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FIRST REPORT ON TRUFFLE-INHABITING FUNGI AND METAGENOMIC COMMUNITIES OF *TUBER AESTIVUM* COLLECTED IN RUSSIA

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Microbial community

Symbionts

Fusarium sp.

Clonostachys sp.

Plectosphaerella sp.

Trichothecium sp.

ABSTRACT

Truffles are one of the least studied groups of fungi in terms of their biological and biotechnological aspects. This study aimed to isolate truffle-inhabiting fungi and assess the metagenomic communities of the most common Russian summer truffle, *Tuber aestivum*. This study is the first to characterize the biodiversity of prokaryotic and eukaryotic organisms living in the truffle *T. aestivum* using molecular analysis and sequencing. Plant pathogens involved in a symbiotic relationship with truffles were identified by sequencing the hypervariable fragments of the 16S rRNA and 18S rRNA genes. In addition, some strains of fungal symbionts and likely pathogens were isolated and recognized for the first time from the truffles. This study also compared and characterized the general diversity and distribution of microbial taxa of *T. aestivum* collected in Russia and Europe. The results revealed that the Russian and European truffle study materials demonstrated high similarity. In addition to the truffles, representatives of bacteria, fungi, and protists were found in the fruiting bodies. Many of these prokaryotic and eukaryotic species inhabiting truffles might influence them, help them form mycorrhizae with trees, and regulate biological processes. Thus, truffles are interesting and promising sources for modern biotechnological and agricultural studies.

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1 Introduction

Truffles belong to the group of hypogean (underground) ascomycetes. This order includes approximately 80–220 species characterized by underground fruiting bodies, usually truffles (Leonardi et al. 2021a). The fruiting bodies of truffles have a round or tuber-like shape, and their texture can be fleshy or cartilaginous. Depending on the species, truffles can vary in size from that of a hazelnut to that of a large potato. The outer part of the truffle forms a skin layer called the peridium, which can be smooth, cracked, or covered with large polyhedral tubercles. When cut, the fleshy tissue inside the truffle has a distinct marbled pattern with alternating light and dark (Taschen et al. 2022). The asci, which contain spores, are located inside the fruiting body on internal veins, forming a layer similar to the hymenium. The asci can also be distributed in a nest-like pattern within the truffle, a characteristic trait. The shape of the asci can be spherical, broadly oval, club-shaped, sac-shaped, or sometimes cylindrical. They can contain one, two, four, or eight spores (Ljubojević et al. 2022). The ascospores are always unicellular, colourless or brown, and globular or elliptical. One ascus can reach 0.08 mm in size, while the spores can reach 0.02 mm under normal conditions. The surfaces of truffles are typically rough or spiky. Spores are released passively when the ascocarp is destroyed or when the truffle is consumed by an animal (Thomas and Thomas 2022).

Truffles obligatorily form mycorrhizae, and they grow near taller plants. Typically, black truffles such as *Tuber melanosporum* and *T. aestivum* grow in forests with deciduous trees such as oak, beech, hornbeam, and hazel. These trees and special soil types provide favourable conditions for truffle growth (Vlahova 2021). White truffles, such as *T. magnatum*, grow in forests with birch, poplar, elm, lime, willow, mountain ash, and hawthorn trees (Leonardi et al. 2021b). Truffles also form mycorrhizae with juniper, fir, and pine trees (Allen and Bennett 2021).

Among the truffles with practical value, the most valuable and important is the black French truffle, which is related to *T. melanosporum* (Perigord truffle) (Oliach et al. 2022). The aroma, taste, and biomedical value of true black truffles drive a constant high demand for these mushrooms (Dogan 2021). Moreover, many pharmaceutical and cosmetic companies produce products with black truffle extracts.

Currently, in the agricultural field, there is an increasing demand to understand the connections between the bacterial communities associated with the ascomata of *Tuber* spp. and those found in the surrounding soils. Additionally, there is a need to investigate the unique microbial diversity present in areas where truffles are cultivated and harvested (Sillo et al. 2022). Due to the symbiotic nature of truffles, artificial growth and planting are complicated compared with these processes for other gastronomic fungi. Microbial communities associated with truffles have been characterized using different microbiological and molecular ecological methods (Perlińska-Lenart et al. 2020a; Bragato et al. 2021). Such studies have focused mainly on bacteria and rarely on the yeasts and fungi inhabiting *T. magnatum*, *T. melanosporum, T. borchii*, and *T. aestivum;* therefore, the fungi and bacteria inhabiting truffles are less characterized. Researchers worldwide do not agree that each truffle species has its unique microbiome. Some studies have shown that the symbiotic composition of truffle mushrooms correlates with the microbiological composition of the soil in which they grow (Liu et al. 2021a). This study aimed to isolate truffle-inhabiting fungi and assess the metagenomic communities of the most common summer truffle (*Tuber aestivum*) collected in Russia.

2 Materials and Methods

2.1 Sampling and description of true truffles

The summer truffle (*Tuber aestivum*) has a large fruiting body, up to the size of a chicken's egg. The shape is irregularly rounded. The peridium is covered with large, hard, slightly striped polyhedral tubercles with black–brown colouring. The gleba of *T. aestivum* is yellowish-white, light brown, or chocolate-coloured when heated. The growth of the fruiting bodies is shallow (near the ground surface), sometimes even in the foliage or under oak, beech, birch, pine, and hazel trees, and especially under hornbeams in clay-lime soil. Because *T. aestivum* is on the Red List of Threatened Species of the Russian Federation, so, permission from the Federal Service for Supervision of Natural Recourses was obtained for this study (Permission No. 183, 70A-10-04-GU/10015 on 23/09/2022).

For this study, truffles ranging in size from 3 to 5 cm were collected in a hornbeam forest in the Matsesta area (Sochi, Krasnodar region, Russia). The humus-calcareous and podzolic yellow earth soil types are predominant in this area. During sampling, the pH ranged from 6.0 to 7.0, and the air temperature ranged from 20 to 27 °C. The fruiting bodies of the truffles were found in August with the help of trained truffle-hunting dogs and were collected using a rake to keep them intact. Then, the truffles were transferred to Irkutsk by air post under temperature-controlled conditions at 6-10 °C, along with some soil and rice to avoid putrefaction.

Truffle dissection and spore microscopy were carried out in the laboratory. The fruiting bodies of the truffles were cleaned with a soft toothbrush and then sterilized with 70% ethyl alcohol. Then, the truffles were fractured, and pieces of the gleba were collected for microbiological and metagenomic analysis. For microscopic analysis, a piece of gleba was homogenized and crushed between glass layers. Observations were made using an immersion system at a magnification of 1000× on a bright-field microscope (Mikromed-3–20, Russia, Saint Petersburg) equipped with a digital camera.

2.2 Isolation of nucleic acids, sequencing, and phylogenetic analysis

Two to five pieces of the gleba were dissected from each truffle. Each piece was approximately 0.5 × 0.5 × 0.5 cm in size. The biomaterials were homogenized in TE buffer (10 mM Tris–HCl, 1 M EDTA) and transferred to BioSpark LLC (Russia, Moscow) for DNA isolation, sequencing, and basic bioinformatic analysis.

Total DNA was extracted using a DNeasy Plant Mini Kit (Art. 6910, Qiagen, Germany, Hilden). Fragments of the 16S rRNA gene containing the hypervariable regions V3–V4 were amplified using a mixture of eubacterial primers. The fungal internal transcribed spacer was amplified using primers for the hypervariable ITS2 region of the 18S rRNA gene. The amplification was performed in 25 µL of a mixture containing 5x KTN-mix (Evrogen, Russia, Moscow) (5 µL), primer mix (2 µL), and 50x SYBR (0.5 µL) in a real-time amplifier CFX96 Touch (Bio-Rad, USA, CA). The nucleotide sequences of the primers and the amplification program are presented in Table 1.

Then, the libraries were synthesized for sequencing. Amplification of the PCR products obtained at the first stage for the purpose of barcoding (indexing) the libraries was carried out in 25 µL of a mixture containing 5x KTN mix (5 µL), the primer mixture (2 µL), and 50x SYBR (0.5 µL) in a real-time amplifier under the following conditions: primary denaturation for 3 min at 95 °C; 7 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 55°C, and elongation for 30 s at 72°C; and a final elongation for 5 min at 72 °C. The Nextera Index Kit was used for amplification with the indices recommended by the manufacturer (Illumina, USA, San Diego).

Sequencing was performed on the Illumina platform. After the second stage, the amplicons were purified using AMPure XP magnetic particles (Kapa Biosystems, United Kingdom, London) at the ratios of 1:0.6 for purification of the PCR products of the 16S rRNA genes and 1:0.7 for purification of the PCR products using primers for amplification of the hypervariable ITS2 region of the 18S rRNA gene. These purified amplicons are ready-made libraries for multiplex sequencing on the Illumina platform. The libraries were mixed and brought to a total concentration of 2 nM. Next, 5 µL of 0.2 M NaOH was added to 5 µL of the mixture and incubated for 5 min. Then, 990 µL of HTI and 1 µL of 12.5 mM predenatured PhyX (Kapa Biosystems, United Kingdom, London) were added to the denatured DNA. Library sequencing was performed on the Illumina MiSeq next-generation sequencer using the paired-end read method, generating at least 10,000 paired reads per sample and using reagents from the MiSeq Reagent Nano Kit v2 and the MiSeq v2 Reagent Kit (Illumina, USA, San Diego) (500 cycles PE).

The obtained sequencing data were processed using QIIME 1.9.1. The program included alignment of the forward and reverse reads and deletion of the technical sequences. Sequences with low quality (less than Q30) and chimeric sequences were removed. The

Table 1 Mixture of primers used for sequencing metagenomic communities

Type of primer	Name	Sequence 5'–3'	Concentration µM	Amplification program
Amplification of the hypervariable region V3-V4 of the 16S rRNA gene				
F	GPro341F	CCTACGGGNBGCASCAG	0.625	95 °C for 3 min (initial denaturation); 95 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s (35 cycles); 72 °C for 5 min (final extension step)
R	GPro806R	GGACTACNVGGGTWTCTAATCC	0.625	
F	NR_16_341F1	TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGTGCCTACGGGNBGCASCAG	2.5	
F	NR_16_341F2	TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCTGCCTACGGGNBGCASCAG	2.5	
F	NR_16_341F3	TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGTCTGCCTACGGGNBGCASCAG	2.5	
R	NR_16_806R1	GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGCCGGACTACNVGGGTWTCTAATCC	2.5	
R	NR_16_806R2	GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGACCGGACTACNVGGGTWTCTAATCC	2.5	
R	NR_16_806R3	GTCTCGTGGGCTCGGAGATGTGTATAAGAG ACAGAACCGGACTACNVGGGTWTCTAATCC	2.5	
Amplification of the hypervariable region ITS2 of the 18S rRNA gene				
F	NR_5.8SR	TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGATCTCGATGAAGAACGCAGCG,	5	95 °C for 3 min (initial denaturation); 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s (7 cycles); 72 °C for 5 min (final extension step).
R	NR_ITS4R	GTCTCGTGGGCTCGGAGATGTGTATAAGA GACAGGCATCCTCCGCTTATTGATATGC	5	

Table 2 The list of bacterial phyla associated with *T. aestivum* collected in different countries.

Fungus	Country	Bacterial diversity (%)					Ref.
		Proteobacteria	Bacteroidetes	Firmicutes	Actinobacteria	Others	
Tuber aestivum	Italy	76.5	4.7	5.15	13.65	-	Monaco et al. 2020
	France	88	-	5	-	7	Vahdatzadeh et al. 2019
	Russia	99.8	0.04	0.09	0.01	0.1	

distribution of sequences by taxonomic units was determined using the SILVA database (version 132) and UNITE v8. A classification algorithm for operational taxonomic units (OTUs) with an open reference and a threshold of 97% was used.

The sequences were compared with the known 16S rRNA and ITS gene sequences and analyzed using BLAST. The data obtained from metagenomic analysis of prokaryotic and eukaryotic communities were registered in the NCBI database as Bioproject PRJNA1026823.

The evolutionary relationships among the taxa were inferred using the maximum likelihood method (Tamura and Nei 1993), with *Morchella* sp. (NCBI ID KY462590.1) serving as an outgroup. This analysis involved 41 nucleotide sequences. The final dataset contained a total of 600 positions. Evolutionary analysis was conducted in MEGA X (Kumar et al. 2018). The nucleotide sequences were deposited in the NCBI database under the accession numbers ON398998 and ON391736–ON391740.

2.3 Isolation and molecular identification of *T. aestivum* fungal symbionts

The gleba pieces that were aseptically dissected from the central part of *T. aestivum* were homogenized and plated on solid nutrient media. Nutrient media such as malt and potato–dextrose agar were used to isolate the fungal symbionts from the truffles. The composition of the nutrient media was as follows: the malt agar included malt extract (20 g/L), glucose (20 g/L), peptone (1 g/L), and agar (20 g/L), while the composition of the potato–dextrose agar was potato extract (20 g/L), glucose (50 g/L), peptone (30 g/L), $MgSO_4$ (5 g/L), K_2HPO_4 (10 g/L), and agar (20 g/L).

Truffle-associated fungal mycelial strains were isolated under thermostatic conditions at 24 °C. Only strains characterized by a white aerial mycelium were collected. Each fungal mycelial colony was transferred into Petri dishes with fresh nutrient media. Then, the isolated fungi were cultivated in liquid nutrient media for DNA isolation. Inoculation of pure cultures was performed in sterile 250–300 mL conical flasks with deflectors with the addition of glass beads. Cultivation was performed under thermostatic conditions at 22-24 °C with constant stirring at 180 rpm on an orbital shaker for 5 days.

The samples were ground using steel balls in a laboratory ball mill to isolate fungal DNA. Next, the potassium acetate method was used for DNA isolation and PCR analysis (Rodríguez et al. 2014). The following primers were used to perform PCR on the obtained fungal DNA samples: ITS5: 5'-GGA AGT AAA AGT CGT AAC AAG-3' (F); ITS7: 5'-ACT CGC CGT TAC TGA GGC AAT-3' (R) (Table 2).

PCR was performed in an automatic amplifier (Biometra, Germany) under the following conditions: denaturation for 5 min at 94 °C (first cycle), annealing for 15 s at 60 °C, and elongation for 40 s and 72 °C (subsequent cycles). The final elongation was performed at 72 °C for 7 min. The total number of cycles was 33. The PCR products were purified using a cleanup kit for purifying DNA from the agarose gel and reaction. The purified PCR products, together with the primers, were sent to Evrogen LLC for sequencing. Nucleotide sequences were sequenced using the Sanger method (Valencia et al. 2013). The contigs were assembled using BioEdit software and then uploaded to the NCBI database for comparison and identification. The nucleotide sequences were aligned with the sequences from the NCBI database that showed the highest similarity using the ClustalW multiple alignment algorithm in the MEGA-11 software package. The evolutionary relationships among the taxa were inferred using the maximum likelihood method (Tamura and Nei 1993), with *Penicillium* sp. (NCBI ID OL838180.1) serving as an outgroup. The phylogenetic tree was constructed with 1000 bootstrap replicates. Bootstrap support values above 70% are indicated in the figures. The nucleotide sequences of truffle-inhabiting fungi were deposited in the NCBI database under accession numbers ON386018.1 - ON386020.1, OR335550, and OR335570.

2.4 Statistical analysis

Metagenomic analysis was performed for 6fruiting bodies of *T. aestivum*. Mean data ± standard deviations are presented in the study. The Shannon diversity index was calculated using QIIME.

3 Results

3.1 Phylogenetic characterization of the truffles collected in Russia

Tuber aestivum, sampled for this study near Sochi, was grown in a hornbeam forest in the Matsesta area (Figure 1). The fruiting bodies and spores of *T. aestivum* are shown in Figure 2.

Figure 1 Photographs of the sampling location; left – *Tuber aestivum* found in leaf litter; right – Hornbeam forest in the Matsesta region, Sochi, Russia 43.57187 N, 39.80306 E

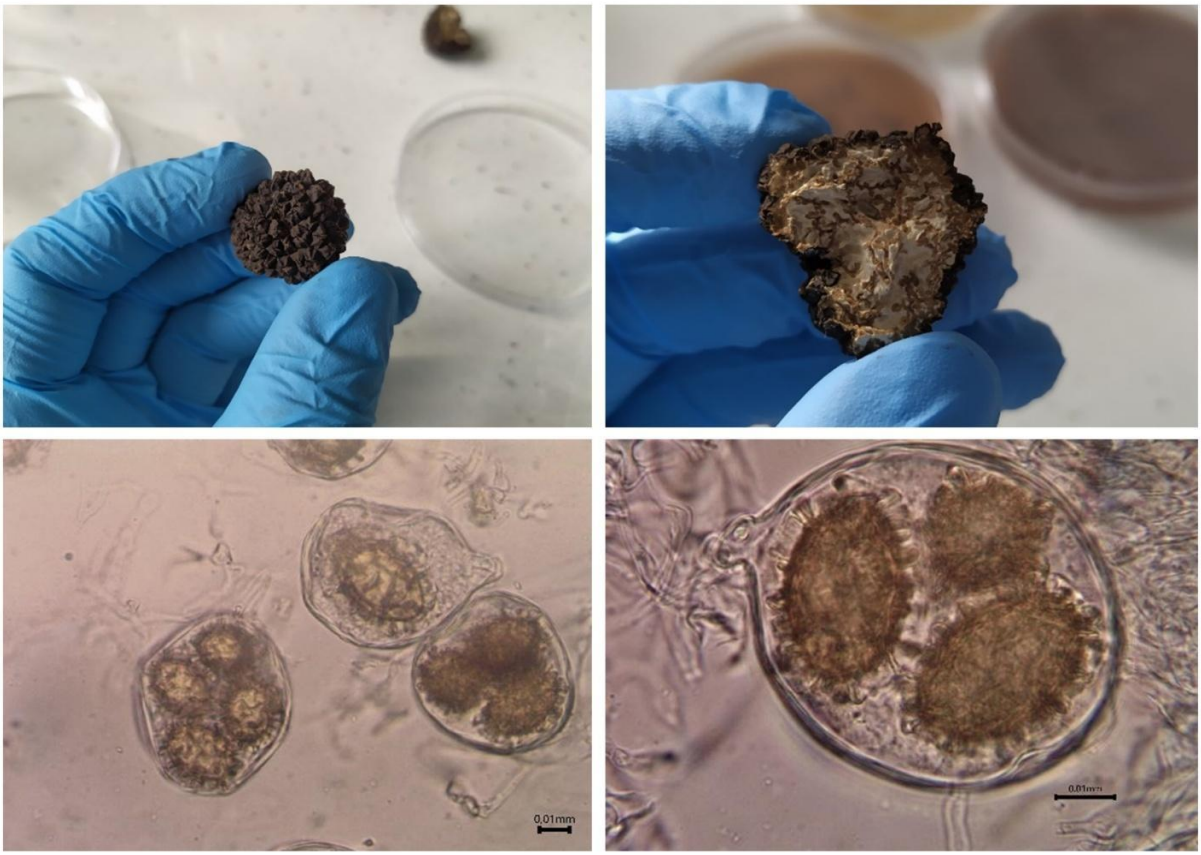

Figure 2 Images of the fruiting body (top) and spores in asci (bottom) of *T. aestivum* used in this study (Scale bars = 0.01 mm).

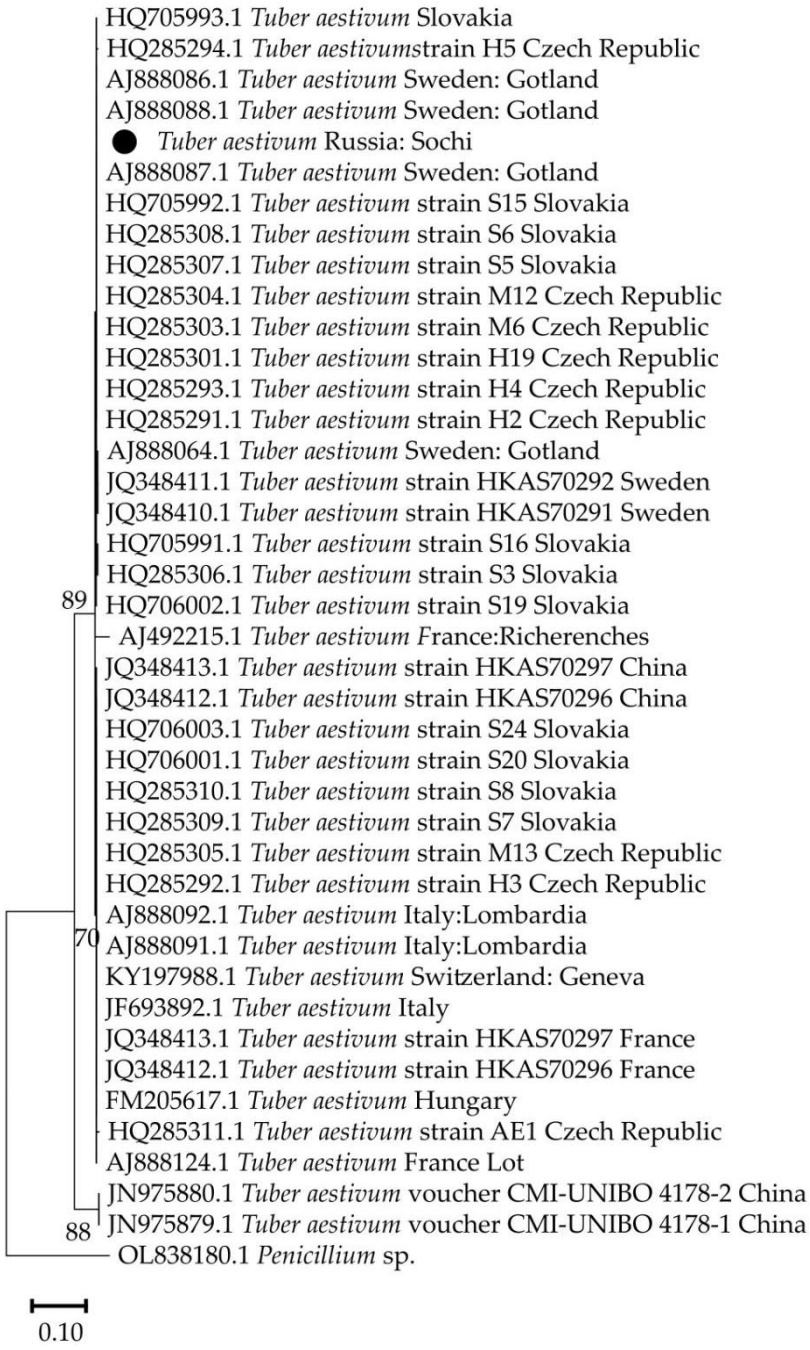

Figure 3 Evolutionary analysis via the maximum likelihood method (the black marker is the strain of *T. aestivum* found in this study).

Based on the results of this study and an analysis of the nucleotide sequences aligned with the nucleotide sequence of this study, the Russian truffle was identified as *Tuber aestivum* (summer truffle). Nucleotide sequences were aligned and compared with those of the studied summer truffle and were used in the analysis (Zambonelli et al. 2012; Zhang et al. 2012; Truong et al. 2017). The phylogenetic analysis was performed based on the fragment of the 18S rRNA gene, the nucleotide sequence of the internal transcribed spacer, and the 5.8S rRNA gene and revealed high similarity between the truffles collected in Russia and the truffles collected in Europe (Czech Republic, Slovakia, etc.). The Russian truffles (*T. aestivum*) were characterized by their high divergence from truffles collected in China (Figure 3).

The evolutionary history was inferred using the maximum likelihood method and the Tamura–Nei model (Tamura and Nei 1993). The percentage of trees in which the associated taxa clustered is shown next to the branches. The initial tree(s) for the heuristic search were obtained automatically by applying the neighbour joining and BioNJ algorithms to a matrix of the pairwise distances estimated using the Tamura–Nei model and then selecting the topology with the superior log likelihood value. The tree is drawn to scale, with the branch lengths measured as the number of substitutions per site. This analysis involved 41 nucleotide sequences, and there were 608 positions in total in the final dataset.

3.2 Characterization of the prokaryotic and eukaryotic communities of truffles collected in Russia

According to the materials used for the metagenomic analysis, the prokaryotic community of *T. aestivum* collected in Russia (Sochi) was represented by the phylum *Proteobacteria* (OTU 99.88 ± 0.11%) and several minor phyla, including *Actinobacteria*, *Bacteroides*, *Cyanobacteria*, and *Firmicutes*. The classes of microorganisms and their distribution are shown in Figure 4. The dominant class was *Gammaproteobacteria*, and representatives of *Pseudomonas* were the dominant group of bacteria inhabiting the ascomata of *T. aestivum*. The major groups of bacteria were represented by bacterial genera such as *Rahnella*, *Enterobacter*, and *Stenotrophomonas*. In addition, several minor groups of bacteria were found in the fruiting bodies of the truffles (OTU <1%). These genera included *Acinetobacter*, *Citrobacter*, *Comamonas*, *Enterohabdus*, *Klebsiella*, *Lactobacillus*, *Massilia*, *Nitrobacter*, *Nocardioides*, *Pedobacter*, *Ralstonia*, *Raoultella*, *Rahnella*, *Rhizobium*, *Sphingobacterium*, and *Staphylococcus*. Among the diverse bacteria, several were identified as *Bacillus anthracis*, *Bacillus cereus*, and *Enterobacter aerogenes.*

The eukaryotic community of the *T. aestivum* collected in Russia (Sochi) contained fungi (OTU 74.3 ± 3.17%), protists (OTU 10.6 ± 4.7), and undescribed taxa (15.06 ± 1.32%) (Figure 5). The fungal community of *T. aestivum* was represented by 13 genera and unclassified taxons: *Alternaria*, *Ascobolus*, *Cladophialophora*, *Holtermanniella*, *Hyaloscypha*, *Malassezia*, *Mycosphaerella*, *Mortierella*, *Penicillium*, *Papulaspora*, *Psathyrella*, *Rhodotorula*, and *Tylospora*. Among the diverse fungi, several were identified as the following species: *Alternaria metachromatica*, *Cladophialophora chaetospira*, *Holtermanniella festucosa*, *Mortierella polygonia*, *Papulaspora equi*, *Penicillium aethiopicum*, *Penicillium bialowiezense*, *Penicillium thomii*, *Psathyrellapygmaea*, and *Rhodotorula dairenensis*.

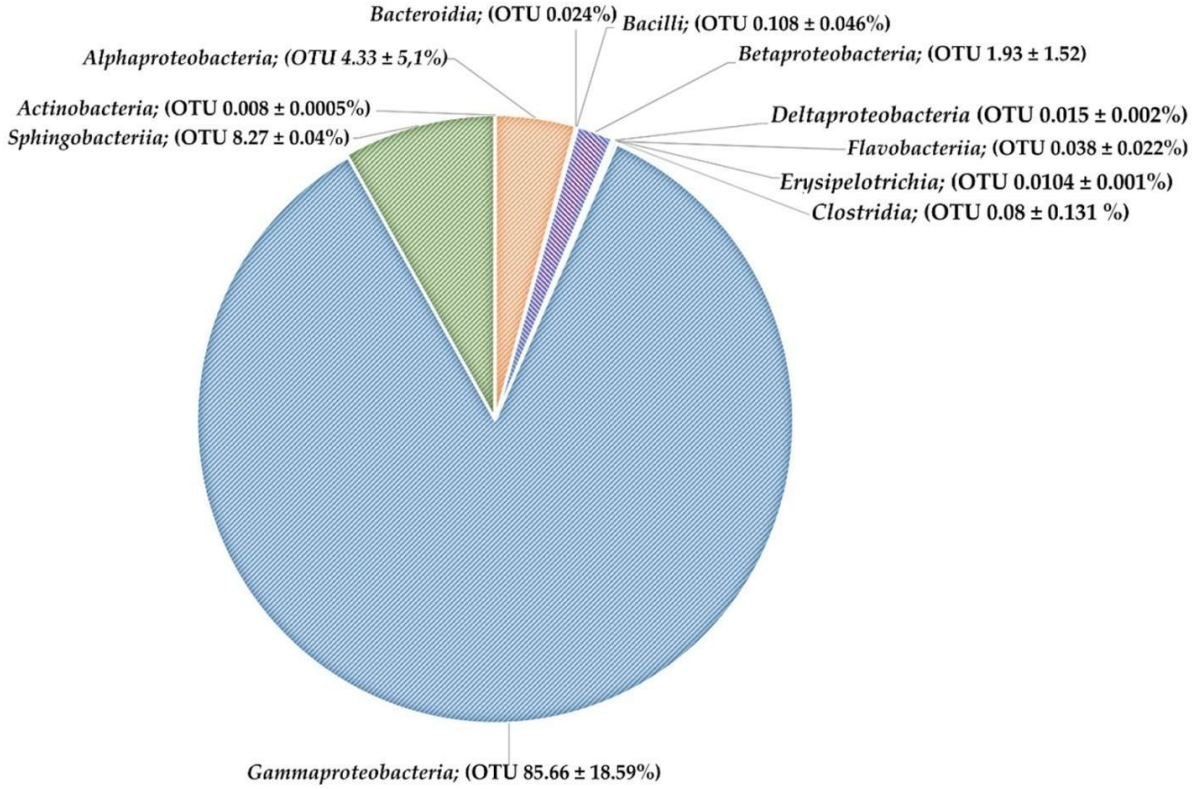

Figure 4 Total distribution of the classes of prokaryotic organisms (OTUs, %) inhabiting *T. aestivum* collected in Russia.

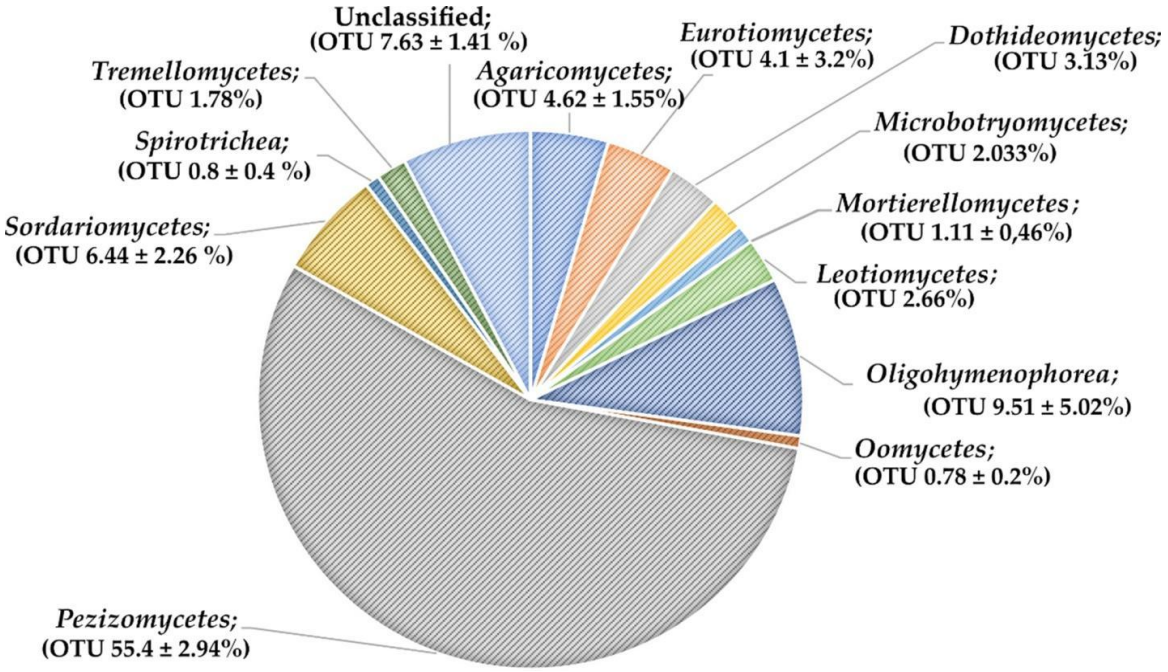

Figure 5 Total distribution of eukaryotic organisms (OTUs, %) found in the ascomata of *T. aestivum* collected in Russia.

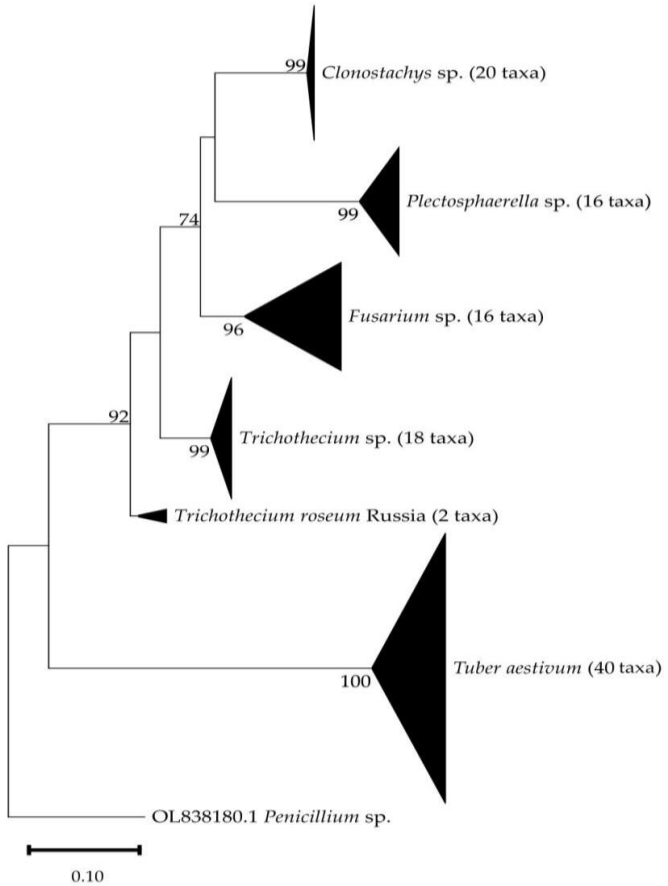

Figure 6 General topology of the phylogenetic tree of the truffle-associated fungi

This study also analyzed the diversity of bacteria, fungi, and other eukaryotes inhabiting the fruiting bodies of truffles. The microorganisms found in Russian, Italian, and French truffles were estimated. The general diversity of the microbial phyla is presented in Table 2. The results suggested a general similarity among the diverse microbial phyla of Russian, Italian, and French truffles.

3.3 Phylogenetic characterization of fungal symbionts isolated from Russian truffles

This study isolated and identified several truffle-inhabiting fungi from black truffles. These strains belong to four genera of mycelial fungi: *Clonostachys* sp., *Fusarium* sp., *Plectosphaerella* sp., and *Trichothecium* sp. The results of the phylogenetic analysis of the sequences are presented in Figures 6–10. The *Clonostachys* sp. LPB202216 strain isolated from *T. aestivum* formed a clade with other *Clonostachys* sp. representatives from the USA, China, Russia, Italy, Mongolia, etc. *Clonostachys* sp. strains from Russia did not constitute a separate clade with each other or with isolated strains (Figure 7).

Another strain, identified as *Fusarium* sp. LPB148, formed a mixed clade with the strains deposited in the NCBI database. The highest similarity was shown for strains from the Netherlands and Switzerland (Figure 8). *Plectosphaerella* sp. LPB202213, which is associated with *T. aestivum* and was isolated during this study, formed a weakly supported clade with a strain found in China (Figure 9).

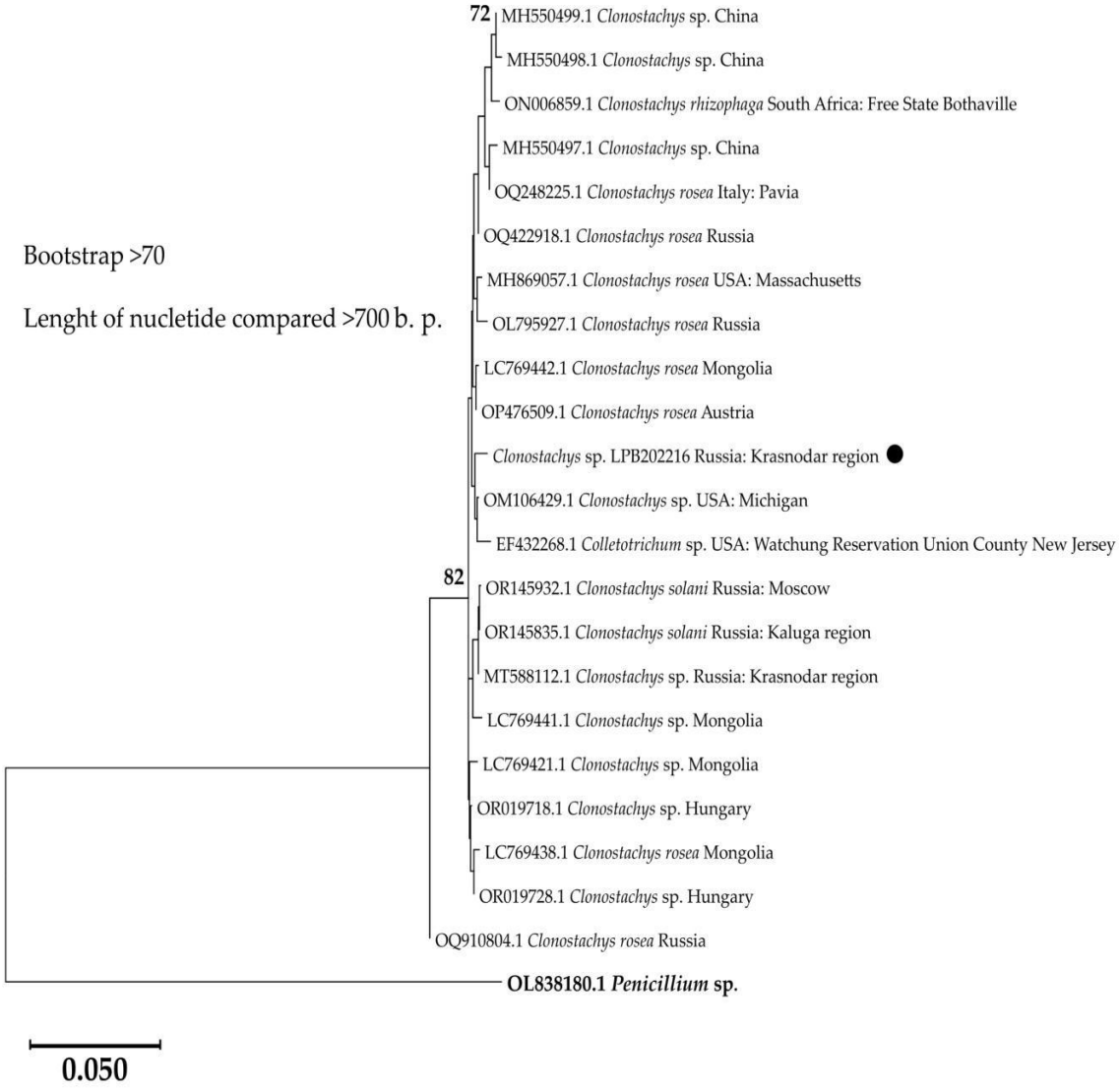

Figure 7 Evolutionary analysis via the maximum likelihood method for *Clonostachys* sp. LPB202216

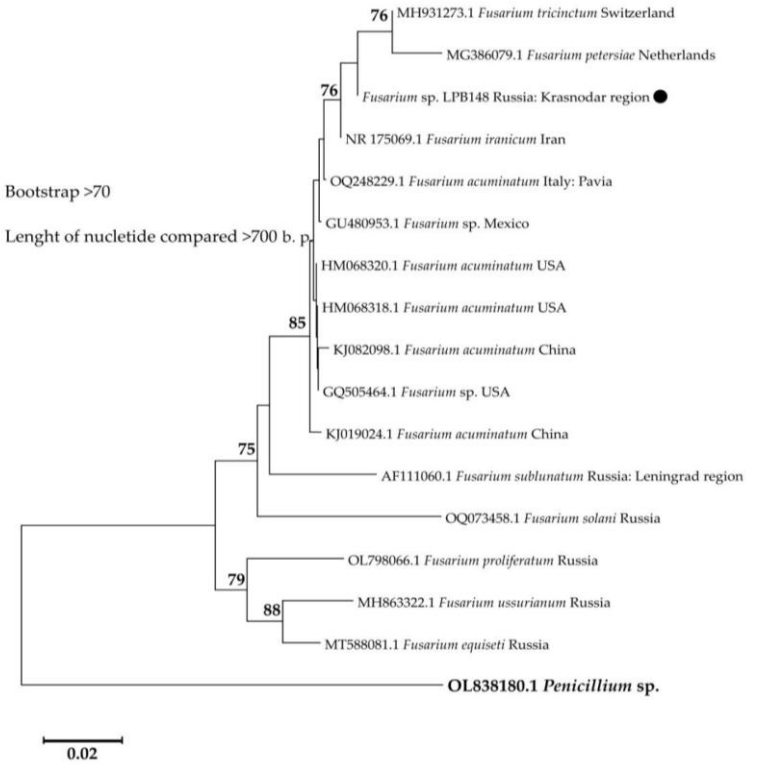

Figure 8 Evolutionary analysis via the maximum likelihood method for *Fusarium* sp. LPB148.

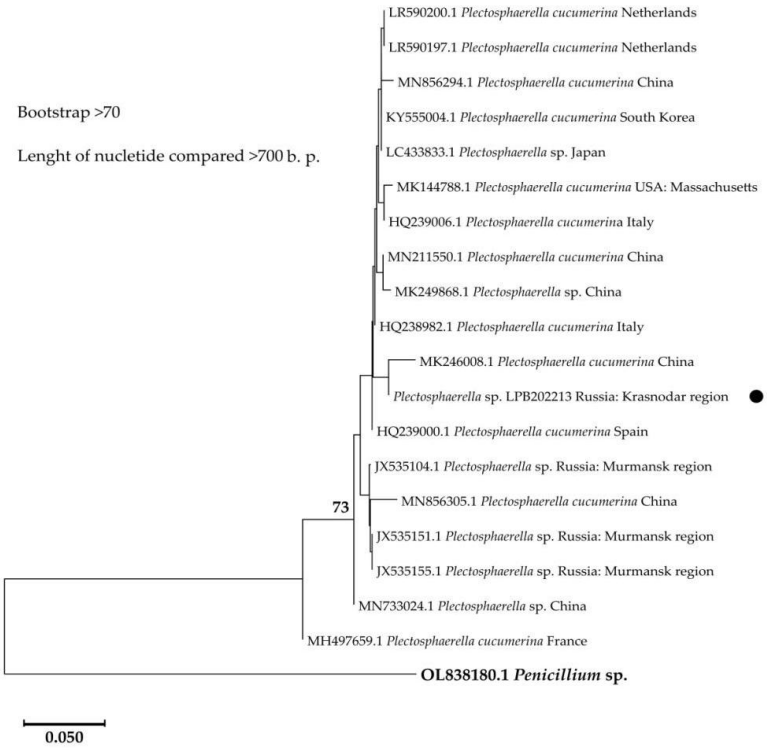

Figure 9 Evolutionary analysis via the maximum likelihood method for *Plectosphaerella* sp. LPB202213.

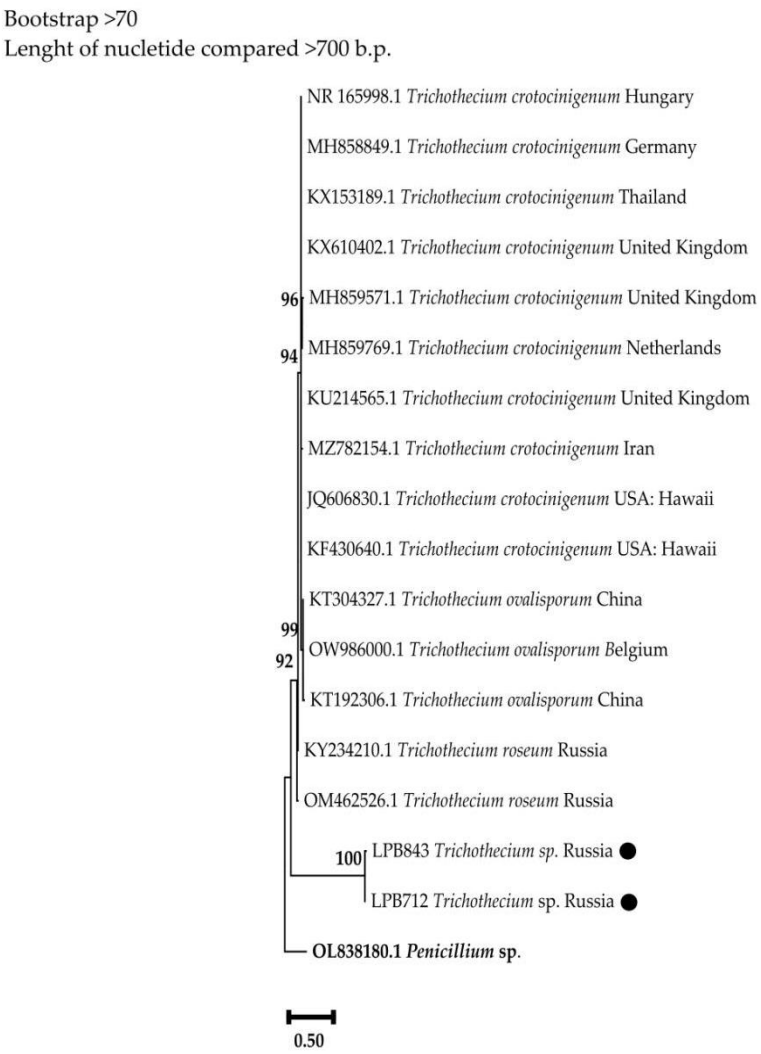

Figure 10 Evolutionary analysis via the maximum likelihood method for *Trichothecium* sp. LPB712 and *Trichothecium* sp. LPB843.

Trichothecium sp. LPB712 and *Trichothecium* sp. LPB843 strains formed a separate clade from the other strains. This indicated a large difference between the isolated truffle-associated strains and those isolated in Russia and other countries' territories (Figure 10). Moreover, the large differences between the isolated strains and the different strains deposited in the NCBI can be explained by new species of *Trichothecium* spp. inhabiting *T. aestivum* in Russia.

4 Discussion

Many published studies focused on the biomedical properties of truffle fruiting bodies in a pure culture of *Tuber* sp. However, the fruiting bodies of truffles contain a consortium of organisms (Das et al. 2022). This can lead to an unstable composition and different therapeutic effects of the extracts obtained from the ascomata (Khalifa et al. 2019; Tejedor-Calvo et al. 2021). Furthermore, the diversity of prokaryotic and eukaryotic organisms in the fruiting bodies of truffles makes obtaining pure cultures problematic (Siebyła and Szyp-Borowska 2021).

Microorganisms that are symbiotic with truffles are understudied, and their biological and biomedical potential has not been described. Several studies on the composition of truffle-associated microbial communities have assessed truffles collected in European countries (Vahdatzadeh et al. 2019; Monaco et al. 2020; Siebyła andSzyp-Borowska 2022; Herero de Aza et al. 2022). However, in Russia, studies on this topic are now being reported for the first time. Thus, for the first time, this study demonstrated that Russian truffles belonging to the genus *T. aestivum* do not form an independent phylogenetic clade. The question of truffle identification was discussed in the study performed by Molinier et al. (2013) demonstrated that European *T. uncinatum* and *T. aestivum* belong to the same species. In addition, it was hypothesized that *T. aestivum* is

most likely self-reproductive. This might explain the high similarity of truffles collected in Russia to other representatives of *T. aestivum*.

The analysis of the symbiotic bacteria genera described in Table 3 revealed that the representatives of *Enterobacter* are typical symbionts of truffles. This bacterium was found in fruiting bodies collected in Russia, Italy, and France. In addition, the results of this study revealed the presence of several bacterial genera only in Russian truffles. These bacteria include the following: *Achromobacter*, *Acinetobacter*, *Comamonas*, *Delftia*, *Enterohabdus*, *Klebsiella*, *Lactobacillus*, *Massilia*, *Rahnella*, *Ralstonia*, *Raoultella*, and *Stenotrophomonas*.

Table 3 Comparative list of bacteria associated with the fruiting bodies of *T. aestivum* collected in different countries.

Genus of bacteria	Known prokaryotic communities of the black truffle *T. aestivum*		
	T. aestivum (region of Sochi, Russia) (current study)	*T. aestivum* (region of Molise, Italy) (Monaco et al. 2020)	*T. aestivum* (region of Var, France) (Vahdatzadeh et al. 2019)
Achromobacter sp.	+	-	-
Acidovorax sp.	-	+	-
Acinetobacter sp.	+	-	-
Bacillus sp.	+	+	-
Bacteroides sp.	+	+	-
Bifidobacterium sp.	-	+	-
Buttiauxella sp.	-	+	-
Bradyrhizobium sp.	-	+	+
Carnobacterium sp.	-	-	+
Chyryseolinea sp.	-	+	-
Citrobacter sp.	+	-	+
Comamonas sp.	+	-	-
Delftia sp.	+	-	-
Devosia sp.	-	+	-
Dongia sp.	-	+	-
Ensifer sp.	-	+	-
Enterobacter sp.	+	+	+
Enterohabdus sp.	+	-	-
Frankia sp.	-	+	-
Hydrogenophaga sp.	-	+	-
Klebsiella sp.	+	-	-
Lactobacillus sp.	+	-	-
Lactococcus sp.	-	-	+
Massilia sp.	+	-	-
Mesorhizobium sp.	-	+	-
Nocardioides sp.	+	+	-
Paenibacillus sp.	-	+	-
Pantoea sp.	-	+	-
Pedobacter sp.	+	-	+
Pedomicrobium sp.	-	+	-

Genus of bacteria	Known prokaryotic communities of the black truffle *T. aestivum*		
	T. aestivum (region of Sochi, Russia) (current study)	*T. aestivum* (region of Molise, Italy) (Monaco et al. 2020)	*T. aestivum* (region of Var, France) (Vahdatzadeh et al. 2019)
Polaromonas sp.	-	+	+
Pseudomonas sp.	+	+	-
Pseudonocaria sp.	-	+	-
Rahnella sp.	+	-	-
Ralstonia sp.	+	-	-
Raoultella sp.	+	-	-
Reyranella sp.	-	+	-
Rhizobium sp.	+	+	-
Rubrobacter sp.	-	+	-
Serratia sp.	+	-	+
Sphingobacterium sp.	+	+	-
Spingomonas sp.	-	+	-
Staphylococcus sp.	+	+	-
Stenotrophomonas sp.	+	-	-
Varivorax sp.	-	-	+

The "+" symbol indicates the presence of this bacterial genus in the truffle microbiome; the "-" symbol indicates the absence of this bacterial genus in the truffle microbiome

Several bacteria were found in European *T. aestivum* but not in Russian truffles, including the following: *Acidovorax*, *Buttiauxella*, *Chyryseolinea*, *Devosia*, *Ensifer*, *Frankia*, *Hydrogenophaga*, *Lactococcus*, *Mesorhizobium*, and *Pantoea*. Representatives of *Bradyrhizobium* and *Polaromonas* were found only in the fruiting bodies of *T. aestivum* sampled in Italy and France (Vahdatzadeh et al. 2019).

During the evaluation of the prokaryotic composition of three samples of the fruiting bodies of the truffle *T. aestivum*, classes belonging to *Gammaproteobacteria* and *Bacilli* were detected in all the samples. A study by Vahdatzadeh et al. (2019) demonstrated that the bacteria associated with T. aestivum classes depended on the product's freshness and storage conditions. A comparison of recently published data and the data obtained in this study allowed us to establish that the classes of bacteria found in the truffle samples are markers of staleness (Ramos-Pereira et al. 2019) despite their fresh appearance and appropriate transport conditions. The diverse fungi found in the fruiting bodies of *T. aestivum* collected in different countries included four common genera: *Malassezia*, *Papulaspora*, *Psathyrella*, and *Penicillium* (Table 4).

In the microbiome of the truffles, we also found fungi of the genus *Penicillium*. The presence of these fungi in the metagenome of food products is a marker of food spoilage (Ramos-Pereira et al. 2019). In addition, the following fungal genera were found in Russian truffles: *Ascobolus*, *Mortierella*, *Holtermanniella*, *Papulaspora*, *Psathyrella*, and *Rhodotorula*. These are soil microorganisms previously isolated from rotting plant residues or ruminant manure. This peculiarity indicates that these microorganisms that enter symbiosis allow the truffles to colonize plant roots more efficiently, utilizing a specific set of cellulases and hydrolases (Siebyła 2022).

Thus, despite the external quality of truffles, molecular analysis revealed a high diversity of pathogenic and nonpathogenic organisms associated with their fruiting bodies. The potential importance of these genera of prokaryotic and eukaryotic organisms is presented in Table 5. The diversity of prokaryotic (Dahal et al. 2021; Rosenberg et al. 2014; Zafar and Saier 2021; Araújo et al. 2021; Ke et al. 2021; Muszyński et al. 2021; Evangelista et al. 2022; Riahi et al. 2022) and eukaryotic (Litvinov 2013; Mesanza et al. 2021; Vohník et al. 2022; Ianiri et al. 2022; Lin et al. 2022) organisms and the high number of pathogenic species in the fruiting bodies of truffles indicate the complexity of the symbiotic interactions that can be observed and studied in the truffle model. Many prokaryotic and eukaryotic organisms mentioned here might influence truffles and help them form effective mycorrhizae with trees.

Table 4 Comparative list of fungi associated with the fruiting bodies of *T. aestivum* collected in different countries.

Genus	Known eukaryotic communities of the black truffle *T. aestivum*	
	T. aestivum (region of Sochi, Russia) (current study)	*T. aestivum* (region of the Nida river, Poland) (Perlińska-Lenart et al. 2020b)
Sphaerodes sp.	-	+
Alternaria sp.	+	-
Ascobolus sp.	+	-
Cladophialophora sp.	+	-
Coprinellus sp.	-	+
Holtermanniella sp.	+	-
Hyaloscypha sp.	+	-
Leptolegnia sp.	-	+
Malassezia sp.	+	+
Mortierella sp.	+	-
Mucor sp.	-	+
Mycosphaerella sp.	+	-
Papulaspora sp.	+	+
Penicillium sp.	+	+
Phlebia sp.	-	+
Piptoporus sp.	-	+
Psathyrella sp.	+	+
Rhodotorula sp.	-	+
Trichoderma sp.	-	+
Tylospora sp.	+	-
Umbelopsis sp.	-	+
Veluticeps sp.	-	+

The "+" symbol indicates the presence of this fungal genus in the truffle microbiome; the "-" symbol indicates the absence of this fungal genus in the truffle microbiome

The truffle-inhabiting fungi isolated in this study were identified as *Fusarium* sp., *Clonostachys* sp., *Plectosphaerella* sp., and *Trichothecium* sp. *Trichothecium crotocinigenum* (also widely known as *Acremonium crotocinigenum*) is responsible for a widespread rot epidemic affecting the cultivation of *T. melanosporum* in Australia. This epidemic has lasted for several years and has resulted in the loss of more than 50% of the truffle harvest in some cases. The pathogenicity of *T. crotocinigenum* has been directly confirmed in the field by inoculating the conidia or gleba of rotten truffles. However, it is worth noting that *T. crotocinigenum* was previously found in healthy ascocarps of *Tuber maculatum* Vittad in the Netherlands (Pacioni and Leonardi 2016). Another similar species, *Trichothecium roseum*, has been isolated from rotten pink Chinese truffles and observed on stored desert truffles, *Terfeziapinoyi* Maire and *Terfeziaclaveryi* Chatin. This mushroom is a truffle symbiont and, presumably, can be useful for combating soil parasites (Satish et al. 2022).

The fungal strain identification results identified a small, studied oligotrophic fungal species belonging to *Plectosphaerella*. Most of the known fungi of the genus *Plectosphaerella* were isolated from soils (Soledad et al. 2023). They are pathogens of several plant species and are described as a component of the core fungal genera associated with forests producing white truffles (Giorgio et al. 2023). However, previously, representatives of *Plectosphaerella* were never reported as organisms living inside the ascomata of truffles, and we found no mention of these fungi as pathogens of truffles, especially black truffles.

Table 5 The potential biological importance of organisms found in association with *T. aestivum*

Organism	Importance
Prokaryotic organisms	
Achromobacter sp.	Soil bacteria, arsenic oxidizers
Acidovorax sp.	Phytopathogens, heavy metal oxidizers
Acinetobacter sp.	Saprophytes, chemoorganotrophs
Bacillus sp.	Saprophytes, plant and animal pathogens
Bacteroides sp.	Saprophytes, human pathogens
Bifidobacterium sp.	Saprophytes, the member of normal human microflora
Buttiauxella sp.	Soil bacteria, endophytes
Bradyrhizobium sp.	Soil nitrogen-fixing bacteria
Carnobacterium sp.	Acidophilic bacteria, producers of bacteriocins
Chyryseobacterium sp.	Soil bacteria, phytopathogenic
Citrobacter sp.	Human and vertebrate pathogens
Comamonas sp.	Denitrifying bacteria
Delftia sp.	Detoxification of heavy metals, ability to decompose herbicides, etc.
Devosia sp.	Nitrogen-fixing nodule bacteria
Desulfovibrio sp.	Oil destructors, metal oxidizers
Dongia sp.	Soil bacteria
Ensifer sp.	Nitrogen-fixing nodule bacteria
Enterohabdus sp.	Vertebrate pathogens
Frankia sp.	Nitrogen-fixing bacteria, plant symbionts
Granulibacter sp.	Human and vertebrate pathogens
Klebsiella sp.	Animal pathogens
Lachnospiraceae sp.	Human intestinal symbionts, destroyer of cellulose
Lactobacillus sp.	Animal pathogens
Lactococcus sp.	Animal pathogens
Massilia sp.	Nitrogen-fixing bacteria, plant symbionts, decomposers of chlorine-based herbicides
Mesorhizobium sp.	Nitrogen-fixing nodule bacteria
Nitrobacter sp.	Soil-nitrifying bacteria
Nocardioides sp.	Detoxifiers, symbionts of algae and vertebrates
Paenibacillus sp.	Destroyers of biopolymers, including cellulose and agar–agar
Pantoea sp.	Pathogen of plants, saprophytes
Pedobacter sp.	Soil bacteria
Pedomicrobium sp.	Oxidizers of manganese
Polaromonas sp.	Psychrophilic bacteria
Pseudomonas sp.	Wide effects and products of biosynthesis
Pseudonocaria sp.	Wide effects and products of biosynthesis

Organism	Importance
Rahnella sp.	Human pathogens in the environment
Ralstonia sp.	Phytopathogens
Raoultella sp.	Human and vertebrate pathogens
Reyranella sp.	Soil bacteria, symbionts of plants
Rhizobium sp.	Nitrogen-fixing nodule bacteria
Ruminococcus sp.	Human pathogens
Serratia sp.	Destroyers of biopolymers, symbionts of plants
Sphingobacterium sp.	Human pathogens
Spingomonas sp.	Plant symbionts, involved in bioremediation processes
Staphylococcus sp.	Human and vertebrate pathogens
Stenotrophomonas sp.	Wide effects and products of biosynthesis
Varivorax sp.	Phytopathogens, nitrogen-fixing bacteria
Eukaryotic organisms	
Alternaria sp.	Soil fungi, phytopathogens
*Ascobolus*sp.	Coprophilous. Found in manure of herbivorous mammals and on plant residues
Cladophialophora sp.	Human pathogens
Coprinellus sp.	Coprophilous. Found in manure of herbivorous mammals and on plant residues
Holtermanniella sp.	Biological destroyers
Hyaloscypha sp.	Symbionts of plant roots
Leptolegnia sp.	Pathogens of insects
Malassezia sp.	Pathogens of humans and mammals
Mortierella sp.	Biological destroyer, pathogens
Mucor sp.	Biological destroyer, pathogens
Mycosphaerella sp.	Phytopathogens
Papulaspora sp.	Producers of amylase
Penicillium sp.	Soil fungi, plant parasites
Phlebia sp.	Phytopathogens
Piptoporus sp.	Biological destroyers
Psathyrella sp.	Found in the manure of herbivores
Rhodotorula sp.	Denitrifiers
Trichoderma sp.	Phytopathogens
Tylospora sp.	Biological destroyers
Umbelopsis sp.	Producers of oils
Veluticeps sp.	Phytopathogens

From an analysis of literary sources, it is known that the fungus *Clonostachys rosea* is of great interest. It is a saprotroph used as an agent for the biological control of phytopathogenic fungi (Funck Jensen et al. 2022). *C. rosea* is short and was mentioned in

(Rennick et al. 2022). However, the role of *Clonostachys* sp. and other truffle-inhabiting fungi in the life of truffles is unclear.

Truffles are special kinds of fungi with unique adaptations and complexes of unknown and rarely studied interactions with other organisms, including fungal symbionts. One of the reasons for having so many fungal symbionts is that they help truffles resist competition from other fungi and microorganisms living in the soil. The variety of fungal symbionts provides the truffle with maximum efficiency in obtaining nutrients for forming fruiting bodies (Garcia-Barreda et al. 2023). Truffles are also known to grow in different ecological conditions, including various types of soils, climatic zones, and plant communities (Mrak et al. 2024). The variety of fungal and bacterial symbionts allows truffles to adapt to these different environmental conditions (Barou et al. 2023). Each fungal symbiont has its own characteristics that help truffles survive under certain climatic conditions and form mycorrhizae with host plants (García-Montero et al. 2024). Thus, the diversity of bacterial and fungal symbionts in truffles plays an important role in their growth and reproduction (Liu et al. 2021b).

Conclusion

In this study, we first demonstrated the diversity of prokaryotic and eukaryotic organisms associated with the Russian truffle *T. aestivum*. The microbiome of the black truffle *T. aestivum* mainly consisted of soil bacteria and fungi. This study also estimated the diversity of microorganisms inhabiting Russian truffles compared with *T. aestivum* collected in Italy and France and found that the general distribution of the microbial taxa was similar. We found a high similarity between Russian and European truffles. Sequencing the hypervariable fragments of the 16S rRNA and 18S rRNA genes allowed us to identify the plant pathogens involved in symbiotic relationships with the truffles. In addition, some strains of fungal symbionts and likely pathogens were isolated and identified in truffles for the first time. Because they are associated with truffles, many species of these prokaryotic and eukaryotic organisms might influence truffles and help them form mycorrhizae with trees. Thus, truffles are interesting and promising sources for modern biotechnological and agricultural studies.

Author Contributions

E.V.M., M.E.D., M.M.M., N.A.I., A.A.V., T.Y.T., V.N.S., A.S.K. and A.Y.B. performed the experiments and analysed the data. E.V.M. and MMM isolated the strains. MED, MMM, and NAI identified the strains. EVM wrote the first draft of the manuscript. DVAG and EVM planned the experiments, analysed the data, and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The sequence data used to support the findings of this study have been deposited in the NCBI Genbank repository (https://www.ncbi.nlm.nih.gov/genbank/).

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Conflicts Of Interest

The authors declare no conflicts of interest.

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