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First Record of Summer Truffle (*Tuber aestivum*) in Portugal

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Abstract: *Tuber aestivum*, commonly known as the summer truffle, is typically found in various parts of Europe where it grows naturally. However, its presence in Portugal was not confirmed until now. The first fruit bodies were collected in April 2024 at stone pine stands (Alenquer and Arruda dos Vinhos, Lisbon) and in June at holm oak stands (Salir, Faro). These specimens are characterized by hypogeous, subglobose, black ascomata with a peridium surface covered with pyramidal warts. Ascopores are subglobose-to-broadly ellipsoid, distinctively ornamented, usually 1–6 per asci. According to the results of the internal transcribed spacer (ITS) rDNA sequence analysis, these specimens form a well-supported group within the Aestivum clade, with *T. aestivum* being the closest phylogenetic taxon. This remarkable discovery opens up new opportunities for truffle exploitation in Portugal thanks to the summer truffle's gastronomical value and high market prices.

Keywords: Ascomycota; Tuberaceae; summer truffle; ectomycorrhizal fungi; taxonomy; phylogeny; natural distribution; fungi diversity

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Citation: Santos-Silva, C.; Brígido, C. First Record of Summer Truffle (*Tuber aestivum*) in Portugal. *Microbiol. Res.* **2024**, *15*, 1494–1504. https://doi.org/10.3390/microbiolres15030101

Academic Editor: Ligang Zhou

Received: 5 July 2024
Revised: 5 August 2024
Accepted: 7 August 2024
Published: 9 August 2024

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1. Introduction

Recently, numerous new species of *Tuber* have been identified and recognized as valid species by the scientific community. It is difficult to assure how many *Tuber* species occur worldwide, since the Global Biodiversity Information Facility (GBIF) estimate that there are around 230 accepted species [1], but the Catalogue of Life only list 196 accepted species [2]. To estimate the number of *Tuber* species on the European continent, various sources were consulted and compared, based on material samples or preserved specimens' records [1–4]. These sources suggest that 51 *Tuber* species have been found in Europe, namely *T. aestivum* (Wulfen) Spreng., *T. albidum* Fr., *T. alcaracense* Ant. Rodr. and Morte, *T. anniae* W. Colgan and Trappe, *T. asa-foetida* Lesp., *T. bellonae* Quél., *T. borchii* Vittad., *T. brumale* Vittad., *T. cistophilum* P. Alvarado, G. Moreno, Manjón, Gelpi and Jaime Muñoz, *T. cryptobrumale* Merényi, T. Varga and Bratek, *T. davidlopezii* Ant. Rodr., Morte and Muñ.-Moh., *T. dryophilum* Tul. and C. Tul., *T. excavatum* Vittad., *T. ferrugineum* Vittad., *T. foetidum* Vittad., *T. fulgens* Quél., *T. gennadii* (Chatin) Pat., *T. gibbosum* Harkn., *T. himalayense* B.C. Zhang and Minter, *T. huidongense* Y. Wang, *T. lacunosum* Mattir., *T. levissimum* Gilkey, *T. lucentum* Bordallo, *T. lusitanicum* Ant. Rodr. and Muñoz-Mohedano, *T. macrosporum* Vittad., *T. maculatum* Vittad., *T. magentipunctatum* Merényi, I. Nagy, Stielow and Bratek, *T. magnatum* Picco, *T. malacodermum* E. Fisch., *T. malenconii* Donadini, Riousset, G. Riousset and G. Chev., *T. melanosporum* Vittad., *T. mesentericum* Vittad., *T. microsporum* Vittad., *T. mutabile* Quél., *T. nitidum* Vittad., *T. oligospermum* (Tul. and C. Tul.) Trappe, *T. panniferum* Tul. and C. Tul., *T. petrophilum* Milenković, P. Jovan., Grebenc, Ivančević and Marković, *T. pseudobrumale* Y. Wang and Shu H. Li, *T. pseudoexcavatum* Y. Wang, G. Moreno, Riousset, Manjón and G. Riousset, *T. puberulum* Berk. and Broome, *T. pulchrosporum* Konstantinidis, Tsampazis, Slavova, Nakkas, Polemis, Fryssouli and Zervakis, *T. rapaeodorum* Tul. and C. Tul., *T. regianum* Montecchi and Lazzari, *T. rufum* Pollini, *T. scleroneuron* Berk. and Broome, *T. scruposum* R. Hesse, *T. sphaerospermum* (Malençon) P. Roux, Guy García and M.C. Roux, *T. stramineum* Ferry and Quél., *T. suave* Pacioni and M. Leonardi, *T. vesicoperidium* L. Fan.

According to GBIF, only eight *Tuber* species occurred in Portugal: *T. borchii*, *T. excavatum*, *T. lacunosum*, *T. maculatum*, *T. oligospermum* and *T. puberulum*, with less than 40 records in total. Although, T. gennadii [5], one of the most common truffles in Portugal, is not referred. In short, from all databases, there is no reference of the occurrence of *T. aestivum* in Portugal.

Tuber aestivum, commonly known as the summer truffle or Burgundy truffle, is highly prized worldwide for its distinctive aroma and flavor. Summer truffles have a milder, more delicate aroma compared to the more intense winter truffles, and its scent is often described as earthy, nutty, and slightly garlicky [6]. High demanded in gourmet and fine dining sectors, summer truffle can be used fresh or in infusions (oils, butter, cheeses) in a wide variety of prepared dishes. The economic value of summer truffle is influenced by its demand versus its availability, as well as by the ascocarps quality, size, and freshness. Fresh summer truffle market prices can vary widely between regions, with lower values in Spain (25 to 70 EUR per kg) and higher in Switzerland (200 to 600 EUR per kg) [7]. Additionally, summer truffles have medicinal properties, such as anti-angiogenic and anti-inflammatory activities [8]. In summary, *T. aestivum* holds significant gastronomical and economic value due to its unique flavor, culinary versatility, and medicinal properties. Although, not as costly as some other truffle varieties, it remains a luxury item that commands a premium price on the market.

T. aestivum belong to Tuberaceae (Pezizales, Ascomycota), a large group of ectomycorrhizal fungi growing in symbiosis with the roots of several vascular plant (Angiosperms: *Betula* spp., *Carpinus* spp., *Castanea* spp., *Corylus avellana*, *Fagus* spp., *Populus* spp., *Quercus* spp. and *Tilia* spp.; Gymnosperms: *Abies* spp., *Cedrus* spp., *Picea* spp. and *Pinus* spp.), fruiting abundantly at matures stands [9–12]. The *T. aestivum* fruit body is hypogeous, dark, globose with an average diameter of 7 cm and not easy to find without trained animals. The optimal soil conditions for its growth and development include specific ranges for soil pH (7.0 to 8.0), organic matter content (2 to 4%), and a C:N ratio (20:1 to 30:1) [12], mainly from loam to silt loam sandy–clayey soils [13]. Concerning climatic conditions, *T. aestivum* prefers mild temperatures (mean annual temperature between +7 °C and +11 °C (max. +18 °C) and moderate precipitation (annual precipitation between 700 mm and 900 mm (min. 400 mm)) [13,14].

T. aestivum natural occurrence exhibits a broad range distribution through Europe, Asia, and North Africa (Figure 1); however, its cultivation is wider and includes other continents. In Portugal, several edible mushrooms and truffles are collected for both personal consumption and retail sale. Mushroom and truffle hunters are typically rural individuals with a deep, intuitive knowledge gained from years of experience. They rely entirely on their expertise, without the aid of animals, like pigs or dogs, to assist in their mushroom search. It is important to note that many hypogeous sporocarps cannot be identified solely by observing the visual characteristics of the terrain or the presence of their host species, as they are buried more than 10 cm deep, leaving no visible trace of their presence. In such cases, the assistance of trained animals to detect them by their scents is necessary. Bearing this in mind, several mycological expeditions with trained dogs were performed in the last years, and many hypogeous fungi were collected (Santos-Silva, unpublished data), some of them novelties for Portugal. Although the authors had identified various *Tuber* species, both fructifications and mycelium, none of them were from the *T. aestivum* group. The main goal of the present study was to reveal, for the first time, the occurrence of the summer truffle in Portugal, and hypothesize about the reasons behind the late detection of this species in this territory. This finding, apart from its ecological importance, opens new opportunities to explore this delicacy in Portuguese gastronomy and can also become an economic asset for the country.

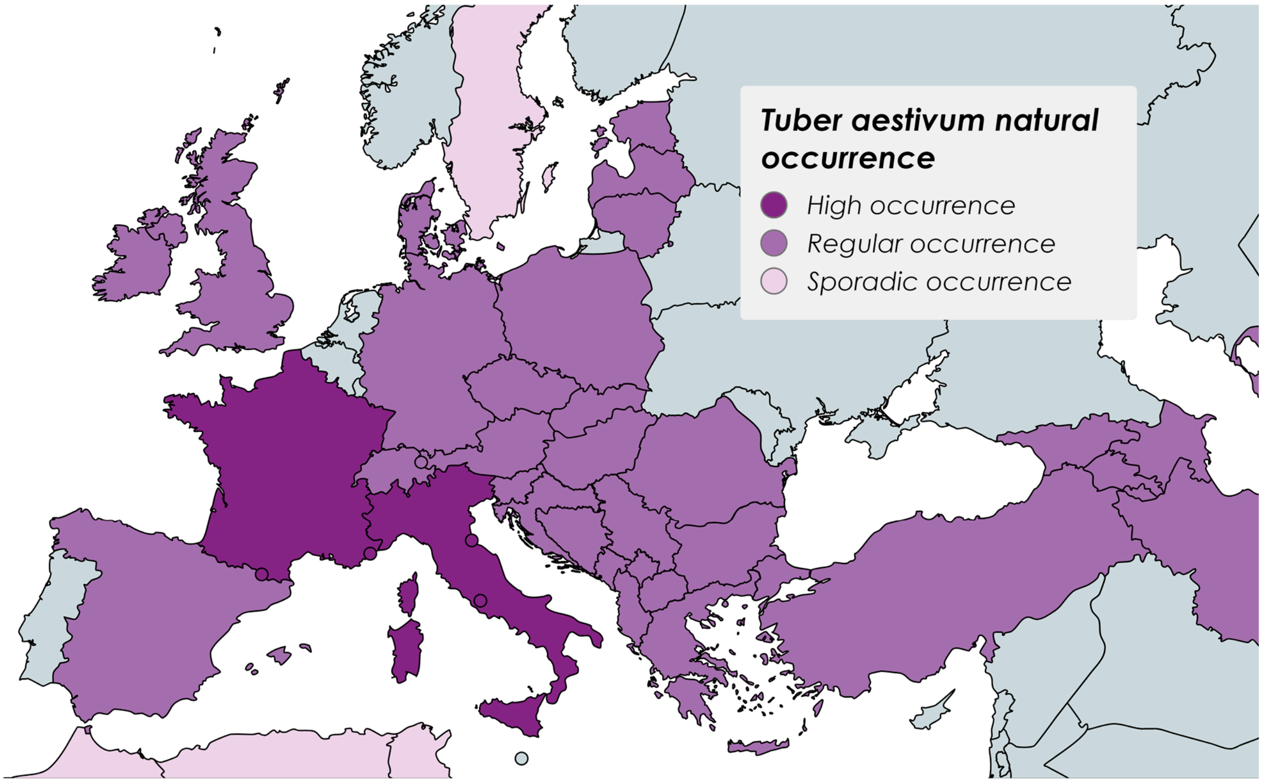

Figure 1. Countries with *Tuber aestivum* natural occurrence. Data retrieved from [15–24], and created with mapchart.net.

2. Materials and Methods

2.1. Truffle Collection and Location

Truffles were collected between April and June 2024, with the assistance of trained dogs, at 3 different locations, in the center (Alenquer and Arruda dos Vinhos) and southern (Salir, Faro) Portugal. Putative tree hosts and organoleptic features of the collected truffles were registered. Fresh truffle specimens were photographed in the field and brought to the laboratory for morphological studies and molecular analysis. Small fragments of each specimen were frozen at −20 °C for DNA amplification, and the remaining were dried at 40 °C and stored in sealed plastic bags, labeled with collection details. The samples were deposited at the Évora University Herbarium (UEVH-FUNGI), Portugal.

2.2. Morphological Study

The external characteristics of ascocarp, including shape, color, and appearance, were recorded in detail from fresh specimens. The ascomata were then dissected, and the morphology of the peridium and gleba was described. Microscopic examination was performed using distilled water and KOH 5%. Spore dimensions were determined based on the measurements of randomly selected (≈100) mature spores, outside asci, mounted in distilled water. Asci and ascospores were examined using a Leica DM750 microscope equipped with a digital camera (Leica ICC50W, Wetzlar, Germany). For species-level determination, ascomata features were compared with descriptions provided by Leonardi et al. [10].

2.3. DNA Extraction, ITS Amplification and Sequencing

Genomic DNA was extracted from approximately 100 mg of fresh ascocarp tissue using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Freiburg, Germany), following the manufacture's protocol. The ribosomal internal transcribed spacer (ITS) region was amplified via a polymerase chain reaction (PCR) using standard primer pairs

ITS4 and ITS5 primers [25]. Each PCR reaction was performed in a total volume of 25 µL, containing 1× DreamTaq^{TM} Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 7.5 pmol of each forward (ITS5) and reverse primers (ITS4), and approximately 20 ng of total DNA. The PCR program for ITS region amplification was as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 54 °C for 45 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. PCR products were visualized on 1% agarose gels in 1× TAE buffer, stained with GreenSafe Premium (NZYtech Lda, Lisbon, Portugal), and the GeneRuler 100 bp Plus DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as a molecular weight marker. The PCR products were purified using the DNA Clean & Concentrator kit (Zymo Research, Freiburg, Germany), and sequenced using the Sanger method with the ITS4 primer by Stab Vida (Lisbon, Portugal).

2.4. Phylogenetic Analyses

The sequence reads were analyzed and edited using BioEdit v.7.2.5 [26]. Trimmed sequences were deposited in the GenBank of the National Center for Biotechnology Information (NCBI) under accession numbers PP928456 to PP928462. For phylogenetic analysis, reference ITS sequences representing the closest relatives of the *Tuber aestivum* were selected, and the sequences of high nucleotide similarity were retrieved from the GenBank using the BLAST algorithm [27]. A total of 70 *Tuber* ITS rDNA sequences were used for phylogenetic analysis by including six new sequences of *T. aestivum* and 64 sequences from GenBank, which correspond to 22 *Tuber* taxa mainly belonging to Aestivum clade and of European distribution (Table 1). *Choiromyces venosus* (Fr.) Th. Fr. (JF300146, JF300147) and *C. magnusii* (Mattir.) Paol. (AH19770) were used as the outgroup.

Table 1. Details of the ITS sequences used for the construction of the phylogenetic tree.

Species	Sample Code	GenBank Accession No.	Origin
Tuber aestivum	S1/2TA	KJ524524	Poland
Tuber aestivum	BG9TA	KJ524523	Poland
Tuber aestivum	TA-GS	KC330220	Poland
Tuber uncinatum	01UNC	FM205618	Spain
Tuber aestivum	I1TA	KJ524528	Italy
Tuber aestivum	HKAS70296	JQ348412	France
Tuber aestivum	E61	AF516783	Italy
Tuber aestivum	HKAS70297	JQ348413	France
Tuber aestivum	E2	AF516781	Italy
Tuber aestivum	E5	AF516791	Italy
Tuber aestivum	E24	AF516792	Italy
Tuber aestivum	HKAS70292	JQ348411	Sweden
Tuber aestivum	HKAS70291	JQ348410	Sweden
Tuber aestivum	2SL	KX028765	Slovakia
Tuber uncinatum	228	AJ492199	Italy
Tuber aestivum	1SL	KX028764	Slovakia
Tuber aestivum	NW1TA	KJ524527	Poland
Tuber aestivum	E17	AY226042	Italy
Tuber aestivum	E60	AF516789	Italy
Tuber aestivum	**UEVH 2005855**	**PP928456**	**Portugal**
Tuber aestivum	**UEVH 2005856**	**PP928457**	**Portugal**
Tuber aestivum	**UEVH 2005859**	**PP928458**	**Portugal**
Tuber aestivum	**UEVH 2005862**	**PP928460**	**Portugal**
Tuber aestivum	**UEVH 2005863**	**PP928461**	**Portugal**
Tuber aestivum	**UEVH 2005864**	**PP928462**	**Portugal**
Tuber sinoaestivum	HKAS59100	JN896356	China
Tuber sinoaestivum	HKAS59101	JN896357	China
Tuber sinoaestivum	HKAS59102	JN896358	China
Tuber sinoaestivum	HKAS59111	JN896352	China

Table 1. *Cont.*

Species	Sample Code	GenBank Accession No.	Origin
Tuber mesentericum	ZB 2228-BPU	OL711606	Hungary
Tuber mesentericum	AQUI 6558	OL711588	Greece
Tuber mesentericum	TmsW088-W047	AJ888044	Sweden
Tuber mesentericum	UASWS1873	KY197989	
Tuber mesentericum	HKAS70294	JQ348414	France
Tuber mesentericum	M1	AF516794	
Tuber mesentericum	M13	AF516793	
Tuber pulchrosporum	GK3801	MK113979	Greece
Tuber pulchrosporum	VK4482	MK113980	Greece
Tuber pulchrosporum	1945 F8517	MK113981	Bulgaria
Tuber pulchrosporum	1961 F0388	MK113982	Bulgaria
Tuber panniferum	JT12835	HM485380	Spain
Tuber malenconii	02MLC	FM205597	Spain
Tuber malenconii	17110	JF908743	Italy
Tuber magnatum	JT19460	HM485374	Italy
Tuber magnatum	Ma2	AF106888	Italy
Tuber magnatum	GB12	JQ925645	Italy
Tuber magnatum	B6	AJ586271	Italy
Tuber magnatum	GO2	AJ586251	Croatia
Tuber macrosporum	HUN_001H	KP738366	Hungary
Tuber macrosporum	ITA_011A	KP738395	Italy
Tuber pustulatum	AQUI 9725	MK211278	Spain
Tuber pustulatum	AQUI 9728	MK211282	France
Tuber malacodermum	JT32319	FJ809889	Spain
Tuber melosporum	AH31737	JN392144	Spain
Tuber umbilicatum	T2_HKAS44316	GU979031	
Tuber huidongense	SDBR-CMU-MTUF006	KT758730	Thailand
Tuber rufum	06RUF	FM205602	Slovenia
Tuber rufum	TUBRUF/141207	FM205636	Slovenia
Tuber ferrugineum	MUB:Fung-0972	MN962719	Spain
Tuber brumale	T.bru-eu03	DQ329360	France
Tuber brumale	GB52	HM485345	Italy
Tuber brumale	GB53	FJ748900	Italy
Tuber indicum	GB236	FJ748906	China
Tuber melanosporum	A3	AF106873	Italy
Tuber pseudoexcavatum	HKAS 39504	AY514310	China
Tuber spinoreticulatum	RH158	GQ221454	USA
Tuber excavatum	M1TE	KJ524534	Poland
Tuber excavatum	NW1TE	KJ524535	Poland
Tuber gennadii	B M1904	HM485361	Italy
Tuber gennadii	A JT9627	HM485360	Spain
Outgroup			
Choiromyces venosus	AH38915	JF300146	Italy
Choiromyces venosus	AH38904	JF300147	Romania
Choiromyces magnusii	AH19770	JF300143	Spain

Sequences newly generated in this study are in bold.

Sequence alignment was performed using the online version of the multiple-sequence alignment program MUSCLE 3.8.425 [28] and checked manually. The alignment comprised 1249 characters, with 550 parsimony-informative sites. This generated alignment was used for phylogenetic analyses and evolutionary model selection using IQ-TREE v 1.6.12 [29,30]. The best evolutionary model for the ITS marker, as selected by ModelFinder [31], was TNe + I + G4. Both maximum likelihood (ML) and Bayesian methods were used for phylogenetic analyses. The resulting tree is the consensus of 1000 replicates of two phylogenetic analyses; an ultra-fast maximum likelihood analysis [32] with 1000 bootstrap replicates, complemented with a Bayesian approximation branch support analysis [33]. The branch lengths of consensus tree were optimized by maximum likelihood on original alignment. The tree was visualized and edited using iTOL v 6.9.1 [34]. The Robinson–Foulds distance between the ML tree and the consensus tree of 2 indicates that no significant conflicts were found between the ML and Bayesian consensus tree topologies.

3. Results

3.1. Taxonomical Characterization

Morphological and anatomical features of the collected specimens agreed with those provided by Leonardi et al. [10] (Figure 2).

Figure 2. *Cont.*

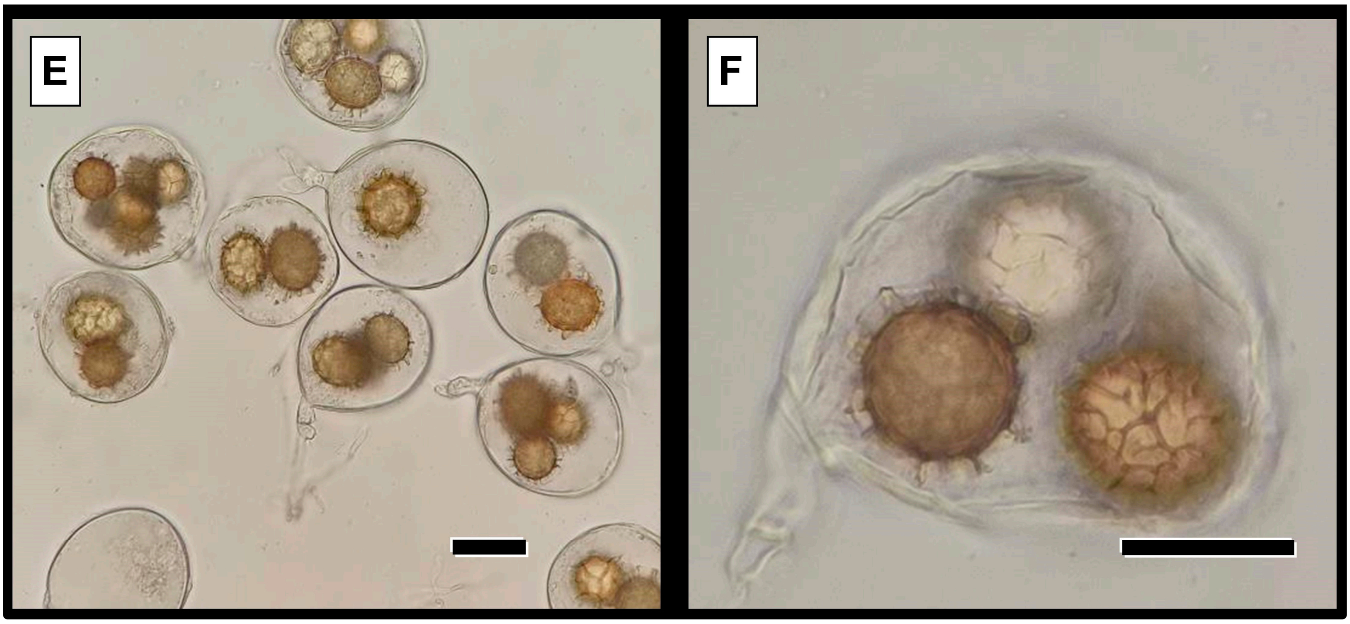

Figure 2. *Tuber aestivum* specimens collected in Portugal. (**A**) Left to right: Giovanni Longo, Pina (dog) and Tanka Sapkota, at 1016 sample location site (https://www.nit.pt/wp-content/uploads/2024/06/49bdcfdd344747a0f30a145d5f6625b1-e1717409790694.jpg, accessed on 28 June 2024). (**B**) Left to right: Celeste Santos-Silva, Larissa Müller and Figo (dog) at 1022 sample location site (Algarve Truffle Group). (**C**) *T. aestivum* ascomata (Larissa Müller). (**D**). *T. aestivum* gleba (Celeste Santos-Silva). (**E**,**F**) *T. aestivum* asci and ascospores (Celeste Santos-Silva). Bars: (**C**,**D**) = 1 cm; (**E**,**F**). = 30 µm.

Family Tuberaceae Dumort.

Genus *Tuber* P. Micheli ex F.H. Wigg.

Tuber aestivum (Wulfen) Spreng.—Portugal, Arruda dos Vinhos (Lisboa), under *Pinus pinea* L. stand, 30-IV-2024, sample n. 1015, UEVH 2005855, GenBank PP928456; Portugal, Arruda dos Vinhos (Lisboa), under *Pinus pinea* L. stand, 20-V-2024, sample n. 1016, UEVH 2005856, GenBank PP928457 (Figure 2A); Portugal, Alenquer (Lisboa), under *Pinus pinea* L. stand, 26-V-2024, sample n. 1019, UEVH 2005859, GenBank PP928458; Portugal, Alenquer (Lisboa), under *Pinus pinea* L. stand, 26-V-2024, sample n. 1020, UEVH 2005860, GenBank PP928459; Portugal, Salir (Loulé, Faro), under *Quercus rotundifolia* Lam. stand, 09-VI-2024, sample n. 1022, UEVH 2005862, GenBank PP928460 (Figure 2B); Portugal, Salir (Loulé, Faro), under *Quercus rotundifolia* Lam. stand, 09-VI-2024, sample n. 1023, UEVH 2005863, GenBank PP928461; Portugal, Salir (Loulé, Faro), under *Quercus rotundifolia* Lam. stand, 09-VI-2024, sample n. 1024, UEVH 2005864, GenBank PP928462.

3.2. Phylogenetic Analyses

Phylogenetic analyses show that our specimens are assigned to the *T. aestivum* nest within the Aestivum clade, which is congruent with the morphological analyses (Figure 3). The consensus tree of Bayesian approximation and maximum likelihood resolves the *T. aestivum*/*T. uncinatum* as an independent, monophyletic and well-supported clade. This species appears as the sister clade of *T. sinoaestivum*. In addition, the ITS sequence of two Portuguese specimens, *T. aestivum* voucher UEVH 2005863 and *T. aestivum* voucher UEVH 2005864, had a 100% nucleotide similarity with the ITS sequence of the *T. aestivum* strain E17 from Italy, while the ITS sequence of *T. aestivum* voucher UEVH 2005859 and *T. aestivum* voucher UEVH 2005862 had a 100% nucleotide similarity with the *T. aestivum* strain E60 from Italy.

Figure 3. Phylogenetic placement of *Tuber aestivum* specimens obtained in this study (bold) in the Aestivum clade. The consensus tree represents a Bayesian approximation with 1000 generations and a maximum likelihood analysis with 1000 bootstrap replicates. The tree is based on the ITS rDNA sequence alignment of 70 sequences assigned to 22 *Tuber taxa*. ITS rDNA sequences of *Choiromyces venosus* and *C. magnusii* were used as the outgroup. Bootstrap supports are shown at the nodes of the branches.

4. Discussion

The Aestivum clade comprises species associated with a wide range of host plants and is found in Europe, North Africa, and Asia [35], and most recently in America [1]. Notable species include *T. aestivum* Vittad. (the type species of this clade), *T. panniferum* Tul. and Tul., *T. malenconii* Donadini, Riousset, G. Riousset and G. Chev., and *T. mesentericum* Vittad., *T. sinoaestivum* Zhang and Liu from China, *T. magnatum* Picco and along with the recently described *T. pulchrosporum* Konstantinidis, Tsampazis, Slavova, Nakkas, Polemis, Fryssouli and Zervakis. *Tuber aestivum* (Wulfen) Spreng. (including *T. uncinatum* Chatin) and *T. sinoaestivum* J.P. Zhang and P.G. Liu specimens can be easily distinguished macroscopically by their blackish peridial surface with prominent pyramidal warts and ascospores bearing a complete reticulum. Both macro- and microscopical characteristics align with the *T. aestivum* descriptions provided by Leonardi et al. [10]. Additionally, molecular analysis revealed that the specimens collected in Portugal cluster with the *T. aestivum* specimens from other locations, confirming their identification as *T. aestivum*. Therefore, surprisingly, this is the first report of *T. aestivum* in mainland Portugal based on morphological and molecular analyses.

Several factors might explain why this is the first time the summer truffle has been reported in Portugal. One possible explanation is the use of trained dogs, which detect truffles underground without any surface indication. However, it is important to note that the initial signs of truffle presence were observed due to soil disturbance caused by wild boars, which unearthed some truffles. Another possible explanation is the exceptional weather conditions preceding the current fruiting season. Precipitation from September 2023 to May 2024 was above the climatological norm for six out of nine months (Figure 4). Notably, October 2024 was the fourth wettest month since 1941. Additionally, air temperatures during this period were higher than average. It is thus likely that the unusually warm and wet weather conditions likely contributed to the atypical fruiting of *T. aestivum* in 2024, which, in combination with animal behavior (wild boars), allowed for the detection of these species. Another possible explanation for the detection of summer truffle only in the present year could be related to shifts in habitat conditions or climate change, which might have made these regions suitable for the presence of this fungus and consequently its fruit body development.

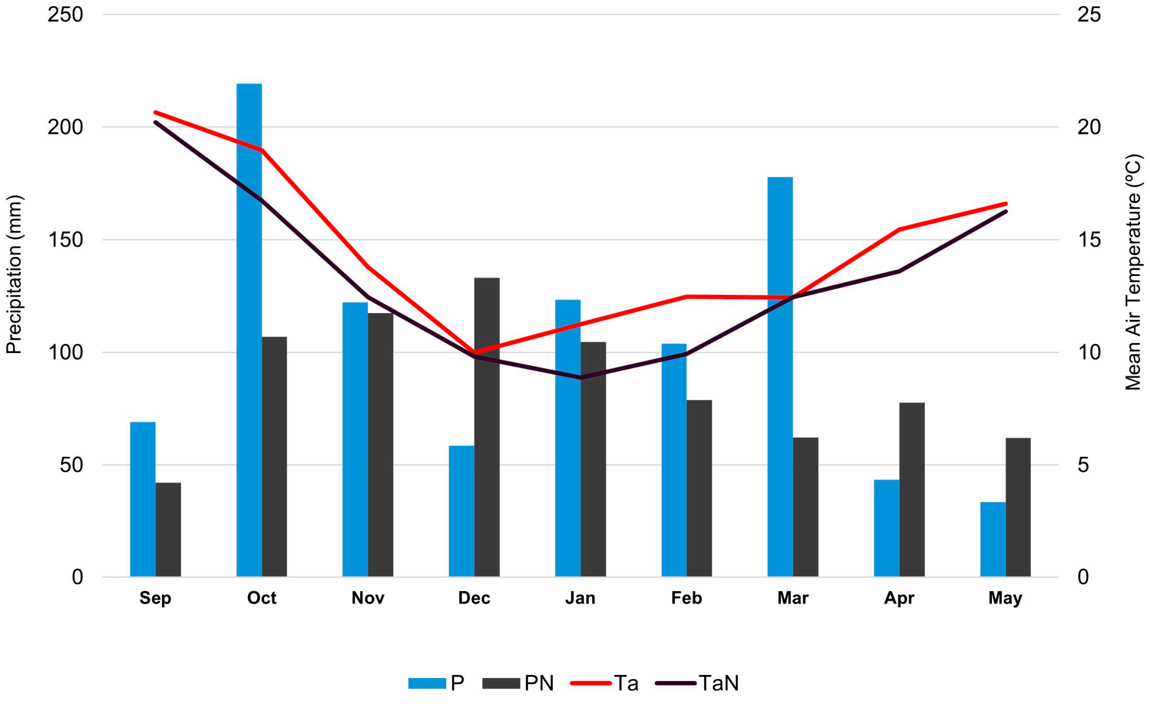

Figure 4. Continental Portugal month precipitation (P) and the mean air temperature (Ta) from September 2023 to May 2024. Climatological standard normals (1941–2023) per month for precipitation (PN) and mean air temperature (TaN) (data from https://www.ipma.pt/pt/publicacoes/, accessed on 26 June 2024).

More studies are being conducted in areas where *T. aestivum* has been discovered to determine the biotic and abiotic soil conditions that may explain the appearance, persistence, and fruiting of summer truffles. These studies could also provide insights into other potential locations for these truffles. Targeted field trips with trained dogs will follow to investigate the presence of *T. aestivum* and identify its possible hosts. Pursuing this line of research can reveal new ecological relationships between truffles, their host trees, and other organisms, and deepen our understanding of the intricate web of life.

Author Contributions: C.S.-S.: conceptualization, specimen collection, morphological analysis, validation, investigation, resources, writing—draft and revision, funding acquisition; C.B.: molecular analyses, validation, investigation, resources, writing—draft and revision. All authors have read and agreed to the published version of the manuscript.

Funding: This work is financed by National Funds through FCT—Foundation for Science and Technology within the scope of project UIDB/05183/2020.

Data Availability Statement: The original contributions presented in the study are included in the article; further inquiries can be directed to the corresponding author.

Acknowledgments: We thank to MED (https://doi.org/10.54499/UIDB/05183/2020 (accessed on 5 August 2024); https://doi.org/10.54499/UIDP/05183/2020 (accessed on 5 August 2024)) and to CHANGE (https://doi.org/10.54499/LA/P/0121/2020 (accessed on 5 August 2024)). We are grateful to Tanka Sapkota and to Larissa Möller, Nelli Eleonore dos Santos (Algarve Truffle Group (ATG)), Figo and Nala (Larissa and Nelli dogs). We sincerely thank Tânia Nobre for her help in refining the English language of the manuscript.

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

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