PCR PRIMERS COMPARISONS FOR A SUCCESSFUL *TUBER* SPP. DNA REGION AMPLIFICATION IN ROUTINE IDENTIFICATIONS

PRIMERJAVA PCR ZAČETNIH OLIGONUKLEOTIDOV ZA USPEŠNO POMNOŽEVANJE DNA REGIJE *TUBER* SPP. PRI RUTINSKI IDENTIFIKACIJI

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ABSTRACT

PCR primers comparisons for a successful *Tuber* spp. DNA region amplification in routine identifications

Since late 20th century DNA sequencing became the method of choice method in precision species identification. The ITS region is one of the official fungal barcoding DNA markers, although in some cases sequencing of the ITS region may, due to misidentification, mislabeling or nomenclature errors in public databases, lead to incorrect or insufficient identification, as is currently a case in the genus *Tuber*. The aim of this study was to test, which ITS primer pairs are most appropriate and optimal for *Tuber* species DNA region amplification. Thereby we (1) compared amplification success for different *Tuber* species using fungal specific primer pair ITS1f and ITS4 and (2) compared amplification success using different ITS primer pair combinations in amplifying DNA region an example species *Tuber aestivum*. Based on results, *Tuber aestivum* was one of the most reluctant *Tuber* species in this study and in most cases failed to amplify with the above primer pair. After comparing different ITS primer pairs, we conclude that the primer pair ITS5 and ITS7 is the most appropriate primer pair for amplification DNA region of *T. aestivum* as it resulted in high amplification success from ectomycorrhizal root tips. Based on sequences, gained from public databases, we found that ITS1f and ITS6 primers have a mismatch in one base pair compared to the target sequence of *Tuber aestivum*, thus resulting in poor or no amplification success. Although primer pair ITS5 and ITS7 in our study was proven to be the most appropriate primer pair in amplifying DNA region *Tuber aestivum* species, further analysis about appropriateness of it for a general barcoding and identification of ectomycorrhiza in complex community samples is needed.

IZVLEČEK

Primerjava PCR začetnih oligonukleotidov za uspešno pomnoževanje DNA regije *Tuber* spp. pri rutinski identifikaciji

Od konca 20. stoletja je določanje nukleotidnega zaporedja DNA postalo ena izmed pogosteje uporabljenih metod za določanje vrst. ITS regija je edna izmed uradnih glivnih DNA markerjev, čeprav lahko določanje nukleotidnega zaporedja le-te, v nekaterih primerih, predvsem zaradi napačne določitve, označevanja oziroma napak v nomenklaturi v javnih bazah podatkov, privede do napačne oziroma nenatančne določitve vrst, kar je trenutno težava pri določitvi vrst iz rodu *Tuber*. Namen te študije je bil testirati kateri pari ITS začetnih oligonukleotidov so najbolj primerni in optimalni za pomnoževanje DNA regij gliv iz rodu *Tuber*. S tem namenom smo v študiji (1) primerjali uspešnost pomnoževanja DNA regije različnih vrst iz rodu *Tuber*, z uporabo glivno specifičnih začetnih oligonukleotidov ITS1f in ITS4 ter hkrati (2) primerjali uspešnost pomnoževanja DNA regije vrste *Tuber aestivum* z uporabo različnih ITS začetnih oligonukleotidov. Na podlagi rezultatov ugotavljamo, da je vrsta *T. aestivum* izmed vseh analiziranih gliv iz rodu *Tuber,* bila najtežavnejša vrsta v naši študiji, saj je v večini primerov pomnoževanje DNA regije te vrste z uporabo glivno specifičnih začetnih oligonukleotidov ITS1f in ITS4 bilo neuspešno. Po primerjavi uspešnosti pomnoževanja z različnimi ITS začetnimi oligonukelotidi ugotavljamo, da sta bila v naši študiji ITS začetna oligonukleotida ITS5 in ITS7 najprimernejša za pomnoževanje DNA regije vrste *T. aestivum*, saj je bila uspešnost pomnoževanja iz ektomikoriznih vršičkov v tem primeru največja. Na podlagi *T. aestivum* nukleotidnih zaporedij pridobljenih iz javnih podatkovnih baz ugotavljamo, da je za začetna oligonukleotida ITS1f in ITS6 značilno

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Keywords: Tuber spp., ITS region, PCR amplification, ITS primers

neujemanje s tarčnim nukleotidnim zaporedjem (*T. aestivum*) v enem baznem paru, kar se lahko odraža bodisi v slabšem pomnoževalnem uspehu ali v nepomnoževanju na splošno. Kljub temu, da v naši študiji ugotavljamo, da sta začetna oligonukleotida ITS5 in ITS7 najprimernejša za pomnoževanje DNA regije glive *T. aestivum,* so potrebne nadaljnje analize, s katerimi bi potrdili splošno primernost omenjenega para ITS5/ITS7 za pomnoževanje DNA regije ne samo vrst iz rodu *Tuber,* temveč za določanje ektomikoriznih glivnih združb na splošno.

Ključne besede: *Tuber* spp., ITS regija, PCR pomnoževanje, ITS začetni oligonukleotidi

1. INTRODUCTION

Functioning of forest ecosystems depends on the interactions between roots of vascular plants and mycorrhizal fungi, which's central role is capturing and retranslocation of soil nutrients and water, and consequently sustaining above-ground vegetation (Smith & Read, 2008). Association between mycorrhizal fungi and vascular plants is one of the key players in soil ecology (Dahlberg, 2001; Allen et al., 2002). Besides the importance of mycorrhizal fungi for functioning and nutrient transport in forest ecosystems, at least 400 mycorrhizal fungal species produce edible sporocarps (fungi) thus representing an important ecosystem service with a high economic interest (Boa, 2004; Bakker et al., 2019). Among edible fungi, truffles are the most appreciated and expensive (Amicucci et al., 1998; Bohannon, 2009). Truffles are ectomycorrhizal fungi, belonging to the genus *Tuber*. There are at least 180 known species of truffles (Bonito et al., 2010; Gryndler et al., 2011), with new being described frequently (Milenković et al., 2016; Grupe et al. 2018), for this reason there is a high interest for their timely and accurate identification.

DNA based methods have in recent decades became a critical research tool in fungal taxonomy, as DNA sequencing in many cases represents the most reliable tool for unequivocal species identification (Kang et al., 2010). Since early 1990 the internal transcribed spacer (ITS) region had been among most frequently sequenced genetic markers for identification of fungi (White et al., 1990) and for analyzing composition and dynamics of ectomycorrhizal communities (Gardes et al., 1991; Kraigher et al., 1995; Horton & Bruns, 2001; Begerow et al., 2010; Schoch et al., 2012). The ITS region is a molecular marker with high power for species-level identification. Due to its widespread use and ease to amplify it, it was selected as one of official fungal barcoding DNA markers (Raja et al., 2017). The average size of the ITS region in fungi is about 550 base-pairs, but may vary considerably among lineages (Feibelman et al., 1994; Schoch et al., 2012). The ITS region is composed of the two variable spacers, namely ITS1 spacer and ITS2 spacer, and of a highly conserved 5.8S ribosomal gene (White et al., 1990). The ITS region molecular marker shows high probability of correct identification at the species level for a broad group of fungi, except in some highly specific genera where its separating power is low (Schoch et al., 2012) or in cases where an intraspecific ITS region variation may lead to fail in a species identification (Linder et al., 2011; Chen et al., 2016; Li et al., 2013). For the genus *Tuber* the current identification based on BLAST analysis of the ITS region sequences (Altschul et al. 1990) may be challenging, due to species misidentification, mislabeling or nomenclature errors in the public databases, and due to insufficiently representation of some taxa in databases (Trappe 2004; Halasz et al., 2005; Iotti et al., 2007). The tendency the *Tuber* ITS region amplification went in direction of designing and using species-specific ITS primer-pairs that may not give satisfactory amplification results over all species in the genus. Thereby, the aim of this study was to estimate an efficient universal fungal ITS primer pair (ITS 1f, ITS4; *sensu* Gardes & Bruns, 1993) to amplify DNA from various *Tuber* species. In species where these primers did not yield sufficient amplification, other available ITS primer pair combinations were tested, both on sporocarps' and ectomycorrhizas' isolated DNA samples.

2. MATERIAL AND METHODS

2.1 Biological material

Sporocarps for testing (Table 1) were selected from the collection at the Slovenian Forestry Institute and several other collections, so as to represent most frequently collected morphological *Tuber* species (Grebenc et al. 2010). For testing the amplification from ectomycorrhizal samples, *Tuber aestivum* root tips from pot-planted and inoculated silver fir seedlings were used (Unuk Nahberger et al., 2020; in prep.). In total, 38 sporocarps were tested in the first step, and 113 ectomycorrhizal roots of *T. aestivum* on silver fir were tested in the second step. Ectomycorrhizal root tips were randomly chosen from silver fir root systems. The identity of *T. aestivum* ectomycorrhiza was confirmed following the methodology and identification key of Agerer (Agerer, 1987-2012).

2.2 DNA extraction

DNA from sporocarps and from ectomycorrhiza was extracted using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) protocol, following manufacturer's instructions.

2.3 PCR amplification

Amplifications were performed in GeneAmp PCR System 9700 (Applied Biosystems, USA) in a total volume 25 µl of the PCR amplification mixture, with volume of extracted DNA 1 ng/µl. The PCR reactions with the pairs of the barcoding primers ITS1f/ITS4 were performed as reported in Sulzbacher et al. (2016). The PCR reactions with the pairs of primers ITS5/ITS7, ITS5/ITS6 (Bertini et al., 1999) were performed using the protocol by Bertini et al. (1999). Based on the test of the barcoding primer (ITS1f/ITS4) with DNA extracts from sporocarps, the primer pairs ITS1f/ITS4, ITS1f/ITS2, ITS5/ITS6 and ITS5/ITS7 were used in amplifying sporocarps' DNA samples that fail to amplify in the barcoding test. The same selection of primers was also used in ectomycorrhizal DNA amplification from *Tuber aestivum* root tips. All PCR reactions were performed five times on all samples to demonstrate the optimality of individual primer pair for amplification.

2.4 Sequencing

PCR products were run on 1.5% agarose gels in 0.5x TBE buffer and visualized with Gel Doc EQ System, PC (Biorad, USA). Amplified DNA fragments were cut out of agarose gels and purified with innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany) following manufacturer's instructions. Purified DNA fragments were sequenced at a commercial sequencing laboratory (Macrogen Inc., Seoul, South Korea). Samples were sequenced in both directions either with the pairs of primers ITS1f/ITS4 (White et al., 1990; Gardes & Bruns, 1993) ITS1f/ITS2 or ITS5/ITS7 (Bertini et al., 1999). The obtained sequences were processed in Geneious version 11.1.4 (https://www.geneious.com, Kearse et al., 2012). Nucleotide base calls with an error probability greater than 5% were trimmed from read ends to improve read quality, while reads were assembled into contigs at 90% base pair similarity. BLASTN algorithm from NCBI website (National Center for Biotechnology Information; https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to assess the similarity of obtained ITS sequences to sequences in GenBank.

2.5 Primers annealing position and mismatch analysis

Sequences with amplified complete ITS region molecular marker, including partial 18S rRNA gene, complete ITS1, 5.8S rRNA and ITS2 genes, and partial 28S rRNA gene, were obtained from GenBank database at the National Centre for Biotechnology (NCBI). Sequences from the database and our newly obtained sequences were analyzed with Geneious version 11.1.4. For alignment, MAFFT alignment program as plugin available for Geneious was used. ITS primers mismatches and annealing position with *Tuber aestivum* sequences from GenBank database were analyzed in Geneious, where all forward and reversed primers were tested with maximum mismatches set at 5 base pairs.

3. RESULTS

All available *Tuber* collections except *T. aestivum* and one collection of *T. mesentericum* yielded sufficient amplification with a fungal barcoding primer pair ITS 1f and ITS4 (Table 1) for downstream applications (e.g. Sanger DNA sequencing) without additional steps. For collections that failed amplification with a fungal barcoding primer pair ITS 1f and ITS4 a further selection of primers for amplification of the partial or complete ITS region of fungi primer pairs ITS1f/ITS4, ITS1f/ITS2, ITS5/ITS6 and ITS5/ITS7 were used to amplify DNA from sporocarps and *T. aestivum* ectomycorrhiza.

As *T. aestivum* showed to be the most difficult one for amplifying with the ITS1f and ITS4 primer pair, further analyzes using different primer pairs were conducted to find the most appropriate and optimal primer pair for amplification of this species.

Based on all together 113 tested *T. aestivum* morphotypes, 27 *Tuber* morphotypes were successfully amplified and sequenced using primer pair ITS1f/ITS4, 61 morphotypes were amplified and sequenced using primer pair ITS5/ITS7 and 25 morphotypes using primer pair ITS1f/ITS2, while primer pair ITS5/ITS6 completely failed in amplification of *T. aestivum*

Table 1: Collections with name, herbarium code, GenBank accession number and reference for each *Tuber* sporocarp samples used for testing the ITS1f and ITS4 barcoding primer pair. + amplification yielded enough DNA for downstream applications, (+) amplification was successful but weak, - amplification failed. MA Fungi – The Herbarium at the Real Jardín Botánico, Madrid, Spain; MES – personal collection of Mattew E. Smith, USA; MS – personal collection of Marcelo Sulzbacher, Brazil; FHS – collection of the Institute for Multidisciplinary Research in Belgrade, Serbia; AP – personal collection of Andrej Piltaver, Slovenia.

Morphological species name	Herbarium code	GenBank accession number	Amplification with ITS1f & ITS4	Reference
Tuber aestivum Vittad.	MA Fungi 54693	FM205622	(+)	Grebenc et al. 2010
Tuber aestivum Vittad.	TUBAES/270211		-	this study
Tuber aestivum Vittad.	TUBAES/060811A		-	this study
Tuber aestivum Vittad.	TUBAES/180812A		(+)	this study
Tuber aestivum Vittad.	TUBAES/060714A		(+)	this study
Tuber aestivum Vittad.	TUBAES/251014B		-	this study
Tuber anniae W.Colgan & Trappe	TUBsp/241013A		+	this study
Tuber borchii Vittad.	TUBBOR/100108	FM205630	+	Marjanović et al. 2010
Tuber brumale Vittad.	TUBBRU/150309	FN433128	+	Grebenc et al. 2010
Tuber brumale var. moschatum (Bull.) Hall, Buchanan, Wang & Cole	TUBBRUfoMOS/250109A	FN433130	+	Grebenc et al. 2010
Tuber excavatum Vittad.	TUBEXC/070309G	FN433148	+	Grebenc et al. 2010
Tuber excavatum Vittad.	TUBEXC/110812A		+	this study
Tuber floridanum Grupe, Sulzbacher & M.E. Sm.	MES654 (Holotype)	MF611781	+	Grupe et al. 2018
Tuber floridanum Grupe, Sulzbacher & M.E. Sm.	MS475	MF611782	+	Grupe et al. 2018
Tuber foetidum Vittad.	FHS-Tmes	FM205704	+	Marjanović et al. 2010
Tuber fulgens Quél.	TUBFUL/221008	FN433154	+	Grebenc et al. 2010
Tuber fulgens Quél.	TUBFUL/041008B	FN433150	+	Grebenc et al. 2010
Tuber himalayense B.C. Zhang & Minter	AP-T71	FM205589	+	this study
Tuber indicum Cooke & Massee	AP-T50A	FM205590	+	this study
Tuber macrosporum Vittad.	FHS-455	FM205663	+	Marjanović et al. 2010
Tuber macrosporum Vittad.	FHS-449	FM205664	+	Marjanović et al. 2010
Tuber maculatum Vittad.	MA Fungi 57008	FM205560	+	Grebenc et al. 2010
Tuber maculatum Vittad.	FHS-399	FM205644	+	Marjanović et al. 2010
Tuber maculatum Vittad.	FHS-426	FM205645	+	Marjanović et al. 2010
Tuber magnatum Pico	TUBMAG/141207	FM205633	+	Marjanović et al. 2010

Tuber magnatum Pico	FHS-465	FM205651	+	Marjanović et al. 2010
Tuber mesentericum Vittad.	TUBMES/060811A		-	this study
Tuber mesentericum Vittad.	TUBMES/020912		(+)	this study
Tuber mesentericum Vittad.	TUBMES/110114B		+	this study
Tuber oligospermum (Tul. & C. Tul.) Trappe	FHS-XX13	FM205683	+	Marjanović et al. 2010
Tuber oligospermum (Tul. & C. Tul.) Trappe	MA Fungi 39553A	FM205505	+	Grebenc et al. 2010
Tuber petrophilum Milenković	BEO 20600	HG810883	+	Milenković et al. 2016
Tuber petrophilum Milenković	BEO 20601	HG810884	+	Milenković et al. 2016
Tuber rufum Pollini	TUBRUF/070911B		+	this study
Tuber rufum fo. *nitidum* (Vittad.) Montecchi & Lazzari)	FHS-XX1	FM205677	+	Marjanović et al. 2010
Tuber rufum fo. *apiculatum* E. Fisch	FHS-353	FM205669	+	Marjanović et al. 2010
Tuber rufum fo. f*errugineum* (Vittad.) Montecchi & Lazzari	TUBRUFvarFER/041008	FN433160	+	Grebenc et al. 2010
Tuber rufum fo.*lucidum* (Bonnet) Montecchi & Lazzari	FHS-471	FM205665	+	Marjanović et al. 2010
Tuber rufum Pollini	TUBRUFvarRUF/070908B	FN433168	+	Grebenc et al. 2010

root tips. Primers pairs showed different concentration of amplified DNA as shown in Figure 1 for selected representative samples. Primer pair ITS5/ITS7 showed the strongest intensities on the agarose gel for most of amplified DNA. Ectomycorrhizal DNA was in most cases difficult to amplify with primers pairs ITS1f/ITS4, ITS1f/ITS2 or with ITS5/ITS6 yielding low or no amplification at all.

3.1 Primers annealing position and mismatch analysis

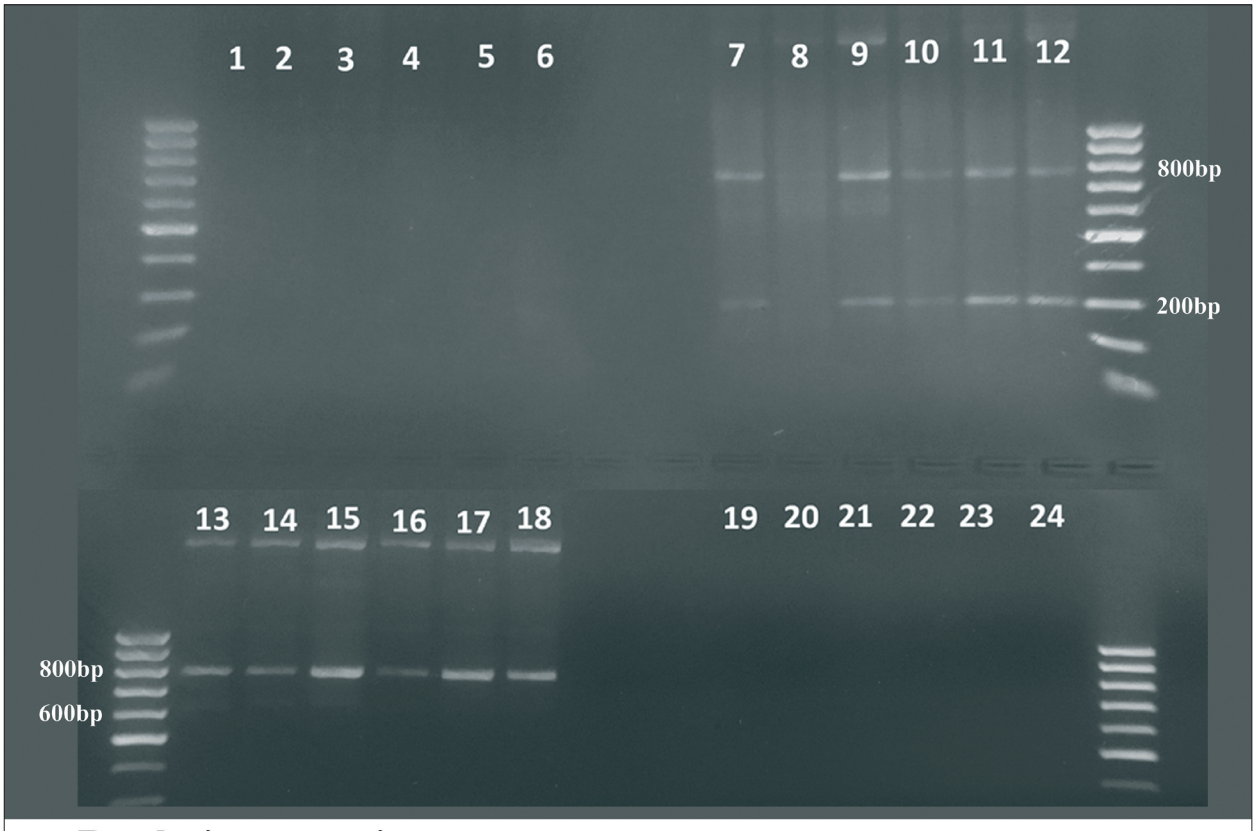
From the GenBank database, sequences with partial 18S rRNA gene, complete ITS1 spacer, 5.8S rRNA gene, ITS2 spacer and partial 28S rRNA gene sequence, were downloaded and analyzed for primers annealing position and nucleotide mismatches. Primers pairs ITS1f/ITS2, ITS1f/ITS4, ITS5/ITS6 and ITS5/ITS7 were included in analysis. The alignment of used sequences, showed that ITS1f primer is located 14 nucleotides upstream ITS5 primer, where several sequences of suitable lengths, showed on ITS1f primer mismatch on the 12th primer nucleotide, with oligo base A and target base G. For primer ITS5 no mismatches were observed. The primers ITS4 and ITS6 are located 35 nucleotides and 8 nucleotides upstream regarding primer ITS7. With the primer ITS2, only complete internal transcribed spacer 1 can be amplified, as it is located in 5.8S rRNA gene. The primer ITS6 had also a mismatch on the 5th primer nucleotide, with oligo base G and target base A, which was confirmed for 42 of 45 analyzed sequences. The amplification product obtained by using the primer pair ITS1f/ITS2 is the smallest, while the product obtained by using the primer pair ITS5/ITS7 is the biggest PCR fragment.

4. DISCUSSION

Due to its variability in length and nucleotide sequence among different fungi, the internal transcribed spacer (ITS) region has been frequently reported as a convenient target region for species delimitation in fungi and molecular identification of ectomycorrhizal fungi (Gardes & Bruns, 1993; Simon et al., 1992; Henrion et al. 1994; Lanfranco et al., 1993; Amicucci et al., 1998; Bertini et al., 1999, Benucci et al., 2011a). Although its broad usefulness for many taxa, repeated fails in amplification with universal barcoding ITS primers were reported for some. Besides technical reasons, such as interference of inhibitors in PCR, or a non-optimal PCR protocols, Bertini et al. (1999) suggested that most often, the problem lies in the primer sequences, as they in their study confirmed a significant interference of high base pair coupling degree with the annealing efficiency.

A fungal barcoding primer pair ITS1f and ITS4 in our study amplified most of the *Tuber* species selected among European, North American and Asian species, with an exception of a broadly distributed European

species *T. aestivum*. This primer pair repeatedly failed to amplify both *T. aestivum* DNA extracted from sporocarps and from ectomycorrhiza, suggesting a negative influence of the primer mismatch in the ITS1f primer sequence as a potential cause of its uselessness in sporocarp and ectomycorrhiza barcoding. Similar lack of amplification was also seen in other primer pair combinations. Primer pair ITS5/ITS6 was not an appropriate primer pair in our study, since the *T. aestivum* amplification using this specific primer pair was not successful. ITS6 primer also showed a mismatch between oligo and target base in comparison to



Results interpretation

Sample ID	ITS1f/ITS2 (lines 1-6)	ITS1f/ITS4 (lines 7-12)	ITS5/ITS7 (lines 13-18)	ITS5/ITS6 (lines 19-24)
E18/22	-	-	+	-
E18/24	-	-	+	-
E18/25	-	(+)	++	-
E18/26 *	-	-	(+)	-
E18/27	-	(+)	++	-
E18/28	-	(+)	++	-

* this DNA extract had 2-times lower DNA concentration comparing to other samples

Figure 1. Partial or complete ITS region amplification success in representative DNA extracts from Tuber aestivum ectomycorrhiza. Primer pairs for partial ITS amplification ITS1f/ITS2 (lines 1-6), the fungal ITS barcoding primer pair ITS1f/ITS4 (lines 7-12), and two alternative primer pairs ITS5/ITS7 (lines 13-18) and ITS5/ITS6 (lines 19-24) were used. Amplified ITS regions obtained with different primers pairs were evaluated and grouped based on intensities of bands (concentrations of DNA after amplification) in: ++ strong intensity; + moderate intensity, (+) weak intensity, and - unsuccessful amplification of the ITS region.

Tuber spp. annealing site sequence, as we have shown in the primers mismatch analysis. *T. aestivum* amplification using primer pair ITS1f/ITS2 also resulted in poor, or no amplification, despite shorter sequences which, such as in primer pair ITS1f/ITS2, should be amplified easier and with better success. Also, in primer pair ITS1f/ITS2 we assume primer mismatch in ITS1f to be the reason for poor amplification outcome. As previously reported by Bertini et al. (1999), less efficient amplification using primer pairs ITS1f/ITS4, ITS1f/ITS2 or ITS5/ITS6 can also be a result of primer to primer interaction, which may have a significant effect on annealing and finally on amplification efficiency of the PCR reaction run under the same reaction conditions.

On the other hand, the primer pair ITS5/ITS7 successfully amplified DNA of *T. aestivum* DNA both from clean sporocarps and from over 70% of all mixed DNA samples of ectomycorrhizal roots. The primer pair ITS5/ITS7 was already reported to be suitable for PCR amplification of *Tuber* species, as allowed amplification even at low DNA quality and concentration (in our study in case of sample E18/26). The primer ITS5 was suggested to be more appropriate than the ITS1 primer, as it forms less nucleotide interactions (Bertini et al., 1999).

In general, designing and optimization of the most efficient pairs of primers for *Tuber* species detection and characterization is of high interest for many biotechnological applications. Appropriate molecular techniques, as is in this case the use of appropriate primers pairs, are very important in the food industry (Strojnik et al. 2020, Šiškovič et al. 2020), in the *in vitro* propagation of mycelia to verify presence of truffles of high economic, or in testing and certifying natural truffle-grounds and truffle plantations (Amicucci et al., 1998, Benucci et al. 2011b). Moreover, the use of appropriate pairs of primers for *Tuber* species is important also in analyzing ectomycorrhizal biodiversity in forest ecosystems (Unuk et al. 2019; Unuk Nahberger et al. 2020; in prep), since only by amplification of all species present a realistic estimation of the ectomycorrhizal species diversity itself can be gained.

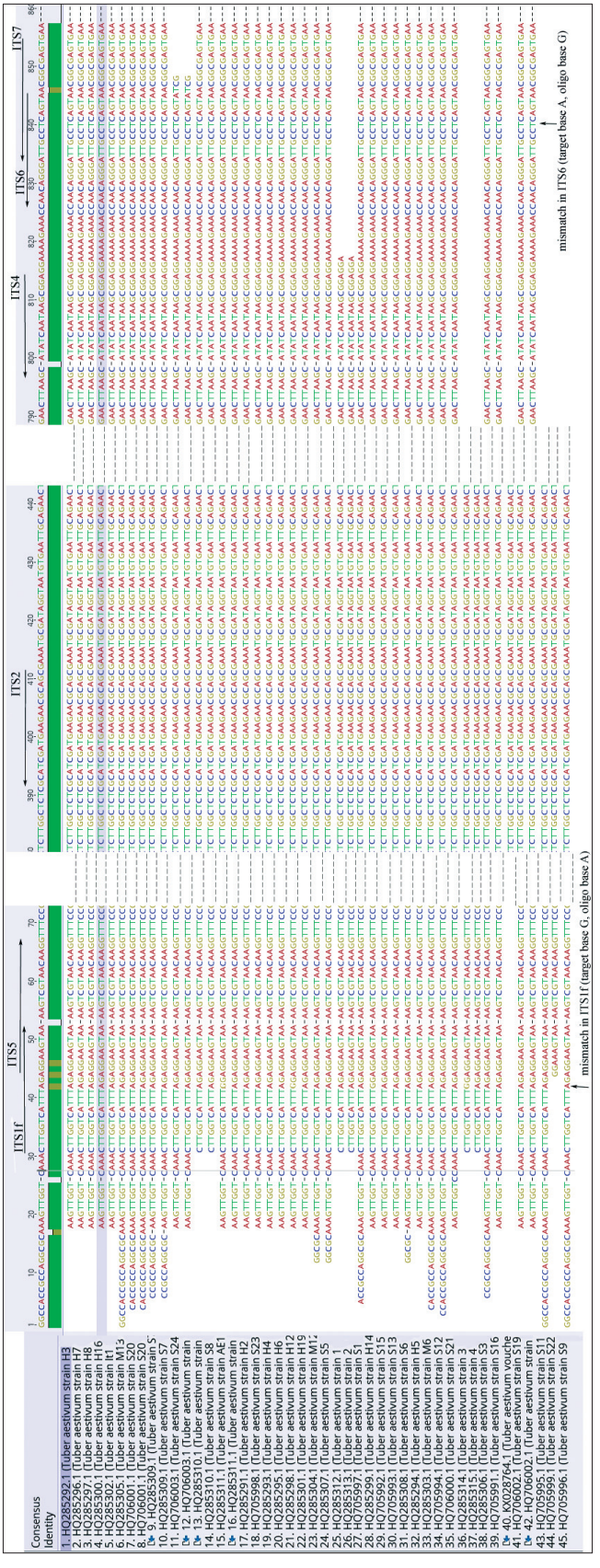


Figure 2: Schematic view of the complete ITS region in fungi, with marked mismatching positions for individual primers in Tuber aestivum sequence.

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REFERENCES

Allen, M.F., Lansing, J., Allen, E.B. 2002: *The Role of Mycorrhizal Fungi in the Composition and Dynamics of Plant Communities: A Scaling Issue*. In: Esser K., Lüttge U., Beyschlag W., Hellwig F. (eds) Progress in Botany. Progress in Botany (Genetics — Physiology — Systematics — Ecology), vol 63. Springer, Berlin, Heidelberg

Agerer, R., 1987-2012: Colour Atlas of Ectomycorrhizae. 1st-12th ed. Einhorn-Verlag, Schwäbisch Gmünd, Germany.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J., 1990: *Basic local alignment search tool*. Journal of molecular biology 215(3), 403-410. DOI: 10.1016/S0022-2836(05)80360-2

Amicucci, A., Zambonelli, A., Giomaro, G., Potenza, L., Stocchi, V., 1998: *Identification of ectomycorrhizal fungi of the genus Tuber by species-specific ITS primers*. Molecular Ecology 7: 273–277. https://doi.org/10.1046/j.1365-294X.1998.00357.x

Bakker, M. R., 1999: *Fine root parameters as indicators of sustainability of forest ecosystems*. Forest Ecology and Management 122: 7–16. DOI: 10.1016/s0378-1127(99)00028-6

Begerow, D., Nilsson, H., Unterseher, M., Maier, W., 2010: *Current state and perspectives of fungal DNA barcoding and rapid identification procedures*. Applied Microbiology and Biotechnology 87: 99–108. https://doi.org/10.1007/s00253-010-2585-4.

Benucci, G. M. N., Raggi, L., Albertini, E., Grebenc, T., Bencivenga, M., Falcinelli, M., Di massimo, G., 2011a: *Ectomycorrhizal communities in a productive Tuber aestivum Vittad. orchard: composition, host influence and species replacement*. FEMS microbiology ecology 76(1), 170-184. DOI: 10.1111/j.1574-6941.2010.01039.x

Benucci, G.M.N., Raggi, L., Di massimo, G., baciarelli-falini, L., Bencivenga, M., Falcinelli, M., Albertini, E., 2011b: *Species-specific primers for the identification of the ectomycorrhizal fungus Tuber acrosporum Vittad*. Molecular diagnostics and DNA taxonomy 11: 378-381. DOI: 10.1111/j.1755-0998.2010.02915.x

Bertini, L., Amicucci, A., Agostini, D., Polidori, E., Potenza, L., Guidi, C., Stocchi, V., 1999: A new pair of primers designed for amplification of the ITS region in Tuber species. FEMS Microbiology Letters 173(1): 239-245. https://doi.org/10.1111/j.1574-6968.1999.tb13508.x

Boa, E. R., 2004: *Wild edible fungi: a global overview of their use and importance to people* (No. 17). Food & Agriculture Organization of the United Nations, FAO, Rome.

Bohannon, J., 2009: *Rooting Around the Truffle Genome*. Science 323 (5917): 1006-1007. DOI: 10.1126/science.323.5917.1006

Bonito, G.M., Gryganski, A.P., Trappe, J.M., Vilglys R., 2010: *A global meta-analysis of Tuber ITS rDNA sequences: species diversity, host associations and long-distance dispersal*. Molecular Ecology 19: 4994-5008. Doi: 10.1111/j.1365-294X.2010.04855.x

Chen, W., Koide, R. T., Adams, T. S., Deforest, J. L., Cheng, L., 2016: *Root morphology and mycorrhizal symbioses together shape nutrient foraging strategies of temperate trees*. Proceedings of the National Academy of Sciences 113(31): 8741–8746. DOI: 10.1073/pnas.1601006113.

Dahlberg, A., 2001: *Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field*. New Phytologist 150: 555-562. https://doi.org/10.1046/j.1469-8137.2001.00142.x

Feibelman, T., Bayman, W., Cibula, W.G., 1994: *Length variation in the internal transcribed spacer of ribosomal DNA in Chanterelles*. Mycological Research 98(6): 614-618. https://doi.org/10.1016/S0953-7562(09)80407-3.

Gardes, M., Bruns, T. D., 1993: *ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts.* Molecular Ecology 2: 113-118.

Gardes, M., White, T.J., Fortin, J.A., Bruns, T.D., Taylor, J.W., 1991: *Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA*. Canadian Journal of Botany 69(1): 180-190. https://doi.org/10.1139/b91-026

Grebenc, T., Bajc, M., Kraigher, H., 2010: *Poledenodobne migracije mikoriznih rastlin in glivnih partnerjev v simbiozi : primer rodu Tuber*. Les: revija za lesno gospodarstvo 62(5): 149-154

Grupe, A. C., Sulzbacher, M. A., Grebenc, T., Healy, R., Bonito, G., Smith, M. E., 2018: *Tuber brennemanii and Tuber floridanum: two new Tuber species are among the most commonly detected ectomycorrhizal taxa within commercial pecan (Carya illinoinensis) orchards*. Mycologia 110(4): 780-790. DOI: 10.1080/00275514.2018.1490121.

Gryndler, M., Hršelová, H., Soukupová, L., Streiblová, E., Valda, S., Borovička, J., Gryndlerová, H., Gažo, J., Miko, M., 2011: *Detection of summer truffle (Tuber aestivum Vittad.) in ectomycorrhizae and in soil using specific primers*. FEMS Microbiology Letters 318: 84– 89. doi:10.1111/j.1574-6968.2011.02243.x

Halász, K., Bratek, Z., Szegő, D., Szabolcs, R., RacZ, I., Lasztity, D., Trappe, J.M., 2005: *Tests of species concepts of the small, white, European group of Tuber spp. based on morphology and rDNA ITS sequences with special reference to Tuber rapaeodorum*. Mycological Progress 4: 281–290. https://doi.org/10.1007/s11557-006-0132-6.

Henrion, B., Chevalier, G., Martin, F., 1994: *Typing truffle species by PCR amplification of the ribosomal DNA spacers*. Mycological Research 98: 37-43. DOI: 10.1016/S0953-7562(09)80333-X

Horton, T., Bruns, T., 2001: *The molecular revolution in ectomycorrhizal ecology: Peeking into the black-box*. Molecular Ecology 10(8): 1855-1871. DOI: 10.1046/j.0962-1083.2001.01333.x

Iotti, M., Amicucci, A., Bonito, G., Bonuso, E., Stocchi, V., Zambonelli, A., 2007: *Selection of a set of specific primers for the identification of Tuber rufum: a truffle species with high genetic variability. FEMS Microbiology Letters* 277(2): 223–231. https://doi.org/10.1111/j.1574-6968.2007.00963.x

Kang, S., Mansfield, M. A., Park, B., Geiser, D. M., Ivors, K. L., Coffey, M. D., Grünwald, N. J., martin, F. N., Lévesque, C. A., Blair, J. E., 2010: *The promise and pitfalls of sequence-based identification of plantpathogenic fungi and oomycetes.* Phytopathology 100:732-737. https://doi.org/10.1094/PHYTO-100-8-0732

Kearse, M., R. Moir, A. Wilson, S. Stones-Havas, M. Cheung, S. Sturrock, S. Buxton, et al. 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12): 1647–1649. https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/bts199.

Kraigher, H., Agerer, R., Javornik, B. 1995: *Ectomycorrhizae of Lactarius lignyotus on Norway spruce, characterized by anatomical and molecular tools*. Mycorrhiza 5: 175–180. https://doi.org/10.1007/BF00203334

Lanfranco, L., Wyss, P., MArzachí, C., Bonfante, P., 1993: *DNA probes for identification of the ectomycorrhizal fungus Tuber magnatum Pico.* FEMS Microbiology Letters 114(3): 245–251, https://doi.org/10.1111/j.1574-6968.1993.tb06581.x

Li, Y., Jiao, L., Yao, Y.-J., 2013: *Non-concerted ITS evolution in fungi, as revealed from the important medicinal fungus Ophiocordyceps sinensis.* Molecular Phylogenetics and Evolution 68(2): 373-379. https://doi.org/10.1016/j.ympev.2013.04.010.

Linder, C.R., Moore, L.A., Jackson, R.B., 2011: *A universal molecular method for identifying underground plant parts to species*. Molecular Ecology, 9(10): 1549-1559. https://doi.org/10.1046/j.1365-294x.2000.01034.x

Marjanović, Ž., Grebenc, T., Marković, M., Glišić, A., Milenković, M. 2010: *Molecular diversity and ecological specificity and molecular diversity of truffles (genus Tuber) originating from mid-west of the Balkan Peninsula*. Sydowia 62(1): 67-87.

Milenković, M., Grebenc, T., Marković, M., Ivančević, B., 2016: *Tuber petrophilum, a new truffle species from Serbia*. Mycotaxon 130(4): 1141-1152. DOI: 10.5248/130.1141.

Raja, H. A., Miller, A. N., Pearce, C. J., Oberlies, N. H., 2017: *Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community.* Journal of Natural Products 80(3): 756-770. DOI: 10.1021/acs.jnatprod.6b01085.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., LevesquE, C.A., Chen, W., 2012: *Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi*. Proceedings of the National Academy of Sciences 109(16): 6241-6246; DOI: 10.1073/pnas.1117018109.

Simon, L., Lalonde, M., bruns, T.D., 1992: *Specific amplification of 18S fungal ribosomal genes from vesicular-arbuscular endomycorrhizal fungi colonizing roots*. Applied and Environmental Microbiolgy 58: 291–295. DOI: 0099-2240/92/010291-05$02.00/0

Smith, S.E., Read, D. 2008: *Mycorrhizal symbiosis* (3rd edition). Academic Press, 800 pp.

Šiškovič, N., Strojnik, L., Grebenc, T., Vidrih, R., Ogrinc, N., 2020: *Differentiation between species and geographical origin of fresh and freeze-dried truffles according to their aromatic profile*. Molecules *(under review).*

Strojnik, L., Grebenc, T., Ogrinc, N., 2020: *Species and geographic variability in truffle aromas*. Food and Chemical Toxicology *(under review).*

Sulzbacher, M. A., Grebenc, T., Cabral, T. S., Giachini, A. J., Goto, B. T., Smith, M. E., Baseia, I. G., 2016: *Restingomyces , a new sequestrate genus from the Brazilian Atlantic rainforest that is phylogenetically related to early-diverging taxa in Trappeaceae (Phallales*). Mycologia 108(5): 954-966. https://www.tandfonline.com/doi/full/10.3852/15-265

Trappe, J.M., 2004: *The ways of herbaria: a cautionary note for users of herbarium collections.* Inoculum 55: 3-4.

Unuk Nahberger, T., Kraigher, H., Grebenc, T., 2020: *Influence of spore inoculums and earthworms on root biomass and morphology and quality assessment of with Tuber eastivum silver fir (Abies alba Mill.) inoculated seedlings.* (in prep.)

Unuk, T., MArtinović, T., Finžgar, D., Šibanc, N., Grebenc, T., Kraigher, H., 2019: *Root-associated fungal communities from two phenologically contrasting silver fir (Abies alba Mill.) groups of trees.* Frontiers in plant science, 10: 214. https://doi.org/10.3389/fpls.2019.00214

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990: *Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics*. PCR Protocols: A Guide to Methods and Applications 18: 315–322. http://dx.doi.org/10.1016/b978-0-12-372180-8.50042-1.