=175). In order to test for additional diagnostic reliability, the motifs were first evaluated against the *Tuber* testing set and then used to scan a GenBank fungal data set that was distinct from *Tuber* spp. and that included diverse fungal lineages using the same software. The motif quality was measured through the calculation of two parameters, including the pattern matching hits: sensitivity, $S_n = [TP/(TP + FN)] \times 100$, referring to the ability to detect true positives (TP) (FN, false negatives), and selectivity, $S_l = [TP/(TP + FP)] \times 100$, referring to the ability of not finding false positives (FP) (50). We considered that a motif was highly specific to a *Tuber* species if it matched most or all the ITS sequences of this species but no other ITS of any other fungal species.

Evaluation of the ITS motif specificity through BLAST analysis. In order to further evaluate the potential use of the ITS sequence motifs for truffle species identification, we directly tested the truffle ITS motifs using the BLAST algorithm against the nonredundant GenBank database of the National Center for Biotechnology Information (nr at NCBI) as well as against the curated “UNITE” database (30) (<http://unite.ut.ee>). The BLAST analysis investigated motifs that showed either conserved or slightly degenerated sites (in non-PROSITE language) obtained from the five *Tuber* species. The BLAST outputs were then analyzed to find only exact or perfect pairwise matches showing significantly high scores and low expect (E) values for each species.

Dot blot hybridization assays. The *T. magnatum* and *T. melanosporum* motifs were synthesized as oligonucleotides (Sigma, Milan, Italy). The motif specificity was tested against *T. magnatum* (accession no. AJ586252) and *T. melanosporum* (accession no. AJ583627), as well as against *T. mesentericum* (accession no. AM407406), *T. indicum* (accession no. AM406672) (two species closely related to *T. magnatum* and *T. melanosporum*, respectively), and *T. maculatum* (accession no. AM406673) (a species distantly related to both species). The total DNA was extracted from 20 mg of ascocarp gleba with the Dneasy plant mini kit (QIAGEN, Milan, Italy), according to the manufacturer's instructions. The ITS was amplified with ITS1f (14) and ITS4 (54) primers according to Henrion et al. (18). Forty nanograms of ITS amplicons was denatured for the dot blots through incubation in a boiling water bath for 5 min and spotted onto a Hybond-N+ membrane (Amersham, Cologno Monzese, Italy). The DNA was cross-linked to the membrane through UV light. The nonradioactive chemiluminescence method, enhanced chemiluminescence (Amersham, Cologno Monzese, Italy), was used for probe labeling, hybridization, and detection, according to the manufacturer's instructions. The quality control of membranes was conducted using the whole *T. magnatum* or *T. melanosporum* ITS as a probe (i.e., positive control). The membranes were prehybridized at 42°C for 1 h in 2 ml of hybridization buffer following an overnight hybridization with 40 ng of each oligonucleotide motif-labeled probe at 42°C. The hybridizations were carried out at least twice to test the reproducibility of the results. The membranes were washed twice in SSC (0.5×; 1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and sodium dodecyl sulfate (0.4%) at 55°C for 10 min; a second wash was done twice in SSC (1×) at room temperature for 5 min. The spots were visualized on a Versadoc imaging system (Bio-Rad, Milan, Italy).

As a further step, the sensitivity of the specific motifs was tested using serial dilutions of *T. magnatum* and *T. melanosporum* ITS spotted onto a membrane and hybridized with specific motifs as described above. The hybridization time was reduced to 3 hours instead of overnight in order to optimize the diagnostic procedure. Finally, the most sensitive motifs of both species were tested for their specificity against a comprehensive collection of 10 *Tuber* species morphologically characterized as *T. magnatum*, *T. melanosporum*, *T. indicum*, *T. mesentericum*, *T. borchii*, *T. puberulum*, *T. macrosporum*, *T. paniferum*, *T. brumale* var. *moschatum*, and *T. maculatum*. The hybridization procedures were performed as described above with 5 ng of the ITS amplicons spotted onto the membranes.

Blind test. In order to test further the potential use of oligonucleotide motifs for the identification of field *T. magnatum* and *T. melanosporum* species, a blind test was carried out on a set of 27 fresh isolates, collected from various geographical locations and identified using morphological characters. The isolates belong to different *Tuber* species (*T. magnatum*, *T. mesentericum*, *T. oligospermum*, *T. borchii*, *T. brumale*, *T. aestivum*, *T. melanosporum*, *T. indicum*), two false

FIG. 1. NJ tree of 202 strains of five *Tuber* species inferred from ITS1, 5.8S rRNA gene, and ITS2 sequences. Each clade gathered ITS accessions of its respective species, which are listed in Table S1 in the supplemental material. BP tests were performed using 1,000 replicates, and their values are reported next to the branch nodes.

truffles (*Choiromyces meanformis* and *Terfezia arenaria*), different mycelia of Pezizomycotina (*Aspergillus uster*, *Aspergillus sclerotium*, *Penicillium restrictum*, *Periconia macrospinosa*, *Curvularia inaequalis*, *Embellisia* sp., and *Pestalotiopsis* sp.), a mycelium of *Phoma* sp., and two Basidiomycota (a *Boletus edulis* fruitbody and a *Coprinus micaceus* mycelium). None of these isolates are available in the GenBank database, and they did not belong to the truffle data set used for the design and validation of the motifs. The isolates were coded (from 1 to 27) to perform the blind test and were considered unknown fungal samples in the hands of naïve operators. Five nanograms of the ITS amplicon was spotted onto each membrane for each sample, and hybridization reactions were carried out using the whole ITS or its selected motifs, as previously described. All the reactions were repeated twice to test the reproducibility of the results.

Phylogenetic tree. The ITS sequences were used for phylogenetic relationship inferences. Multiple sequence alignments were performed using CLUSTALW software (49) utilizing default settings. Ambiguously aligned regions were excluded from the 5' and 3' ends using the BIOEDIT program (16). The phylogenetic analysis was performed using a distance method with the MEGA 3.1 program (26). The distance matrix and the neighbor-joining (NJ) tree were based on Kimura's two-parameter (K2P) model (22, 41). Gaps were treated as missing data. Branch robustness was estimated through bootstrap (BP) analyses of 1,000 replicates (13).

RESULTS

Sequence data to design the ITS motifs from the *Tuber* spp.

A total of 823 ITS entries were retrieved from the EMBL database. In this set, we found 28 *Tuber* species represented by 526 accessions, all with forward sequences without undetermined characters. The remaining 297 ITS accessions included either non-*Tuber* sequences (276) or *Tuber* sequences with reverse (3) or undermined (18) characters. These data were omitted from the subsequent analysis.

The first high-quality training set gathered the five well-studied *Tuber* species, 15 to 90 sequences by species, represented by 202 entries (see list in Table S1 in the supplemental material). The NJ tree elaborated on the ITS of the training set distinguished five phylogenetic clades, corresponding to the five *Tuber* species (Fig. 1). The sequences of each species were correctly placed in the same tree clade together with their respective reference sequences. Each phylogenetic clade showed confident BP values except for the *T. indicum* sequences (BP = 64) (Fig. 1). These data can be explained by the fact that *T. indicum* is closely related to *T. melanosporum* and has a high genetic variability (see below and reference 51 for details). All the other sequences (i.e., 324) were placed in the testing set (see Table S2 in the supplemental material).

Design and validation of the ITS sequence motifs from intraspecific variability of the *Tuber* spp. We identified hundreds of sequence motifs from the ITS areas of the five *Tuber* species (data not shown). These were then screened and validated against the training set using the pattern matching tool, which allowed a final selection to be made of 46 representative short sequence motifs of sizes inferior or equal to 50 nucleotides (Table 1). Each *Tuber* sp. showed several oligonucleotide motifs (7 to 11 per species) located in either the ITS1 or ITS2 loci (two to eight per loci). *T. magnatum* and *T. melanosporum* showed more conserved motifs (six to nine per species, with one haplotype) than the variable motifs (two per species, with two or three haplotypes). *T. aestivum*, *T. indicum*, and *T. mesentericum*

TABLE 1. Features, hits, sensitivity, and selectivity of the ITS sequence motifs obtained from five European and Chinese *Tuber* species

Species motif code	Position in ITS	Length (bp)	No. of training set hits/202	No. of testing set hits/324 ^a	No. of fungal set hits/5,000	Sensitivity S _n (%)	Selectivity S _i (%)	Sequence motif in PROSITE format (from 5' to 3' ends)
<i>T. magnatum</i>								
Tma-ITS1-1	42–86	30	30	36	0	100	100	T-C-C-T-G-T-G-T-A-C-C-A-T-G-C-C-A-T-G-T-T-G-C-T-T-C-C-C-C-A
Tma-ITS1-2	42–106	50	30	36	0	100	100	T-C-C-T-G-T-G-T-A-C-C-A-T-G-C-C-A-T-G-T-T-G-C-T-T-C-C-C-C-A-G-G-G-T-C-A-C-G-G-T-C-A-C-T-G-T-G-C-C-G
Tma-ITS1-3	91–135	30	30	36	0	100	100	G-C-C-T-G-G-A-G-A-G-G-C-A-C-A-G-T-C-C-C-A-A-A-A-C-T
Tma-ITS1-4	123–167	30	30	36	0	100	100	T-G-T-C-A-T-C-C-T-A-C-C-A-G-C-A-G-T-C-T-G-A-G-A-A-A-G-G-C
Tma-ITS1-5	156–199	29	30	36	0	100	100	T-C-A-G-G-C-C-T-G-A-C-A-A-A-C-T-T-A-A-A-C-A-G-T-T-A-A-A
Tma-ITS2-1	324–368	30	30	36	0	100	100	T-G-T-T-T-G-A-G-C-G-T-C-A-C-T-G-A-A-A-A-C-C-C-A-C-T-C-A-C-G
Tma-ITS2-2	360–404	30	30	36	0	100	100	C-G-T-G-G-T-G-C-T-G-A-T-G-G-A-G-G-T-G-G-C-T-T-G-G-T-A-C-C-G
Tma-ITS2-3	360–424	50	30	36	0	100	100	C-G-T-G-G-T-G-C-T-G-A-T-G-G-A-G-G-T-G-G-C-T-T-G-G-T-A-C-C-G-C-A-G-C-G-G-C-G-G-T-G-G-C-C-G-G-T-C-C
Tma-ITS2-4	382–425	29	30	36	0	100	100	T-G-G-T-A-C-C-G-C-A-G-C-G-G-C-G-T-G-G-C-C-G-G-G-T-C-C-A
Tma-ITS2-5	382–445	49	30	36	0	100	100	T-G-G-T-A-C-C-G-C-A-G-C-G-C-G-G-T-G-G-C-C-G-G-G-T-C-C-A-C-C-[CT]-T-G-T-C-G-A-A-T-T-T-C-A-T-T-G-G-G
Tma-ITS2-6	493–524	17	30	36	0	100	100	A-G-T-C-A-G-G-C-C-[CT]-G-G-T-C-C-T-G
<i>T. melanosporum</i>								
Tmel-ITS1-1	51–80	30	90	82 (A: 3)	0	98.3	100	G-C-T-T-C-C-A-C-A-G-G-T-T-A-A-G-T-G-A-C-C-A-T-G-C-T-G-G-C-A
Tmel-ITS1-2	51–100	50	90	82 (A: 3)	0	98.3	100	G-C-T-T-C-C-A-C-A-G-G-T-T-A-A-G-T-G-A-C-C-A-T-G-C-T-G-G-C-A-C-C-T-G-T-G-G-A-G-A-T-C-T-T-A-T-G-T
Tmel-ITS1-3	77–106	30	90	82 (A: 3)	0	98.3	100	G-G-C-A-C-C-T-G-T-G-G-A-G-A-T-C-T-C-T-A-T-G-T-T-T-A-A-C-G
Tmel-ITS1-4	120–149	30	90	83 (A: 2)	0	99.0	100	G-C-T-G-T-C-T-G-A-G-C-T-G-G-C-C-A-T-G-T-G-T-C-[AG]-G-A-T-T-T-A
Tmel-ITS1-5	212–241	30	117	119 (A: 2)	0	99.0	73.3	A-T-G-[AC]-G-A-T-A-G-G-T-A-A-T-G-T-G-A-A-T-T-G-C-A-G-A-A-T-T-C
Tmel-ITS2-1	417–466	50	90	81 (A: 2) (B: 2)	0	99.0	100	A-G-G-C-A-T-G-A-A-C-G-A-[CT]-G-G-A-C-T-T-T-A-T-A-A-C-G-G-T-T-A-T-A-A-G-A-C-C-T-G-G-A-T-C-A-G-T-C-A-C
Tmel-ITS2-2	458–487	30	90	83 (B: 2)	0	100	100	A-T-C-A-G-T-C-A-C-A-A-G-T-C-T-T-G-T-C-T-G-G-T-C-C-T-T-A-C-C
Tmel-ITS2-3	458–507	50	90	82 (A: 1) (B: 2)	0	99.4	100	A-T-C-A-G-T-C-A-C-A-A-G-T-C-T-T-G-T-C-T-G-G-T-C-C-T-T-A-C-C-T-T-A-A-G-A-C-C-[CT]-C-C-A-T-C-C-[GT]-A-G-A
Tmel-ITS2-4	475–495	20	91	85 (B: 2)	0	100	98.3	C-T-G-G-T-C-C-T-T-A-C-C-T-T-A-A-G-G-A-C
<i>T. indicum</i>								
Ti-ITS1-1	13–138	19	117	120	0	100	25.4	C-A-T-G-T-T-G-C-T-T-C-C-A-C-[AG]-G-G-[CT]-T
Ti-ITS1-2	60–189	19	27	34 (A: 4)	0	93.2	100	T-C-T-[CT]-C-A-C-[AG]-T-T-A-A-C-A-G-A-G-T-A
Ti-ITS1-3	92–221	19	117	123	0	100	25.2	T-C-T-G-A-G-C-T-G-G-C-C-A-[CT]-G-T-G-T-C
Ti-ITS2-1	242–401	49	117	118 (A: 1)	0	98.3	25.3	G-C-C-C-T-T-T-G-G-T-A-T-T-C-C-T-T-A-G-G-G-C-A-T-G-C-C-T-G-T-T-C-G-A-G-C-G-T-C-A-C-T-A-C-A-C-A-C-C
Ti-ITS2-2	256–416	50	27	35 (A: 3)	0	95.0	100	C-T-T-A-G-G-G-C-A-T-G-C-C-T-G-T-T-C-G-A-G-C-G-T-C-A-C-T-A-C-A-C-A-C-C-T-T-A-T-C-A-C-A-A-[CT]-A-T-T-T-G
Ti-ITS2-3	306–436	20	117	123	0	100	25.2	T-G-G-T-C-T-T-G-G-C-A-G-G-A-G-T-G-A-A-T
Ti-ITS2-4	306–466	50	27	35 (A: 2)	0	97.0	100	T-G-G-T-C-T-T-G-G-C-A-G-G-A-G-T-G-A-A-T-T-x(0,1)-G-C-T-[AG]-G-T-C-T-A-T-G-[AG]-A-A-[AG]-T-A-T-T-C-C-A-G-C-[TC]-G-T-[CA]-C
Ti-ITS2-5	307–447	31	117	122	0	100	25.3	G-G-T-C-T-T-G-G-C-A-G-G-A-G-T-G-A-A-T-T-x(0,1)-G-C-T-[AG]-G-T-C-T-A-T-T-[CG]-A-T-G-T-C-T-G-G-T-C-x(1,2)-T-[A-T]-A-[CGT]-[CG]-[ACT]
Ti-ITS2-6	438–568	20	27	38	0	100	100	A-T-G-T-C-T-G-G-T-C-[CT]-x(0,1)-T-[AT]-A-x(0,3)-C-C-T-T-A-[AGT]-G-G-A
Ti-ITS2-7	440–577	27	27	38	0	100	100	T-C-[CT]-x(0,1)-T-[AT]-A-x(0,3)-C-C-T-T-A-[AGT]-G-G-A-x(0,1)-C
Ti-ITS2-8	448–579	21	117	121	0	100	25.4	

Continued on following page

TABLE 1—Continued

Species motif code	Position in ITS	Length (bp)	No. of training set hits/202	No. of testing set hits/324 ^a	No. of fungal set hits/5,000	Sensitivity S _n (%)	Selectivity S _i (%)	Sequence motif in PROSITE format (from 5' to 3' ends)
<i>T. aestivum</i>								
Ta-ITS1-1	15–83	30	40	76 (A: 1) (B: 1)	0	99.0	100	G-G-C-G-G-C-C-A-C-C-C-A-G-A-G-C-A-C-C-A-A-A-C-C-A-C-A-G-C-T
Ta-ITS1-2	15–103	50	40	76 (A: 1) (B: 1)	0	99.0	100	G-G-C-G-G-C-C-A-C-C-C-A-G-A-G-C-A-C-C-A-A-A-C-C-A-C-A-G-C-T-G-C-G-T-A-C-A-A-T-G-C-C-A-[CT]-G-T-T-G-C-T
Ta-ITS1-3	178–250	30	40	78	0	100	100	G-G-A-C-T-G-T-x(0,2)-C-T-G-A-C-[CG]-T-G-G-G-C-C-x(0,1)-A-C-A-T-T-G-G-[CG]
Ta-ITS2-1	349–419	30	55	79	0	100	87.0	G-G-C-A-T-T-C-C-T-C-A-G-G-G-C-A-T-G-C-C-T-G-T-C-C-G-A-G-C-G
Ta-ITS2-2	447–515	30	40	69 (A: 1) (B: 8)	0	100	100	G-C-G-G-C-C-C-[CG]-G-T-C-C-T-C-C-C-C-A-C-C-C-C-G-C-[CT]-G-A-A-A-T
Ta-ITS2-3	497–565	30	40	68 (A: 1) (B: 8)	0	99.0	100	T-G-G-C-G-G-A-C-C-C-G-A-C-T-T-G-G-T-A-G-A-C-G-C-T-G-T-G-G-T
Ta-ITS2-4	497–586	50	40	65 (A: 2) (B: 10)	0	98.0	100	T-G-G-C-G-G-A-C-C-C-G-A-C-T-T-G-G-T-A-G-A-C-G-C-T-G-T-G-G-T
Ta-ITS2-5	524–593	30	40	62 (A: 2) (B: 14)	0	98.0	100	G-G-T-T-A-G-C-G-T-G-[AG]-A-G-C-A-G-T-C-G-C-G-G-G-C-C-C-A-T-T-G
<i>T. mesentericum</i>								
Tmes-ITS1-1	77–132	30	15	1	0	100	100	T-C-C-C-x(0,1)-G-G-G-[AC]-C-G-x(1,2)-G-G-C-C-[AG]-T-[CG]-G-[CT]-[CT]-G-C-C-C-G-[CT]-C
Tmes-ITS1-2	77–152	51	15	1	0	100	100	T-C-C-C-x(0,1)-G-G-G-[AC]-C-G-x(1,2)-G-G-C-C-[AG]-T-[CG]-G-[CT]-[CT]-G-C-C-C-G-[CT]-C-G-x(0,1)-G-C-G-A-G-[AT]-C-[AG]-C-x(0,1)-[AC]-[CGT]-[CG]-[TG]-T-G-G-[AC]-T
Tmes-ITS2-1	379–433	29	53	80	0	100	12.0	G-G-G-C-A-T-G-C-C-T-G-T-T-C-C-G-A-G-C-G-T-C-A-G-T-[AG]-C-A-A-A
Tmes-ITS2-2	385–440	30	15	3	0	100	89.0	G-C-C-x(0,2)-T-C-x(0,1)-G-A-G-C-G-T-C-A-G-T-[AG]-C-A-A-A-C-C-C-[CG]-C-T-C
Tmes-ITS2-3	412–467	30	15	1	0	100	100	C-T-C-A-C-[AG]-G-x(2,3)-C-[AC]-[CT]-G-T-G-G-T-C-[AC]-T-G-G-[CT]-G-G-A-G-G-T
Tmes-ITS2-4	412–487	50	15	1	0	100	100	C-T-C-A-C-[AG]-G-x(2,3)-C-[AC]-[CT]-G-T-G-G-T-C-[AC]-T-G-G-[CT]-G-G-A-G-G-T-G-G-[AG]-[TG]-[AGC]-x(0,2)-G-[AC]-G-[CT]-C-G-[CG]-G-C-T-[GC]-[GC]-C
Tmes-ITS2-5	566–611	20	15 + 13	0 (B: 1)	0	100	100	T-C-C-C-[AGT]-C-C-C-G-C-C-[CT]-C-[AG]-A-G-[ACG]-[CG]-C-C

^a In parentheses, numbers of unmatched accessions with a nucleic base(s) lacking in the sequence motifs (A) or with partial ITS sequences (B; not included in S_n calculations). Accession no. (with bp in parentheses) by species: *T. melanosporum*, A, AF106874 (620), AF106873 (618), AF106876 (620), AF106878 (620), AF106879 (620), U95174 (449) and Y09789 (442), and B, U95174 (449) and Y09789 (442); *T. indicum*, A, AF106881 (620), AF106p84 (624), U89362 (620), DQ375517 (570), DQ375518 (574), DQ375519 (574), DQ375512 (570), and DQ375524 (576); *T. aestivum*, A, AJ492216 (643), AJ888120 (608), AJ888126 (595), and AJ516789 (701), and B, AJ492211 (440), AJ492213 (427), AJ492217 (463), AJ888078 (559), AJ888079 (559), AJ888080 (432), AJ888081 (417), AJ888084 (441), AJ888100 (563), AJ888103 (565), AJ888104 (455), AJ888112 (549), AJ888128 (454), and AJ888129 (432); *T. mesentericum*, B, AJ888043 (538).

instead showed more variable motifs (five to nine per species with at least two haplotypes) than the conserved motifs (zero to two per species with one haplotype or no haplotypes). All the motifs obtained from the 5.8S rRNA gene were rejected because they lacked specificity among the *Tuber* spp. (data not shown).

In silico identification of truffle species based on pattern matching ITS motifs against *Tuber* and other fungal data sets. The motifs of the white truffle, *T. magnatum*, matched sequences belonging to the *T. magnatum* species of both the training and testing sets, which originated from Italy and Croatia (e.g., motif “Tma-ITS1-1,” TP = 66, FN = 0, S_n = 100%) (Table 1). They were also highly selective (FP = 0, S_i = 100%) with unmatching ITS of other *Tuber* or fungal species of data sets. All the *T. melanosporum* motifs were found to be highly sensitive through the matching of the *T. melanosporum* sequences of *Tuber* data sets collected from France, Italy, and Spain and their corresponding sets (TP = 171 to 173, FN = 0 to 3, S_n = 98 to 100%). These motifs (except two) also showed a high selectivity against all the other truffle and fungal species

(FP = 0, S_i = 100%). Only two highly sensitive *T. melanosporum* motifs (“Tmel-ITS1-5” and “Tmel-ITS2-4,” S_n = 99 to 100%) were found to be less selective (FP = 3 to 63, S_i = 73 to 98%) through matching of *T. indicum* accessions of the *Tuber* sets, as well as *T. himalayense* and *T. sinense* sequences of the testing set. All the *T. indicum* motifs were found to be highly sensitive and matched the *T. indicum* TP strains of both *Tuber* data sets collected from China (TP = 55 to 59, FN = 0 to 4, S_n = 93 to 100%). All these motifs also matched the *T. himalayense* and *T. sinense* sequences (five to six accessions) of the testing set. However, five of them showed high selectivity and did not match the FP strains of the data sets, i.e., all accessions belonging to other *Tuber* and fungal species (FP = 0, S_i = 100%). Six highly sensitive *T. indicum* motifs (“Ti-ITS1-1,” “Ti-ITS1-3,” “Ti-ITS2-1,” “Ti-ITS2-3,” “Ti-ITS2-5,” and “Ti-ITS2-8,” S_i = 98 to 100%), which also retrieved *T. himalayense* and *T. sinense* accessions, showed a low selectivity through the matching of nearly all the *T. melanosporum* sequences from the *Tuber* data sets (FP = 171 to 175, S_i = 25%).

All the *T. aestivum* motifs matched the *T. aestivum* sequences with high sensitivity (90 to 105%) of both the *Tuber* data sets originating from eight European countries (Italy, Sweden, France, Spain, Hungary, the United Kingdom, Denmark, and Germany) (TP = 89 to 105, FN = 0 to 2, S_n = 97 to 100%). These motifs also retrieved sequences of *T. uncinatum* and one unidentified *Tuber* ectomycorrhiza (accession no. AY286194) of the testing set. Seven of them also showed a high selectivity and did not match the FPs of the other *Tuber* and fungal species (FP = 0, S_i = 100%). A single sensitive motif of *T. aestivum* ("Ta-ITS2-1," S_n = 100%), which also retrieved *T. uncinatum* accessions, showed a low selectivity through the matching of *T. mesentericum* strains of both *Tuber* data sets (FP = 16, S_i = 88%).

Similarly, all the *T. mesentericum* motifs matched the *T. mesentericum* sequences from both the training and testing sets originating from Italy and Sweden (TP = 15 to 16, FN = 0, S_n = 100%). Five of these seven motifs did not match either the *Tuber* species or the other fungal species present in the data set (FP = 0, S_i = 100%). Interestingly, the "Tmes-ITS2-5" motif, located at the 3' end of ITS2, was found to be repeated in 13 *T. mesentericum* accessions but not in other *Tuber* specimens. Two highly sensitive motifs of *T. mesentericum* ("Tmes-ITS2-1" and "Tmes-ITS2-2," S_n = 100%) matched the *T. aestivum*, *T. uncinatum*, and *T. panniferum* accessions from the training and/or testing sets (FP = 117, S_i = 12% and FP = 2, S_i = 89%, respectively).

Evaluation of truffle assignment through BLAST ITS motifs against GenBank and UNITE databases. A total of 33 ITS1 and ITS2 motifs (either conserved or with one or two mismatches) were tested by BLAST analysis against the generalized GenBank database (see Table 2).

The *T. magnatum* motifs showed exact or perfect BLAST matches with the *T. magnatum* sequences from Italy and Croatia (best hits, 67 to 68; 97 to 100% of identity; E values, $6e^{-07}$ to $5e^{-19}$). All the motifs were also able to recognize two unidentified *Tuber* ectomycorrhizas (accession no. AJ879687 and AJ879679) (34). Seven out of nine *T. melanosporum* motifs exactly or perfectly matched sequences of *T. melanosporum* European strains (best hits, 172 to 176; 96 to 100% of identity; E values, $1e^{-06}$ to $5e^{-19}$). The remaining two *T. melanosporum* motifs, which showed exact or perfect, significant matches with *T. melanosporum* strains, crossed with Chinese *T. indicum* sequences and/or those of other fungal species ("Tmel-ITS1-5" and "Tmel-ITS2-4"; best hits, 176 to 259; 96 to 100% of identity; E values, $1e^{-06}$ and 0.03, respectively). Similarly, two *T. indicum* motifs significantly and perfectly matched Chinese strains identified as *T. indicum* (55 to 56), but also entries of the Chinese *T. himalayense* and *T. sinense* (6) ("Ti-ITS1-2" and "Ti-ITS2-2"; best hits, 61 to 62; 89 to 98% of identity; E values, 6.2 and $4e^{-18}$, respectively). However, four *T. indicum* motifs perfectly matched the Chinese *T. indicum* (58 to 59), *T. himalayense* and *T. sinense* (6) strains, as well as the European *T. melanosporum* sequences (175 to 177) (best hits, 239 to 242; 93 to 98% of identity; E values, 0.79 to $2e^{-18}$). Six *T. aestivum* motifs matched the European strains of *T. aestivum* exactly or perfectly (92 to 103), *T. uncinatum* (14 to 15), and two unidentified *Tuber* ectomycorrhizas (accession no. AY286194 and AY833041) (21, 45) (best hits, 109 to 120; 93 to 100% of identity; E values, $9e^{-06}$ to $4e^{-18}$). Only a single *T. aestivum*

motif, which also gave a significant exact match with *T. aestivum* (107), *T. uncinatum* (15), and unidentified *Tuber* ectomycorrhizas (2), also crossed with all the *T. mesentericum* strains (16) from Europe ("Ta-ITS2-1"; best hits, 140; 100% of identity; E values, $2e^{-07}$). Similarly, one *T. mesentericum* motif showed a perfect match with a constant E value with all the *T. mesentericum* strains (16), but also with *T. aestivum*/*T. uncinatum* (122) and *T. panniferum* (1) ("Ta-ITS2-1"; best hits, 139; 96% of identity; E value, $4e^{-06}$).

Some motifs did not show an exact match for each *Tuber* species and gave distinct E values with rare haplotypes and/or did not match with a limited number of partial sequences (see Table 2, labels A and B, respectively). Most motifs did not match any distantly related *Tuber* or non-*Tuber* species available in the GenBank database with the same identity and E values (Table 2). In addition, no motif showed any significant match or BLAST hits with sequences of the curated "UNITE" ectomycorrhizal database.

Specificity and sensitivity of the *T. melanosporum* and *T. magnatum* ITS motifs tested by dot blot assays. The whole *T. magnatum* and *T. melanosporum* ITS amplicons, used as probes (i.e., positive controls), showed hybridization signals with the five *Tuber* species (Fig. 2A and B). Five out of the 11 *T. magnatum* tested motifs exclusively hybridized with *T. magnatum* ITS ("Tma-ITS1-1," "Tma-ITS1-3," "Tma-ITS1-4," "Tma-ITS1-5," and "Tma-ITS2-5") (Fig. 2A). When tested against serial dilutions, the "Tma-ITS2-5" motif was the most sensitive and revealed up to 32 pg of DNA spotted on the membrane (see Fig. S1 in the supplemental material). Moreover, this motif was found to be highly specific, since it only hybridized *T. magnatum* when tested against 10 *Tuber* species (Fig. 2C). In the case of *T. melanosporum*, one motif out of nine only hybridized *T. melanosporum* ("Tmel-ITS2-1") (Fig. 2B). This motif revealed up to 0.8 ng of DNA spotted onto the membrane (see Fig. S1 in the supplemental material) and was highly specific, since it hybridized with *T. melanosporum* but not with other *Tuber* species tested (Fig. 2C). The remaining *T. magnatum* and *T. melanosporum* motifs crossed with at least one nontargeted species with distinct hybridization signal intensities or did not give any hybridization reaction, including their respective target species (Fig. 2A and B).

Use of oligonucleotide motifs for the detection of *T. melanosporum* and *T. magnatum* in a blind test. The potential use of oligonucleotide motifs for the identification of field *T. melanosporum* and *T. magnatum* species was tested in a blind test of 27 isolates with the whole *T. magnatum* ITS as the control and two specific and sensitive motifs (i.e., "Tmel-ITS2-1" and "Tma-ITS2-5") (see results in Fig. 3). The hybridization reaction with whole *T. magnatum* ITS gave a positive signal for all the spotted amplicons belonging to Ascomycota or Basidiomycota. However, when hybridized with the "Tma-ITS2-5" motif as probes, only spots 1 and 16 gave a reproducible positive signal. Similarly, when hybridized with the "Tmel-ITS2-1" motif, only spots 15 and 17 showed a reproducible positive reaction. The comparison of the results with the known identifications revealed that spots 1 and 16 corresponded to the DNA of the two *T. magnatum* isolates spotted onto the membrane, and spots 15 and 17 corresponded to the DNA of the two *T. melanosporum* isolates spotted onto the membrane.

TABLE 2. BLAST outputs of *Tuber* ITS sequence motifs against NCBI GenBank database

Species motif code	Length (bp)	No. of best hits ^a	Identity (%)	E value	Sequence (from 5' to 3' ends)
<i>T. magnatum</i>					
Tma-ITS1-1	30	68	100	2e ⁻⁰⁷	TCCTGTGTACCATGCCATGTTGCTTCCCCA
Tma-ITS1-2	50	68	100	5e ⁻¹⁹	TCCTGTGTACCATGCCATGTTGCTTCCCCA GGGTACCGGTCACTGTGCCG
Tma-ITS1-3	30	67 (A: 1)	100	2e ⁻⁰⁷	GCCTGGGAGGAGGGACAGTCCCAAAAACT
Tma-ITS1-4	30	67 (A: 1)	100	2e ⁻⁰⁷	TGTCATCCTACCAGCAGTCTGAGAAAGGGC
Tma-ITS1-5	29	68	100	6e ⁻⁰⁷	TCAGGCCTGACAACTTAAACAGTTAAAA
Tma-ITS2-1	30	68	100	2e ⁻⁰⁷	TGTTTGAGCGTCACTGAAAAACCACTCACG
Tma-ITS2-2	30	68	100	2e ⁻⁰⁷	CGTGGTGCTGATGGAGGTGGCTTGGTACCG
Tma-ITS2-3	50	68	100	5e ⁻¹⁹	CGTGGTGCTGATGGAGGTGGCTTGGTACCG CAGCGGCGGTGGCCGGGTCC
Tma-ITS2-4	29	68	100	6e ⁻⁰⁷	TGGTACCGCAGCGGCGGTGGCCGGGTCCA
Tma-ITS2-5	49	68	97	1e ⁻¹⁷	TGGTACCGCAGCGGCGGTGGCCGGGTCC ACCTGTGCGAATTTTCATTGGG
<i>T. melanosporum</i>					
Tmel-ITS1-1	30	174 (A: 3)	100	2e ⁻⁰⁷	GCTTCCACAGGTTAAGTGACCATGCTGGCA
Tmel-ITS1-2	50	174 (A: 3)	100	5e ⁻¹⁹	GCTTCCACAGGTTAAGTGACCATGCTGGCA CCTGTGGGAGATCTCTATGT
Tmel-ITS1-3	30	174 (A: 3)	100	2e ⁻⁰⁷	GGCACCTGTGGGAGATCTCTATGTTAACGG
Tmel-ITS1-4	30	175 (A: 2)	96	1e ⁻⁰⁶	GCTGTCTGAGCTGGCCATGTGTCRGATTTA
Tmel-ITS1-5	30	259 (B: 2)	96	1e ⁻⁰⁶	ATGGMGATAGGTAATGTGAATTGCAGAATTC
Tmel-ITS2-1	50	172 (A: 4) (B: 1)	98	4e ⁻¹⁸	AGGCATGAACGAYGGACTTTATAAACG GTTA TAAGACCTGGATCAGTCAC
Tmel-ITS2-2	30	173 (B: 4)	100	2e ⁻⁰⁷	ATCAGTCACAAGTCTTGTCTGGTCCTTACC
Tmel-ITS2-3	50	172 (A: 1) (B: 4)	96	3e ⁻¹⁷	ATCAGTCACAAGTCTTGTCTGGTCCTTACC TTAAGGACCYCCATCCKAGA
Tmel-ITS2-4	20	176 (B: 4)	100	0.03	CTGGTCTTACCTTAAGGAC
<i>T. indicum</i>					
Ti-ITS1-2	19	61 (A: 1) (B: 3)	89	6.2	TCTYACRTTAACAGAGTA
Ti-ITS1-3	19	242	94	0.79	TCTGAGCTGGCCAYGTGTC
Ti-ITS2-1	49	239 (A: 1)	100	2e ⁻¹⁸	GCCCTTTGGTATTCTTAGGGCATGCCTGTT CGAGCGTCACTACACACC
Ti-ITS2-2	50	62 (A: 2) (B: 1)	98	4e ⁻¹⁸	CTTAGGGCATGCCTGTTGAGCGTCACTACA CACCTTATCACAAYATTG
Ti-ITS2-3	20	242	100	0.03	TGGTCTTGGCAGGAGTGAAT
Ti-ITS2-5	31	242	93	0.023	GGTCTTGGCAGGAGTGAATTNGCTRGCTAT
<i>T. aestivum</i>					
Ta-ITS1-1	30	120 (A: 2) (B: 4)	100	2e ⁻⁰⁷	GGCGGCCACCCAGAGCACCAAAACCACAGCT
Ta-ITS1-2	50	120 (A: 2) (B: 4)	98	4e ⁻¹⁸	GGCGGCCACCCAGAGCACCAAAACCACAGCT GCGTACAATGCCAYGTTGCT
Ta-ITS2-1	30	140 (B: 2)	100	2e ⁻⁰⁷	GGCATTCTCAGGGCATGCCTGTCCGAGCG
Ta-ITS2-2	30	113 (A: 3) (B: 9)	93	9e ⁻⁰⁶	GCGGCCCSGTCTCCCCACCCGCGYGAAT
Ta-ITS2-3	30	115 (A: 1) (B: 9)	100	2e ⁻⁰⁷	TGGCGGACCCGACTTGGTAGACGCTGTGGT
Ta-ITS2-4	50	112 (A: 4) (B: 9)	98	4e ⁻¹⁸	TGGCGGACCCGACTTGGTAGACGCTGTGGT TAGCGTGRAGCAGTCGCGGG
Ta-ITS2-5	30	109 (A: 6) (B: 11)	96	1e ⁻⁰⁶	GGTTAGCGTGRAGCAGTCGCGGGGCCATTG
<i>T. mesentericum</i>					
Tmes-ITS2-1	29	139	96	4e ⁻⁰⁶	GGGCATGCCTGTCCGAGCGTCAGTRCAA

^a Letters and numbers in parentheses represent the total number of accessions which did not show exact matches (A) or did not match (B) with sequence motifs. Accession no. (with bp in parentheses) by species: *T. magnatum*, A, AJ879687 (621); *T. melanosporum*, A, AF106874 (620), U95174 (449), Y09789 (442), AJ786645 (410), AF106878 (620), AF106879 (620), AF106876 (620), and AF106873 (618), and B, U95174 (449), Y09789 (442), AF106876 (620), AJ786646 (464), and AJ786645 (410); *T. indicum*, A, DQ375519 (574), DQ375517 (570), and DQ375512 (570), and B, AF106881 (620), AF106884 (624), U89362 (620), and DQ375518 (574); *T. aestivum*, A, AJ492216 (643), AJ492215 (693), AJ492214 (181), AJ492212 (443), AJ888120 (608), AJ888126 (595), AJ888128 (457), AJ888112 (549), AJ888103 (565), AJ888100 (563), AJ888079 (559), AJ888078 (559), and AF516789 (701), and B, AJ492211 (440), AJ492212 (443), AJ492213 (427), AJ492214 (181), AJ492217 (463), AJ888128 (454), AJ888080 (432), and AJ888081 (417).

DISCUSSION

Truffle species identification and evolutionary inference generally use molecular and phylogenetic approaches on the ITS as target genomic loci for fungal diagnostics. This paper has investigated the development of short ITS sequence motifs, as DNA oligonucleotide bar codes, for an unambiguous and easy identifi-

cation of five well-characterized and studied truffle species from Europe and Asia.

Identification of *T. magnatum* and *T. melanosporum* through oligonucleotide bar codes. *T. magnatum* (the white Piedmont truffle) and *T. melanosporum* (the black Perigord truffle) motifs were found to be more conserved than variable, reflecting

FIG. 2. Dot blot analysis of *T. magnatum* (A) and *T. melanosporum* (B) ITS motifs against ITS PCR products from five different *Tuber* spp. The whole *T. magnatum* ITS was used as a probe, which gave a positive, unspecific hybridization signal. Specific motifs are in bold print. The membranes were exposed for 20 min. (C) Dot blot analysis of “Tma-ITS2-5” and “Tmel ITS2-1” motifs against 10 different *Tuber* species. The whole *T. melanosporum* ITS was used as a probe, which gave a positive, unspecific hybridization signal. The membranes were exposed for 2 min.

well their bottleneck population genetic diversity (3, 31, 35). All the *T. magnatum* motifs exclusively identified all the *T. magnatum* sequences in silico, avoiding any overlapping with morphologically similar taxa, such as the European white truffles *T. maculatum*, *T. borchii*, *T. dryophilum*, and *T. puberulum* (2, 30, 32, 55), as well as with some phylogenetically similar taxa, such as *T. mesentericum* and *T. aestivum* (52). Similarly, most of the *T. melanosporum* motifs detected in silico all the *T.*

melanosporum sequences but not its close relatives, such as the Chinese black truffle, *T. indicum* (Fig. 1) (37, 38, 51, 52, 56). With the dot blot experiments, we demonstrated that five *T. magnatum* motifs hybridized only *T. magnatum* ITS, whereas only one *T. melanosporum* motif was specific. It was not possible to correlate the number of mismatches observed in silico and the specificity in the dot blot analysis (data not shown). For example, the *T. melanosporum*-specific motif “Tmel-ITS2-1”

FIG. 3. Dot blot hybridization signals of 27 field isolates used in the blind test. The ITS amplicons of the isolates were coded from 1 to 27 and spotted on each membrane. The whole *T. magnatum* ITS as well as the “Tma-ITS2-5” and “Tmel-ITS2-1” motifs were used as probes. The coded spots correspond to the following species: 1, *T. magnatum*; 2 and 3, *Choiromyces meanformis*; 4 and 5, *T. mesentericum*; 6, *Terfezia arenaria*; 7, *T. oligospermum*; 8 and 9, *T. borchii*; 10 and 11, *T. brumale*; 12, *Boletus edulis*; 13 and 14, *T. aestivum*; 15, *T. melanosporum*; 16, *T. magnatum*; 17, *T. melanosporum*; 18, *T. indicum*; 19, *Aspergillus uster*; 20, *Aspergillus sclerotium*; 21, *Penicillium restrictum*; 22, *Periconia macrospinosus*; 23, *Curvularia inaequalis*; 24, *Embellisia* sp.; 25, *Pestalotiopsis* sp.; 26, *Phoma* sp.; and 27, *Coprinus micaceus*. The membranes were exposed for 2 min.

has about 10 mismatches out of 50 nucleotides against the *T. indicum* sequences (a 20% difference). However, the “Tmel-ITS1-1” motif, which presents a similar level of mismatches (6 out of 30 nucleotides, i.e., a 30% difference), hybridized the *T. indicum* ITS (Fig. 2B). These data confirmed the previous results and showed that the specific hybridization reactions were not always found to be consistent, with small numbers of nucleotide mismatches (27, 48), suggesting that other factors, such as base compositions, may influence the hybridization reaction and stability of oligonucleotides (48).

The specific hybridization signals of the “Tma-ITS2-5” and “Tmel-ITS2-1” oligonucleotide motifs to *T. magnatum* and *T. melanosporum*, respectively, were also confirmed in a blind test that included Ascomycota and Basidiomycota fresh isolates. Moreover, the motifs recognized one GenBank (Fig. 2C) sequence and two fresh isolates (Fig. 3) of each species, indicating that the identification of the *T. magnatum* and *T. melanosporum* isolates at the species level was not affected by the sequences of the motifs. Both motifs are highly conserved and showed only a single nucleic variation, and their corresponding species have a very low intraspecific genetic variability (3, 31, 35).

We therefore propose that the “Tma-ITS2-5” and “Tmel-ITS2-1” motifs can be used as new tools to identify *T. magnatum* and *T. melanosporum*, respectively. Both satisfy the criteria necessary to be used as oligonucleotide bar codes, i.e., (i) they are highly specific for their respective target species, (ii) they efficiently bind both target sequences allowing low quantities of their targets to be detected in complex mixtures, and (iii) they display similar hybridization behavior (46). We did not examine motifs from other *Tuber* taxa, because we focused on the two most economically important truffles. Specific oligonucleotide identification was applied to distinguish either pathogenic or saprotrophic fungal species (7, 44, 48). To our knowledge, this is the first report that has presented sequence motifs of ITS loci for an unambiguous identification of edible mycorrhizal fungi.

Assignment of *T. indicum*, *T. aestivum*, and *T. mesentericum* through oligonucleotide bar codes. Compared with *T. magnatum* and *T. melanosporum*, the *T. indicum*, *T. aestivum*, and *T. mesentericum* motifs were found to be more variable than the conserved sequence motifs. However, it was possible to design ITS motifs which recognized these species in silico. The distinction between *T. indicum* and *T. melanosporum* species (see above) based on short sequence motifs was unexpected, because they are difficult to distinguish through molecular approaches. On the other hand, all the *T. indicum* motifs detected six specimens of two Chinese black truffles, *T. hima-*

layense and *T. sinense* (51). This result is congruent with the phylogenetic analyses, which placed both species in the *T. indicum* cluster, confirming that they belong to the same *T. indicum* species (51).

Most of the *T. aestivum* motifs covered the intraspecific variability of this species well and were able to identify most (if not all) the *T. aestivum* and *T. uncinatum* isolates, but not *T. mesentericum*, a black truffle, which is a sister to *T. aestivum* (36, 53). Similarly, the *T. mesentericum* motifs identified all the *T. mesentericum* accessions, and most of them were not found on the *T. aestivum* sequences. We have therefore confirmed the result of some recent molecular and phylogenetic investigations, which ascertained that *T. aestivum* and *T. uncinatum* belong to the same species or are synonyms (36, 53). These authors indicated that spore reticulum height is not a diagnostic key to distinguish *T. aestivum* from *T. uncinatum*.

The evolutionary relationships of truffles are reflected through oligonucleotide bar codes. A critical point in taxonomy or bar code identification is the distinction between closely related species (42). This difficulty was overcome for several *Tuber* motifs. However, other motifs were found to be specific to a group of species of *T. melanosporum* and *T. indicum* or *T. aestivum*, *T. mesentericum*, and/or *T. pinniferum*. For example, two *T. melanosporum* and six *T. indicum* motifs matched both their respective European and Chinese isolates. This cross-matching was also obtained with some *T. melanosporum* motifs after the hybridization reaction. These data are consistent with similar morphological characteristics and close phylogenetic relationships of both species. Similar results were observed with other genomic loci of both *Tuber* species (38), as well as with the ITS oligonucleotides between the target and nontarget fungal species, such as *Verticillium* and *Pythium* (27, 48). On the other hand, the current data also showed that *T. indicum*, which has a great intraspecific variability, contains several short ancestral haplotypes that are very similar to and in common with *T. melanosporum*, a species that has a low intraspecific variability. Wang and colleagues (51) suggested that a common ancestor of both species, probably from India, migrated towards Europe in *T. melanosporum* and towards China in *T. indicum*.

Riouiset et al. (39) grouped *T. magnatum* in the *T. magnatum* group with white truffles such as *T. borchii* and *T. maculatum* on the basis of morphological criteria. However, Wang et al. (52) showed that *T. magnatum* clustered with black truffles such as *T. mesentericum* and *T. aestivum* after phylogenetic analysis. We have here confirmed that *T. magnatum* is phylogenetically related to *T. mesentericum*, since three motifs cross-

hybridized with this species, whereas only one cross-hybridized with *T. maculatum*.

In conclusion, the present in silico identification of the five *Tuber* spp. with ITS sequence motifs is consistent with investigations made using traditional approaches (i.e., by morphology), as well as through molecular fingerprinting and/or phylogeny of genomic loci such as ITS and β -tubulin (see GenBank entries) (29, 31, 35, 36, 38, 40, 51–53, 56). The specific motifs identified the *Tuber* species not only from the ascocarp sequences but also from the sequences of unidentified soil-borne ectomycorrhizas (either complete or partial), suggesting that the ITS motifs can be used in a wide range of ecological studies to monitor the hypogeous *Tuber* species without cultivation. ITS sequence motifs can therefore be used for the different phases of the truffle life cycle as well as for systematic, environmental, and food control investigations. The approach provides a powerful new tool for an accurate assignment of unknown truffle species and is the first step toward the realization of oligonucleotide bar codes of *Tuber* spp. In the comparative genomic and metagenomic age, truffle DNA bar coding also offers new ways of understanding their life cycles more clearly, cycles which are still largely unknown.

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