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Design and Validation of qPCR-Specific Primers for Quantification of the Marketed *Terfezia claveryi* and *Terfezia crassiverrucosa* in Soil

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Abstract: Desert truffle crop is a pioneer in southeastern Spain, a region where native edible hypogeous fungi are adapted to the semiarid areas with low annual rainfall. *Terfezia claveryi* Chatin was the first species of desert truffle to be cultivated, and has been increasing in recent years as an alternative rainfed crop in the Iberian Peninsula. However, its behaviour in the field has yet not been investigated. For this purpose, specific primers were designed for the soil DNA quantification of both *T. claveryi* and *Terfezia crassiverrucosa* and a real-time qPCR protocol was developed, using the ITS rDNA region as a target. Moreover, a young desert truffle orchard was sampled for environmental validation. The results showed the highest efficiency for the TerclaF3/TerclaR1 primers pair, 89%, and the minimal fungal biomass that could be reliably detected was set at 4.23 µg mycelium/g soil. The spatial distribution of fungal biomass was heterogeneous, and there was not a direct relationship between the quantity of winter soil mycelium and the location/productivity of desert truffles. This protocol could be applied to tracking these species in soil and understand their mycelial dynamics in plantations and wild areas.

Keywords: ITS; *Terfezia claveryi*; *Terfezia crassiverrucosa*; qPCR; desert truffles; rDNA; ascomycetes; mycelium



Citation: Arenas, F.; Morte, A.; Navarro-Ródenas, A. Design and Validation of qPCR-Specific Primers for Quantification of the Marketed *Terfezia claveryi* and *Terfezia crassiverrucosa* in Soil. *J. Fungi* **2022**, *8*, 1095. <https://doi.org/10.3390/jof8101095>

Academic Editors: Qiang-Sheng Wu, Qing Yao, Abeer Hashem and Anoop Kumar Srivastava

Received: 19 September 2022

Accepted: 12 October 2022

Published: 17 October 2022

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1. Introduction

Terfezia claveryi Chatin is an edible mycorrhizal hypogeous fungi belonging to the *Pezizaceae* family that establish mycorrhizal symbiosis with some plants of the *Helianthemum* genus [1]. Its natural habitats are arid and semiarid environments with a low annual rainfall inputs, mild winters, and warm summers, mainly encompassing countries of the Mediterranean geographical region [2,3]. *T. claveryi* was the first desert truffle to be cultivated [4], and it is known to be one of the most appreciated desert truffle species on the market [5], together with other known desert truffles (mainly *Terfezia boudieri* Chatin, *Tirmania nivea* (Desf.) Trappe and *Tirmania pinoyi* (Maire) Malençon [6]). In addition, desert truffles are not only an important economic resource, but contain high nutritional and antioxidant properties [7,8], including bioactive compounds with potential health benefits such as antimicrobial, anti-inflammatory, hepatoprotective, and antitumor activities [9–12].

Recently, the area cultivated with the desert truffle *T. claveryi* has been increased in semiarid areas of Spain [5,13], becoming an alternative agricultural crop thanks to low water requirements for cultivation [14]. Until now, some abiotic factors or agroclimatic parameters associated with plant management and the control of fungal fruiting have been studied [14–16]. Although this knowledge on mycorrhizal plant phenology could helped to stabilise annual fluctuations in yield ascocarps production [17], there are still high fluctuations within the same plantation, resulting in productive and non-productive

areas or “patches” [18]. The analysis of ecology, phenology, and interannual fluctuations on mycelial development are also essential for the proper management of mycorrhizal plants producing truffles or mushrooms [19–21].

Molecular strategies such as restriction fragment length polymorphism (RLFP), denaturing gradient gel electrophoresis (DGGE) or amplified rDNA restriction analysis (ARDRA) [22] have been used for years to track or monitor edible inoculated fungal species in the field or to identify them in other applications and bioprocesses [23–30]. Among PCR-based methods, the use of species-specific primers in a quantitative real-time PCR (qPCR) approach has been widely applied to trace root and soil mycelia of different mycorrhizal fungi-producing truffles or mushrooms with high socioeconomic impact, such as *Tuber melanosporum* [31–33], *Tuber magnatum* [34–36], *Tuber aestivum* [37,38], *Lactarius deliciosus* [39–42], *Tricholoma matsutake* [43], and *Boletus edulis* [42,44,45]. In addition, the use of this technique has been used to quantify the presence of mycelium in black truffle plantations, both in the first years to control the establishment of plantations, and to subsequently evaluate its relationship with the productivity of the crop [20].

Soil properties such as pH (acid or alkaline) and the host plant species lead to the fruiting of different species of desert truffle [1,5]. In recent years, several studies on the genus *Terfezia* have been published to clarify and update the phylogenetic relationships among the new species and those already described within the genus [46–54]. These studies showed intraspecific genetic variations in the nrDNA-ITS sequence of *Terfezia* spp., including the identification of some cryptic species [55–57], in which only molecular data are required and used for species identification [20,56].

Traditionally *T. claveryi* and *Terfezia crassiverrucosa* Zitouni-Haouar, G. Moreno, Manjón, Fortas & Carlavilla have been collected and marketed together in alkaline soils, because no key differences in distribution, host plant, macroscopy, taste, and flavour characteristics can be found [49]. In fact, they are species very similar morphologically and phylogenetically [49]. Consequently, both species share their habitat in plantations and wild areas and have been called “*turmas*” indistinctly by gatherers. For this reason, and from now on, when the term *turmas* is used in this study, we refer to both marketed *Terfezia* species in Spanish alkaline soils (*T. claveryi* and *T. crassiverrucosa*). The internal transcribed spacer (ITS) region from ribosomal DNA (ITS1-5.8S-ITS2) has extensively been used as a universal DNA barcode marker for *Fungi* [58]. This region was selected to design specific primers for the detection and quantification of *T. claveryi* and *T. crassiverrucosa* DNA in soil by real-time quantitative PCR (qPCR). Thus, the objectives of this study are as follows: (a) design and check a set of specific primers for the quantification of DNA of these *turmas* in soil by qPCR approach; and (b) apply this strategy to determine how mycelium is distributed and spread in a desert truffle plantation.

2. Materials and Methods

2.1. Environmental Sampling

A desert truffle plantation with mycorrhized *H. almeriense* × *T. claveryi* plants (located in Torre-Pacheco, Murcia, Spain, 37°46′29.7″ N, 0°57′1.2″ W) was used to investigate the mycelium spreading of the inoculated species in the soil. The plantation was established in 2016, and it was extended again twice more with new mycorrhized plants in 2018 and 2019. This plantation started to be productive one year after the first round of planting in March 2017. The plants were established in rows separated 0.75 m and 1 m between rows. A suitable management of the cultivation and watering according to the recommendations described in [5,13,14,17,59] was followed.

In total, 36 soil samples were collected in February 2020 at an equal distance from the surrounding plants and at a depth of 10–15 cm. They were maintained at 10 °C until they were transported to the laboratory and kept at –20 °C until processing. Before DNA extraction, soil samples were dried at room temperature for 24–48 h. As detailed in Figure 1, 18 samples were from the three rows planted in 2016, 12 from the two rows planted in 2018, and 6 from the one row planted in 2019. There was a separation of 2–2.5 m between the

samples within each row, and a distance of 1–1.5 m from one row to the next. Moreover, a soil sample was taken as a negative control from a non-productive area outside the plantation, free of *H. almeriense* mycorrhizal plants. During the fruiting season in spring 2020, 3, 19, and 6 ascocarps of similar weights were collected from 2016, 2018, and 2019 planting areas, respectively.

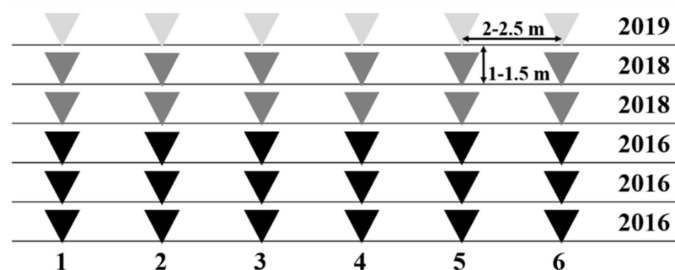


Figure 1. Diagram of sampling points (pyramid marks) in different years of plantation establishment.

2.2. Soil DNA Extraction

Soil samples were carefully sieved through 500 μm mesh to remove any root fragments, stones, or plant material debris. Then, genomic DNA was extracted in duplicate from 0.25 g of each sample, previously well homogenized, using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. All DNA was eluted in 100 μL of elution buffer (10 mM Tris) and stored at $-20\text{ }^{\circ}\text{C}$ until processing. The concentrations of DNA extractions were measured using a NanoDrop ND-2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the quality was examined by 260/280 nm and 260/230 nm optical density ratios.

In the same way, DNA extracted from a mixture of 113.1 mg *T. claveryi* active mycelium (T7 strain), from a pure culture in MMN-O liquid medium [60], and 0.1543 g of negative control soil (twice autoclaved), was used for the generation of the standard curve.

2.3. Design of Specific Primers for Turmas

ITS-rDNA (ITS1-5.8S-ITS2) sequences of *T. claveryi*, *T. crassiverrucosa* and other desert truffle species from GenBank and RefSeq databases (Table S1) were used for primers design by two different web-based software programs: ABI PRISM Primer Express v3.0.1 (Applied Biosystems, Waltham, MA, USA) and ProbeFinder v2.50 (Universal ProbeLibrary, UPL, Assay Design Center) (Roche Molecular Systems, Pleasanton, CA, USA). Multiple sequence alignments were carried out using the MUSCLE algorithm [61] to delimit specific regions for optimal primer selection using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms v10.0.5 [62].

Customized primer criteria were established according to the following SYBR Green qPCR assay requirements and recommendations [63–65]: melting temperature $55\text{--}62\text{ }^{\circ}\text{C}$ (opt. $60\text{ }^{\circ}\text{C}$), GC content 40–55% (opt. 50%), primer size 15–30 nt (opt. 20 nt), amplicon size 75–150 nt (opt. 100 nt), and GC clamp 1 nt. Primer sets generated were examined for cross- and self-dimers and hairpin formations (Beacon Designer software, PREMIER Biosoft's, Palo Alto, MA, USA). Those with ΔG values of -3.5 kcal/mol and below were avoided. Moreover, amplicon checking for secondary structures was carried out using the UNAFold web tool (IDT, Integrated DNA technologies, Coralville, IA, USA), adjusting the Mg concentration to 3 mM. All structures formed had to meet a T_m (melting temperature) less than the qPCR annealing temperature and values of ΔG above -9 kcal/mol . Furthermore, the oligonucleotides and the obtained amplicons were evaluated in silico for specificity using Megablast search at NCBI GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>; accessed on 1 April 2020) [66].

Direct PCR amplifications from dried ascoma of fungal reference materials (Table 1) were performed in a FlexCycler (Analytik Jena GmbH, Jena, Germany) according to the protocol described by Bonito [67]. Each 25 μL reaction volumes was amplified with ITS1F-

ITS4 primer pair [68,69] and it was composed of 0.4 mM for each primer, 0.2 mM for each dNTP, 2.0 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.04% BSA and 1.25 U of Taq DNA polymerase (Invitrogen). The parameters of the thermal cycler were: initial denaturation for 2 min at 94 °C, 40 cycles consisting of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, and a final extension for 5 min at 72 °C. PCR products were purified using the EZNA Cycle-Pure-Kit (Omega Bio-Tek), according to the manufacturer's instructions, and sequenced at the Molecular Biology Service of the University of Murcia. In order to check in vitro specificity, DNA extracts of different species of desert truffles were used as templates (Table 1) under qPCR conditions.

Table 1. Fungal reference materials used in this study.

| Taxon | Specimen ID ¹ | GenBank Accession Number |
|---|--------------------------|--------------------------|
| <i>Terfezia albida</i> Ant. Rodr., Muñoz-Mohedano & Bordallo | j574 | OP458226 |
| <i>Terfezia eliocrocae</i> Bordallo, Morte & Honrubia | j579 | OP458228 |
| <i>Terfezia olbiensis</i> (Tul. & C. Tul.) Sacc. | j588 | OP458229 |
| <i>Terfezia claveryi</i> Chatin | j592 | OP458224 |
| <i>Terfezia claveryi</i> Chatin | j596 | OP458223 |
| <i>Terfezia claveryi</i> Chatin | j597 | OP458222 |
| <i>Terfezia claveryi</i> Chatin | j216 | OP458220 |
| <i>Terfezia claveryi</i> Chatin | j73 | OP458219 |
| <i>Terfezia crassiverrucosa</i> Zitouni-Haouar, G. Moreno, Manjón, Fortas, & Carlavilla | j53 | OP458218 |
| <i>Terfezia crassiverrucosa</i> Zitouni-Haouar, G. Moreno, Manjón, Fortas, & Carlavilla | j235 | OP458221 |
| <i>Tirmania pinoyi</i> (Maire) Malençon | j601 | MG920185.1 |
| <i>Tirmania nivea</i> (Desf.) Trappe | j590 | OP458225 |
| <i>Terfezia grisea</i> Bordallo, V. Kaounas & Ant. Rodr. | j485 | KP189333 |
| <i>Terfezia fanfani</i> Mattir. | j484 | OP458230 |
| <i>Terfezia pseudoleptoderma</i> Bordallo, Ant. Rodr. & Muñoz-Mohedano | j478 | OP458231 |
| <i>Terfezia arenaria</i> (Moris) Trappe | j466 | OP458227 |
| <i>Terfezia boudieri</i> Chatin | j371 | OP458234 |
| <i>Tirmania honrubiae</i> Morte, Bordallo & Ant. Rodr. | j366 | OP458233 |
| <i>Terfezia fanfani</i> Mattir. | L14 | HM056219 |
| <i>Terfezia extremadurensis</i> Muñoz-Mohedano, Ant. Rodr. & Bordallo | j96 | OP458232 |
| <i>Terfezia pini</i> Bordallo, Ant. Rodr. & Muñoz-Mohedano | j151 | OP458235 |
| <i>Picoa</i> sp. Vittad. | j442 | OP458217 |
| <i>Picoa</i> sp. Vittad. | j17 | OP458215 |
| <i>Picoa</i> sp. Vittad. | j59 | OP458214 |
| <i>Picoa</i> sp. Vittad. | j41 | OP458213 |
| <i>Picoa</i> sp. Vittad. | j45 | OP458212 |
| <i>Picoa</i> sp. Vittad. | j20 | OP458216 |
| <i>Geopora</i> sp. Harkn. | R21b | OP458210 |
| <i>Geopora</i> sp. Harkn. | R23 | OP458209 |
| <i>Geopora</i> sp. Harkn. | j121 | OP458211 |

¹ Herbarium of University of Murcia (MUB-FUNGI).

2.4. Quantitative Real-Time PCR Conditions

A standard curve was generated from 1/10 dilutions of purified DNA standard (amounts of *T. claveryi* mycelium in soil) with nuclease-free water. Then, the efficiency of the real-time PCR was calculated for each primer pair selected from the value of the slope of the calibration curve [70] (generated as: $E = (10^{(-1/\text{slope})} - 1) \times 100$), and the primer concentration was optimised in the range of 50 to 200 nM for the chosen combination of primers. In addition, the minimum amount of mycelium detected by this qPCR protocol was established.

Real-time SYBR-Green-dye-based PCR amplification was carried out for in vitro tests and experimental samples in 96-well plates using a QuantStudio™ 5 Flex (Applied Biosystems, Waltham, MA, USA) instrument. Each amplification was performed on 10 µL reaction volumes containing 5 µL of Power SYBR Green PCR Master Mix (2×) (Thermo

Fisher Scientific), 0.1 µL of each primer at 10 µM, 3.8 µL of nuclease-free water, and 1 µL of 1/5 diluted DNA template. The thermal cycle protocol was 50 °C for 2 min and 95 °C for 10 min at hold stage followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s at PCR stage. After that, melting curve analysis was used to delete from the analysis those samples with non-target sequences and secondary structures. Three replicates for each standard DNA dilution, for each sample and for a no template control (NTC), were included for each run. Then, C_T (cycle threshold) values were automatically converted to quantities of *turmas* mycelium in soil (mg mycelium/g soil) by QuantStudio Design & Analysis software v1.4.

2.5. Statistical Analysis

Statistical analyses were performed using the *stats* package in the R software environment (<https://www.R-project.org/>; accessed on 20 May 2022) [71]. Soil mycelium data were evaluated by Grubbs' test to determine whether one of the values was a significant outlier from the rest (<https://www.graphpad.com/quickcalcs/Grubbs1.cfm>; accessed on 20 May 2022). Differences among groups of samples were compared using Kruskal–Wallis tests with the *kruskal.test* function. When the test was significant, post hoc analysis was performed using the *dunnTest* function in the *FSA* package [72]. Correlations between soil-detected mycelium and harvested truffles were analysed by Poisson regression using the *glm* function.

3. Results and Discussion

3.1. In Silico Primer Screening

The ITS rDNA region is the most commonly used fragment for fungal species identification and as a target for soil fungal diversity studies; however, it shows different intraspecific variability in all groups of fungi and high length polymorphism [58,73,74]. In addition, even though many mycologists advocate LSU region as alternative, the ITS region shows greater efficiency in species discrimination [58]. The consensus sequence was generated from the independent *turmas* sequences (Table 1) aligned by MEGA X software. This sequence was used as a DNA template, resulting in three sets of designed primers (Table 2) based on in silico analyses. The specificity of the primers and the amplicons produced was also confirmed against the sequences of GenBank and RefSeq databases (Table S1).

Table 2. Set of primers designed and tested.

| Primer Set | Sequence (5' → 3') | Length (nt) | T _m (°C) | GC (%) | Amplicon (nt) |
|------------|-------------------------------|-------------|---------------------|--------|---------------|
| TerclaF1 | ATAGGGCATGCCTGTCTGAG | 20 | 60.0 | 55 | 106 |
| TerclaR1 | TGGAGGGCAACTTAATACACAGT | 23 | 59.2 | 43 | |
| TerclaF2 | TAACTGTGTATTAAGTTGCCCTCCAG | 26 | 59.0 | 42 | 120 |
| TerclaR2 | GAGTTGAGGCAAGTACAATCAATCATAAC | 28 | 59.2 | 39 | |
| TerclaF3 | GCTCCCCCTCACTCAAGTAT | 20 | 59.1 | 55 | 79 |
| TerclaR1 | TGGAGGGCAACTTAATACACAGT | 23 | 59.2 | 43 | |

T_m = melting temperature; GC = guanine–cytosine.

ITS regions from multiple alignments of *turmas* and desert truffle sequences showed short and limited sections located within the ITS2 region for the optimal design of specific primers. This made it difficult to obtain primers automatically, and only the primer set TerclaF1/R1 was generated by ProbeFinder software. Moreover, some of the considerations for proper primer composition made the design even more complicated, because when SYBR Green dye is used as fluorescence marker, the presence of primer dimers, the formation of secondary structures, or non-specific amplifications may induce the detection of false signals [64,75]. All this forced the manual design of the primer set TerclaF2/R2 and primer set TerclaF3/R1, using the parameters already set as closely as possible.

3.2. Selection and Validation of qPCR-Specific Primers

In vitro specificity was also confirmed for the three set of primers designed, and non-amplifications were found for other fungal species (Table 1). However, the set TerclaF3/R1 provided lower Ct values, with the same amount of *turmas* DNA template as the sets TerclaF1/R1 and TerclaF2/R2. Careful focus was taken with non-specific amplifications of other desert truffle species (*T. albida*, *T. grisea*, *T. eliocrocae*, *Picoa* sp. and *Geopora* sp.), because they can share the habitat and the host plant with *turmas* [1,2,47,56,76]. Moreover, other *Terfezia* species from acid soils under non-*Helianthemum* sp. host plants were tested for cross-validation.

DNA serially diluted of the standard sample (10-fold dilutions) were performed and a calibration curve was constructed from 10^{-1} to 10^{-5} dilutions for three sets of primers designed. The results showed the highest efficiency for primer set TerclaF3/R1, 89% (Figure 2), followed by primer set TerclaF2/R2 and TerclaF1/R1 (64% and 58%, respectively). Moreover, coefficients of determination (R^2) were always greater than 0.99 in all curves.

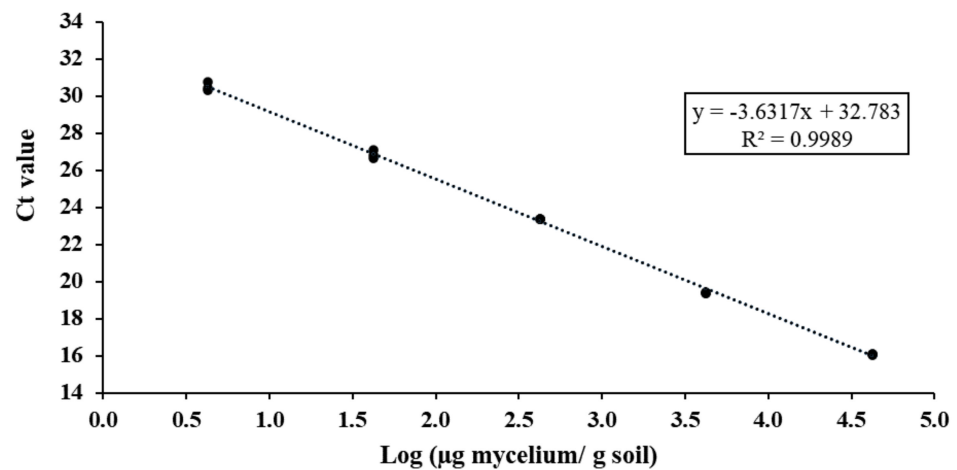


Figure 2. Real-time qPCR standard curve for *T. clavaryi* DNA quantification in soil. The curve was generated by plotting the Ct values obtained from 10-fold serial dilutions of DNA standard sample against the logarithm of the quantity of mycelium in soil ($\mu\text{g}/\text{g}$). Efficiency for the primer set TerclaF3/R1 was 89%.

Finally, the primers combination chosen were TerclaF3/R1 for optimal real-time qPCR assay using SYBR green fluorescence dye, and they were used for subsequent analyses. In addition, the primer concentration was adjusted to 100 nM, and PCR inhibitors were observed when using pure soil DNA extraction as DNA template. Thus, 1/5 dilutions of each soil DNA extraction were sufficient to avoid inhibition in qPCR reactions. This was an important check point in order to prevent a drop in the efficiency of the samples analysed [70].

The minimal fungal biomass that could be reliably detected was set at $4.23 \mu\text{g}$ mycelium/g soil, because below this value, reproducibility was lost (Figure 2). Sensitivity levels were different to a greater or lesser degree for other ectomycorrhizal fungi, due to the different strategies used for standard DNA and calibration curve. The detection limit for extraradical mycelium of the edible fungi *L. deliciosus*, *Rhizopogon roseolus* and *Rhizopogon luteolus* was 10-fold lower ($0.48 \mu\text{g}$ mycelium/g soil) from the DNA extraction of fresh mycelium in soil [40]. However, in a previous study, *L. deliciosus* was detected at up to $2 \mu\text{g}$ mycelium/g soil [39], and *B. edulis* was detected at around $39 \mu\text{g}$ mycelium/g soil [44]. Later, minimal quantities of *L. deliciosus* and *B. edulis* fungal biomass were detected: 1 and $4 \mu\text{g}$ mycelium/g soil, respectively [42]. In cases where pure in vitro culture of mycelium is difficult to achieve, such as in *Tuber* species [77], immature ascocarps have been used for standard DNA extraction [32–34,38]. Gryndler et al. [37] linked ITS rDNA copies in the PCR product with the biomass of *T. aestivum* mycelium for absolute quantification; however, this method has been

questioned for comparison studies because there is a large variability in the number of copies of this gene between fungal species [78].

Real-time qPCR protocols could also be affected by the DNA extraction process, in which the quality of the experiment varies depending on the amount of DNA obtained and contaminants co-extracted [79,80]. However, researchers have commonly added control soil to the extraction DNA procedure in order to generate site-specific calibration curves [32,35]. Furthermore, although TaqMan-based qPCR assays that include hydrolysis probes avoid the detection of non-specific products, SYBR-Green-dye-based techniques have shown the same high-performance results when appropriate qPCR protocols are followed [63,81].

3.3. Spatial Dynamic of *Turmas* Mycelium in a Desert Truffle Orchard

A four-year-old desert truffle orchard was sampled for environmental validation of the primer pair selected, TerclaF3/R1. Mycorrhized plants, inoculated with *T. claveryi* spores, were planted in three different years (2016, 2018, and 2019) (Figure 1); therefore, mycelia from three different ages could be cohabiting. Soil samples were collected in winter, before the fruiting season (spring) of desert truffles in the Mediterranean area [1]. Moreover, winter is the plant's physiology stage of maximum activity over the year [15]. *H. almeriense* shows a high photosynthetic rate and gas exchange together with a vigorous vegetative growth and flower bud production [15].

Mycelial distribution in the plantation is shown in Figure 3, in which a high variability in fungal biomass in soil between the samples can be appreciated. The range of fungal biomass detected and quantified was from 0.079 to 4.798 mg mycelium/g soil, and only 2 of the 36 samples were undetected. The specificity was also confirmed through checking melting curves after PCR cycles.

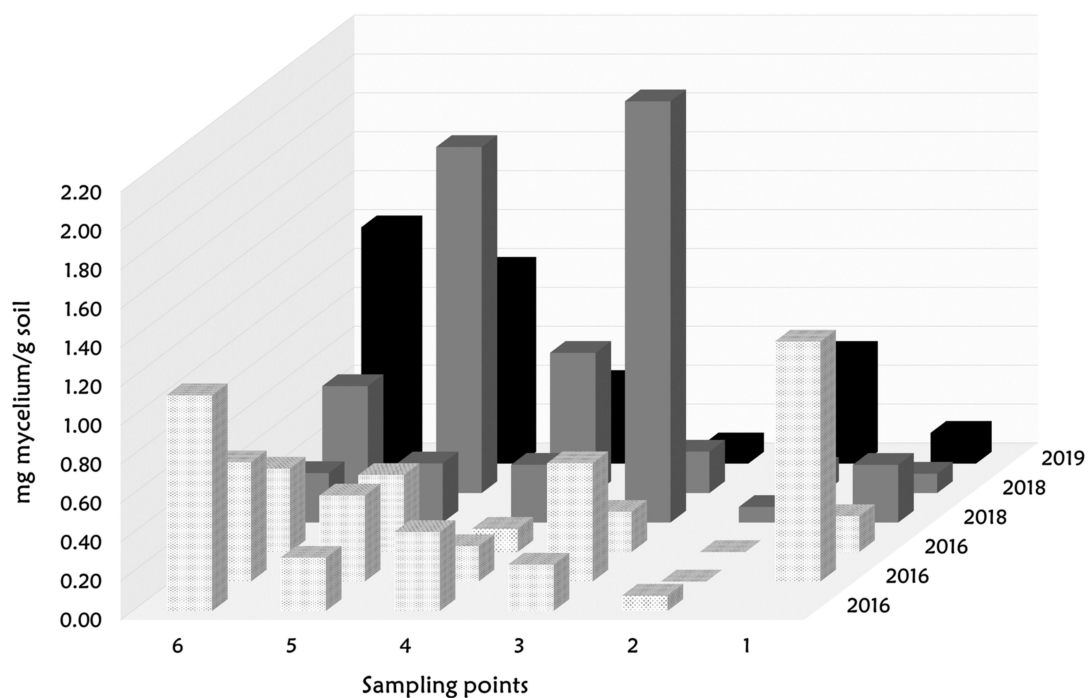


Figure 3. Distribution of *turmas* mycelium in the different planting years (2019 in black, 2018 in grey, and 2016 in white raster) across sampling points (1–6).

No differences in soil fungal biomass were found between years of the planting area (Kruskal–Wallis chi-squared = 0.7417, $df = 2$, p -value = 0.6901) (Table 3). However, significant differences were found between the sampling points (Kruskal–Wallis chi-squared = 12.188, $df = 5$, p -value = 0.0323) (Table 3).

Table 3. Mycelia detected in soil for each variable in desert truffle orchard.

| Variable | Samples (N) | Mean Fungal Biomass (Mg Mycelium/g Soil) | SD | Significance Level (p -Value < 0.05) |
|-----------|-------------|--|-------|---|
| Year 2016 | 18 | 0.386 | 0.350 | a |
| Year 2018 | 12 | 0.574 | 0.684 | a |
| Year 2019 | 6 | 0.577 | 0.451 | a |
| SP-1 | 5 | 0.394 | 0.474 | ab |
| SP-2 | 6 | 0.142 | 0.215 | a |
| SP-3 | 6 | 0.588 | 0.792 | ab |
| SP-4 | 6 | 0.358 | 0.214 | ab |
| SP-5 | 6 | 0.700 | 0.592 | b |
| SP-6 | 6 | 0.695 | 0.383 | b |

SP: sampling point; SD: standard deviation.

In contrast to the idea of finding a pattern over the years, we detected a heterogeneous mycelial spreading in that plantation, which does not seem to respond to the year of planting. In winter, *turmas* mycelium may concentrate in those areas where the plant requires nutritional support, either due to sub-optimal soil conditions or due to increased plant needs. Moreover, agroclimatic parameters may also have an effect on mycelial development.

In accordance with the desert truffle life cycle [82,83], the first rainfalls of late summer and early autumn promote primordia formation, which is associated with the high production of ascocarps in the next spring [14,15]. As well as in the genus *Tuber* [84,85], *T. clavaryi* exhibited a heterothallic lifestyle, which requires the combination of mycelia with different mating type genes in order to form fruiting bodies [82]. Thus, another reason for the heterogeneity of soil mycelium detected in our case study could be related to the mating type mycelia frequency across plantation, but there are still no studies in desert truffles and, thus far, no clear evidence has been found in the genus *Tuber* either. The detection of both mating types was correlated with productive trees in black truffle plantations [86,87], but in other studies, mating type frequency was extended randomly across plantation, and it was not significantly related to black truffle ascocarps harvested [88,89].

The amount of fungal biomass in winter showed no significant relationship with the amount of ascocarps harvested by year (3, 19, and 11 ascocarps collected in 2016, 2019, and 2018, respectively; Poisson regression p -value = 0.573). It seems that the middle age section accumulated higher desert fruiting bodies. Although the plantation was very successful in coming into production in the first year after planting, it did not reach its maximum productivity, which would occur around the eighth year [14]. In natural and cultivated experiments of black truffle, significant differences were found between mycelial abundance and productive areas [31–33,87]. *T. magnatum* mycelium was significantly higher in the surrounding fruiting areas [35]. Other ectomycorrhizal fungi, such as *L. deliciosus* and *B. edulis*, showed a non-correlation between the productivity of the different plots with the soil fungal biomass [42], but the soil mycelium was strongly related to climatic parameters. The results derived from this assay should be analysed with prudence, because long-term annual studies across season are necessary to explain the mycelial behaviour of desert truffles in soil, as discussed above for other mycorrhizal fungi.

Although the plantation of our study was irrigated and the competing vegetation had been eliminated, other strategies, such as mechanical tilling practices [90–92], should be investigated in order to maintain a youthful plantation and avoid the loss of crops after a few years. Therefore, it is necessary to explore the mycelial dynamics over time, because the plantation may be at risk of ageing, with mycelium and mycorrhizae displaced and no ascocarp production.

4. Conclusions

In conclusion, the selected primers designed within ITS regions are sufficiently accurate to develop a real-time qPCR protocol for the quantification of fungal biomass of

T. claveryi and *T. crassiverrucosa* in soil samples. The TerclaF3/R1 primer set was tested and validated for the SYBR-Green-based qPCR assay. Moreover, the preliminary study on soil samples from a desert truffle plantation showed no correlation between winter soil fungal biomass and truffle productivity in spring. However, the amount of fungal soil mycelium seemed to trend to decrease with the years, indicating that, after a certain time, the plantation could become unproductive. In-depth knowledge of mycelial dynamics over the years would help us to develop proposals for plantation management to extend the useful life of plantations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jof8101095/s1>, Table S1: Accession number from GenBank and RefSeq databases (NCBI) of the species used for *turmas* primers design.

Author Contributions: Conceptualization, F.A. and A.M.; methodology, F.A. and A.N.-R.; formal analysis, F.A. and A.N.-R.; investigation, all authors; writing—original draft preparation, F.A.; writing—review and editing, all authors; funding acquisition, A.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by MCIN/AEI/10.13039/50110001103, grant number PID2020-115210RB-I00, and FEDER and Fundación Séneca, Agencia de Ciencia y Tecnología of the Region of Murcia (Spain), grant number 20866/PI/18.

Data Availability Statement: All sequences used during this experimental study are available from GenBank and accession numbers are given in Tables 1 and S1.

Acknowledgments: The authors are grateful to Tomás López for allowing the sampling in his desert truffle plantation and providing the desert truffle production data and to Angel Guarnizo Serrudo for his support in statistical analyses.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Morte, A.; Pérez-Gilabert, M.; Gutiérrez, A.; Arenas, F.; Marqués-Gálvez, J.E.; Bordallo, J.J.; Rodríguez, A.; Berná, L.M.; Lozano-Carrillo, C.; Navarro-Ródenas, A. Basic and Applied Research for Desert Truffle Cultivation. In *Mycorrhiza-Eco-Physiology, Secondary Metabolites, Nanomaterials*; Varma, A., Prasad, R., Tuteja, N., Eds.; Springer: Cham, Switzerland, 2017; pp. 23–42.
- Bradai, L.; Bissati, S.; Chenchouni, H. Desert Truffles of the North Algerian Sahara: Diversity and Bioecology. *Emir. J. Food Agric.* **2014**, *26*, 425–435. [[CrossRef](#)]
- Shavit, E. The History of Desert Truffle Use. In *Desert Truffles. Soil Biology*; Kagan-Zur, V., Roth-Bejerano, N., Sitrit, Y., Morte, A., Eds.; Springer: Berlin/Heidelberg, Germany, 2014; pp. 217–241.
- Honrubia, M.; Gutiérrez, A.; Morte, A. Desert Truffle Plantation from South-East Spain. In Proceedings of the Edible Mycorrhizal Mushrooms and Their Cultivation: Proceedings of the Second International Conference on Edible Mycorrhizal Mushrooms, Christchurch, New Zealand, 3–6 July 2001; pp. 3–5.
- Morte, A.; Gutiérrez, A.; Ródenas, A.N. Advances in Desert Truffle Mycorrhization and Cultivation. In *Mushrooms, Humans and Nature in a Changing World. Perspectives from Ecological, Agricultural and Social Sciences*; Pérez-Moreno, J., Guerin-Laguette, A., Arzú, R.F., Yu, F.-Q., Eds.; Springer: Cham, Switzerland, 2020; pp. 205–219.
- Volpato, G.; Rossi, D.; Dentoni, D. A Reward for Patience and Suffering: Ethnomycology and Commodification of Desert Truffles among Sahrawi Refugees and Nomads of Western Sahara. *Econ. Bot.* **2013**, *67*, 147–160. [[CrossRef](#)]
- Murcia, M.A.; Martínez-Tomé, M.; Jiménez, A.M.; Vera, A.M.; Honrubia, M.; Parras, P. Antioxidant Activity of Edible Fungi (Truffles and Mushrooms): Losses during Industrial Processing. *J. Food Prot.* **2002**, *65*, 1614–1622. [[CrossRef](#)] [[PubMed](#)]
- Murcia, M.A.; Martínez-Tomé, M.; Vera, A.; Morte, A.; Gutiérrez, A.; Honrubia, M.; Jiménez, A.M. Effect of Industrial Processing on Desert Truffles *Terfezia claveryi* Chatin and *Picoa juniperi* Vittadini: Proximate Composition and Fatty Acids. *J. Sci. Food Agric.* **2003**, *83*, 535–541. [[CrossRef](#)]
- Wang, S.; Marcone, M.F. The Biochemistry and Biological Properties of the World's Most Expensive Underground Edible Mushroom: Truffles. *Food Res. Int.* **2011**, *44*, 2567–2581. [[CrossRef](#)]
- Martínez-Tomé, M.; Maggi, L.; Jiménez-Monreal, A.M.; Murcia, M.A.; Marí, J.A.T. Nutritional and Antioxidant Properties of *Terfezia* and *Picoa*. In *Desert Truffles. Soil Biology*; Kagan-Zur, V., Roth-Bejerano, N., Sitrit, M.A., Eds.; Springer: Berlin/Heidelberg, Germany, 2014; pp. 261–273.
- Patel, S.; Rauf, A.; Khan, H.; Khalid, S.; Mubarak, M.S. Potential Health Benefits of Natural Products Derived from Truffles: A Review. *Trends Food Sci. Technol.* **2017**, *70*, 1–8. [[CrossRef](#)]

12. Dahham, S.S.; Al-Rawi, S.S.; Ibrahim, A.H.; Abdul Majid, A.S.; Abdul Majid, A.M.S. Antioxidant, Anticancer, Apoptosis Properties and Chemical Composition of Black Truffle *Terfezia claveryi*. *Saudi J. Biol. Sci.* **2018**, *25*, 1524–1534. [[CrossRef](#)]
13. Morte, A.; Arenas, F.; Marqués-Gálvez, J.E.; Berna, L.M.; Guarnizo-Serrudo, Á.L.; Gutierrez, A.; Rodriguez, A.; Navarro-Ródenas, A. Turmiculture Project: Desert Truffle Crop against Climate Change and for Rural Development. In Proceedings of the X International Workshop of Edible Mycorrhizal Mushrooms (IWEMM10), Suwa City, Japan, 20–29 October 2019.
14. Andrino, A.; Navarro-Ródenas, A.; Marqués-Gálvez, J.E.; Morte, A. The Crop of Desert Truffle Depends on Agroclimatic Parameters during Two Key Annual Periods. *Agron. Sustain. Dev.* **2019**, *39*, 51. [[CrossRef](#)]
15. Marqués-Gálvez, J.E.; Morte, A.; Navarro-Ródenas, A. Spring Stomatal Response to Vapor Pressure Deficit as a Marker for Desert Truffle Fruiting. *Mycorrhiza* **2020**, *30*, 503–512. [[CrossRef](#)]
16. Marqués-Gálvez, J.E.; Navarro-Ródenas, A.; Peguero-Pina, J.J.; Arenas, F.; Guarnizo, A.L.; Gil-Pelegrián, E.; Morte, A. Elevated Atmospheric CO₂ Modifies Responses to Water-Stress and Flowering of Mediterranean Desert Truffle Mycorrhizal Shrubs. *Physiol. Plant* **2020**, *170*, 537–549. [[CrossRef](#)]
17. Morte, A.; Arenas, F.; Marqués-Gálvez, J.E.; Andrino, A.; Guarnizo, Á.L.; Gutiérrez, A.; Berná, L.M.; Pérez-Gilabert, M.; Rodríguez, A.; Navarro-Ródenas, A. Desert Truffles (*Terfezia* spp.) Breeding. In *Advances in Plant Breeding Strategies: Vegetable Crops*; Al-Khayri, J.M., Jain, S.M., Johnson, D.V., Eds.; Springer: Cham, Switzerland, 2021; pp. 479–504. ISBN 978-3-030-66969-0.
18. Arenas, F.; Navarro-Ródenas, A.; Marqués-Gálvez, J.E.; Ghignone, S.; Mello, A.; Morte, A. Different Patterns in Root and Soil Fungal Diversity Drive Plant Productivity of the Desert Truffle *Terfezia claveryi* in Plantation. *Environ. Microbiol.* **2021**, *23*, 5917–5933. [[CrossRef](#)]
19. Hall, I.R.; Yun, W.; Amicucci, A. Cultivation of Edible Ectomycorrhizal Mushrooms. *Trends Biotechnol.* **2003**, *21*, 433–438. [[CrossRef](#)]
20. Zambonelli, A.; Iotti, M.; Boutahir, S.; Lancellotti, E.; Perini, C.; Pacioni, G. Ectomycorrhizal Fungal Communities of Edible Ectomycorrhizal Mushrooms. In *Edible Ectomycorrhizal Mushrooms: Current Knowledge and Future Prospects*; Zambonelli, A., Bonito, G.M., Eds.; Springer: Berlin/Heidelberg, Germany, 2012; pp. 105–124.
21. Navarro-Ródenas, A.; Berná, L.M.; Lozano-Carrillo, C.; Andrino, A.; Morte, A. Beneficial Native Bacteria Improve Survival and Mycorrhization of Desert Truffle Mycorrhizal Plants in Nursery Conditions. *Mycorrhiza* **2016**, *26*, 769–779. [[CrossRef](#)]
22. Anderson, I.C.; Cairney, J.W.G. Diversity and Ecology of Soil Fungal Communities: Increased Understanding through the Application of Molecular Techniques. *Environ. Microbiol.* **2004**, *6*, 769–779. [[CrossRef](#)]
23. Séjalón-Delmas, N.; Roux, C.; Martins, M.; Kulifaj, M.; Bécard, G.; Dargent, R. Molecular Tools for the Identification of *Tuber melanosporum* in Agroindustry. *J. Agric. Food Chem.* **2000**, *48*, 2608–2613. [[CrossRef](#)]
24. Zarivi, O.; Cesare, P.; Ragnelli, A.M.; Aimola, P.; Leonardi, M.; Bonfigli, A.; Colafarina, S.; Poma, A.M.; Miranda, M.; Pacioni, G. Validation of Reference Genes for Quantitative Real-Time PCR in Périgord Black Truffle (*Tuber melanosporum*) Developmental Stages. *Phytochemistry* **2015**, *116*, 78–86. [[CrossRef](#)]
25. Leonardi, M.; Ascione, S.; Pacioni, G.; Cesare, P.; Pacioni, M.L.; Miranda, M.; Zarivi, O. The Challenge for Identifying the Fungi Living inside Mushrooms: The Case of Truffle Inhabiting Mycelia. *Plant Biosyst.* **2018**, *152*, 1002–1010. [[CrossRef](#)]
26. Rizzello, R.; Zampieri, E.; Vizzini, A.; Autino, A.; Cresti, M.; Bonfante, P.; Mello, A. Authentication of Prized White and Black Truffles in Processed Products Using Quantitative Real-Time PCR. *Food Res. Int.* **2012**, *48*, 792–797. [[CrossRef](#)]
27. Jomura, M.; Kuwayama, T.; Soma, Y.; Yamaguchi, M.; Komatsu, M.; Maruyama, Y. Mycelial Biomass Estimation and Metabolic Quotient of *Lentinula edodes* Using Species-specific QPCR. *PLoS ONE* **2020**, *15*, e0232049. [[CrossRef](#)]
28. Suz, L.M.; Martín, M.P.; Colinas, C. Detection of *Tuber melanosporum* DNA in Soil. *FEMS Microbiol. Lett.* **2006**, *254*, 251–257. [[CrossRef](#)]
29. Bertini, L.; Rossi, I.; Zambonelli, A.; Amicucci, A.; Sacchi, A.; Cecchini, M.; Gregori, G.; Stocchi, V. Molecular Identification of *Tuber magnatum* Ectomycorrhizae in the Field. *Microbiol. Res.* **2006**, *161*, 59–64. [[CrossRef](#)]
30. Hortal, S.; Pera, J.; Galipienso, L.; Parladé, J. Molecular Identification of the Edible Ectomycorrhizal Fungus *Lactarius deliciosus* in the Symbiotic and Extraradical Mycelium Stages. *J. Biotechnol.* **2006**, *126*, 123–134. [[CrossRef](#)]
31. Suz, L.M.; Martín, M.P.; Oliach, D.; Fischer, C.R.; Colinas, C. Mycelial Abundance and Other Factors Related to Truffle Productivity in *Tuber melanosporum*-*Quercus ilex* Orchards. *FEMS Microbiol. Lett.* **2008**, *285*, 72–78. [[CrossRef](#)]
32. Parladé, J.; De la Varga, H.; De Miguel, A.M.; Sáez, R.; Pera, J. Quantification of Extraradical Mycelium of *Tuber melanosporum* in Soils from Truffle Orchards in Northern Spain. *Mycorrhiza* **2013**, *23*, 99–106. [[CrossRef](#)]
33. Queralt, M.; Parladé, J.; Pera, J.; De Miguel, A.M. Seasonal Dynamics of Extraradical Mycelium and Mycorrhizas in a Black Truffle (*Tuber melanosporum*) Plantation. *Mycorrhiza* **2017**, *27*, 565–576. [[CrossRef](#)]
34. Iotti, M.; Leonardi, M.; Oddis, M.; Salerni, E.; Baraldi, E.; Zambonelli, A. Development and Validation of a Real-Time PCR Assay for Detection and Quantification of *Tuber magnatum* in Soil. *BMC Microbiol.* **2012**, *12*, 93. [[CrossRef](#)]
35. Iotti, M.; Leonardi, M.; Lancellotti, E.; Salerni, E.; Oddis, M.; Leonardi, P.; Perini, C.; Pacioni, G.; Zambonelli, A. Spatio-Temporal Dynamic of *Tuber magnatum* Mycelium in Natural Truffle Grounds. *PLoS ONE* **2014**, *9*, e115921. [[CrossRef](#)]
36. Iotti, M.; Leonardi, P.; Vitali, G.; Zambonelli, A. Effect of Summer Soil Moisture and Temperature on the Vertical Distribution of *Tuber magnatum* Mycelium in Soil. *Biol. Fertil. Soils* **2018**, *54*, 707–716. [[CrossRef](#)]
37. Gryndler, M.; Trilčová, J.; Hršelová, H.; Streiblová, E.; Gryndlerová, H.; Jansa, J. *Tuber aestivum* Vittad. Mycelium Quantified: Advantages and Limitations of a QPCR Approach. *Mycorrhiza* **2013**, *23*, 341–348. [[CrossRef](#)]

38. Todesco, F.; Belmondo, S.; Guignet, Y.; Laurent, L.; Fizzala, S.; Le Tacon, F.; Murat, C. Soil Temperature and Hydric Potential Influences the Monthly Variations of Soil *Tuber aestivum* DNA in a Highly Productive Orchard. *Sci. Rep.* **2019**, *9*, 12964. [[CrossRef](#)]
39. Parladé, J.; Hortal, S.; Pera, J.; Galipienso, L. Quantitative Detection of *Lactarius deliciosus* Extraradical Soil Mycelium by Real-Time PCR and Its Application in the Study of Fungal Persistence and Interspecific Competition. *J. Biotechnol.* **2007**, *128*, 14–23. [[CrossRef](#)] [[PubMed](#)]
40. Hortal, S.; Pera, J.; Parladé, J. Tracking Mycorrhizas and Extraradical Mycelium of the Edible Fungus *Lactarius deliciosus* under Field Competition with *Rhizopogon* spp. *Mycorrhiza* **2008**, *18*, 69–77. [[CrossRef](#)] [[PubMed](#)]
41. Hortal, S.; Pera, J.; Parladé, J. Field Persistence of the Edible Ectomycorrhizal Fungus *Lactarius deliciosus*: Effects of Inoculation Strain, Initial Colonization Level, and Site Characteristics. *Mycorrhiza* **2009**, *19*, 167–177. [[CrossRef](#)] [[PubMed](#)]
42. De la Varga, H.; Águeda, B.; Ágreda, T.; Martínez-Peña, F.; Parladé, J.; Pera, J. Seasonal Dynamics of *Boletus edulis* and *Lactarius deliciosus* Extraradical Mycelium in Pine Forests of Central Spain. *Mycorrhiza* **2013**, *23*, 391–402. [[CrossRef](#)] [[PubMed](#)]
43. Yamaguchi, M.; Narimatsu, M.; Fujita, T.; Kawai, M.; Kobayashi, H.; Ohta, A.; Yamada, A.; Matsushita, N.; Neda, H.; Shimokawa, T.; et al. A QPCR Assay That Specifically Quantifies *Tricholoma matsutake* Biomass in Natural Soil. *Mycorrhiza* **2016**, *26*, 847–861. [[CrossRef](#)]
44. De la Varga, H.; Águeda, B.; Martínez-Peña, F.; Parladé, J.; Pera, J. Quantification of Extraradical Soil Mycelium and Ectomycorrhizas of *Boletus edulis* in a Scots Pine Forest with Variable Sporocarp Productivity. *Mycorrhiza* **2011**, *22*, 59–68. [[CrossRef](#)] [[PubMed](#)]
45. Parladé, J.; Martínez-Peña, F.; Pera, J. Effects of Forest Management and Climatic Variables on the Mycelium Dynamics and Sporocarp Production of the Ectomycorrhizal Fungus *Boletus edulis*. *For. Ecol. Manag.* **2017**, *390*, 73–79. [[CrossRef](#)]
46. Aviram, S.; Roth-Bejerano, N.; Kagan-Zur, V. Two ITS Forms Co-Inhabiting a Single Genet of an Isolate of *Terfezia boudieri* (Ascomycotina), a Desert Truffle. *Antonie Van Leeuwenhoek Int.* **2004**, *85*, 169–174. [[CrossRef](#)]
47. Bordallo, J.-J.; Rodríguez, A.; Kaounas, V.; Camello, F.; Honrubia, M.; Morte, A. Two New *Terfezia* Species from Southern Europe. *Phytotaxa* **2015**, *230*, 239–249. [[CrossRef](#)]
48. Bordallo, J.J.; Rodríguez, A.; Santos-Silva, C.; Louro, R.; Muñoz-Mohedano, J.; Morte, A. *Terfezia lusitanica*, a New Mycorrhizal Species Associated to *Tuberaria guttata* (Cistaceae). *Phytotaxa* **2018**, *357*, 141–147. [[CrossRef](#)]
49. Zitouni-Haouar, F.E.H.; Carlavilla, J.R.; Moreno, G.; Manjón, J.L.; Fortas, Z. Genetic Diversity of the Genus *Terfezia* (Pezizaceae, Pezizales): New Species and New Record from North Africa. *Phytotaxa* **2018**, *334*, 183–194. [[CrossRef](#)]
50. Crous, P.W.; Wingfield, M.J.; Burgess, T.I.; Hardy, G.E.S.J.; Gené, J.; Guarro, J.; Baseia, I.G.; García, D.; Gusmão, L.F.P.; Souza-Motta, C.M.; et al. Fungal Planet Description Sheets: 716–784. *Pers. Mol. Phylogeny Evol. Fungi* **2018**, *40*, 240–393. [[CrossRef](#)]
51. Crous, P.W.; Wingfield, M.J.; Lombard, L.; Roets, F.; Swart, W.J.; Alvarado, P.; Carnegie, A.J.; Moreno, G.; Luangsa-Ard, J.; Thangavel, R.; et al. Fungal Planet Description Sheets: 951–1041. *Pers. Mol. Phylogeny Evol. Fungi* **2019**, *43*, 223–425. [[CrossRef](#)]
52. Moreno, G.; Manjón, J.L.; Alvarado, P. A New *Terfezia* from Spain. *Bol. Soc. Micol. Madr.* **2019**, *43*, 55–60.
53. Rodríguez, A.; Navarro-Ródenas, A.; Arenas, F.; Muñoz-Mohedano, J.M.; Morte, A. Solving the Identity of *Terfezia trappei* (Pezizaceae, Ascomycota). *Phytotaxa* **2019**, *411*, 230–236. [[CrossRef](#)]
54. Vizzini, A.; Arenas, F.; Rodríguez, A.; Mello, A.; Lainé, P.; Muñoz-Mohedano, J.M.; Morte, A. Typification of *Terfezia fanfani* (Ascomycota, Pezizaceae). *Phytotaxa* **2019**, *387*, 73–76. [[CrossRef](#)]
55. Kovács, G.M.; Balázs, T.K.; Calonge, F.D.; Martín, M.P. The Diversity of *Terfezia* Desert Truffles: New Species and a Highly Variable Species Complex with Intrasporocarpic nrDNA ITS Heterogeneity. *Mycologia* **2011**, *103*, 841–853. [[CrossRef](#)]
56. Bordallo, J.-J.; Rodríguez, A. Cryptic and New Species. In *Desert Truffles. Soil Biology*; Kagan-Zur, V., Roth-Bejerano, N., Sitrit, Y., Morte, A., Eds.; Springer: Berlin/Heidelberg, Germany, 2014; pp. 39–53. ISBN 978-3-642-40095-7.
57. Louro, R.; Santos-Silva, C.; Nobre, T. What Is in a Name? *Terfezia Classification Revisited. Fungal. Biol.* **2019**, *123*, 267–273. [[CrossRef](#)]
58. Schoch, C.L.; Seifert, K.A.; Huhndorf, S.; Robert, V.; Spouge, J.L.; Levesque, C.A.; Chen, W.; Consortium, F.B. Nuclear Ribosomal Internal Transcribed Spacer (ITS) Region as a Universal DNA Barcode Marker for Fungi. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 6241–6246. [[CrossRef](#)]
59. Oliach, D.; Morte, A.; Sánchez, S.; Navarro-Ródenas, A.; Marco, P.; Gutiérrez, A.; Martín-Santafé, M.; Fischer, C.; Albisu, L.M.; García-Barreda, S.; et al. Las Trufas y Las Turmas. In *Los Productos Forestales No Madereros en España: Del Monte a La Industria*; Sánchez-González, M., Calama, R., Bonet, J.A., Eds.; INIA, Ministerio de Economía Industria y Competitividad: Madrid, Spain, 2020; pp. 283–324. ISBN 9788474985856.
60. Arenas, F.; Navarro-Ródenas, A.; Chávez, D.; Gutiérrez, A.; Pérez-Gilabert, M.; Morte, A. Mycelium of *Terfezia claveryi* as Inoculum Source to Produce Desert Truffle Mycorrhizal Plants. *Mycorrhiza* **2018**, *28*, 691–701. [[CrossRef](#)]
61. Edgar, R.C. MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [[CrossRef](#)]
62. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [[CrossRef](#)]
63. Thornton, B.; Basu, C. Rapid and Simple Method of QPCR Primer Design. In *PCR Primer Design. Methods in Molecular Biology*; Basu, C., Ed.; Humana Press Inc.: Totowa, NJ, USA, 2015; Volume 1275, pp. 173–179.
64. Rodríguez, A.; Rodríguez, M.; Córdoba, J.J.; Andrade, M.J. Design of Primers and Probes for Quantitative Real-Time PCR Methods. In *PCR Primer Design. Methods in Molecular Biology*; Basu, C., Ed.; Humana Press Inc.: Totowa, NJ, USA, 2015; Volume 1275, pp. 31–56.

65. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clin. Chem.* **2009**, *55*, 611–622. [[CrossRef](#)]
66. Morgulis, A.; Coulouris, G.; Raytselis, Y.; Madden, T.L.; Agarwala, R.; Schäffer, A.A. Database Indexing for Production MegaBLAST Searches. *Bioinformatics* **2008**, *24*, 1757–1764. [[CrossRef](#)]
67. Bonito, G. Fast DNA-Based Identification of the Black Truffle *Tuber melanosporum* with Direct PCR and Species-Specific Primers. *FEMS Microbiol. Lett.* **2009**, *301*, 171–175. [[CrossRef](#)]
68. White, T.J.; Bruns, T.; Lee, S.; Taylor, J. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In *PCR Protocols: A Guide to Methods and Applications.*; Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., Eds.; Academic Press: New York, NY, USA, 1990; Volume 18, pp. 315–322.
69. Gardes, M.; Bruns, T.D. ITS Primers with Enhanced Specificity for Basidiomycetes—Application to the Identification of Mycorrhizae and Rusts. *Mol. Ecol.* **1993**, *2*, 113–118. [[CrossRef](#)]
70. Kralik, P.; Ricchi, M. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Front. Microbiol.* **2017**, *8*, 108. [[CrossRef](#)]
71. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. 2021. Available online: <https://www.R-project.org/> (accessed on 20 May 2022).
72. Ogle, D.H.; Doll, J.C.; Wheeler, P.; Dinno, A.; FSA: Fisheries Stock Analysis. R Package Version 0.9.3. 2022. Available online: <https://github.com/FishR-Core-Team/FSA> (accessed on 20 May 2022).
73. Nilsson, R.H.; Kristiansson, E.; Ryberg, M.; Hallenberg, N.; Larsson, K.H. Intraspecific ITS Variability in the Kingdom Fungi as Expressed in the International Sequence Databases and Its Implications for Molecular Species Identification. *Evol. Bioinform.* **2008**, *4*, EBO-S653. [[CrossRef](#)]
74. Chemidlin Prévost-Bouré, N.; Christen, R.; Dequiedt, S.; Mougél, C.; Lelièvre, M.; Jolivet, C.; Shahbazkia, H.R.; Guillou, L.; Arrouays, D.; Ranjard, L. Validation and Application of a PCR Primer Set to Quantify Fungal Communities in the Soil Environment by Real-Time Quantitative PCR. *PLoS ONE* **2011**, *6*, e24166. [[CrossRef](#)]
75. Singh, A.; Pandey, G.K. Primer Design Using Primer Express ® for SYBR Green- Based Quantitative PCR. In *PCR Primer Design. Methods in Molecular Biology*; Basu, C., Ed.; Humana Press Inc.: Totowa, NJ, USA, 2015; Volume 1275, pp. 153–164.
76. Bordallo, J.J.; Rodríguez, A.; Muñoz-Mohedano, J.M.; Suz, L.M.; Honrubia, M.; Morte, A. Five New *Terfezia* Species from the Iberian Peninsula. *Mycotaxon* **2013**, *124*, 189–208. [[CrossRef](#)]
77. Hall, I.R.; Zambonelli, A.; Wang, Y. The Cultivation of Mycorrhizal Mushrooms—Success and Failure. In Proceedings of the International Conference on Mushroom Biology and Mushroom Products, Nantong, China, 18–21 June 2009.
78. Landeweert, R.; Veenman, C.; Kuyper, T.W.; Fritze, H.; Wernars, K.; Smit, E. Quantification of Ectomycorrhizal Mycelium in Soil by Real-Time PCR Compared to Conventional Quantification Techniques. *FEMS Microbiol. Ecol.* **2003**, *45*, 283–292. [[CrossRef](#)]
79. Johnson, G.; Nolan, T.; Bustin, S.A. Real-Time Quantitative PCR, Pathogen Detection and MIQE. *Methods Mol. Biol.* **2013**, *943*, 1–16. [[CrossRef](#)] [[PubMed](#)]
80. Bustin, S.; Huggett, J. QPCR Primer Design Revisited. *Biomol. Detect. Quantif.* **2017**, *14*, 19–28. [[CrossRef](#)] [[PubMed](#)]
81. Tajadini, M.; Panjehpour, M.; Javanmard, S. Comparison of SYBR Green and TaqMan Methods in Quantitative Real-Time Polymerase Chain Reaction Analysis of Four Adenosine Receptor Subtypes. *Adv. Biomed. Res.* **2014**, *3*, 85. [[CrossRef](#)]
82. Marqués-Gálvez, J.E.; Miyauchi, S.; Paolocci, F.; Navarro-Ródenas, A.; Arenas, F.; Pérez-Gilabert, M.; Morin, E.; Auer, L.; Barry, K.W.; Kuo, A.; et al. Desert Truffle Genomes Reveal Their Reproductive Modes and New Insights into Plant–Fungal Interaction and Ectendomycorrhizal Lifestyle. *New Phytol.* **2021**, *229*, 2917–2932. [[CrossRef](#)]
83. Arenas, F.; López-García, Á.; Berná, L.M.; Morte, A.; Navarro-Ródenas, A. Desert Truffle Mycorrhizosphere Harbors Organic Acid Releasing Plant Growth–Promoting Rhizobacteria, Essentially during the Truffle Fruiting Season. *Mycorrhiza* **2022**, *32*, 193–202. [[CrossRef](#)]
84. Selosse, M.A.; Schneider-Maunoury, L.; Taschen, E.; Rousset, F.; Richard, F. Black Truffle, a Hermaphrodite with Forced Unisexual Behaviour. *Trends Microbiol.* **2017**, *25*, 784–787. [[CrossRef](#)]
85. Martin, F.; Kohler, A.; Murat, C.; Balestrini, R.; Coutinho, P.M.; Jaillon, O.; Montanini, B.; Morin, E.; Noel, B.; Percudani, R.; et al. Périgord Black Truffle Genome Uncovers Evolutionary Origins and Mechanisms of Symbiosis. *Nature* **2010**, *464*, 1033–1038. [[CrossRef](#)]
86. Chen, J.; De la Varga, H.; Todesco, F.; Beacco, P.; Martino, E.; Le Tacon, F.; Murat, C. Frequency of the Two Mating Types in the Soil under Productive and Non-Productive Trees in Five French Orchards of the Périgord Black Truffle (*Tuber melanosporum* Vittad.). *Mycorrhiza* **2021**, *31*, 361–369. [[CrossRef](#)]
87. Zampieri, E.; Rizzello, R.; Bonfante, P.; Mello, A. The Detection of Mating Type Genes of *Tuber melanosporum* in Productive and Non Productive Soils. *Appl. Soil Ecol.* **2012**, *57*, 9–15. [[CrossRef](#)]
88. Splivallo, R.; Vahdatzadeh, M.; MacIá-Vicente, J.G.; Molinier, V.; Peter, M.; Egli, S.; Uroz, S.; Paolocci, F.; Deveau, A. Orchard Conditions and Fruiting Body Characteristics Drive the Microbiome of the Black Truffle *Tuber aestivum*. *Front. Microbiol.* **2019**, *10*, 1437. [[CrossRef](#)]
89. Oliach, D.; Colinas, C.; Castaño, C.; Fischer, C.R.; Bolaño, F.; Bonet, J.A.; Oliva, J. The Influence of Forest Surroundings on the Soil Fungal Community of Black Truffle (*Tuber melanosporum*) Plantations. *For. Ecol. Manag.* **2020**, *469*, 118119. [[CrossRef](#)]

90. Olivera, A.; Fischer, C.R.; Bonet, J.A.; de Aragón, J.M.; Oliach, D.; Colinas, C. Weed Management and Irrigation Are Key Treatments in Emerging Black Truffle (*Tuber melanosporum*) Cultivation. *New For.* **2011**, *42*, 227–239. [[CrossRef](#)]
91. Piñuela, Y.; Alday, J.G.; Oliach, D.; Castaño, C.; Bolaño, F.; Colinas, C.; Bonet, J.A. White Mulch and Irrigation Increase Black Truffle Soil Mycelium When Competing with Summer Truffle in Young Truffle Orchards. *Mycorrhiza* **2021**, *31*, 371–382. [[CrossRef](#)]
92. Fischer, C.; Oliach, D.; Bonet, A.; Colinas, C. *Best Practices for Cultivation of Truffles*; Forest Sciences Centre of Catalonia: Solsona, Spain; Yaşama Dair Vakıf: Antalya, Turkey, 2017; ISBN 978-84-697-8163-0.