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Review

Overview of molecular methods for quick identification of *Tuber* species

Antonella Amicucci[1], Bianca Ranocchi[1]

[1]Department of Biomolecular Sciences, Urbino University; via Saffi 2, 61029 Urbino, Italy

Corresponding author e-mail: antonella.amicucci@uniurb.it

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Abstract
Truffles represent a resource of great value all over the world. The various species are widespread and occupy various habitats. Methods to identify their value in terms of marketing, cultivation, and the ecological–forestal field are needed. The traditional classification of *Tuber* species is based on their morphology; nevertheless, the macroscopic and microscopic characteristics of the fruiting body, spores, and ectomycorrhizae may be absent or insufficiently discriminating. This problem is amplified when dealing with valuable truffle species, which are often replaced by truffles with lower organoleptic qualities and commercial value. Today, molecular biology can help to correctly identify them through several widely used methods that are fast, specific, and sensitive. Molecular methods are mostly PCR-based, with primers designed on various targets, genes, or regions widely variable between species but sufficiently conserved within the same species. The DNA regions analyzed for identifying truffles include ribosomal internal transcribed spacer (ITS), ribosomal large subunit (LSU), β-tubulin, and EF-α elongation factor. This review summarizes the main and most reliable DNA-based molecular methods for identifying *Tuber* species. The methods are listed schematically and clearly for quick consultation and use in all areas of study in which monitoring, and verification of the species is essential.

Keywords
Truffle, ectomycorrhizae, processed food, ITS region, PCR

Introduction

Truffles are the fruiting bodies of ectomycorrhizal fungi belonging to the genus *Tuber*, order Pezizales, and division Ascomycota (Læssøe and Hasen, 2007; Trappe et al., 2009). They form an ectomycorrhizal symbiosis on the roots of different tree species, including beech, oak, birch, poplar, hazel, hornbeam, and pine (Harley and Harley, 1987). Some *Tuber* species are edible and appreciated for their intense and superb aroma (Pelùsio et al., 1995; Mello et al., 2006) acting as cue for animals which are thus able to spread their spores (Splivallo et al., 2011).

Amongst the known 200 *Tuber* species (Bonito et al., 2013), the most expensive species is *Tuber magnatum* Picco, whose ascomata retail prices vary from EUR 1000/kg to EUR 6000/kg in Italy but can reach higher prices outside of Italy (Hall et al., 2007). For these reasons, truffles represent a notable economic resource for Italy. In 2018 (latest data available), the economic value of truffle exports was around 49.2 million euros for fresh or frozen truffles and around 13.7 million euros for preserved truffles (Istat, 2019).

Moreover, valuable edible truffles have a fundamental role in various economic fields, including gastronomy, tourism, retail markets, and truffle cultivation (Thomas, 2014; Büntgen et al., 2017) and Europe accounts for 85% of the world truffle export market (Hamzić Gregorčič et al., 2020). Another important aspect is the conservation of natural forests; for example, the European *Tuber melanosporum* Vittad. can grow in natural areas without the utilization of machinery and chemicals, and their plantations represent a firebreak thanks to low plantation densities, soil tilling, and, therefore, the herbicidal effect of the fungus (Büntgen et al., 2017).

The unequivocal identification of the species is fundamental, as it guarantees all phases of mycorrhizal seedlings production, cultivation, trade, and the research experimental models setting up (Zambonelli et al., 2021). In the production of mycorrhized seedlings, it is essential to verify the identity of the truffles used for inoculation, the presence of the selected species, and to minimize the contamination risk of mycorrhized roots by competitive fungi. It is important to monitor the presence of specific *Tuber* mycelium in the greenhouse before and after transplanting in the field of mycorrhized seedlings to verify the presence of competitive fungi. For example, *Pulvinula constellatio* Berk. and Br. Boud. are ascomycete fungi that can create ectomycorrhizal infections in plants with *Tuber* spp. in greenhouses (Amicucci et al., 2001). Regarding commerce, *T. melanosporum* may be exchanged for the Chinese truffle *Tuber indicum* Cooke & Massee because it is morphologically very similar, but the latter has very low quality (Lazzari et al., 1995). *T. magnatum* can be the subject of fraud or confused with similar species, especially when the salient morphological features for identification are missing and at the mycorrhizal level where it is typically contaminated by other species (Iotti et al., 2012; Mello et al., 2001). Over the years, numerous researchers have defined the distinctive morphological characters of the species (Amicucci et al., 1998; Pacioni et al., 1999; Mello et al., 2000; Paolocci et al., 2004; Kovács et al., 2006; Marozzi et al., 2020). Although morphological methods are fundamental, taxonomically and morphologically similar species may have different organoleptic and commercial value. Moreover, sometimes some distinctive morphological features are absent: immature ascomata lack spores, mycelia are poorly defined among the many species, mycorrhizae can lose their typical ornamentations, and cooking or other food preparation processes can alter their distinctive characteristics (Iotti et al., 2012).

For these reasons, for decades, the ever-growing interest in this precious fungus has prompted many researchers to carry out increasingly refined research to find molecular markers capable of recognizing truffles in all phases of their life cycle. Numerous molecular markers have been developed to identify the most economically important truffle species to date (Gardes et al., 1991; Mello et al., 1999; Paolocci et al., 1999). The molecular methods have become indispensable to complement the traditional methods, overcome the lack of distinctive elements, increase the reliability of identification, and speed up the analysis.

This review aims to illustrate and list the molecular methods used for identification. The development of the PCR technique enabled the molecular identification of truffles, starting with the

work by Gardes et al. in the 1990s (Gardes et al., 1991). With the PCR technique, many researchers screened target DNA sequences conserved within a species, but variable among different species (Mello et al., 1999).

The genome sequencing of different *Tuber* species has also provided various advantages by expanding the distinctive target DNA's availability, hypervariable regions, and specific mating types of genes. One of the regions most used for evolutionary, diagnostic, and taxonomic studies is the ribosome internal transcribed spacer region (ITS) of nuclear rDNA. The ITS region is located between ribosomal rRNA 18S and 28S genes and includes the gene for the 5.8S rRNA (Jeandroz et al., 2008). ITS regions are present in multiple copies within the same genome, and the intraspecific homogeneity in *Tuber* is highly conserved; these characteristics made this target suitable for designing species-specific primers for several years (Ciarmela et al., 2022), although intragenomic variability has recently emerged in some fungi (Bradshow et al., 2023).

Furthermore, β-tubulin is a good barcoding region for designing *Tuber*-genus-specific primers (Zampieri et al., 2009). At the same time, elongation factor 1-α was employed for designing new primer pairs specific for each *Tuber* phylogenetic lineage and for phylogeographic analyses (Leonardi et al., 2021). Finally, the microsatellite regions have also been studied to find species-specific markers. However, these targets have proved to be more versatile for characterizing intraspecific variability and have no longer been considered for these purposes (Amicucci et al., 2002; Murat et al., 2011; Molinier et al., 2013; Molinier et al., 2016).

Molecular methods based on ITS region

ITS (Internal transcribed spacer) is a non-coding ribosomal DNA region interposed to ribosomal genes 18S e 28S, as shown in Figure 1, and repeated in clusters in all eukaryote genomes.

Fig. 1. – Scheme of the ITS region of fungi.

The repeated units of transcribed rDNA regions are separated by intergenic spacers (IGS) (Ciarmela et al., 2002; Jeandroz et al., 2008). An IGS separates the large subunit (LSU) rRNA gene (encoding 5.8S and 28S rRNA) in one repeat from the small subunit (SSU) rRNA gene (encoding 18S rRNA) in the adjacent downstream repeat (Ciarmela et al., 2002; Jeandroz et al., 2008). This IGS region, expressing extremely intraspecies variability, allows studies of intraspecies and taxonomic variability but is not a suitable target for developing species-specific markers (Ciarmela et al., 2002).

Restriction Length Fragment Polymorphism Analyses (RFLP)

ITS may present polymorphisms of length and sequence and is amplifiable by using universal primers (White et al., 1990; Bruns et al., 1991; Gardes et al., 1993). The length of the ITS region is quite similar between the different species, while the restriction fragment length polymorphism analysis (RFLP) produces characteristic profiles. The ITS amplicons are digested with specific restriction endonucleases selected from the restriction maps of the ITS sequences of each species to detect

polymorphisms. The digested DNA is separated by gel electrophoresis, from which the pattern RFLP is produced (Bertini et al., 1998; Paolocci et al., 1999; Bertini et al., 2006). The variable lengths of cleavage fragments are characteristic of a sequence of DNA. The restriction analysis of the ITS region allows obtaining species-specific patterns for different species of truffles: electrophoretic separation allows the comparison of restriction profiles produced by different species. The results of this method are obtained quickly and are easy to interpret. Table 1 summarizes the enzymes that can be used to identify white truffle species.

Table 1 - Enzymes specific for white truffle species.

Species	Enzymes													
	*Msp*I	*Rsa*I	*Ava*II	*Eco*RI	*Nco*I	*Hinf*I	*Taq*I	*Mbo*I	*Cla*I	*Alu*I	*Kpn*I	*Stu*I	*Sfi*I	*Sca*I
T. magnatum	+	+	+	+	+	+	+	-	+	-	+	+	+	-
T. borchii	+	+	+	+	-	+	+	-	+	+	-	-	-	-
T. puberulum	+	+	+	-	-	-	-	-	-	-	-	-	-	+
T. dryophilum	+	+	+	-	+	-	-	-	-	-	-	-	-	-
T. maculatum	+	+	+	-	-	+	-	+	-	+	-	-	-	-

Species-specific enzyme = +; non-species-specific enzyme = -

As regards the species *T. melanosporum* and *T. indicum,* the research of Paolocci et al. reports the possibility of distinguishing these two species through ITS restriction analysis using the *Rsa*I or *Ssp*I enzymes (Paolocci et al., 1997). The sizes of the restriction fragments, which are useful for recognition, are shown in Table 2.

Table 2 - ITS size fragments by *Rsa*I and *Ssp*I

Species	Size fragments (bp) by enzyme *Rsa*I	Size fragments (bp) by enzyme *Ssp*I
T. melanosporum	191/429	Not cut
T. indicum B1	123/497	418/202
T. indicum B2	Not cut	418/202
T. indicum A	153/276/195	376/248

ITS specific primers

The ITS regions of the different *Tuber* species were sequenced, analyzed, and aligned through a dedicated computer program. The sequence alignment analyses allowed the detection of species-specific regions, and with the support of web platforms, specific primer pairs were selected, respecting the specificity of pairing, the length, the GC content, and avoiding inverted repeated sequences. Table 3 lists the specific primer pairs available for *Tuber* species to date.

Table 3 - List of specific primer pairs available for *Tuber* species and conditions used for PCR analysis.

Tuber species		Primer name	Primer sequence (5'-3')	Frag. length (bp)	Thermocycling parameters	Accession No. GenBank	Reference
T. magnatum	SCAR (RAPD)	TAR I	CCCCAACGCGTAAAAGAGTA	145	25 cycles: 94 °C 45 s, 53 °C 45 s	---	Amicucci et al., 1997
		TAR II	CGTATCGGTGTCTTGCCAGTG				
	ITS-rDNA	TmagI	GGATGCGTCTCCGAATCCTGAAT	173	25 cycles: 94 °C 20 s, 62 °C 15 s	AF003911	Amicucci et al., 1998
		TmagII	TCGGGCCCTTTCTCAGACTGCTG				
	ITS-rDNA	P7	TCCTACCAGCAGTCTGAGAAAGGGC	434	40 cycles: 94 °C 45 s, 65 °C 45 s, 72 °C 45 s	---	Mello et al., 1999; Mello et al. 2006
		M3	TGAGGTCTACCCAGTTGGGCAGTGG				
	ITS-rDNA	MG09 I	TCACTTGGGGCGTCCGAGAT	158	30 cycles: 94 °C 15 s, 52 °C 15 s	---	Rossi et al., 2000
		MG09 II	ACTGCTGGAAACGGGGAATG				
	ITS-rDNA	TmgITS1for	GCGTCTCCGAATCCTGAATA	106	25 cycles: 95 °C 20 s, 60 °C 30 s, 72 °C 40 s	---	Iotti et al., 2012
		TmgITSrev	ACAGTAGTTTTTGGGACTGTGC				
	ITS-rDNA	Tmag3	TTAACTGTTTAAGTTTGTCAGGC	151	35 cycles: 94 °C 30 s, 56 °C 30 s, 72 °C 2 min	FM205629	Rizzello et al., 2012
		Tmag4	CCTGAATATCTCCTGTGTACCAT				
	ITS-rDNA	ITSMAGN	GTCACTGAAAACCCACTCACG	230	25 cycles: 95 °C 30 s, 63 °C 30 s, 72 °C 45 s	AF325174	Rubini et al., 2001
		ITSBACK3	TGAGGTCAACCCAGTTGGACAGT				
	ITS-rDNA	TmagI	GGATGCGTCTCCGAATCCTGAAT	635	30 cycles: 94 °C 20 s, 62 °C 15 s, 72 °C 30 s	AF003911	Amicucci et al., 2000
		ITS4	TCCTCCGCTTATTGATATGC				
	β-tubulin	Tubmagnf	CCTCCCAATTTGCAATACAC	282	25 cycles: 94 °C 30 s, 61 °C 30 s, 72 °C 30 s	FN252812	Zampieri et al., 2010
		Elytubr	AAAGACGAAGTTATCTGGCCTGA				
T. borchii	ITS-	TboI	TGTATGGGATGCCCTATCGGAC	397	30 cycles: 94 °C	AF003920	Amicucci et al., 1998

	rDNA		T		20 s, 55 °C 15 s, 72 °C 30 s		
		TboII	CTATTACCACGGTCAACTTC				
	ITS-	TBA	TGCCCTATCGGACTCCCAAG	432	40 cycles: 94 °C 45 s, 65 °C 45 s, 72 °C 45 s	---	Mello et al., 1999
	rDNA	TBB	GCTCAGAACATGACTTGGAG				
	ITS-	rTboII	GAAGTTGACCGTGGTAATAG		30 cycles: 94 °C 20 s, 62 °C 15 s, 72 °C 30 s	AF003920	Amicucci et al., 2000
	rDNA	ITS4	TCCTCCGCTTATTGATATGC	185			
	SCAR (RAPD)	TB I	AGAGAATACATGCGGTAGTGT	363	30 cycles: 94 °C 30 s, 58 °C 30 s, 72 °C 40 s	U94390	Bertini et al., 1998
		TB II	CTCGCTTGGAGATAATAACAG				
	SCAR (TBF gene)	TBF1for	TCACCAAGCACTAACTCCTCTCT	699	30 cycles: 94 °C 30 s, 53 °C 30 s, 72 °C 45 s	U83996.1	De Bellis et al., 1998
		TBF2rev	CCAAACCGAATCAAACAACTAAT				
T. maculatum	ITS-	TmacI	GACACAGGCTCCCGATAAAACAC	407	25 cycles: 94 °C 20 s, 62 °C 15 s, 72 °C 30 s	AF003909	Amicucci et al., 1998
	rDNA	TmacII	CAGCAGCACTGATAGCCCCG				
		rTmacII	CGGGGCTATCAGTGCTGCTG	225	30 cycles: 94 °C 20 s, 62 °C 15 s, 72 °C 30 s	AF003909	Amicucci et al., 2000
		ITS4	TCCTCCGCTTATTGATATGC				
T. dryophilum	ITS-	TdryI	ATCGGGCTCCCAAGCAAAACA	510	30 cycles: 94 °C 20 s, 62 °C 15 s, 72 °C 30 s	AF003917	Amicucci et al., 1998
	rDNA	TdryII	TCTACTACCATGGTTCACTTT				
T. puberulum	ITS-	TpuI	TCTGTTACCAGGGTCCACATT	368	30 cycles: 94 °C 15 s, 55 °C 30 s	AF003918	Amicucci et al., 1998
	rDNA	TpuII	GGCTTCTGGGTTGAGGTGTTT				
	ITS-	TpuI	TCTGTTACCAGGGTCCACATT	560	30 cycles: 94 °C 20 s, 62 °C 15 s, 72 °C 30 s	AF003918	Amicucci et al., 2000
	rDNA	ITS4	TCCTCCGCTTATTGATATGC				
T. rufum	ITS-	Ru1f	TGCTTTCCCAGGTGGTTGG	566	30 cycles: 94 °C 20 s, 63 °C 20 s,	AF106892, AY112894	Iotti et al., 2007
	rDNA	ITS4	TCCTCCGCTTATTGATATGC				

		Ru2f	TTGC TTTCCCAGGGAATTGG	572	72 °C 30 s		
		ITS4	TCCTCCGCTTATTGATATGC				
T. macrosporum	ITS-rDNA	Tmacr For	CGTCGCTCATCAAAGCAGTC	248	33 cycles: 94 °C 20 s, 61 °C 20 s, 72 °C 20 s	AF106885, FM205663, FM205664, AY112895	Benucci et al., 2011
		Tmacr Rev	CCGCCAGTACCACCAGGAG				
T. melanosporum	ITS-rDNA	ITSML	TGGCCATGTGTCAGATTTAGTA	436	23 cycles: 94 °C 30 s, 63 °C 30 s, 72 °C for 45 s	U89359	Paolocci et al., 1997
		ITS4LNG	TGATATGCTTAAGTTCAGCGGG				
		Mela-fw	ACGACGGACTTTATAAACGGTTATAA	141	30 cycles: 95 °C 20 s, 60 °C 60 s	U89359	Schelm et al., 2020
		Mela-rv	AGCGGGTATCCCTCCCTGATT				
	ITS-rDNA	T.mel_for	TTGCTTCCACAGGTTAAGTGA	351	30 cycles: 94 °C 2 min, 55 °C 30 s, 72 °C 30 s	GQ917052	Bonito, 2009
		T.mel_rev	TAAAGTCCGTCGTTCATGC				
T. brumale	ITS-rDNA	ITSB	CAATGTCAGAGCCAATCTAATGC	700	23 cycles: 94 °C 30 s, 63 °C 30 s, 72 °C 45 s	AF001010	Paolocci et al., 1999
		ITS4LNG	TGATATGCTTAAGTTCAGCGGG				
T. indicum	ITS-rDNA	ITSCHCH	AACAACAGACTTTGTAAAGGGTTG	140	23 cycles: 94 °C 30 s, 63 °C 30 s, 72 °C 45 s	U89360, U89361, U89362	Paolocci et al., 1999
		ITS4LNG	TGATATGCTTAAGTTCAGCGGG				
		Indi-fw	AACAACAGACTTTGTAAAGGGTT	146	30 cycles: 95 °C 20 s, 60 °C 60 s	U89359	Schelm et al., 2020
		ITS4LNG	TGATATGCTTAAGTTCAGCGGG				
T. aestivum/uncinatum	ITS-rDNA	UncI	TGGGCCGCCGAAAACTTG	405	27 cycles: 95 °C 1 min, 59 °C 45 s, 72 °C 45 s	AJ492199-210	Mello et al., 2002
		UncII	CTGACGAGATGCCCCGGA				
		Tu1sekvF	AGAGCACCAAACCACAG	496-502	34 cycles: 95 °C 60 s; 52, 59 or	AJ492216 AJ888120	Grindler et al., 2011

		Tu2sekvR	ACCACAGCGTCTACCAA		63.5 °C 45 or 40s, 72°C 45 or 40 s		
Multiplex:	ITS-rDNA	TmagI	GGATGCGTCTCCGAATCCTGAAT	635	30 cycles: 94 °C 20 s, 62 °C 15 s, 72 °C 30 s	AF003911, AF003918,	Amicucci et al., 2000
T. magnatum,		TpuI	TCTGTTACCAGGGTCCACATT	560		AF003909,	
T. puberulum,		rTmacII	CGGGGCTATCAGTGCTGCTG	225		AF003920	
T. maculatum,		rTboII	GAAGTTGACCGTGGTAATAG	185			
T. borchii		ITS4 (Reverse)	TCCTCCGCTTATTGATATGC				
Multiplex:	ITS-rDNA	ITSML	CGTCGCTCATCAAAGCAGTC	440	23 cycles: 94 °C 30 s, 63 °C 45 s, 72 °C 45 s	U89359, AF001010	Paolocci et al., 1999
		ITSB	CAATGTCAGAGCCAATCT	700			
T. melanosporum,		ITSCHCH	AACAACAGACTTTGTAAAGGGTTG	140		U89360-62	
T. brumale,							
T. indicum		ITS4LNG (Reverse)	TGATATGCTTAAGTTCAGCGGG				

In some cases, such as in soil analyses, the PCR reaction, albeit with specific primers, may not show the presence of the species of interest due to the high amount of different species in the soil. In this case, Nested PCR can be used. A first amplification can be carried out with ITS universal primers, and subsequently, a second reaction can be performed using as a template a small amount of the first reaction product and a pair of primers internal to the previous target (Green and Sambrook, 2019).

Table 4 - List of specific primers and probes for qPCR for truffles.

Species	Primer/Probe	Sequence 5' – 3'	Product size	Cycles	Reference
T. melanosporum	Mela – fw	ACGACGGACTTTATAAACGGTTATAA	141	95 °C 4 min; 30 cycles: 95 °C 20 s, 60 °C 60 s; 72 °C 10 min	Schelm et al., 2020
	Mela – rv	AGCGGGTATCCCTCCCTGATT			
	probe	Cy5–GACCTGGATCAGTCACAAGTCTTGTCTGGT-BHQ2			
	Tmelfwd	TCTCTGCGTATCACTCCATGTTG	61	95 °C 30 s; 40 cycles: 95 °C 5 s, 60 °C 34 s	Parladé et al., 2013
	Tmelrev	TCCCACAGGTGCCAGCAT			
	STQTmel	*6FAM*-TTCCACAGGTTAAGTGAC-*MGB*			
	T.mel_for*	TTGCTTCCACAGGTTAAGTGA	351	95 °C 10 min; 45 cycles: 95 °C 30 s, 56 °C 30 s, 72 °C 30 s, 95 °C 15 s, 60 1 min, and 95 °C 15 s	Zampieri et al., 2012
	T.mel_rev*	TAAAGTCCGTCGTTCATGC			
T. magnatum	TmgITS1for	GCGTCTCCGAATCCTGAATA	106	95 °C 6 min; 25 cycles: 95 °C 20 s, 60 °C 30 s, 72 °C for 40 s; 72 °C 7 min	Iotti et al., 2012
	TmgITS1rev	ACAGTAGTTTTTGGGACTGTGC			
	TmgITS1prob	TGTACCATGCCATGTTGCTT			
	TmgITS2for	AAACCCACTCACGGAATCAC	99	95 °C 6 min; 25 cycles: 95 °C 20 s, 60 °C 30 s, 72 °C for 40 s; 72 °C 7 min	Iotti et al., 2012
	TmgITS2rev	CGTCATCCTCCCAATGAAA			
	TmgITS2prob	GTACCAAGCCACCTCCATCA			
	Tmag3*	TTAACTGTTTAAGTTTGTCAGGC	151	95 °C 10 min; 40 cycles: 95 °C 30 s, 56 °C 30 s, 72 °C 30 s	Rizzello et al., 2012
	Tmag4*	CCTGAATATCTCCTGTGTACCAT			
T. indicum/ T.himalayense	Indi – fw	AACAACAGACTTTGTAAAGGGTT	146	95 °C 5 min; 30 cycles: 95 °C 20 s, 60 °C 60 s; 72 °C 10 min	Schelm et al., 2020
	ITS4LNG	TGATATGCTTAAGTTCAGCGGG			
	probe	HEX-GGACCTAGATCAGTCACAAGTCATGTCTGG-BHQ2			

* SYBR Green Reaction

In the context of identification, quantitative Polymerase Chain Reaction (qPCR) is therefore convenient for studying the truffle distribution in the soil to clarify the unknown aspects of their biology, the unresolved problems in productivity, and the factors leading to the spreading and development of the mycelium in the soil (Suz et al., 2006; Zampieri et al., 2010). The quantification of a specific species of *Tuber* is even more helpful to achieve such purposes. Table 4 lists the specific primers and probes for truffles designed to date.

The specific primers selected can be employed to set up a multiplex PCR. A reaction is carried out with a set of primers to analyze complex samples in which DNA mixtures from different species may be present simultaneously. The multiplex PCR allows the simultaneous identification of different truffle species in a single amplification reaction. This method has the advantage of saving time, reagents, and amount of target DNA. In addition, at the same time, it is possible to identify truffles and related ectomycorrhiza in a single amplification (Paolocci et al., 1999; Amicucci et al., 2000). Therefore, multiplex PCR is useful for studying the ecology of different species of *Tuber* and symbiotic relationships (Paolocci et al., 1999). The selection of primers must, however, be very accurate to work with the optimal amplification conditions; in particular, primers must have similar melting temperatures and must not hybridize with each other.

RAPD technique and SCAR markers

RAPD (Random Amplified Polymorphic DNA) is a modified PCR technique in which a single and short (generally 10 bp) random oligonucleotide is used. The annealing temperature is very low to allow the binding of the primer to the genomic DNA (Williams et al., 1990; Babu et al., 2021). The result of this amplification is a specific pattern of the individual that depends on the distribution of complementary sequences to those of the primer along the genome. RAPD is cost-effective, quick, and easy, and no information about the target organism's genome is needed. Through random-primed amplification, nucleotide sequence polymorphisms are detected simultaneously for multiple samples. Despite its great potential, this method has significant criticalities (Williams et al., 1990). These include, for example, the high risk of contamination and the low reproducibility. Moreover, RAPD markers are not locus-specific because they are all dominant. For this reason, the nucleotide profiles obtained with random primers are used to identify species-specific markers; once they have been detected, they are purified from the agarose gel, and sequenced, and primers are drawn from them to be used in specific PCR. The markers obtained from RAPD fragments are named SCARs (Sequence Characterized Amplified Region) and are locus-specific PCR-based markers (Potenza et al., 1994; Amicucci et al., 1997; Bertini et al., 1998; Paolocci et al., 2000; Rossi et al., 2000; Mello et al., 2006). The developed primer pairs specific for *Tuber* spp. are listed in Table 3.

RAPD analysis was among the first to be used to study the genetic diversity among *Tuber* spp. because of its advantages. Thanks to the use of specific markers, the technique becomes strategic for the identification of *Tuber* spp. in all stages of the biological cycle, so it allows the detection of possible commercial frauds and guarantees certified products.

Methods for DNA extraction-free analysis

A DNA isolation procedure must precede all the methods described. Generally, the methods are laborious and time-consuming. In recent years, direct PCRs have been developed to skip the isolation phase and immediately apply the selected PCR.

In particular, in 2005, Iotti et al. developed a new technique for identifying ectomycorrhizae directly, quickly, and precisely by PCR omitting the DNA extraction. The process consists of adding BSA to stabilize the PCR reactions and prevent the adhesion of enzymes. The mycorrhized tips of each sample were separated from other tissues and cleaned. The ECM manipulation was done in Petri dishes containing sterile distilled water to prevent PCR contamination. A small portion of each morphotype was then transferred directly to the PCR tube containing sterile water. Analysis was performed on fresh and frozen (-80 °C) mycorrhizae (Iotti et al., 2005). This method minimizes contamination risks and doesn't use harmful substances.

Furthermore, for successful truffle cultivation, it is crucial to use the appropriate species of truffle for the inoculum. The inoculum used for truffle cultivation comes from truffle fruitbodies. It is a spore substrate, so it is necessary to have a molecular method for identifying spores.

The protocol of Bonito consists in a direct amplification from fruitbodies of *T. melanosporum* (Bonito, 2009). Fresh fruitbodies were cut and dried, then they were amplified by direct PCR using the T.mel_for – T.mel_rev primer set (Bonito, 2009).

These methods speed up the identification process and also require only minimal quantities of starting material, facilitating analysis when only a few mycorrhizae, or a few spores, are available. It also has the advantage of safeguarding the carpophore which will then eventually be placed on the market. Preserving the original structure of the truffle preserves its quality and improves environmental sustainability.

Applications in processed truffle food

To ensure high-quality *Tuber* species that are used in food products, molecular techniques are necessary for accurate identification. These applications can be used in truffle traceability, from harvest to table. The ability to record the origin and route of each truffle allows for greater transparency in the supply chain. This not only ensures product quality but also promotes ethical and sustainable business practices. The combined use of molecular markers and advanced analytical techniques allows truffles to be traced at the molecular level in food, ensuring the quality and authenticity of the final product. It is important in the food sector to combat counterfeiting and ensure that consumers receive authentic, high-quality products. The applications of molecular methods to identify truffle species in food products are exposed in Amicucci et al. (2002), in Rizzello et al. (2012), and also in Tejedor-Calvo et al. (2023). These papers show specific cases of identification in processed truffle-based food products: cream, fresh pasta, truffle-flavored rice, oil, salt, and cheese. In one specific case, a species of Chinese truffle was detected, rather than *T. melanosporum*, as incorrectly reported on the package (Amicucci et al., 2002).

Commercial fraud is a big problem in the marketing of truffles and mycorrhizal seedlings. However, it is even more prevalent in truffle-based food products because the temperatures and treatments involved in the processing of truffles can damage the elements that are important for their

identification, which represent a fundamental and unique tool in the truffle industry. Truffle-based food products represent a significant portion of the overall truffle market.

Whole-genome sequencing

Despite the complexity and high cost, genomics has become an applied science in the fungal world as well, aimed at better understanding the biology and characterizing the species in a more definite way. The genomic sequencing technique aims at predicting and annotating the set of genes present in the organism and also the non-coding sequences (Pareek et al., 2011). In fact, genome sequencing includes the sequencing of the entire exome (i.e., all coding regions of each gene) and all intergenic regions, which represent the majority of DNA (Kuo et al., 2014). The knowledge of the genome determines its structure and provides useful information to understand the function of genes (Pareek et al., 2011; Köser et al., 2012). In addition, it lays the foundations for understanding the interactions between the organism and the environment in which it lives and other organisms, as well as the biochemical and transcriptomics properties, which is why it represents a turning point in understanding the biology of mycorrhizal fungi such as truffles.

The first sequences were based on the application of the method based on Sanger biochemistry, which, over the years, has undergone changes and innovations, resulting in the achievement of a large-scale automated sequencing method. The first fungal genomes were sequenced via the Sanger method but with the Whole-Genome Shotgun approach (Heather and Chain, 2016; Gryganskyi et al., 2023). The WGS approach is a more advantageous method than clone-by-clone because it greatly reduces sequencing times and costs. DNA is initially fragmented into several random segments of defined sizes, which are then sequenced using the Sanger method (Köser et al., 2012). Many fragmentations and sequencing cycles are performed to obtain high overlapping DNA fractions that allow the reconstruction of the entire genome through alignment and assembly programs. This strategy has the advantage of generating sequence reads from both insert ends and it produces highly redundant sequence coverage (Edwards et al., 1990). The first fungal genome to be completely sequenced was *Saccharomyces cerevisiae* in 1996; the sequencing of the first filamentous fungus, *Neurospora crassa*, was completed in 2003 (Galagan et al., 2003; Otero et al., 2010).

The interest in sequencing the genomes of truffles was born out of simple basic knowledge, but it has had and will continue to have enormous importance in deepening knowledge of its biology. It has provided and will provide fundamental knowledge for the study of processes related to its biological cycle: the establishment of symbiosis with the plant, carpophore formation, and diffusion of mycelium in the soil. Furthermore, whole-genome sequencing will allow the identification of new targets useful for identification. It will also be able to provide information on the process of synthesis of aromas, which are highly appreciated and distinctive among the different species. Table 5 provides the main information on the truffle genomes characterized to date, with the related links to access them.

More organisms belonging to *Tuber* are being sequenced, in addition to the already published genomes which started with that of *T. melanosporum* (Martin et al., 2010). As part of the "1000 Fungal Genomes Project (1KFG): Deep Sequencing of Ecologically-relevant Dikarya" (CSP1974), the genome and transcriptome of *Tuber canaliculatum* Gilkey (a type of truffle native to eastern North America) and *Tuber gibbosum* Harkn. (Oregon white truffle, California, Washington and British Columbia) have been sequenced (Bonito et al., 2010; Bonito et al., 2013; Benucci et al., 2016).

Table 5 - *Tuber* genomes sequenced.

Tuber species	Size Mb	Predicted protein-coding gene number	Repeated sequences % or Transposable elements	Web site	Reference
T. melanosporum	125	7496	57.73	www.genoscope.cns.fr/tuber	Martin et al., 2010
T. magnatum	192	9,433	58	https://mycocosm.jgi.doe.gov/Tubma1/Tubma1.home.html	Murat et al., 2018b
T. borchii	97.18	12,346	47,12	http://genome.jgi.doe.gov/Tubbor1	Murat et al., 2018a
T. aestivum	145	9,344	49.5	https://mycocosm.jgi.doe.gov/Tubae1/Tubae1.home.html	Murat et al., 2018b
T. brumale	171.44	12,380	61,5	https://mycocosm.jgi.doe.gov/Tubbr1_1/Tubbr1_1.home.html	Morin et al., 2021
T. indicum	110.49	11,870	47,1	https://mycocosm.jgi.doe.gov/Tubbr1_1/Tubbr1_1.home.html	Morin et al., 2021

Conclusions and perspectives

Truffles belong to the genus *Tuber* and are among the most prized mushrooms in the world. Trade, agriculture, and research sectors have developed around this valuable product, and due to its economic value, it is important to guarantee its origin, safety, traceability, and authenticity. Many researchers are studying to understand the unknown aspects of truffle biology and to develop effective cultivation methods, especially for *T. magnatum*, which is among the most precious species. The application of molecular biology techniques is useful not only for these purposes but also for certifying seedlings, guaranteeing the marketed products, and monitoring the presence of the species of interest.

This review presents the molecular targets and methods for rapidly identifying and quantifying the most important *Tuber* species. It is a scheme of molecular tools that researchers and others can apply to unambiguously identify mycorrhizae, mycelial cultures, and carpophores. It is essential to guarantee the reliability of the study models and the mycorrhization systems and to evaluate the presence of the precious *Tuber* in food products. All this is fundamental for evolutionary and ecological studies, studies on the mechanisms underlying the biological cycle, to understand the interactions with other microorganisms and the reactions to biotic and abiotic factors.

We reported the validated and most cited protocols in scientific identification papers, but in perspective, the emerging technique of digital PCR has recently begun to be applied to environmental studies; it can provide a very sensitive identification and quantification method, and many researchers are already working on it, using the same targets and primers, but with much greater sensitivity. The principle of high-level sample partitioning ensures that dPCR results are precise and accurate, even when starting from very low target copy numbers (Whale et al., 2013). The proposed methods

summarized in this review may not be exhaustive for taxonomic studies, as some species, other than *Tuber*, have shown intraspecific variability of some targets used.

Moreover, new sequencing technologies will make it easier to discern the genomes of many other species of *Tuber* in addition to those already sequenced, providing new knowledge useful for identifying and characterizing the species. There will also be new fascinating strategies with the advent of artificial intelligence (AI) and machine learning through software applications capable of quickly and accurately recognizing species-specific patterns in DNA sequences (Picek et al., 2022). This provides significant potential for studying and understanding mycology and opening up new applications in agriculture, industry, and medicine (Picek et al., 2022).

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