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RESEARCH ARTICLE

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The Uneven Distribution of Mating Type Genes in Natural and Cultivated Truffle Orchards Contributes to the Fructification of *Tuber indicum*

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ABSTRACT

The aim of this study was to investigate the pattern of distribution of mating type (*MAT*) genes of *Tuber indicum* in ectomycorhizosphere soils from natural *T. indicum*-producing areas and cultivated truffle orchards and ascocarp samples from different regions. Quantitative real-time PCR and multiplex PCR were used to weight the copy numbers of *MAT1-1-1* and *MAT1-2-1* in natural truffle soils and cultivated orchard soils. The effect of limestone on the pattern of truffle *MAT* genes and the correlation between soil properties and the proportion of *MAT* genes were also assessed. These results indicated that an uneven and nonrandom distribution of *MAT* genes was common in truffle-producing areas, cultivated truffle orchards, and ascocarps gleba. The competition between the two mating type genes and the expansion of unbalanced distribution was found to be closely related to truffle fructification. Limestone treatments failed to alter the proportion of the two mating type genes in the soil. The content of available phosphorus in soil was significantly correlated with the value of *MAT1-1-1/MAT1-2-1* in cultivated and natural ectomycorhizosphere soils. The application of real-time quantitative PCR can provide reference for monitoring the dynamic changes of mating type genes in soil. This study investigates the distributional pattern of *T. indicum MAT* genes in the ectomycorhizosphere soil and ascocarp gleba from different regions, which may provide a foundation for the cultivation of *T. indicum*.

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1. Introduction

The truffle *Tuber* spp. establishes ectomycorrhizal (ECM) symbioses with plant roots (e.g., poplar, oak, willow, and shrubs) [1–3]. The fruit bodies, referred to as truffles, are well-known for their intense aroma, nutritional attributes, and biological activity worldwide [4–6]. The Asian black truffle *T. indicum* is the most important commercial black truffle in China for the rich flavors of the completely mature ascocarp.

The sequencing of the European black truffle *T. melanosporum* genome and subsequent studies have revealed that *T. melanosporum* is heterothallic [7–11]. Thereafter, it has been proven that *T. indicum* is heterothallic as well [12]. A single heterothallic ascomycete presents one of two alternative mating type (*MAT*) genes, *MAT1-1-1* or *MAT1-2-1*, and the life cycle can only be completed when different strains with different mating type genes become fused. This process is a precondition for truffle fructification; in other words, the distribution patterns of *MAT*s in truffle populations are the key determinants of successful fruit-body initiation [13,14]. However, it seems contradictory that the distribution patterns of *T. melanosporum* strains in their hosts are likely the result of competition between different *MAT*s. Murat et al. [15] found each single ECM manifested only one of the two *MAT* genes, and all of the *T. melanosporum* ECMs collected from the same host plant shared the same *MAT* genes. Rubini et al. [16] investigated the distribution of the two *MAT*s in ECMs from a natural *T. melanosporum* orchard and a nursery-inoculated glasshouse; this study revealed that the ECMs from individual hosts or from sites close to each other (in the range of 3–30 m), as well as the sampling soils from the glasshouse, shared identical *MAT* profiles. Linde and Selmes [17] found that only approximately half of host trees had both *MAT*s of *T. melanosporum*, with *MAT1-1-1* predominating in established trees in Australia, suggesting a competitive advantage for *MAT1-1-1* strains. It is widely believed that ascomycete hyphae need partners

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exhibiting different mating type genes to complete their life cycle. Thus, it is unreasonable that the distribution patterns of *T. melanosporum* strains are uneven beneath the same ground. To our knowledge, no research has been performed to investigate if this uneven *MAT* gene distribution occurs in *T. indicum* soils.

Meanwhile, the importance of soil physical and chemical properties associated with *T. melanosporum*, such as pH and carbonates, has been reported. Several statistical studies have indicated that a high concentration of active carbonate in the soil and neutral or slightly acidic pH favors *T. melanosporum* fruit-body production [18,19]. However, soil properties relating to *T. indicum* were not adequately investigated. In addition, the correlations between mating type genes and soil properties have not yet been assessed, so it is not yet clear whether it is possible to change the *MAT* gene distribution by manipulating specific soil properties.

So far, few successful truffle orchards have been established in mainland China [20]. A *T. indicum* cultivated orchard area was built in Yanbian County in Panzhihua in 2009 with the aim of establishing a truffle orchard in China through cooperation between the Sichuan Academy of Agricultural Sciences and the Panzhihua Academy of Agricultural and Forestry Sciences. Although the ectomycorrhiza of *Castanea mollissima* Blume. or *Corylus heterophylla* Fisch. infected with *T. indicum* have been observed to be well colonized, to date no fruiting bodies have been found in this orchard. In this paper, we investigated the distribution pattern of *MAT* genes in ascocarp gleba from different regions. The distribution of *MAT* genes in soils of cultivated and natural truffle orchards from similar environments was also investigated using a real-time PCR analysis to determine the relationships between the *MAT* distribution pattern in soil and truffle yield to determine if the fructification of truffles was relative to the *MAT* distribution. Also, the physical and chemical properties of soil were detected to analyze the correlations between these properties and the *MAT* genes. To our knowledge, this is the first report on the pattern of distribution of *MAT* genes of *T. indicum* in ectomycorhizosphere soils of natural *T. indicum*-producing areas and cultivated truffle orchards, as well as in ascocarps from different regions. These results may provide guidance for the cultivation of *T. indicum*.

2. Materials and methods

2.1. Sampling location

Ectomycorhizosphere soils of natural *T. indicum*-producing areas and cultivated truffle orchards were collected from Yanbian County, Panzhihua City (N26°05′–26°21′, E101°38′–101°41′, Sichuan Province, China) in May 2015. The mean annual temperature in Panzhihua was 19–21 °C, and the mean annual precipitation was 760–1200 mm. The main host plant of *T. indicum* in the natural truffle-producing areas is *Pinus yunnanensis* and it grows in sandy loam soil. The altitude of sampling sites ranged from 1620 to 2429 m. Truffles have been found at these sites since 2005.

The *T. indicum* orchard was built in Yanbian County, Panzhihua City since 2009, which is located near each other geographically in an ecological environment similar to that of natural *T. indicum*-producing areas. Although the ectomycorrhizae of *C. mollissima* Blume and *Co. heterophylla* Fisch infected with *T. indicum* in the cultivated orchards have been thoroughly studied, no ascocarps had yet been found during the past years. Some of the host trees had calcareous rocks stacked underneath them to investigate the effect of limestone on the distributional pattern of *MAT* genes.

Thirty-four ascocarps of *T. indicum* were collected from Sichuan and Yunnan Provinces in southwestern China, to investigate the pattern of distribution of *MAT* genes in truffle gleba of ascocarps. Information about the collection sites and number of samples from each site is shown in Figure 1 and Table 3.

2.2. Sampling method, storage and pretreatment

A total of seven ectomycorhizosphere soil samples from natural *T. indicum*-producing areas and thirteen soil samples from the cultivated orchard were collected (Tables 1 and 2), namely the Control, Ti-S-1, Ti-S-2, Ti-S-3, Ti-S-4, Ti-S-5, Ti-S-6, Art-Control, Cm-Ti-1, Cm-Ti-2, Cm-Ti-3, Cm-Ti-li-1, Cm-Ti-li-2, Cm-Ti-li-3, Ch-Ti-1, Ch-Ti-2, Ch-Ti-3, Ch-Ti-li-1, Ch-Ti-li-2, and Ch-Ti-li-3. "Cm-" means that samples were collected from *C. mollissima* Blume, while "Ch-" came from *Co. heterophylla* Fisch. The term "Control" means that the soil samples were collected from the site at which no ascocarp of *T. indicum* had ever been found in natural field, and Art-Control means the soil samples were collected from the sites at which no truffles were inoculated but were very close to where they were planted (less than 10 m). Soil samples from natural truffle-producing areas were classified into three groups according to yield. The ascocarp yield at Ti-S-1 and Ti-S-2 were ranged from 10 to 100 g per square meter (g/m^2), the yield of Ti-S-3 and Ti-S-4 were ranged from 100 to 200 g/m^2, and the yield of Ti-S-5 and Ti-S-6 were more than 200 g/m^2. They were grouped into low-producing, middle-producing, and high-producing grounds, respectively (Table 2). The ascocarp yield from these areas was calculated based

Figure 1. Ectomycorhizosphere soil and ascocarp sampling sites in southwestern China. CJ: Chengjiang County; HD: Huidong County; HL: Huili County; JY: Jinyang County; WS: Weishan County; XGLL: Xianggelila County; YS: Yongsheng County.

Table 1. Hosts information, soil treatments and copy numbers of *MAT* genes in soil of cultivated truffle orchards.

Average yield (g/m^2)	Host	Treatments	Soil sample ID	*MAT1-1-1* copy number	*MAT1-2-1* copy number	*MAT1-1-1/MAT1-2-1* ratio
0	/	none	Art-Control	0	0	/
0	*C. mollissima*	no calcareous rock	Cm-Ti-1	326.43 ± 26.59	362.42 ± 62.51	0.74 ± 0.10a
			Cm-Ti-2	312.30 ± 73.20	549.17 ± 129.01	
			Cm-Ti-3	346.26 ± 11.30	463.10 ± 46.26	
		calcareous rock stacked under hosts	Cm-Ti-li-1	411.65 ± 8.70	2250.52 ± 224.10	0.74 ± 0.29a
			Cm-Ti-li-2	501.72 ± 50.44	444.15 ± 153.62	
			Cm-Ti-li-3	559.98 ± 119.00	622.03 ± 87.08	
	Co. heterophylla	no calcareous rock	Ch-Ti-1	387.97 ± 44.01	581.07 ± 95.36	0.54 ± 0.07ab
			Ch-Ti-2	428.59 ± 54.52	989.23 ± 30.00	
			Ch-Ti-3	422.39 ± 28.90	819.83 ± 370.266	
		calcareous rock stacked under hosts	Ch-Ti-li-1	429.49 ± 19.26	2657.21 ± 329.51	0.23 ± 0.04b
			Ch-Ti-li-2	435.96 ± 65.27	1927.02 ± 259.48	
			Ch-Ti-li-3	460.00 ± 76.45	1564.61 ± 287.17	

Different letters in a column indicate a significant difference between treatments ($p < .05$).
Art-Control: soil sample from uninoculated ground; Cm-Ti-1, Cm-Ti-2, Cm-Ti-3: soil samples of *T. indicum*-inoculated from *C. mollissima* Blume not treated with stacking of calcareous rock; Cm-Ti-li-1, Cm-Ti-li-2, Cm-Ti-li-3: soil samples of *T. indicum*-inoculated from *C. mollissima* Blume stacking of calcareous rock; Ch-Ti-1, Ch-Ti-2, Ch-Ti-3: soil samples of *T. indicum*-inoculated from *Co. heterophylla* Fisch not treated with stacking of calcareous rock; Ch-Ti-li-1, Ch-Ti-li-2, Ch-Ti-li-3: soil samples of *T. indicum*-inoculated from *Co. heterophylla* Fisch with stacking of calcareous rock.

Table 2. Host information, soil treatment, and copy numbers of *MAT* genes in soil from natural *T. indicum*-producing areas.

Average yield (g/m^2)	Host	Treatments	Soil sample ID	*MAT1-1-1* copy number	*MAT1-2-1* copy number	*MAT1-1-1/MAT1-2-1* ratio
0	*P. yunnanensis*	natural-truffle-producing	Natural-control[a]	0	0	/
10 to 100	*P. yunnanensis*	areas without any	Ti-S-1	126.08 ± 23.60	0.85 ± 1.46	148.33
		treatment	Ti-S-2	1123. 10 ± 260.74	93.00 ± 61.42	12.08
100 to 200			Ti-S-3	0	3144.64 ± 904.20	0
			Ti-S-4	0	4803.28 ± 556.09	0
More than 200			Ti-S-5	90.19 ± 32.71	113.95 ± 35.35	0.79
			Ti-S-6	0	4792.80 ± 1245.33	0

[a]Natural-control: soil sample from non-truffle ground; Ti-S-1 and Ti-S-2: *T. indicum*-soils from low productive grounds; Ti-S-3 and Ti-S-4: *T. indicum*-soils from middle producing grounds; Ti-S-5 and Ti-S-6: *T. indicum*-soils from highly productive grounds.

on the average yield of the last three years by a local guide. Three replicates, a total of 200 g, of soil samples were randomly collected from each sample site. The soils were checked to make sure forest debris was discarded and the samples were then kept in sterile sealed bags on ice for less than 24 h until transfer to refrigerator at −20 °C for preservation.

2.3. Soil property analysis

Soil samples from the same site were mixed to analyze their properties after being air-dried and sieved to obtain the <2 mm fraction. The pH in water (1:2.5 soil/water ratio) was measured by dissolving air-dried soil. Organic matter content was estimated by the Tyurin method [21]. Total nitrogen was measured by the Kjeldahl method [22]. The alkali solution diffusion method was used to determine effective nitrogen. The baking soda leaching-molybdenum antimony colorimetric method and ammonium acetate extraction-flame photometry [23] was performed to measure available phosphorus and available potassium, respectively. The exchangeable cations of Ca^{2+} and Mg^{2+} were determined described as Andrzej et al. [24].

Table 3. Information of sample sources, total number of samples, and two different gleba mating types.

Collection sites		*MAT1-1-1*	*MAT1-2-1*	Number of samples
Huili County	Sichuan Province	2	4	6
Jinyang County	Sichuan Province	4	9	13
Huidong County	Sichuan Province	0	5	5
Xianggelila County	Yunnan Province	2	1	3
Chengjiang County	Yunnan Province	1	0	1
Yongsheng County	Yunnan Province	1	2	3
Weishan County	Yunnan Province	3	0	3
Total number		13	21	34

2.4. DNA extraction and PCR reactions

DNA extraction from 200 mg soil was carried out with an E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA) following the manufacturer's guidelines. The concentration and quality of the extracted DNA were determined using a micronucleic acid measuring instrument K5500 (Kaiao, Beijing, China). 1 to 10 ECMs from one plant root were selected under a Greenough stereo microscope, (model Leica S8 APO, Leica Microsystems, Wetzlar, Germany). Universal primers ITS1/ITS2 were used for ITS region amplification to identify ECMs collected from Panzhihua [25]. The i3/i4 and i5/i6 primers [12] were used for MAT genes of *T. indicum* real-time PCR amplification in soil samples, and *MAT 1-1-1* and *MAT1-2-1* genes were cloned into *E. coli* strain JM109 using standard protocols for absolute quantification real-time PCR. Real-time PCR reactions were determined in 20 µL containing 10 µL SYBR® Premix Ex TaqTM II (Takara, Japan), 4 µM of each primer, 4 ng of DNA, and then distilled water was added to a total volume of 20 µL. The real-time PCR cycling conditions included an initial denaturation at 95 °C for 3 min, followed by 39 cycles of denaturing at 95 °C for 30 s, annealing at 65 °C for 20 s, and extension at 72 °C for 30 s; fluorescence was obtained every 0.5 s. At least three replicates per sample were used. Real-time PCR was conducted in a CFX96™ Real-Time PCR detection system (Bio-Rad, Hercules, CA). The P19/P20 and P1/P2 primers [12] were used for MAT gene amplification in the gleba of ascocarps. PCR reactions were performed in a total volume of 50 µL containing 25 µL of 2 × Taq PCR master mix (Tiangen, China), 25 µLM of each primer, and 10 ng of template DNA, to which distilled water was added until a total volume of 50 µL was reached. The MAT gene PCR amplification program contained an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. PCR products were detected by electrophoresis through a 1.5% agarose gel in 0.5 × TAE buffer, and visualized under UV light. Gene copy numbers were calculated according to Ct values and standard sample DNA copies.

2.5. Statistical analysis

Data are presented as means ± standard deviation (SD) of three biological replicates for each treatment. A least significant difference test was performed using the one-way ANOVA, and Spearman's rank-order correlation was used to determine correlations between the *MAT1-1-1*/*MAT1-2-1* and soil properties.

3. Results

3.1. Distribution of mating type genes in orchard and natural-truffle-producing soils

For real-time PCR, standard curves were generated; eight serial dilutions containing 10^8–10^1 positive sense standard sample DNA copies were prepared (15–20 ng/µL). The standard curve covered a dynamic range of six or seven log units of concentration and showed a strong linear relationship, with high coefficient of determination (R^2) of 0.999 and a high amplification efficiency (87.7%–94.0%).

Both *MAT1-1-1* and *MAT1-2-1* were successfully detected using absolute quantification real-time PCR in ectomycorhizosphere soil from the cultivated truffle orchard (Table 1). No *MAT1-1-1* gene amplification was obtained in Ti-S-3, Ti-S-4, or Ti-S-6. *MAT1-2-1* gene amplification was successfully obtained in all soil samples from natural truffle-producing areas (Table 2), indicating the unbalanced distribution of two kinds of mating type genes in those areas. In the cultivated truffle orchard, where truffle production was 0, *MAT1-2-1* gene was more abundant than the *MAT1-1-1* in most samples. Their ratio of *MAT1-1*/*MAT1-2-1* ranged from 0.16 to 1.12. Compared to *MAT* genes in the cultivated truffle orchard, the two *MAT* genes in soils from natural truffle-producing areas were highly uneven. In low-producing areas, the proportion of *MAT1-1-1* gene to *MAT1-2-1* was as high as 12.08 and 148.33, and *MAT1-1-1* gene occupies a dominant position relative to the *MAT1-2-1* gene. In soil in which truffle yield was more than 100 g/m^2, *MAT1-2-1* gene occupied the dominant position, and even in some samples, only *MAT1-2-1* gene could be detected. These results indicated that ascocarps production is closely related to the competition between two mating type genes and the expansion of the unbalanced distribution of two kinds of mating type genes.

MAT1-1-1/*MAT1-2-1* values were also calculated to assess the distribution situation of two *MAT* genes in cultivated orchard soils of different host species (Table 1). The value of *MAT1-1-1*/*MAT1-2-1* was greater in *C. mollissima*–*T. indicum* ground soil than in *Co. heterophylla*–*T. indicum* ground soils, indicating that the distribution of mating type genes might be associated with the hosts. Limestone plays an important role in the success of truffle fructification in orchards. No significant correlations were found between limestone treatment and the distribution of *MAT* genes.

3.2. Mating types of T. indicum gleba

The patterns of distribution of *MAT* genes in ascocarps gleba from different truffle-producing areas were investigated in this paper. Mating type genes of all 34 ascocarps gleba were amplified using changes in PCR reaction cycles and the concentration of DNA template (Table 3). Among 34 ascocarps, twenty-one of their gleba presented *MAT1-2-1* mating type, thirteen of their gleba presented *MAT1-1-1* mating types. Samples from each site showed non-random distribution of two mating types. For example, samples from Huidong and Weishan presented only one mating types, *MAT1-2-1* were obviously greater than *MAT1-1-1* in samples from Jinyang and Huili (Table 3).

3.3. Characteristics of T. indicum-producing soils in natural and cultivated orchard soils

The pH of the ectomycorhizosphere soil samples collected from the natural truffle-producing areas and cultivated orchards were neutral or acidic (pH: 6.10–7.19) (Table 4). In total, most of both natural and cultivated soil properties were significantly different from their matched controls. Sand content value in all natural samples was significantly higher than that of the natural-control. The value of total nitrogen, available calcium (ACa), and silt was lower than that of the natural-control. Clay values in natural samples were significantly higher than that of the natural-control in most samples with Ti-S-1 as an exception. For the cultivated orchards soils, available calcium in soils of truffle cultivated orchards was increased by calcareous rock stacked under hosts (Table 4). However, there was no significant correlation between available calcium and the value

Table 4. Physical and chemical properties of natural *T. indicum*-producing soils and cultivated plantation soils.

Sample types	No.	pH	Sand (100%)	Clay (100%)	Silt (100%)	OM (g/kg)	TN (g/kg)	AN (mg/kg)	AP (mg/kg)	AK (mg/kg)	ACa (cmol/kg)	AMg (cmol/kg)
Natural *T. indicum*-producing soils	natural-control	7.07 ± 0.06ab	22.69 ± 0.39c	12.46 ± 1.02c	64.85 ± 0.95a	47.82 ± 1.6bc	2.59 ± 0.21a	123.65 ± 2.0ab	23.04 ± 0.56b	142.29 ± 2.20bc	4.59 ± 0.08a	0.5 ± 0.02b
	Ti-S-1	6.85 ± 0.08b	50.45 ± 0.58a	11.5 ± 0.13c	38.05 ± 0.72c	55.08 ± 0.6b	1.49 ± 0.02c	129.44 ± 1.5ab	35.9 ± 0.42a	141.11 ± 1.62bc	1.22 ± 0.01e	0.46 ± 0.017b
	Ti-S-2	6.10 ± 0.07c	52.45 ± 0.61a	22.28 ± 0.26b	25.27 ± 0.87d	49.78 ± 0.58bc	1.71 ± 0.02bc	137.17 ± 1.6ab	30.91 ± 0.36ab	120.16 ± 1.45cd	3.2 ± 0.03bc	0.6 ± 0.02a
	Ti-S-3	6.62 ± 0.08bc	32.65 ± 0.38b	29.46 ± 0.34a	37.89 ± 0.72c	35.97 ± 0.42cd	2.03 ± 0.02b	108.19 ± 1.2bc	17.57 ± 0.20bc	113.44 ± 1.32d	3.42 ± 0.04bc	0.37 ± 0.01c
	Ti-S-4	7.18 ± 0.08a	35.03 ± 0.41b	28.82 ± 0.34a	36.15 ± 0.74c	51.94 ± 0.60bc	2.4 ± 0.03a	86.94 ± 1.0c	14.78 ± 0.17c	163.64 ± 1.91a	2.27 ± 0.03c	0.29 ± 0.01d
	Ti-S-5	6.55 ± 0.08bc	32.4 ± 0.38b	14.99 ± 0.17c	52.61 ± 0.55b	44.79 ± 0.52c	2.52 ± 0.03a	100.46 ± 1.2bc	29.38 ± 0.34ab	113.44 ± 1.30d	3.45 ± 0.04bc	0.43 ± 0.02b
	Ti-S-6	6.98 ± 0.08ab	33.98 ± 0.39b	20.17 ± ± 0.23b	45.85 ± 0.62bc	46.45 ± 0.54c	1.77 ± 0.02bc	97.62 ± 1.1c	28.99 ± 0.33ab	132.81 ± 1.50c	2.64 ± 0.03c	0.47 ± 0.02b
T. indicum orchard soils	Art-control	7.19 ± 0.08a	27.87 ± 0.32bc	15.29 ± 0.18c	56.84 ± 0.50ab	29.5 ± 0.34d	1.56 ± 0.02c	117.85 ± 1.4b	15.26 ± 0.18c	153.75 ± 1.80b	1.78 ± 0.02d	0.33 ± 0.01c
	Cm-Ti	6.28 ± 0.08c	19.51 ± 0.23c	12.71 ± 0.14c	67.78 ± 0.37a	62.33 ± 0.72a	1.62 ± 0.02c	141.04 ± 1.6a	32.74 ± 0.38a	86.72 ± 1.00e	1.52 ± 0.02d	0.46 ± 0.02b
	Cm-Ti-li	7.04 ± 0.08ab	30.12 ± 0.35bc	29.4 ± 0.34a	40.48 ± 0.69c	43.12 ± 0.50cd	1.89 ± 0.02bc	88.87 ± 1.0c	24.48 ± 0.28b	154.15 ± 1.80b	4.51 ± 0.05a	0.37 ± 0.01c
	Ch-Ti	6.71 ± 0.08b	28.08 ± 0.32bc	30.05 ± 0.35a	41.87 ± 0.67c	55.76 ± 0.65b	1.61 ± 0.02c	133.6 ± 1.5ab	33.6 ± 0.39a	171.54 ± 2.00a	3.47 ± 0.04bc	0.45 ± 0.02b
	Ch-Ti-li	6.92 ± 0.08ab	32.25 ± ± 0.37b	12.46 ± 0.14c	55.28 ± 0.52ab	61.45 ± 0.71a	2.38 ± 0.03ab	88.87 ± 1.0c	20.45 ± 0.24b	151.38 ± 1.70b	3.72 ± 0.04b	0.4 ± 0.02c

Different letters in a column indicate a significant difference between treatments ($p < .05$).

Natural control: soil sample from non-truffle ground; Ti-S-1, Ti-S-2: *T. indicum*-soils from low productive grounds; Ti-S-3, Ti-S-4: *T. indicum*-soils from moderately productive grounds; Ti-S-5, Ti-S-6: *T. indicum*-soils from highly productive grounds; Art-control: soil sample from non-inoculated ground; Cm-Ti: soil samples of *T. indicum*-inoculated from *C. mollissima* Blume not treated with stacking of calcareous rock; Cm-Ti-li: soil samples of *T. indicum*-inoculated from *C. mollissima* Blume treated with of stacking of calcareous rock; Ch-Ti: soil samples of *T. indicum*-inoculated from *Co. heterophylla* Fisch not treated with stacking of calcareous rock; Ch-Ti-li: soil samples of *T. indicum*-inoculated from *Co. heterophylla* Fisch with the treatment of stacking calcareous; ACa: available calcium; AK: available potassium; AMg: available magnesium; AN: available nitrogen; AP: available phosphorus; OM: organic matter; TK: total potassium; TN: total nitrogen; TP: total phosphorus. Each value is the mean of 3 replicates (±SD). Values followed by different lowercase letters indicate significant differences ($p < .05$) between samples in a line. Sand, the soil mechanical composition of sand particles; Clay, the soil mechanical composition of clay; and Silt, the soil mechanical composition of silt.

Table 5. Spearman correlation coefficient between soil properties and mating type gene distribution in cultivated plantation soils.

	pH	Sand	Silt	Clay	OM	TN	AN	AP	AK	ACa	AMg
MAT1-1-1/MAT1-2-1	−0.505	0.142	−0.431	−0.025	0.154	−0.419	0.515	0.732*	−0.210	−0.142	0.477

ACa: available calcium; AK: available potassium; AMg: available magnesium; AN: effective nitrogen; AP: available phosphorus; OM: organic matter; TN: total nitrogen.
*Significant at $p < .05$.

of |(*MAT1-1-1*/*MAT1-2-1*)| in cultivated and natural samples (Table 5). Available phosphorus was found to be significantly closely correlated with the *MAT1-1-1*/*MAT1-2-1* in ectomycorhizosphere soil.

4. Discussion

Several studies have reported that active carbonate favors the growth of *T. melanosporum* because active carbonate constitutes an important reserve of exchangeable calcium ions (Ca^{2+}) [18,26]. Riousset et al. [27] reported that *T. indicum* developed in soils free from calcium carbonate, with a moderate pH, and rich in organic matter. However, other authors indicated that *T. indicum* inhabits calcareous soils. García found that *T. indicum* ectomycorrhizae develop well in calcareous substrates that are rich in active carbonate [28]. Granetti et al. [29] indicated that *T. indicum* inhabits calcareous substrates with a pH that varies from 5.5 (due to organic matter) to 8.5. Fourré et al. [30] reported that *T. indicum* occurs on calcareous plateaus at 2000–3000 m. In our study, *T. indicum* inhabited neutral or acidic soils (pH 6.10–7.18). It is quite interesting to see in our study that active calcium in natural samples was significantly ($p < .05$) lower than that of the control, indicating that *T. indicum* might adapt to a wide range of calcium conditions in soil. Most of natural and cultivated soil properties were found significantly different from their corresponding controls, which indicated that the colonization of truffle mycelia could affect the properties of ectomycorhizosphere soil. Compared to the controls, the natural truffle producing soils have higher sand and clay content, which may be beneficial to oxygen acquisition for truffle production and discharge of excess water. Soils treated with stacking calcareous rock had a relatively higher exchangeable Ca^{2+} compared to samples without limestone treatment, and correlation analysis showed that exchangeable Ca^{2+} in soils showed no significant correlation with the ratio of *MAT1-1-1*/*MAT1-2-1* in soils. However, available phosphorus was found significantly correlated with the *MAT1-1-1*/*MAT1-2-1* in ectomycorhizosphere soil. The result provides a potential way to affect the proportion of the mating type genes in ectomycorhizosphere soil. In the cultivated orchards, *T. indicum* are associated with some indigenous host trees, such as pines, *C. mollissima* and Asian *Quercus* [31]. In our study, *C. mollissima* and *Co. heterophylla* were considered due to their relative high colonization rate with *T. indicum*. No significant difference was found for soil properties between the soils of the two host trees. However, the value of *MAT1-1-1*/*MAT1-2-1* in *C. mollissima*–*T. indicum* ground soils was greater than in *Co. heterophylla*–*T. indicum* ground soils, indicating that the distribution of mating type genes might be associated with the hosts. Further investigation is needed to study the relationships between *MAT* gene distribution of *T. indicum* and its hosts.

The Asian black truffle *T. indicum* was proven to be heterothallic by Belfiori in 2013 [12]. The distribution pattern of European black truffle *T. melanosporum* strains on their hosts was found to share an identical *MAT* profile with the underground soil, which is not conducive to the formation of its ascocarps. The presence of two *MAT* idiomorphs (*MAT1-2* and *MAT1-1*) in the *T. melanosporum* orchard was determined by Osting and Tedersoo [14], and *MAT*-gene determination was successful in *T. melanosporum* root tip samples in their study. They reported that each DNA sample of *T. melanosporum* analyzed displayed the presence of either the *MAT1-2* or the *MAT1-1* idiomorph. Previous studies indicated that although black truffle spores of both mating types coexisted on young seedlings, one of the mating types was progressively supplanted over time [32]. These findings are consistent with the results of this study, which showed that in natural-truffle-producing-areas, the *MAT1-2-1* gene could be detected in only three of the collected soil samples (Ti-S-3, Ti-S-4, and Ti-S-6), suggesting that the *MAT1-1-1* gene may be excluded over time. Soil samples were also collected to investigate the distribution of *T. melanosporum* mycelia with different *MAT*s by Murat et al. [15], in which at least one *MAT* gene was amplified in 45 of the 48 soil DNA samples studied. In our study, we found that ascocarp production was closely related to the competition between two mating type genes and the expansion of the unbalanced distribution of two kinds of mating type genes, which supported previous studies on *T. melanosporum* soils. Furthermore, ascocarps gleba from each site showed a nonrandom distribution of the two mating types. For example, samples from Huidong and Weishan contained only one mating type, *MAT1-2-1*, which was more plentiful than *MAT1-1-1* in samples from Jinyang and Huili. Absolute quantitative real-time PCR has been reported to be sensitive and accurate and can be used to detect genes in environmental samples with low copy numbers [33]. The application of absolute quantification real-time PCR technology in this study has helped us calculate the distribution of *MAT* genes in ectomycorhizosphere soil and the ratio of the two mating types. This suggests that monitoring the dynamic changes in *MAT* genes in the soil and ECMs may have good prospects for use.

In conclusion, the phenomenon of uneven and nonrandom distribution of *MAT* genes in truffle-producing areas or ascocarps gleba was here found to be shared across different soil groups. The competition between the two mating type genes and the subsequent expansion of the unbalanced distribution is considered to be closely related to truffle fructification. Limestone treatments failed to alter the proportion of the two mating type genes in the soil. The use of real-time quantitative PCR can provide a reference for monitoring the dynamic changes of mating type genes in soil. Further studies are needed to investigate the competition, fusion, and uneven distribution of mating type genes and the associated environmental factors that may promote fructification in truffles.

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