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 oligonukleotidmi 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...
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 (Dahlgren et al., 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); therefore 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 research is 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 ITS3/ITS7 were used to amplify DNA from sporocarps and T. aestivum ectomycorrhiza.

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 Matthew 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 borellii Vittad.     | TUBBOR/100108  | FM205630 (+)              |                               | Marjanovič 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-Times      | FM205704 (+)              |                               | Marjanovič 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 & Masse | AP-T50A | FM205590 (+) | this study |
| Tuber macrosporum Vittad.  | FHS-455        | FM205663 (+)              |                               | Marjanovič et al. 2010 |
| Tuber macrosporum Vittad.  | FHS-449        | FM205664 (+)              |                               | Marjanovič et al. 2010 |
| Tuber maculatum Vittad.    | MA Fungi 57008 | FM205560 (+)              |                               | Grebenc et al. 2010 |
| Tuber maculatum Vittad.    | FHS-399        | FM205644 (+)              |                               | Marjanovič et al. 2010 |
| Tuber maculatum Vittad.    | FHS-426        | FM205645 (+)              |                               | Marjanovič et al. 2010 |
| Tuber magnatum Pico        | TUBMAG/141207  | FM205633 (+)              |                               | Marjanovič 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/ITS4, ITS1f/ITS2 or with ITS5/ITS6 yielding low or no amplification at all.

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                          | GenBank Accession  | Sequence | Amplification |
|----------------------------------|-------------------|----------|---------------|
| Tuber magnatum Pico             | FHS-465           | FM205651 | +             |
| Tuber mesentericium Vittad.     | TUBMES/060811A    | -        | this study    |
| Tuber mesentericium Vittad.     | TUBMES/020912     | (+)      | this study    |
| Tuber mesentericium Vittad.     | TUBMES/110114B    | +        | this study    |
| Tuber oligospermum (Tul. & C. Tul.) Trappe | FHS-XX13           | FM205683 | +             |
| Tuber petrophilum Milenkovic     | BEO 20600         | HG810883 | +             |
| Tuber petrophilum Milenkovic     | BEO 20601         | HG810884 | +             |
| Tuber rafum Pollini             | TUBRUF/070911B    | -        | this study    |
| Tuber rafum fo. nitidum (Vittad.) Montecchi & Lazzari | FHS-XX1   | FM205677 | +             |
| Tuber rafum fo. apiculatum E. Fisch | FHS-353           | FM205669 | +             |
| Tuber rafum fo. terrugineum (Vittad.) Montecchi & Lazzari | TUBRUFvarFER/041008 | FN433160 | +             |
| Tuber rafum fo. lucidum (Bonnet) Montecchi & Lazzari | FHS-471            | FM205665 | +             |
| Tuber rafum Pollini             | TUBRUFvarRUF/070908B | FN433168 | +             |
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

![Image of gel electrophoresis](image.png)

**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.
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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., Lütting U., Betschlag 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 acroporum 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

Grebenšek, Y., 2013: Non-concerted ITS evolution in fungi, as revealed from the important medicinal fungus Tuber magnatum Pico. FEMS Microbiology Letters 114(3): 245–251, https://doi.org/10.1111/j.1574-6968.1993.tb06581.x

Grebenc, T., Baic, M., Kragher, H., 2010: Podeolenodobne migracije mikoriznih rastlin in glvih partnerjev v simbioti: 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.

Gyndler, M., Hršelová, H., Soukupová, L., Streiblová, E., Valda, S., Borovička, J., Gyndlerová, 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.1574-6968.2010.02243.x

Haláš, 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 rufum. Mycological Progress 4: 1647–1649. https://doi.org/10.1111/j.1574-6968.2007.00963.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.1574-6968.2007.09636.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. 10.1093/bioinformatics/bts199. 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/10.1111/j.1365-294x.2000.01034.x

Marjanović, Ž., Grebenšek, 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., Grebenšek, 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.jpnp.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 Microbiology 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.