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Shiro-like Structure Formation of Chinese *Tricholoma matsutake* Strain YN1 in *Pinus armandii* and *Pinus elliottii* Seedlings

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Abstract: *Tricholoma matsutake* is one of the edible ectomycorrhizal fungi with great economic and ecological values. Artificially mycorrhized seedlings inoculated with *T. matsutake* strains from Finland and Japan have been widely reported. However, few reports on the morphological characteristics of mycorrhizae synthesized from the Chinese *T. matsutake* strain are available to date. Here, we find that the *T. matsutake* strain YN1 from Yunnan province, China, can form well-developed ectomycorrhizae in symbiosis with both exotic (*Pinus elliottii*) and Chinese native (*Pinus armandii*) species. The symbiotic structures of ectomycorrhizae included obvious mantle sheaths and Hartig nets visible under a microscope. The ectomycorrhizae appeared earlier in a larger quantity in *P. elliottii* than in *P. armandii* in the culture-plate symbiotic system. The ectomycorrhizae obviously promoted the growth of potted *P. armandii* and *P. elliottii*, accompanied by the formation of shiro-like structures. Our results provide references for the effective management of ectomycorrhizae synthesis in both *P. armandii* and *P. elliottii* seedlings to form shiro-like structures, and also provide a new perspective for the afforestation and mushroom cultivation research on *T. matsutake*.

Keywords: ectomycorrhizae; *Pinus armandii*; *Pinus elliottii*; *Tricholoma matsutake*; shiro-like structures

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1. Introduction

Tricholoma matsutake usually occurs on leaf litter on the forest floor around the roots of non-symbiotic or symbiotic host plants, and can form mycorrhizal associations with the roots of several tree species. *T. matsutake* tends to be symbiotic with *Pinus densiflora* [1–3]. Matsutake was once known as *Tricholoma nauseosum* in northern Europe [4–6]. *T. matsutake* is distributed in the northern hemisphere, mainly in Asia (China, Bhutan, Japan, North and South Korea, and Russia), North America (Canada and eastern USA), and Europe (Austria, Czechia, France, Finland, Germany, Norway, Switzerland, and Sweden) [1,6–9]. *T. matsutake* (S. Ito & S. Imai) Singer is named "song rong" or "song koumo" in East Asia, and it is an edible ectomycorrhizal mushroom with high commercial value. In China, wild *T. matsutake* is mainly found in southwestern China, including Guizhou, Sichuan, Yunnan, and Tibet provinces, and northeastern China, including Jilin and Heilongjiang provinces [10,11]. *T. matsutake* has become rare or even extinct in its main habitats, such as Japan, North and South Korea, and China, due to excessive commercial collection and damage to natural habitats, especially the damage to the soil mycelium aggregate (hereafter referred to as shiro) [1,12]. Since 1999, it has been rated as a secondarily endangered species in China [13]. In 2020, it was listed as one of the world's endangered species by the International Union for Conservation of Nature (IUCN) for the first time. The IUCN Red List of Threatened Species 2020: e. T76267712A177054645. https://dx.doi.org/10.2305/IUCN.UK.2020-3.RLTS.T76267712A177054645.en, accessed on 6 April 2022. Therefore, *T. matsutake* is a globally endemic

species. Additionally, it is urgent to protect and rationally develop the natural population of *T. matsutake*.

Some studies have reported that *T. matsutake* and its host plants (*P. densiflora*) can form mycorrhizal structures under aseptic or open conditions [14–18]; however, few studies on *T. matsutake* have been conducted in China [13,19]. In Europe, *T. matsutake* is primarily symbiotic with *Pinus sylvestris* and *Picea abies*, and in Japan, *Abies veitchii* and *Tsuga diversifolia* have been reported to be the hosts of *T. matsutake* [14]. In northeastern China as well as North and South Korea, *Pinus pumila*, *Pinus thunbergii*, and *Quercus mongolica* have been found to be symbiotic with *T. matsutake* [20]. In southwestern China and Bhutan, *Castanopsis orthacantha*, *Pinus yunnanensis*, *Pinus wallichiana*, *Pinus armandii*, *Pasania* spp., *Quercus aquifolioides*, and *Lithocarpus* spp. have been reported to be the host plants of *T. matsutake* [20–24]. In addition to these natural hosts, *T. matsutake* can also be symbiotic with artificially selected tree species *Cedrela hererae* (Meliaceae) and *Betula platyphylla* var. *japonica* in vitro [22,23]. In China, *T. matsutake* is mostly collected from *Pinus koraiensis*, *P. densiflora*, *P. armandii*, and *P. yunnanensis*, among which *P. armandii* and *P. yunnanensis* are mainly distributed in Yunnan province. Additionally, *P. densiflora* has been reported to be a major host plant of *T. matsutake* under experimental conditions in northeastern China, Japan, North Korea, and South Korea [1,3,15–18].

Numerous studies have been conducted in Japan and Finland to synthesize the mycorrhizae of *T. matsutake* under aseptic conditions [14,15]. However, few studies have been conducted to culture *T. matsutake* in the rhizosphere of Chinese native trees, such as *P. yunnanensis*, *P. densiflora*, and *P. armandii*. There are many types of host plants that are symbiotic with *T. matsutake* in nature. In Finland, *T. matsutake* strains can form ectomycorrhizae in native *P. sylvestris* and *P. abies* seedlings in vitro; however, in Japan, *T. matsutake* strains cannot perform this activity [14]. Therefore, the excellent host plants of *T. matsutake* strains may vary in regions, which is supported by the findings of Vaario et al. [25] that there were some significant differences in *T. matsutake* ectomycorrhizae among two strains of *T. matsutake* from different origins and four *P. sylvestris* clones from different mother trees.

In this study, the morphological and anatomical features of ectomycorrhizal and shiro-like structures of *T. matsutake* in both *P. armandii* and *P. elliottii* are investigated. The mycorrhization level of exotic *P. elliottii* is evaluated and compared with that of *P. armandii*, a native species widely distributed in central and southwestern China [26]. The results also show that the *P. elliottiii* (exotic tree) might be a potentially excellent host tree of *T. matsutake*, which can form well-developed ectomycorrhizae with the *T. matsutake* strain YN1. *P. elliottii* originates from the United States and has been widely cultivated all over the world [27]. Due to its rapid growth, strong adaptability, and high resin content, *P. elliottii* has been rapidly and widely introduced in more than 12 provinces in China for afforestation [28,29]. Considering this, the symbiotic relationship between the China native *T. matsutake* strain YN1 and exotic pine trees needs to be further explored.

2. Materials and Methods

2.1. Sampling, Mycelial Isolation, and Inoculant Preparation of Tricholoma matsutake

Three samples of *T. matsutake* were collected from coniferous forests in Nanhua county, Yunnan province, China, dried with silica gel, and preserved at the Institute of Applied Mycology at Huazhong Agricultural University (Figure 1a,b). The *T. matsutake* YN1 strain was isolated from a sporocarp of one of the three fresh samples. The mycelia of *T. matsutake* were cultured on P5 medium (pH 5.8) according to the formula reported by Chen et al. [30]. After being cultured in the dark at 24 °C for 2 months, the mycelial culture was used as a solid inoculant for mycorrhiza synthesis (Figure 1c,d). Subsequently, 8 to 10 small mycelial discs (diameter: 6 mm) from *T. matsutake* initially cultured colonies were placed into sterile 250 mL glass flasks with 100 mL of liquid P5 medium to culture the liquid mycelia in the dark at 24 °C. The obtained liquid mycelium culture was added into seedling pots as a supplementary liquid inoculum to maintain the activity and density of *T. matsutake* mycelia 45 days after the mycorrhizal seedlings' transplantation.

Figure 1. Morphology of *Tricholoma matsutake* sporocarps and mycorrhization of the strain YN1 isolated from the sporocarp YN1. (**a**) *T. matsutake* sporocarps on the forest floor; (**b**) morphology of *T. matsutake* YN1 sporocarp; (**c**) morphology of *T. matsutake* strain YN1 mycelia at day 120 post-culture in P5 (Pachlewski) medium; (**d**) mycelial ball of *T. matsutake* strain YN1 at day 60 post-culture in liquid P5 medium in a 150 mL glass flask; (**e**) *Pinus armandii* seedlings inoculated with *T. matsutake* strain YN1 at day 60 post-culture on MS medium in a square Petri dish; (**f**) *Pinus elliottii* seedlings inoculated with *T. matsutake* strain YN1 cultured at day 60 post-culture on MS medium in a round Petri dish.

2.2. Axenic Culture of Pinus armandii and Pinus elliottii Seedlings in Petri Dishes

Seeds of *P. armandii* and *P. elliottii* were purchased from the Forestry Bureau of Hubei Province, Wuhan, China, and kept at 4 °C until utilization. The disinfection of these two Pinaceae seeds was performed according to the method reported by Chen et al. [30]. The sterilized seeds of *P. armandii* and *P. elliottii* were first placed in Petri dishes containing 1.0% water–agar medium and cultured at 24 °C in the dark for 6 days. The seeds germinated at 24 °C in the light for 7 days. After germination, sterile seedlings were selected and transplanted into mycorrhizal induction plates.

2.3. Aseptic Synthesis of Mycorrhizae in Petri Dishes

Ectomycorrhizae were synthesized in large plastic Petri dishes (a 15 cm diameter round plate or 12 cm × 12 cm square plate). The Petri dishes were filled with 20 mL of 1/2 MS agar medium without sucrose (Sigma-Aldrich Corporation, St. Louis, MO, USA) [31]. The surface of the 1/2 MS culture medium was covered with a layer of sterile cellophane membrane in order to prevent the roots of *P. armandii* or *P. elliottii* from growing into the medium. The aseptic mycorrhizal synthesis of the two *Pinus* seedlings was conducted as described by Chen et al. [30]. The seedlings without the inoculation of *T. matsutake* were used as the control. All the plates were placed at a 45-degree angle in the growth chamber for ectomycorrhizal development. During the ectomycorrhizal synthesis process, the plate part containing roots was covered with aluminum foil, and the ectomycorrhizae were cultured at a regime of 23 °C/17 °C and a 12 h light/12 h dark cycle for 3 months. All the experiments were conducted with at least four replicates.

2.4. Ectomycorrhizal Observation

Ectomycorrhizae were observed under a stereomicroscope (Olympus SZX16, Osaka, Japan). Mycorrhizal cross-sections were prepared as follows. Some selected putative mycorrhizal root tips were cut into 2–4 mm segments; fixed with FAA fixative consisting of 10% (*v*/*v*) formaldehyde, 50% (*v*/*v*) absolute ethanol, and 5% (*v*/*v*) acetic acid; dehydrated by a graded series of ethanol; and embedded using paraffin (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) [16–32]. The root tips were continuously sliced with a semi-automatic microtome (Thermo Scientific Microm HM340E, Waltham, MA, USA) to a thickness of 10 μm, and the sections were attached to microscope slides. The sections were stained with 1% safranin and 0.1% fast green, referring to Chen et al. [30]. The samples were observed under an optical microscope (Olympus BX51, Osaka, Japan) and images were captured using a MicroPublisher 5.0 RTV camera attached to the microscope.

2.5. Cultivation of Mycorrhizal Seedlings

The *P. elliottii* and *P. armandii* mycorrhizal seedlings inoculated with *T. matsutake* were transplanted into white plastic pots with a bottom diameter of 10.5 cm, upper diameter of 15.5 cm, and height of 14.1 cm. The pots were filled with 2.1 kg of autoclaved (0.11 Mpa, 121 °C, 2 h) brown soil collected from Shizishan Hill at Huazhong Agricultural University with a mixed matrix (including peat, vermiculite, and perlite) bought from Nanhu flower and tree market with at least 4 pots per treatment. The brown soil was thoroughly mixed with the matrix at a volume ratio of 1:1 to improve the air permeability, and the final pH value of the substrate mixed with sterilized soil was about 6.0. The seedlings without the inoculation of the *T. matsutake* strain were used as the blank control. All the treated seedlings were placed in a greenhouse where the phot flux density was 982 μmol/m^2/s, the day/night temperature was 24/20 °C, and the relative humidity was 85% from 2 April 2016 to 13 December 2017. After blending with some sterilized water, 5 mL of liquid cultured mycelial inoculum (after filtration) was supplemented to the root system of mycorrhizal seedlings 3 times at an interval of every 15 days to prevent the decline in the mycorrhizal structure of *T. matsutake*. Moreover, it was necessary to set a control with the same amount of sterilized inoculum to the root system of non-mycorrhizal seedlings.

2.6. Molecular Identification of Sporocarps, Mycelium Cultures, Mycorrhizae, and Shiro-like Structures

ITS Fragment Amplification by PCR

The *T. matsutake* sporocarps, mycelium cultures, mycorrhizae, and shiro-like structures were ground with an electric tissue grinding set (OSE–Y50, TIANGEN Co., Ltd., Beijing, China) to extract DNA following the protocol of CTAB (hexadecyltrimethylammonium bromide) [31]. The optimized polymerase chain reaction (PCR) was conducted using the primer pair ITS1F/ITS4R according to the previously reported method [13,31,33]. PCR without a DNA template was used as a negative control to avoid possible contamination. The

PCR products were sequenced using amplified primers at Quintarabio Co., Ltd. (Wuhan, China). The representative contigs were selected and aligned to the fungal sequences against GenBank by blast. The original sequences in the present study were submitted to GenBank with accession numbers of MF521898 (YN1 isolate), OR121471 (YN1 parent sporocarp), OR128362 (YN1 mycorrhiza), and OR226259 (YN1 shiro-like structures).

2.7. Data Analysis

A total of 22 ITS sequences (S1) of *Tricholoma* were subjected to phylogenetic analyses, including 4 sequences from our study, and the remaining 18 sequences were obtained from the literature on identified *Tricholoma* with their sequences submitted to GenBank [5,14,17,18,23,31]. *Catathelasma ventricosum* was used as an outgroup. Detailed information of the analyzed sequences is provided in Table 1. The sequences were aligned using MEGA7.0. The aligned data were analyzed by ML (maximum likelihood) using a rapid bootstrapping algorithm with 1000 replicates to construct a phylogenetic tree.

Table 1. Sequences of mycorrhiza, strains, and sporocarps used for phylogenetic analyses in this study.

Species	Voucher	GenBank Accession No. ITS	Mycorrhiza/ Shiro/Strain/Sporocarp	Country
Tricholoma matsutake	YN1	MF521898	Strain	China
T. matsutake	YN1	OR121471	Sporocarp	China
T. matsutake	YN1	OR128362	Mycorrhiza	China
T. matsutake	YN1	OR226259	Shiro	China
T. matsutake	Uncultured *Tricholoma*	GU134497	Strain	China
T. matsutake	AT-2195	AB968622	Strain	Japan
T. matsutake	EF	GQ904716	Strain	Finland
T. matsutake	Tn5	AB188553	Strain	Italy
T. matsutake	Tn6	AB188554	Strain	Switzerland
Tricholoma bakamatsutake	Tb1	AF204807	Strain	Japan
T. bakamatsutake	B1	AB036898	Strain	Japan
T. bakamatsutake	IFO30663	AF241515	Strain	South Korea
Tricholoma robustum	-	AB078341	Unknown	Japan
T. robustum	Tr3	AB289665	Strain	Japan
Tricholoma portentosum	615	AB036896	Strain	Japan
T. portentosum	KMS304	AF349686	Sporocarp	America
Tricholoma flavovirens	DED5365	AF377181	Strain	America
T. flavovirens	trh545	AF458449	Sporocarp	America
Tricholoma fulvocastaneum	TN6941	AB289668	Strain	Unknown
T. fulvocastaneum	MR26	AB036901	Strain	Japan
T. fulvocastaneum	WK1N	AF204808	Strain	Japan
T. fulvocastaneum	-	DQ067895	Sporocarp	Thailand
Catathelasma ventricosum	PBM2403	DQ486686	Basidiomata	Czech Republic

The data of pine seedlings' shoot heights (S2) were statistically analyzed with one-way ANOVA (and nonparametric or mixed) multiple comparisons in GraphPad Prism v9.0.0 software, and the significance level was set as 0.05. The column chart of this article was also created using GraphPad Prism v9.0.0 for Windows (S3).

3. Results

3.1. Mycorrhizal System in Culture Plate

The colony of *T. matsutake* strain YN1 is white, whose marginal hyphae are flat and smooth. Its colony growth rate is very slow at about 0.5 mm/day (diameter). The hyphae of strain YN1 are denser in the center than at the edges, and the aerial hyphae often gather into bundles (Figure 1c). The liquid mycelia of *T. matsutake* strain YN1 can be rapidly cultured (60 days/glass flasks), and its mycelial ball is as dense as cotton (Figure 1d). In the pure-culture system, the lateral root tips of *P. elliottii* seedlings were colonized by

T. matsutake strain YN1 on large plastic Petri dishes (round or square) on day 60 after inoculation; however, the clonization of *P. armandii* seedlings by this strain was less evident at the same time (Figure 1e,f). The obvious "Y-type" mycorrhizal morphology of the lateral root tips in *P. armandii* seedlings was observed on day 75 after inoculation. All the seedlings developed excellent ectomycorrhizae on day 120 after inoculation with *T. matsutake* strain YN1 in vitro, whereas no ectomycorrhizae were observed on the root tips of the control seedlings without inoculation (Figure 2).

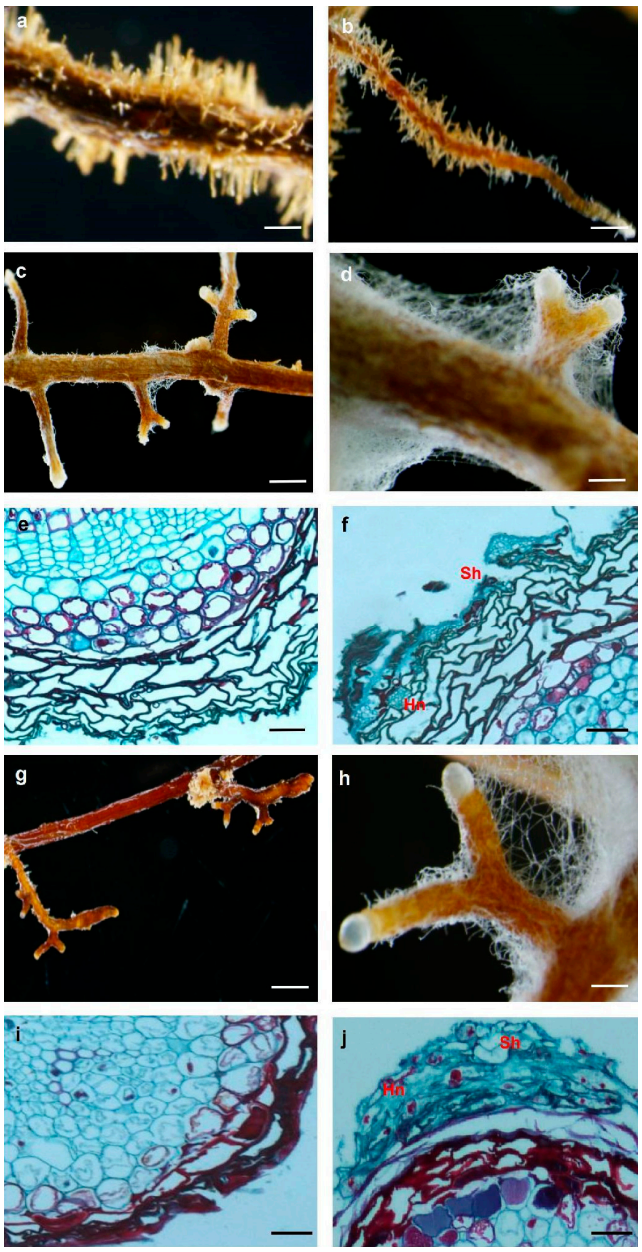

Figure 2. Morphological features of mycorrhizae of *Pinus armandii* and *Pinus elliottii* seedlings. The lateral roots of the control *P. armandii* (**a**) and *P. elliottii* (**b**) seedlings without inoculation. Morphology of the *Tricholoma matsutake* mycorrhizal tips of the lateral roots of *P. armandii* (**c**,**d**) and *P. elliottii* (**g**,**h**) seedlings on day 120 post-culture. "Y-type" mycorrhizal structures in *P. armandii* (**d**) and *P. elliotti* (**h**) seedlings. Cross-sections of the control *P. armandii* (**e**) and *P. elliottii* (**i**) seedlings without inoculation. Cross-sections of the mycorrhizal tips of *P. armandii* (**f**) and *P. elliotti* (**j**) seedlings. The dark red represents the stained plant cell walls. Blue-green indicates fungal sheath and Hartig net. Sh, fungal sheath; Hn, Hartig net. Bars = 2 mm (**a**,**b**,**c**,**g**); bars = 0.5 mm (**d**,**h**); bars = 50 μm (**e**,**f**,**i**,**j**).

3.2. Morphological Features of the Mycorrhiza of Pinus armandii and Pinus elliottii Seedlings

Although the lateral roots of the control seedlings (*P. armandii* and *P. elliottii*) without inoculation were densely covered with root hairs, the root hairs of *P. armandii* seedlings were larger and stronger than those of *P. elliottii.* The color of *P. armandii* seedlings was yellowish-brown, which was darker than that of *P. elliottii* (Figure 2c,g), and its root epidermal cells were also more irregular than those of *P. elliottii* (Figure 2i,j). After colonization by *T. matsutake* strain YN1, the root hairs on the lateral roots of *P. elliottii* and *P. armandii* seedlings gradually decreased, until they disappeared, and then mycorrhizal structures slowly developed with the main and lateral root surfaces covered with hairy extraradical mycelium (Figure 2a,b,e,f). Compared with the control seedlings (*P. armandii* and *P. elliottii*) (Figure 2i,j), *P. armandii* (Figure 2a,b,d) and *P. elliottii* (Figure 2e,f,h) seedlings inoculated with *T. matsutake* exhibited more obvious morphological features of ectomycorrhizae. Many swollen parts and "Y-type" mycorrhizal structures and increasingly developed Hartig net were observed on the lateral roots, and thick-walled fungal sheaths formed an obvious mantle to wrap the root surface (Figure 2b,d,f,h). The ectomycorrhizae of *P. elliottii* were formed earlier (at least 2–3 weeks earlier) and were more abundant than those of *P. armandii.* Due to the irregularity of its root epidermal cells, the Hartig net of *P. armandii* mycorrhizal seedlings seemed incomplete, compared with that of *P. elliottii* mycorrhizal seedlings.

3.3. Transplantation of Mycorrhizal Seedlings

Compared with the non-mycorrhizal seedlings, mycorrhizal seedlings (*P. elliottii* and *P. armandii*) inoculated with *T. matsutake* exhibited faster growth 18 months after transplantation into the white plastic pots (Figure 3a,c). The heights of the mycorrhizal seedlings were 2.07 (*P. armandii*)- and 1.85 (*P. elliottii*)-times as high as that of the non-mycorrhizal seedlings, respectively (Figure 3e). Whether inoculated or not, the *P. elliottii* seedlings grew obviously faster than the *P. armandii* seedlings under the same conditions. The colonies of mycorrhizal seedlings inoculated with *T. matsutake* were often located 5–10 cm below the matrix soil with irregular shapes, and the soil mycelium aggregate designated as shiro [17,24,31]. In these shiro-like structures (Figure 3b,d), the gaps among tree roots, soil particles, and rock particles were colonized by *T. matsutake* mycelium, and the soil turned from gray to gray–white and became loose and powdery with an obvious mushroom smell. However, the soil moisture content in the shiro-like structure of *P. elliottii* mycorrhizal seedlings decreased more than that of *P. armandii* mycorrhizal seedlings (Figure 3b,d).

High similarities in the mycorrhizal morphology was observed between *P. elliottii* and *P. armandii* seedlings, both of which are uniaxial pinnate, bifurcate (Y-type), irregularly branched, or single rod-shaped specimens. The carbonization of the mycorrhizal tip cortex was observed in the mycorrhizal seedlings (*P. armandii* and *P. elliotti*) (Figure 3b,d), which conformed to typical features of *T. matsutake* mycorrhizae in the granite-based soil [17]. The mycorrhizal front end was straight or slightly curved, and the white-to-pale-yellow mycorrhizal surface was coated with a large number of cotton flocculent hyphae. These hyphae were entangled with each other and they were hard to peel from the seedling roots. The surface of young and tender mycorrhizae was smooth and shiny, more or less rod-shaped, plump, and mostly light-yellowish brown, whereas the surface of mature mycorrhizae was often swollen, deep black, almost carbonaceous, and easy to break. The dead mycorrhiza was nearly hollow and its surface was rough, shriveled, and dark brown (Figure 3b,d). Our observation was consistent with the report by Vaario et al. [26].

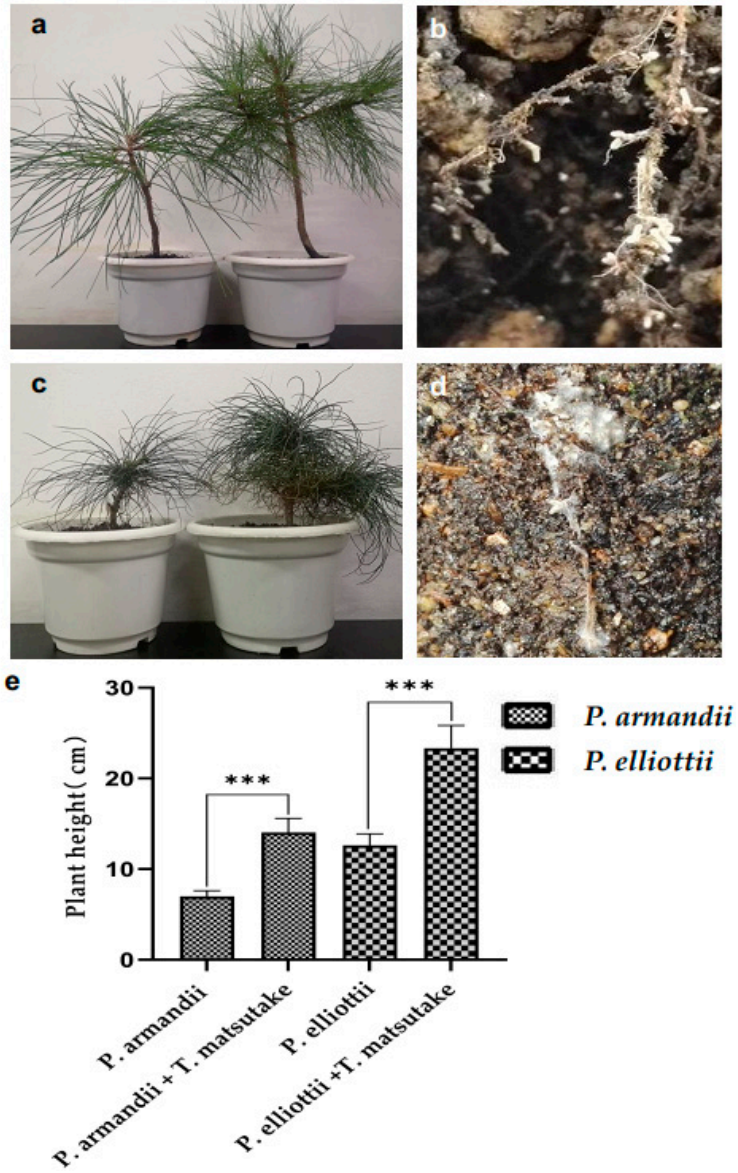

Figure 3. Effects of *Tricholoma matsutake* ectomycorrhiza on plant height and lateral root development of *Pinus elliottii* and *Pinus armandii* seedlings. (**a**,**c**) Ectomycorrhizal and non-mycorrhizal *P. elliottii* (**a**) and *P. armandii* (**c**) seedlings 18 months after transplantation. On the left is the non-mycorrhizal seedling and on the right is the mycorrhizal seedling inoculated with *T. matsutake*. (**b**,**d**) Shiro-like structures of mycorrhizal *P. elliottii* (**b**) and *P. armandii* (**d**) seedlings inoculated with *T. matsutake*. (**e**) Effects of *T. matsutake* ectomycorrhiza on plant height. Significant differences are detected by *t*-tests in GraphPad Prism v9.0.0. Data are expressed as the mean ± SE of 4 biological replicates (n = 4). ***, $p < 0.0001$. All these treatments were repeated four times with similar results.

3.4. Identification and Phylogenetic Analysis of the Strains and Its Relevant Materials

The ITS sequence of *T. matsutake* strain YN1 (GenBank acc. MF521898) from Yunnan province, China, was identical to that of its parent sporocarp (GenBank acc. OR121471), which was firstly verified by the NCBI–BLAST search to ensure its accuracy. Maximum likelihood (ML) phylogenetic trees were constructed based on the ITS sequences. The ITS sequences of *P. elliottii* and *P. armandii* seedling mycorrhizae (GenBank acc. OR128362) and shiro-like structures (GenBank acc. OR226259) exhibited 100% similarity to the *T. matsutake* YN1 strain. The ITS sequences of the *T. matsutake* YN1 sporocarp, YN1 strain, YN1 mycorrhizae, and YN1 shiro-like structures were compared with the those of Switzerland strain Tn6 AB188554 [5], Finland strain GQ904716 [14], Italy strain Tn5 AB188553, Japan

strain AB968622 [21], and published northeastern China ITS sequence GU134497. The results show that no4. differences in ITS sequences (642 base pairs) can be observed among these strains, mycorrhizae, sporocarps, and shiro-like structures. The phylogenetic analysis showed that the strain YN1, sporocarp, mycorrhiza, and shiro-like structure of *T. matsutake* were clustered together; however, they were distinguished from their related species. The above results confirm that the strain YN1, the parent sporocarp, and the mycorrhizae of *P. elliottii* and *P. armandii* belong to *T. matsutake* (Figure 4).

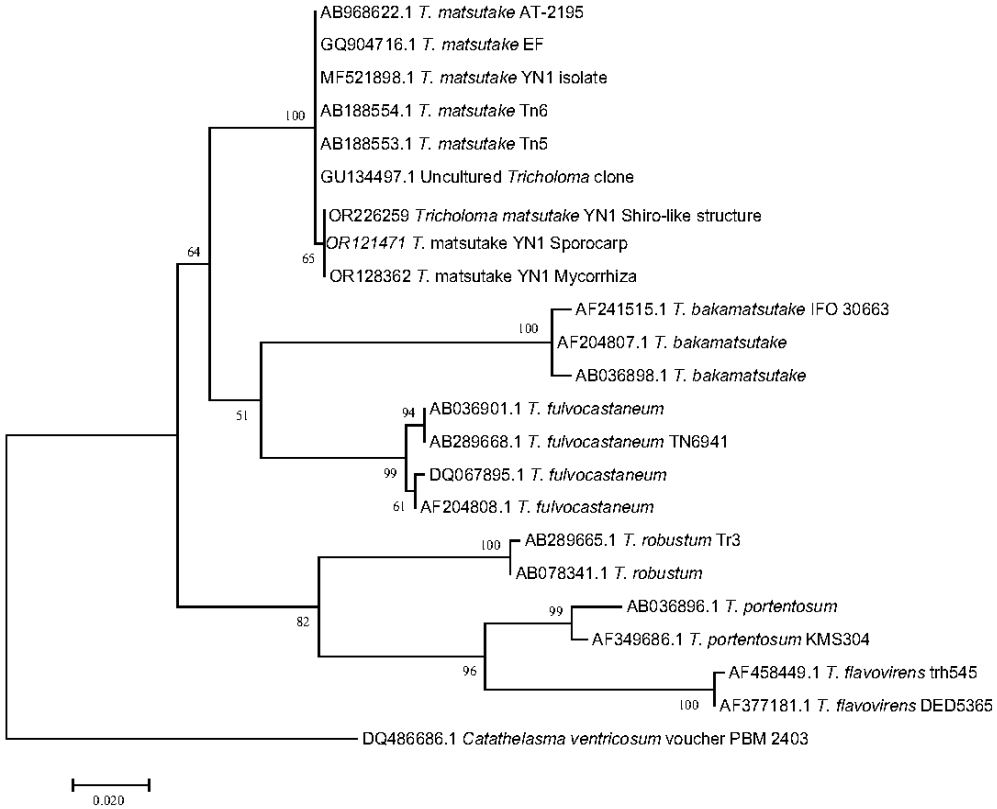

Figure 4. Phylogenetic relationships between *Tricholoma matsutake* and its related species by analyzing *T. matsutake* strains, mycorrhizae, sporocarps, and shiro-like structures. The phylogenetic tree was constructed by the method of ML (maximum likelihood) and plotted using the MEGA 7 program.

4. Discussion

Because of its high economic and ecological value, the domestication and cultivation of *T. matsutake* on a large scale have always been performed in China, and the related research has been widely conducted in Japan and other countries. However, the artificial domestication of *T. matsutake* in a short time range is challenging. The research on the domestication and cultivation of *T. matsutake* in Japan began in the early 20th century [21], involving germinating spores [22,33], obtaining pure culture mycelia [22], synthesizing ectomycorrhizae [22,33], forming shiro-like structure in an open-pot culture system containing a granite-based soil substrate [17,33], and artificially cultivating sporocarps of three *T. matsutake* close allies (*T. portentosum*, *Tricholoma terreum*, and *Tricholoma saponaceum*) in open pots [34,35]. Unfortunately, although the transplantation of mycorrhizal seedlings was successful, they failed to produce a large number of sporocarps of *T. matsutake* in their plantations.

Despite continuous efforts, the genetic characteristics of *T. matsutake*, the shiro-like structure and sporocarp formation, and the symbiotic mechanism with host plants remain largely unclear. To date, there have been few reports on inducing *T. matsutake* mycorrhizae under sterile conditions in China. The main obstacle is how to separate pure-culture hyphae from *T. matsutake* sporocarps, and the special nutrient requirements of *T. matsutake* make it

difficult for its mycelia to grow in a culture plate [15]. After culturing it in P5 medium, *T. matsutake* mycelia were successfully obtained from YN1 parent sporocarp tissues collected from a mixed forest (coniferous and broad-leaved) in southwestern China [21]. Our ITS sequences and phylogenetic analysis confirmed strain YN1 and mycorrhizal tips as *T. matsutake*. Our data indicate that using the liquid culture of mycelia as the inoculum can effectively solve the problem of the slow growth of the mycelia. Compared with solid-culture mycelia, liquid-culture mycelia grew better with a relatively simple operation.

The *T. matsutake* mycorrhiza synthesis technology is the basis of its mycorrhizal cultivation on the roots of host plants in the forest. The lack of a suitable culture system was the main factor limiting the successful induction of mycorrhizae in the culture plate. For the rapid and low-cost cultivation of mycorrhizal fungi, the open-pot system is a good choice to obtain inoculated ectomycorrhizal seedlings. Using this open-pot system, several species formed ectomycorrhizal mushrooms under greenhouse culture conditions, including *Cantharellus cibarius* [35–38], *T. portentosum*, *T. terreum*, and *T. saponaceum* [35,36,38]. However, the open-pot system is not suitable for the mycorrhizal induction of *T. matsutake*. In Finland, mycorrhizal seedlings were successfully synthesized using a sterile tube system filled with slant agar and clay beads [14]. Despite its obvious advantages, this sterile tube system seems to be time-consuming, and the mycorrhizal structure is easily destroyed by treatment. In this study, we used a modified pure-culture agar plate system [30], which was extremely intuitive and easy to use, and by using this system, mycorrhizae were effectively synthesized from Chinese *T. matsutake* strain YN1. With the help of a supplementary liquid inoculant, the *T. matsutake*-colonized pine seedlings were successfully acclimatized to open pots after being taken out from the pure-culture agar plates, and the shiro-like structures were formed 18 months after the mycorrhizal seedlings were transplanted to the nursery.

In addition to the culture systems, host specificity is another factor limiting successful mycorrhization, as mycorrhization requires the combination of the host plant species and *T. matsutake* strain YN1 in the culture plate [18]. It has been reported that local tree species are more suitable for local *T. matsutake* [27]. However, our study showed that the colonization of the Chinese local *T. matsutake* strain YN1 in the exotic tree *P. elliottii* seemed to be stronger than that in the local tree *P. armandii* (Figure 2c–f,h and Figure 3a,c); although, there was no significant difference in the morphological characteristics of mycorrhizae between *P. armandii* and *P. elliottii* seedlings (Figure 3b,d). The mycelium in *Pinus elliottii* (4–5 layers of epidermal cells) seemed to be thicker than that in *Pinus armandii* (2–3 layers of epidermal cells), which can be observed from the image in Figure 2.

Both *P. armandii* and *P. elliottii* are economically and ecologically important tree species, and they are widely planted in southwestern and central China. *P. elliottii* is thought to have originated from America and its neighborhood [27], and it was introduced into China in the late 1940s [28], while *P. armandii* is reported to be one of the local natural host trees of *T. matsutake* in southwestern China [21]. In this study, the local Chinese *T. matsutake* strain YN1 was found to efficiently synthesize the mycorrhizae with the exotic pine *P. elliottii*, which may be related to the origin and evolution of *T. matsutake* species in China. It has been reported that the ancestor of *T. matsutake* distributed in Asia at present came from North America through the Bering Strait [7], which may partially explain the good symbiotic relationship between exotic pine *P. elliottii* seedlings and the Chinese *T. matsutake* strain.

5. Conclusions

This study systematically investigated the mycorrhizal synthesis of the Chinese *T. matsutake* strain YN1 colonized in two commercially important pine species (*P. armandii* and *P. elliottii*) for the first time. The growth rate of *P. elliottii* seedlings was significantly faster than that of *P. armandii* seedlings under the same conditions, especially in the hot and humid climate, suggesting that *P. elliottii* was an excellent host plant. Additionally, the mycorrhizal seedlings inoculated with *T. matsutake* grew and developed better than the control (non-mycorrhizal seedlings), which might act as a form of afforestation and trending technology

in China in the future. We also found that shiro-like structures were formed 18 months after transplanting the mycorrhizal seedlings, and these shiro-like structures exhibited great potential for the practical cultivation of *T. matsutake*. Although the mycorrhizal synthesis technology of *T. matsutake* seems to be developing slowly in China, the biological research and its mushroom cultivation of *T. matsutake* will be accelerated in the future.

6. Patents

A Chinese patent (CN108260470 B) stemmed from the study reported in this manuscript, which is entitled "A method for improving the mycorrhizal seedlings of *T. matsutake* and its application", filed on 14 January 2018 and authorized on 14 August 2020 by the Patent Office of the People's Republic of China. The patent inventors were Heng Kang, Xin Chen, Chunye Mou, and Yinbing Bian from Huazhong Agricultural University.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14071439/s1. S1. The ITS sequence of *T. matsutake* Strains and Its Relevant Materials. S2. Pine Seedings height. S3. The height of mycorrhizal seedlings and the non-mycorrhizal seedlings.

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Data Availability Statement: All the data supporting the findings of this study are included in this article.

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