Traditional knowledge, diversity and cultivation of commercial ectomycorrhizal fungi in Southwest China, and impact of fungal communities on plantation establishment

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TESI DOCTORAL

Traditional knowledge, diversity and cultivation of commercial ectomycorrhizal fungi in Southwest China, and impact of fungal communities on plantation establishment

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RELATED WORK AND MANUSCRIPTS

The following manuscripts derived from this thesis are:

I. **Ran Wang**, Fu-Qiang Yu, Jesús Pérez-Moreno, Carlos Colinas, 2021. A new edible *Rhizopogon* species from Southwest China, and its mycorrhizal synthesis with two native pines. *Mycorrhiza*, 31: 85–92.

II. **Ran Wang**, Alexis Guerin-Laguette, Ruth Butler, Lan-Lan Huang, Fu-Qiang Yu, 2019. The European delicacy *Tuber melanosporum* forms mycorrhizae with some indigenous Chinese *Quercus* species and promotes growth of the oak seedlings. *Mycorrhiza*, 29: 649–661.

III. **Ran Wang**, Mariana Herrera, Wenjun Xu, Peng Zhang, Jesús Pérez Moreno, Carlos Colinas, Fuqiang Yu. Ethnomycological study on wild mushrooms and ectomycorrhizal fungi diversity in Pu'er Prefecture, Southwest Yunnan, China. *Manuscript has been submitted to Journal of Ethnobiology and Ethnomedicine*.

IV. **Ran Wang**, Shanping Wan, Juan Yang, Fuqiang Yu. *Choiromyces sichuanensis* sp. nov., a new edible pig truffle species from Southwest China, and its mycorrhizal synthesis with native trees. *Manuscript has been submitted to Mycorrhiza.*

V. **Ran Wang**, Yanliang Wang, Alexis Guerin-Laguette, Peng Zhang, Carlos Colinas, Fuqiang Yu. A successful establishment of exotic *Pinus radiata* seedlings depends on interaction effect of co-introduced *Lactarius deliciosus* or local ectomycorrhizal fungal communities. *Manuscript has been submitted to Frontiers in Microbiology.*

CONTRIBUTION IN OTHER ARTICLES AND MANUSCRIPTS

I. Mariana Herrera, **Ran Wang**, Peng Zhang, Fu-Qiang Yu, 2022. The ectomycorrhizal association of *Tricholoma matsutake* and two allied species, *T. bakamatsutake* and *T. fulvocastaneum*, with native hosts in subtropical China. *Mycologia*, 114: 303–318.

II. Yan-Liang Wang, **Ran Wang**, Bin Lu, Alexis Guerin-Laguette, Xin-Hua He, Fu-Qiang Yu, 2021. Mycorrhization of *Quercus mongolica* seedlings by *Tuber melanosporum* alters root carbon exudation and rhizosphere bacterial communities. *Plant and Soil*, 467: 391–403.

III. Dong Liu, Jesus Perez-Moreno, Peng Zhang, **Ran Wang**, Fu-Qiang Yu, 2021. Distinct compartmentalization of microbial community and potential metabolic function in the fruiting body of *Tricholoma matsutake*. *Journal of Fungi*, 7: 586.

IV. Lan-Lan Huang, Alexis Guerin-Laguette, **Ran Wang**, Fu-Qiang Yu, 2020.

Characterization of *Tuber indicum* (Pezizales, Tuberaceae) mycorrhizae synthesized with four host trees exotic to China. *Symbiosis*, 82: 215–224.

CONGRESS PROCEEDINGS OR TRAINING COURSES

I. Attended the 2021 National workshop on Microbial diversity and Phylogenetics which was co-hosted by Guizhou University/Center for Biochemical Engineering of Guizhou province and Mycological Diversity and Systematics Committee of Chinese Society of Fungi, and hold in Guiyang from September 26th to 29th, 2021.

II. Attended the 5th training course of Ethnobotany which was hold by Ethnobotanical research group of Kunming Institute of Botany, Chinese Academy of Sciences from July 12th to 15th, 2020. All learning contents have been completed according to schedule.

III. Attended the 10th International Workshop on Edible Mycorrhizal Mushrooms (IWEMM 10) hold in Suwa, Nagano, Japan in 20th October-29th, 2019 and made an oral presentation "Diversity of commercial wild mushrooms in Yunnan, China and cultivation of the ectomycorrhizal genera *Lactarius* and *Tuber*".

IV. Completed an online non-credit course "How to write and publish a scientific paper (Project-center course)" in 2019.

V. Attended the summit forum of Modern fungi taxonomy and molecular ecology, and the training courses of new technology and application which was hold by Mycological Society of China, National key laboratory and public technical service center of Institute of Microbiology, Chinese Academy of Sciences in Beijing from December 17th to 20th, 2018.

VI. Acted as interpreter (simultaneous translation for the English speaking speakers) in the 6th China international truffle festival that took place from November 30th to December 2nd, 2018 in Yongren, Yunnan, P.R. China.

ABSTRACT

Wild edible fungal fruiting bodies, or mushrooms, known as “delicacies from the mountains”, are a natural forest resource widely acknowledged for their nutritional, medicinal, economic, and even cultural value. Most of high-priced wild edible mushrooms are ectomycorrhizal fungi (EMF) which form a symbiotic relationship with trees and play an important role in the ecosystem. Southwest China is rich in fungal diversity and cultural diversity, but there are few researches on ethnomycology. In addition, extensive utilization of wild edible mushrooms, especially the EMF, threatens the fungal diversity. Filling the gaps in ethnomycological knowledge will be beneficial to assess species availability as a prelude to conservation and ecological sustainability in southwest China. Focusing on EMF cultivation, especially new commercial high-valued EMF, and myco-silviculture will alleviate pressures on wild mushroom populations and on the environment. The present thesis targeted research into fungal diversity and sustainable utilization of wild edible mushrooms by investigating local mycological knowledge, fungal taxonomy, mycorrhizal inoculation and the effect of fungal communities on plantation establishment, which will provide new sources of high-value mushrooms and provide insights into the mechanism of plant establishment during ecosystem restoration.

In the presented thesis, a semi-structured interview with mushroom vendors in markets and with mushroom collectors in natural habitats in Yunnan, China, was conducted from 2019 to 2021. Wild edible fungi were collected from forests and markets, while morphological and molecular techniques were used to identify fungal species. Then we described two new high-value commercial ectomycorrhizal fungi by morphological and molecular phylogenetic analyses, and mycorrhizal symbiosis of economic trees with two new ectomycorrhizal fungi was examined. In addition, exotic value truffle species, *Tuber melanosporum*, inoculated to six Chinese oak species in the greenhouse was studied. Ectomycorrhizal development was monitored for up to 32 months to acclimatize the mycorrhizae obtained in glasshouse conditions and assess which oak species could establish a persistent mycorrhizal symbiosis with *T. melanosporum*. In the end, we studied interaction effects of *Lactarius deliciosus* and local fungal communities at the establishment of exotic *Pinus radiata* seedlings. Strategies of introduced *P. radiata* seedlings colonized with an ectomycorrhizal fungus (EMF), *L. deliciosus*, were studied to understand whether it formed familiar or new associations with local EMF in a new habitat over a period of 2.5 years. This will help us know how *P. radiata* and *L. deliciosus* could be successfully established.

The results showed that (i) Pu’er Prefecture is rich in local mycological knowledge and fungi diversity, and it is necessary to continue the research of ethnomycology and develop the rational management of wild edible mushrooms. (ii) The new species *Rhizopogon songmaodan* sp. nov. belongs to the subgenus *Versicolores*. Abundant ectomycorrhizae were produced four monts after inoculation. *R. songmaodan* could be a good candidate for cultivation in southwest China. (iii) New species *Choiromyces*

sichuanensis sp. nov. was easily distinguished from other Chinese *Choiromyces* species by morphological and molecular phylogenetic analyses. The synthesized mycorrhizae between pine plants with *C. sichuanensis* was first reported and well developed. (iv) Controlled mycorrhization of five indigenous Chinese oak species by *T. melanosporum* was successful and had positive effects on the growth of three of these species in the nursery, 24 months after inoculation. Two species, *Q. mongolica* and *Q. longispica*, appear to be promising symbionts for gourmet truffle cultivation projects in China, while potentially contributing to afforestation and ecosystem conservation. (v) Introduced *Pinus radiata* seedlings could be successfully established in new habitat by forming mycorrhizae with *Lactarius deliciosus* or forming familiar associations with local EcMF. Co-introduced *L. deliciosus* could persist in the Xifeng plantation and not be replaced by local EcMF. A local EcMF, *Suillus* sp. in the local habitat formed association with *P. radiata*.

RESUMEN

Los cuerpos fructíferos de hongos comestibles silvestres, setas y trufas, conocidos como "delicias de la montaña", son un recurso forestal natural ampliamente reconocido por su valor nutricional, medicinal, económico e incluso cultural. La mayoría de los hongos comestibles silvestres de alto precio son hongos ectomicorrízicos (EMF) que forman una relación simbiótica con los árboles y juegan un papel importante en el ecosistema. El suroeste de China es rico en diversidad de hongos y diversidad cultural, pero hay pocas investigaciones sobre etnomicología. Además, la utilización extensiva de hongos silvestres comestibles, especialmente los EMF, amenaza la diversidad fúngica. Llenar los vacíos en el conocimiento etnomicológico será beneficioso para evaluar la disponibilidad de especies como preludio de la conservación y la sostenibilidad ecológica en el suroeste de China. Concentrarnos en el cultivo de EMF, especialmente los nuevos EMF comerciales de alto valor, y la mico-silvicultura, aliviará las presiones sobre las poblaciones de hongos silvestres y sobre el medio ambiente. La presente tesis se centró en la investigación de la diversidad fúngica y la utilización sostenible de los hongos silvestres comestibles investigando el conocimiento micológico local, la taxonomía fúngica, la inoculación de micorrizas y el efecto de las comunidades fúngicas en el establecimiento de plantaciones, lo que proporcionará nuevas fuentes de hongos de alto valor y brindará información sobre el mecanismo de establecimiento de plantas durante la restauración del ecosistema.

En la tesis presentada, se realizó una entrevista semiestructurada con vendedores de hongos en mercados y recolectores de hongos en hábitats naturales en Yunnan, China, de 2019 a 2021. Se recolectaron hongos silvestres comestibles de bosques y mercados, mientras que se utilizaron técnicas morfológicas y moleculares para identificar especies de hongos. Luego describimos dos nuevos hongos ectomicorrízicos comerciales de alto valor mediante análisis filogenéticos morfológicos y moleculares, y se examinó la simbiosis micorrícica de árboles de alto rendimiento económico con dos nuevos hongos ectomicorrízicos. Además, se estudió la especie de trufa de exótica, *Tuber melanosporum*, de alto valor en los mercados, inoculada

en seis especies de roble chino en el invernadero. Se controló el desarrollo de ectomicorrizas durante un máximo de 32 meses para aclimatar las micorrizas obtenidas en condiciones de invernadero y evaluar qué especies de robles podrían establecer una simbiosis micorrízica persistente con *T. melanosporum*. Al final, estudiamos los efectos de interacción de *Lactarius deliciosus* y las comunidades fúngicas locales en el establecimiento de plántulas exóticas de *Pinus radiata*. Se estudiaron las estrategias de plántulas de *P. radiata* introducidas colonizadas con un hongo ectomicorrícico (EMF), *L. deliciosus*, para comprender si formaba asociaciones familiares o nuevas con EMF local en un nuevo hábitat durante un período de 2,5 años. Esto nos ayudará a saber cómo se podrían establecer con éxito *P. radiata* y *L. deliciosus*.

Los resultados mostraron que (i) la prefectura de Pu'er es rica en conocimiento micológico local y diversidad de hongos, y es necesario continuar con la investigación de la etnomicología y desarrollar el manejo racional de los hongos silvestres comestibles. (ii) La nueva especie *Rhizopogon songmaodan* sp. nov. Pertenece al subgénero Versicolores. Se produjeron abundantes ectomicorrizas cuatro meses después de la inoculación. *R. songmaodan* podría ser un buen candidato para el cultivo en el suroeste de China. (iii) La nueva especie *Choiromyces sichuanensis* sp. nov. se distinguió fácilmente de otras especies chinas de *Choiromyces* mediante análisis filogenéticos, morfológicos y moleculares. Las micorrizas sintetizadas entre plantas de pino con *C. sichuanensis* se obtuvieron por primera vez y se desarrollaron bien. (iv) La micorrización controlada de cinco especies autóctonas de roble chino por *T. melanosporum* fue exitosa y tuvo efectos positivos en el crecimiento de tres de estas especies en el vivero, 24 meses después de la inoculación. Dos especies, *Q. mongolica* y *Q. longispica*, parecen ser simbiontes prometedores para los proyectos de cultivo de trufa gourmet en China, al tiempo que contribuyen potencialmente a la forestación y la conservación del ecosistema. (v) Las plántulas de *Pinus radiata* introducidas podrían establecerse con éxito en un nuevo hábitat formando micorrizas con *Lactarius deliciosus* o formando asociaciones familiares con EcMF local. *L. deliciosus* cointroducido podría persistir en la plantación de Xifeng y no ser reemplazada por EcMF local. Un ECMF local, *Suillus* sp. en el hábitat local formó asociación con *P. radiata*.

RESUM

Els cossos fructífers de fongs comestibles silvestres, tòfones o bolets, coneguts com a "delícies de les muntanyes", són un recurs forestal natural àmpliament reconegut pel seu valor nutricional, medicinal, econòmic i fins i tot cultural. La majoria dels bolets silvestres comestibles d'alt preu són fongs ectomicorízics (EMF) que formen una relació simbiòtica amb els arbres i tenen un paper important en l'ecosistema. El sud-oest de la Xina és ric en diversitat de fongs i diversitat cultural, però hi ha poques investigacions sobre etnomicologia. A més, l'ús extensiu de bolets comestibles silvestres, especialment els EMF, amenaça la diversitat de fongs. Omplir els buits en el coneixement etnomicològic serà beneficiós per avaluar la disponibilitat d'espècies com a preludi de la conservació i la sostenibilitat ecològica al sud-oest de la Xina. Tot i centrar-se en el cultiu de EMF, especialment els nous EMF comercials de gran valor i la mico-silvicultura alleujaran les pressions sobre les poblacions de bolets

silvestres i sobre el medi ambient. La present tesi va dirigir la investigació sobre la diversitat de fongs i la utilització sostenible dels bolets comestibles silvestres mitjançant la investigació del coneixement micològic local, la taxonomia dels fongs, la inoculació de fongs micorízicos i l'efecte de les comunitats de fongs en l'establiment de les plantacions, que proporcionaran noves fonts de bolets d'alt valor i proporcionaran informació sobre el mecanisme d'establiment de plantes durant la restauració dels ecosistemes.

A la tesi presentada, es van dur a terme entrevistes semiestructurades amb venedors de bolets als mercats i amb recol·lectors de bolets en hàbitats naturals de Yunnan, Xina, del 2019 al 2021. Es van recollir fongs comestibles silvestres de boscos i mercats, mentre que es van utilitzar tècniques morfològiques i moleculars per identificar espècies de fongs. A continuació, vam descriure dos nous fongs ectomicorízics comercials d'alt valor mitjançant anàlisis filogenètiques morfològiques i moleculars, i es va examinar la simbiosi micorízica d'arbres econòmics amb dos nous fongs ectomicorízics. A més, es va estudiar la espècie exotic de tòfona Tuber melanosporum, de alt valor als mercats inoculada a sis espècies de roure xinès a l'hivernacle. Es va controlar el desenvolupament ectomicorízic fins a 32 mesos per aclimatar les micorizes obtingudes en condicions d'hivernacle i avaluar quines espècies de roure podrien establir una simbiosi micorízica persistent amb *T. melanosporum*. Al final, vam estudiar els efectes d'interacció de *Lactarius deliciosus* i les comunitats de fongs locals en l'establiment de plàntules exòtiques de *Pinus radiata*. Es van estudiar estratègies de plàntules de *P. radiata* introduïdes colonitzades amb un fong ectomicorízic (EMF), *L. deliciosus*, per entendre si va formar associacions familiars o noves amb els EMF locals en un nou hàbitat durant un període de 2,5 anys. Això ens ajudarà a saber com es podrien establir amb èxit *P. radiata* i *L. deliciosus*.

Els nostres resultats van demostrar que (i) la prefectura de Pu'er és rica en coneixements micològics locals i diversitat de fongs, i és necessari continuar la investigació d'etnomicologia i desenvolupar la gestió racional dels bolets comestibles salvatges. (ii) La nova espècie *Rhizopogon songmaodan* sp. nov. pertany al subgènere Versicolores. Es van produir abundants ectomicorizes quatre mesos després de la inoculació. *R. songmaodan* podria ser un bon candidat per al cultiu al sud-oest de la Xina. (iii) La nova espècie *Choiromyces sichuanensis* sp. nov. es va distingir fàcilment d'altres espècies xineses de *Choiromyces* per anàlisis morfològiques i filogenètiques moleculars. Les micorizes sintetitzades entre plantes de pi amb *C. sichuanensis* es van informar per primera vegada i van estar ben desenvolupades. (iv) La micorrització controlada de cinc espècies autòctones de roure xinès per *T. melanosporum* va tenir èxit i va tenir efectes positius en el creixement de tres d'aquestes espècies al viver, 24 mesos després de la inoculació. Dues espècies, *Q. mongolica* i *Q. longispica*, semblen ser simbionts prometedors per a projectes de cultiu de tòfona gourmet a la Xina, alhora que poden contribuir a la forestació i la conservació dels ecosistemes. (v) Les plàntules de *Pinus radiata* introduïdes es podrien establir amb èxit en un nou hàbitat formant micorizes amb *Lactarius deliciosus* o formant associacions familiars amb EcMF local. *L. deliciosus* cointroduït podria persistir a la plantació de Xifeng i no ser substituït per EcMF local. Un EcMF local, *Suillus* sp. a l'hàbitat local va formar associació amb *P. radiata*.

INTRODUCTION

The topography of southwestern China is complex and the altitude differences are great. The whole region is deeply influenced by a subtropical monsoon climate and a plateau mountain climate, especially by two warm and humid air flows from the Indian Ocean and the Pacific Ocean. Consequently, there is abundant rainfall, numerous rivers and the tropical, subtropical, subalpine and alpine temperate vegetation in the region supports a wealth of biological resources. In China, this region is known as the Kingdom of animals, plants and fungi. Wild edible fungal fruiting bodies, or mushrooms, known as "delicacies from the mountains", are a natural forest resource widely acknowledged for their nutritional, medicinal, economic, and even cultural value (Bao 2004, Barros et al. 2008, Moerman 2008, Li et al. 2021). Local knowledge has been developed regarding wild edible mushrooms, their ecology, phenology, and morphology, which has permitted their use (Montoya et al. 2003; Zent et al. 2004, Ruan-Soto et al. 2018). More than 800 species of edible mushrooms have been identified in southwest China, ranking it at the top of fungal diversity in China. Moreover, new fungal resources continue to be discovered in the region (Cui et al. 2018, Ge et al. 2020, Han et al. 2020).

Local traditional knowledge allows people to recognize wild edible fungi (WEF) species, their temporality, their habitat, and, most importantly, the sustainable way to use the mushrooms in their forests. Ethnomycology is a relatively new area of research that investigates traditional knowledge, as well as cultural and environmental effects, of the association between human societies and fungi (Wasson 1957). Despite the historical use of fungi since antiquity, ethnological studies of wild mushrooms are a relatively new phenomenon (Chen et al. 2003, Yilmaz et al. 2016). Ethnomycological knowledge is a key tool for forest conservation to predict anthropic harvesting pressure on WEF and support the management and sustainable utilization of wild fungi (Franco-Maass et al. 2016). And the preservation of these knowledge is fundamental to avoiding poisonings (Ruan-Soto et al. 2018). Filling the gaps in ethnomycological knowledge will be beneficial to assess species availability as a prelude to conservation and ecological sustainability in southwest China.

In recent years, gathering, processing and selling WEF has become a major industry, which have played an increasingly important role for poverty alleviation in many remote and mountainous areas in southwest China (Sitta et al. 2012, Wang et al. 2019, Duan 2020, Zhang 2020). The Yunnan Province in southwestern China, in particular, has an important tradition of consumption and trade of mushroom (Sitta et al. 2012). The annual production of WEF in Yunnan amounts to about 80,000 t (Yang 2016) of commercial fungi, including truffles, matsutake, porcini, chanterelles, milk agaric etc. Most of high-priced wild edible fungi are ectomycorrhizal fungi (EMF). Wang and Liu (2002) studied systematically the trade of fungi in Yunnan markets and showed that about 81.2% of the WEF species are EMF. In nature, the number of EMF species is estimated to be between 20,000−25,000 (Alice 2016). It is a key component in forest

ecosystem, which provides plants with mineral nutrients, water, or defense against pathogens, whereas plants supply EMF with energy-rich carbon compounds in return (Allen 1991, Liang et al. 2002, Kumar and Atri 2018) (Fig 1). However, the high economic value of WEF has been driving forest-dependent communities to completely devote their resources to hunting mushrooms for immediate cash thanks to an endless market demand (Mortimer et al. 2012, Hall et al 2003). Disorderly digging and hunting, habitat loss, and vegetation deterioration (Fig 2a, b) is threatening the survival of fungal populations and the forests that support them (Yu et al. 2002, Donnini et al. 2013, Zhao et al. 2021, Ma et al. 2020). The above situation motivated research foci to new and better techniques for inoculating, transplanting and establishing mycorrhizal tree seedlings more widely to provide new sources of these valued mushrooms. And discovery of more new valued mushroom species will contribute to the sustainable use of WEF resources. In addition, mycorrhizal fungi can promote survival, establishment and growth of young trees in newly established forest plantation (Xu et al. 2001, Zhang et al. 2017) and it could be a useful tool in the afforestation or reforestation (Krpata 2007, Sousa et al. 2011). Therefore, the cultivation of EMF and integration with myco-silviculture practices has the potential to alleviate pressures on wild mushroom populations and on the environment (Savoie and Largeteau 2011).

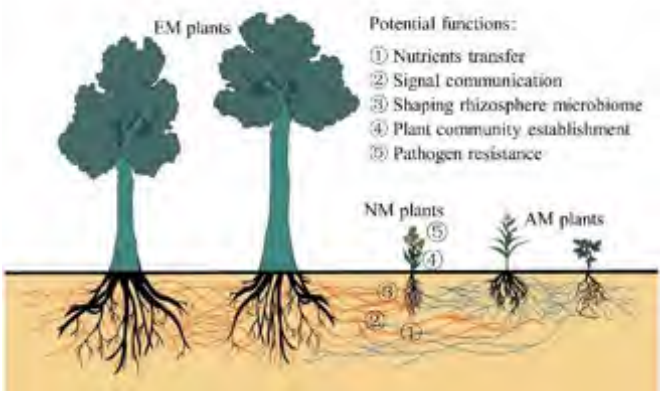

Figure 1 A conceptual diagram showing potential common mycorrhizal linkages or networks among arbuscular mycorrhizal (AM), ectomycorrhizal (EM) and/or non- mycorrhizal (NM) plants (from Wang et al., 2022)

Figure 2: a−c Disorderly digging truffle in Yunnan (c immature *Tuber indicum*). d Eucalyptus trees instead of native forests in center of Yunnan. e Established rubber plantations with large area in south of Yunnan. (a, b Fuqiang Yu; c https://mp.weixin.qq.com/s/DSFtEGnB46OKszJOhZE4kg)

Many previous studies indicated that seedling responses to mycorrhizal inoculation is is positive, neutral, or negative depending on environmental factors or on EMF species or host plant used (Dunabeitia 2004, Flores-Renteria 2018). Truffles have a broad host spectrum (Wang 2012) and their cultivation promotes economic restoration and reforestation of rural lands and land-use stability to a large extent in European countries (Bonet et al. 2006). So far, more than 50 *Tuber* spp. have been found in China (Chen 2007; Chen et al. 2011; Wan 2017) and some Chinese truffle plantations have been established in China especially in Guizhou, Yunnan and Sichuan Provinces, southwest China (Wang 2012) (Fig. 3). Besides work on Chinese truffles, mycorrhizal synthesis between exotic truffles and Chinese trees were also tried in China. The Périgord black truffle, *Tuber melanosporum* Vittad. (Ascomycota, Pezizales), also known as "the black diamond of French cuisine", is highly appreciated because of its special taste and aroma (Mello et al. 2006). It is probably the most economically important truffle in Europe (Donnini et al. 2013) and its commercial value is much higher than those of other black truffle species. Known from the majority of European countries, *T. melanosporum* characteristically lives in calcareous soils. And some reports indicated that *T. melanosporum* is strictly calcicolous and grows in calcareous soils with a C/N relationship close to 10 (Lulli et al., 1999, Castrignano et al., 2000). In southwestern China, especially in its southern and eastern parts where typical karst landscapes are well developed (southeastern Yunnan, Guizhou, northern Guangxi), soil between rocks is poor for crops (Fig. 4). The vast mountainous regions would make truffle cultivation prosperous in southwest China. In previous studies, mycorrhizae were synthesized successfully by inoculating *T. melanosporum* onto different Chinese indigenous trees,

including *Castanopsis hystrix* (Chen 2002), *Pinus yunnanensis* (Lin et al. 2013) and *P. armandii* (Su et al. 2017). Partner selection in the mycorrhizal symbiosis is thought to be a key factor stabilizing the mutualism (Werner 2014). *Tuber*-associated host trees may have economical value as timber (pine, oak), food (pecan, hazelnut), and in fuel production (poplar, oak) (Del Lungo et al. 2006; Benucci et al. 2012). They also have an important ecological value in nature as major reforestation species. Truffle cultivation is still a new topic in China. To assess which tree species could establish a persistent mycorrhizal symbiosis with *T. melanosporum* in greenhouse could provide potential candidates for cultivating this high-value truffle species in China in the future.

Figure 3 Ascocarps of *Tuber indicum* were obtained from Guizhou plantation in 2018

Figure 4: Karst rocky desertification in Guizhou (Fuqiang Yu)

Wang et al (2013) recommended that the use of exotic tree species versus native species in afforestation should be site dependent, in degraded land with poor soils, exotics are better than natives in nutrient retention. A number of exotic tree species from the genera *Pinus*, *Eucalyptus* and *Populus* have been introduced to China since 1980's, which have become the dominant tree species in timber plantations with easy propagation, strong adaptability, short growth cycle and high-quality wood (Song, 1983; Wang et al., 2006; Chen et al., 2018; Farooq et al., 2021). Suitable mutualists play important roles in naturalization of introduced plants (Keane and Crawley, 2002; Pringle et al., 2009; Jurkien et al., 2020; Moyano et al., 2020; Ning et al., 2020). It has been long recognized that the absence of co-evolved EMF in fields is a major obstacle to successful establishment of introduced plants (Mikola, 1970; Poynton, 1979; Richardson et al., 2000; Pringle et al., 2009, Nunez and Dickie, 2014). *Pinus radiata* D. Don is a native species to the central coast of California, the United States. It has a lowest mortality

and a best growth rate during early establishment where other forest species are difficult to be established (Bi et al., 2003). Since 1990's, *P. radiata* has become the main planted tree species in New Zealand. One plantation of *P. radiata* from New Zealand has been established in Guizhou province, southwest of China since 2018. Mycorrhizal seedlings have been obtained between pines and the genus *Lactarius* Pers. (Wang et al., 2019), an economically valuable edible fungi *Lactarius deliciosus* culture was originally from fruiting bodies in New Zealand's *P. radiata* plantation (Wang et al., 2002, Guerin-Laguette et al., 2014). In May 2018, mycorrhizal and non-mycorrhizal seedlings of exotic *P. radiata*, and non-mycorrhizal seedlings of native *P. massoniana* Lamb. were respectively planted in Xifeng County, Guizhou Province. Success of plantations is clearly related to the environment, the mycorrhizal status of the host trees over the years from inoculated seedlings to fungi-producing trees. Understanding on how an introduced EMF *L. deliciosus* or local EMF help an exotic *P. radiata* to be successfully established in a new habitat will provide insights into the mechanism of exotic plant establishment during ecosystem restoration.

In the present research, (i) we studied the areas in southwest China, especially in Pu'er Prefecture in the southern part of Yunnan province which has the highest diversity of both cultures and fungi. We aimed to gather ethnomycological knowledge and update the knowledge regarding the fungal species used by ethnic groups in southwest China, while the fungal diversity in natural habitats was also documented. (ii) Then we collected new valued commercial mushrooms from markets. We aimed to identify them by morphological and molecular phylogenetic analyses, and examine the feasibility of inoculating seedlings in the greenhouse. (iii) In addition, we assessed the selection of the suitable host plants for *Tuber melanosporum*. We aimed to acclimatize the mycorrhizae obtained in glasshouse conditions and assess which oak species could establish a persistent mycorrhizal symbiosis with *T. melanosporum* and, therefore, be potential candidates for cultivating this high-value truffle species in China in the future. (v) In the end, the strategies to introduce exotic timber pine *P. radiata* seedlings either colonized with an ectomycorrhizal fungus (EcMF) or form familiar/new associations with local EcMF in a new habitat were studied. We aimed to know how *P. radiata* could be successfully established over a period of 2.5 years and provides insights into a better understanding of the interaction effects between host trees and fungi in the afforestation. Above researches will provide scientific information and insights, and the foremost pathway to achieve the sustainable development goals of wild edible mushrooms.

OBJECTIVES

The objectives of the presented thesis are:

1. To fill the gaps in ethnomycological knowledge in Pu'er Prefecture, Yunnan, China, update and supplement the information on fungal taxa present in markets and natural habitats, especially ectomycorrhizal fungi (EMF), and collect ectomycorrhizal fungi isolates and provide help for sustainable utilization of wild fungi resources.

2. To identify a high value of commercial *Rhizopogon* species by morphological and molecular phylogenetic analyses and provide a phenotypic key to related species, and examine the feasibility of inoculating seedlings of two *Pinus* species, *Pinus armandii* and *P. yunnanensis* (the main forest and economic trees in Yunnan province) with spores of new *Rhizopogon* species in the greenhouse.

3. To identify a high value commercial *Choiromyces* species by morphological and molecular phylogenetic analyses and discuss its relationships with other *Choiromyces* species, and examine the feasibility of inoculating seedlings of two *Picea* species, *Picea likiangensis* and *Pi. crassifolia*, and one *Pinus* species, *Pinus armandii* with spores of this new *Choiromyces* species in the greenhouse.

4. To acclimatize the mycorrhizal seedlings obtained in glasshouse conditions and assess which native oak species could establish a persistent mycorrhizal symbiosis with *T. melanosporum* and, therefore, be potential candidates for cultivating this high-value truffle species in China in the future.

5. To study the strategies of introduced *Pinus radiata* seedlings colonized with an ectomycorrhizal fungus (EMF), *Lactarius deliciosus*, or formed familiar/new associations with local EMF in a new habitat to know how *P. radiata* could be successfully established over a period of 2.5 years in China.

THESIS STRUCTURE

The structure of the thesis to accomplish the aforesaid objectives includes ethnomycological study on wild mushrooms and ectomycorrhizal fungi diversity (Chapter I), taxonomic research and mycorrhizal synthesis study on new commercial ectomycorrhizal fungal species (Chapter II and Chapter III), a test for *Tuber melanosporum* and native Chinese oak species forming stable mycorrhizal symbioses (Chapter IV) and, finally, the interaction effects of co-introduced *Lactarius deliciosus* and local fungal communities on the establishment of exotic *Pinus radiata* seedlings (Chapter V).

First, aiming to investigate mycological culture diversity and fungal diversity in Yunnan Province (Chapter I), ethnomycological knowledge was surveyed, the information on fungal taxa present in markets and natural habitats was updated and supplemented, and ectomycorrhizal fungi isolates were collected for future studies.

Second, we focused on taxonomy and mycorrhiza formation of two new high-valued commercial wild mushroom species (Chapter II and Chapter III). Our second studies provide fundamental data about the species diversity and mycorrhizal research of this genus for further studies. And successful inoculation of this new commercial mushroom species and selected tree species would develop an effective pathway to produce both mushrooms and timber under either planted or natural forests.

Third, we aimed to test whether *Tuber melanosporum* and native Chinese oak species could form stable mycorrhizal symbioses (Chapter IV). In this study, ectomycorrhizal development was monitored for up to 32 months and seedling growth was assessed two years after inoculation.

Finally, we analyzed the interaction effect of EMF, including co-introduced *Lactarius deliciosus* and local EcMF communities, on the establishment of exotic *Pinus radiata* seedlings in Guizhou, southwest China to understand on how an introduced EMF *Lactarius deliciosus* or local EcMF could help an exotic *P. radiata* to be successfully established in a new habitat (Chapter V).

METHODOLOGY

Site description (Fig. 5)

Chapter I

The investigation took place in Pu'er Prefecture, the largest prefecture in Yunnan Province, southwest China with a total area of 45,385 square kilometers. It is located between 22°02' N~24°50' N and 99°09' E~102°19' E, and the Tropic of Cancer runs across the middle of the prefecture. About 62.8% of Pu'er is forested where the main type of vegetation is broad-leaved forest, mixed forest (*Alnus*, *Castanopsis*, *Olea*, *Pinus*, *Quercus*) and *Pinus* forests. Pu'er is one of the most culturally diverse prefectures, with fourteen ethnic groups inhabiting this area. Our investigation was carried out in five nationality autonomous counties (Lancang Lahu, Menglian Dai Lahu Wa, Mojiang Hani, Ning'er Hani Yi, Ximeng Wa) and one homonymous municipality, Pu'er (Figure 1), all located south of the Tropic of Cancer.

Chapter II

The *Rhizopogon* samples were collected from markets and natural *Pinus armandii* Franch. forests in Aziying County, Yunnan Province and Duge Village, Huidong County, Liangshan Yi Autonomous Prefecture, Sichuan Province, China. The locations are 25.3866 ºN, 102.8839 ºE, alt. 2,710 m and 26.72640815 ºN 102.66346161 ºE, alt. 2338 m. *Rhizopogon* mycorrhiza samples were collected from natural *Pinus armandii* Franch. forest in Aziying County, Yunnan Province.

Chapter III

Fresh specimens were purchased from markets in Songpan County, Tibetan Qiang Autonomous Prefecture of Ngawa, Sichuan, China. The location is between 32°06' N~ 33°09′, 102°38′~104°15′ E.

Chapter V

The plantation (0.67 ha) is located in Xifeng County (27° 21′ N, 106°41′ E, alt. 1213 m above the sea level), Guizhou, southwest China, where has an annual rainfall of ~1,200 mm. The plantation area was an abandoned farmland where shrubs and weeds (dominated by *Pyracantha* sp. and Fabaceae) had grown for more than five years. The native *Pinus massoniana* (PM) forests are 1,000 m from the plantation.

Figure 5: Location of all sites

Experiment design, methods and analyses:

Chapter I

Our investigation was carried out in five nationality autonomous counties and one homonymous municipality, Pu'er, all located south of the Tropic of Cancer. Semi-structured interviews were carried during the mushroom season (July to October) in three consecutive years (2019 to 2021) in established mushroom markets, mobile markets and street-stalls beside county highways or village roads. Wild edible fungi were collected from forests to record the fungal resources and diversity in Pu'er prefecture by using the random line transect method. Fresh fruiting bodies of ectomycorrhizal fungi from markets and natural habitats were used to make pure cultures from the flesh of cap on prepared agar medium. Collections purchased from markets and collected from natural habitats were identified through taxonomical and molecular studies.

Chapter II

Sporocarps of new species *Rhizopogon songmaodan* sp. nov. were obtained from markets and forests. Microscopic characteristics were described from fresh specimens. Ectomycorrhizas were collected from *P. armandii* forest and separated under the stereomicroscope (Leica S8AP0) in order to select, photograph, and characterize morphotypes macroscopically (Agerer 1987–2002). Mycorrhizal synthesis via spore inoculation between *R. songmaodan* and two native pine species, *Pinus armandii* and *P. yunnanensis* was carried out in a greenhouse study. DNA of basidiomata and ectomycorrhizal (ECM) tips were extracted using Aidlab™ kit (Beijing). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified from DNA extracts using the ITS1F/ITS4 primer pair (White et al. 1990; Gardes and Bruns 1993). A total of 40 ITS sequences including 10 newly generated in this study and 30 retrieved from GenBank formed the dataset. Maximum likelihood (ML) phylogenetic analyses were performed to infer phylogenetic relationships of taxa.

Chapter III

Specimens of *Choiromyces sichuanensis* sp. nov. were purchased from markets. Gross morphology was described based on the fresh ascomata, and microscopic examination was later conducted using dry material according to Yang and Zhang (2003). Mycorrhizal synthesis via spore inoculation between *C. sichuanensis* and three native tree species, *Picea likiangensis*, *Pi. crassifolia* and *Pinus armandii* was carried out in a greenhouse study. Total DNA was extracted from dried ascomata and mycorrhizal tips using a modified CTAB procedure (Gardes and Bruns 1993). The ITS region of nuclear ribosomal DNA (nrDNA) was amplified using primers ITS1F and ITS4. The phylogenetic relationships of taxa were inferred using Maximum Likelihood (ML) (Stamatakis 2006) and Bayesian Inference (BI) (Ronquist and Huelsenbeck 2003). Macro-morphological and anatomical characters of *C. sichuanensis* mycorrhizae using a stereomicroscope (Leica S8AP0, Leica Microsytems, Wetzlar, Germany) and a compound light microscope (Leica DM2500, Leica Microsytems, Wetzlar, Germany) were identified following the methods of Agerer (2006) and Giraud (1988).

Chapter IV

Mycorrhizal synthesis via spore inoculation between *Tuber melanosporum* and six *Quercus* species which are well-developed root system and can adapt to different habitats and altitudes was conducted in greenhouse. Six, nine, twelve and twenty-four months after inoculation, all seedlings of each oak species were monitored for mycorrhiza formation. The macro-morphological and anatomical characters of *T. melanosporum* ectomycorrhizae were identified following the methods of Agerer (2006) and Giraud (1988). Cross- and longitudinal sections were made by a freezing microtome (Leica CM3050S). Genomic DNA of three to 10 pooled mycorrhizal tips displaying morphology characteristic of *T. melanosporum* were extracted using Aidlab™ kit (Beijing) and ITS region of the ribosomal DNA was amplified from DNA extracts using the ITS1F/ITS4 primer pair. Two years after inoculation, the effects of

mycorrhization on plant growth for *Q. longispica*, *Q. mongolica* and *Q. senescens* were analyzed by three variables: canopy diameter, numbers of leaves (both one measurement per pot) and mean leaf dimension (leaf length+leaf width)/2, with one measurement per leaf for each pot.

Chapter V

In plantation, seedlings' height was measured every six months from November 2018 to May 2021 and it was recorded every six months (T1~T6). The survive rate of seedlings was also recorded one year after planting. In March 2021, five plants of each tree species were randomly selected and soil layer was lightly excavated at 0.5 m and 1.0 m from the trunk to observe mycorrhiza or fine roots. Soil samples, mycorrhizal tips and roots were used for Illumina MisSeq sequencing. Healthy mature pine needles were sampled. The content of carbon (C), nitrogen (N) and total phosphorus (P), potassium (K), calcium (Ca), iron (Fe) and manganese (Mn) of the dried needles were measured. A greenhouse bioassay experiment of local soil (Ning et al., 2020) to assess the divergent of ectomycorrhizal fungal communities between exotic and native pine seedlings was also conducted.

Chapter I

Ethnomycological study on wild mushrooms and ectomycorrhizal fungi diversity in Pu'er Prefecture, Southwest Yunnan, China

Ethnomycological study on wild mushrooms and ectomycorrhizal fungi diversity in Pu'er Prefecture, Southwest Yunnan, China

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Abstract

Background: Yunnan is rich in fungal diversity and cultural diversity, but there are few researches on ethnomycology. In addition, extensive utilization of wild edible fungi (WEF), especially the ectomycorrhizal fungi, threatens the fungal diversity. Hence, this study aims to contribute to the ethnomycological knowledge in Pu'er Prefecture, Yunnan, China including information on the fungal taxa presented in markets and natural habitats, with emphasis in ectomycorrhizal fungi (EMF).

Methods: Semi-structured interviews with mushroom vendors in markets and with mushroom collectors in natural habitats was conducted. Information related to local names, habitat, fruiting time, species identification, price, cooking methods and preservation methods of wild edible mushrooms were recorded. Wild edible fungi were collected from forests and morphological and molecular techniques were used to identify fungal species.

Results: A total of 11 markets were visited during this study. The 101 species collected in the markets, belonged to 22 families and 39 genera, and about 76% of them were EMF. A wealth of ethnomycological knowledge was recorded and we found that participants in the 45−65 age group were able to judge mushroom species more accurately. Additionally, men usually had a deepest mushroom knowledge than women. A total of 283 species, varieties and undescribed species were collected from natural

habitats, and about 70% of them were EMF. Mushroom species and recorded amounts showed correspondence between markets and the natural habitats on different months. **Conclusion:** The present study shows that Pu'er Prefecture is rich in local mycological knowledge and fungal diversity. However, it is necessary to continue the research of ethnomycological studies and to design and conduct dissemination of local knowledge in order to preserve it, since it currently remains mainly among the elderly population.
Key words: Ethnomycology; Fungal diversity; Pu'er; South of the Tropic of Cancer

Background

Wild edible fungal fruiting bodies, or mushrooms, known as "delicacies from the mountains", are a natural forest resource widely acknowledged for their nutritional, medicinal, economic, and cultural value [1-4]. China is one of the most important mushroom producers in the world in terms of the total volume of trade and commercialized fungal species. The Yunnan Province in southwestern China, in particular, has an important tradition of consumption and mushroom trade [5]. In China there are about 900 species of wild edible fungi (WEF), 90% of which are present in Yunnan and utilized by local people as both a source of food and income [6]. Most of the main mushroom markets, with a large variety of species, are located in the central regions of the province because of the dense population, convenient transportation and high market demand, while countless small mushroom markets with unique fungal species are scattered throughout Yunnan in mountainous areas which are inhabited by a number of ethnic groups [7] where gathering of WEF and mushroom industry has become an important tool for poverty alleviation [8-10].

The rural population of Yunnan has a wealth of traditional knowledge related to WEF and is familiar with many species as well as their uses and ecology. The traditional mycological knowledge, generally gathered by the indigenous communities in their long interaction with nature, is an important part of human cultural heritage [11-15]. Ethnomycology is a relatively new area of research that investigates traditional knowledge, as well as cultural and environmental effects, of the association between human societies and fungi [16]. Yunnan is the province with the largest number of ethnic groups in China, each minority with their own culture, language, history and, of course, different uses for wild forest fungi. Brown [17] investigated Yi ethnomycological knowledge in four communities in Nanhua County, Yunnan Province, which showed that documenting ethnomycological knowledge highlights the importance of fungi in local ecosystems and livelihoods. Ethnomycological knowledge is a key tool for forest conservation to predict anthropic harvesting pressure zones of WEF and support the management and sustainable utilization of wild fungi [18]. For example, documenting the fungal biodiversity which has a local use, would allow to design and implement strategies to cultivate the most important WEF in specific areas and at the same time to integrate this cultivation into production systems which contribute to the recycling of local agricultural wastes, providing at the same time nutritious and healthy food. Additionally, the record of the local ethnomycological

knowledge would allow to increase the promotion of responsible use and to design preservation techniques for the most valuable WEF, in order to maintain this important natural resource as a livelihood opportunity in rural areas. Additionally, the documentation and preservation of traditional mycological knowledge is fundamental to avoid poisonings [19]. However, compared with local folk knowledge related to plants and animals, ethnomycological knowledge started late and remains scarce [20, 21].

The annual production of WEF in Yunnan amounts to about 80,000 t [6]. The largest market share of commercial fungi, either in terms of monetary value or of quantity, including truffles (*Tuber indicum* Cooke & Massee, *Tuber sinoaestivum* J.P. Zhang & P.G. Liu), matsutake (*Tricholoma matsutake* (S. Ito & S. Imai) Singer), porcini (*Boletus edulis* Bull.), chanterelles (*Cantharellus cibarius* Fr.) and milk agaric (e.g., *Lactarius deliciosus* (L.) Gray, *Lactifluus volemus* (Fr.) Kuntze). Most of high-priced WEF are ectomycorrhizal fungi (EMF) which form a symbiotic relationship with trees and play an important role in the ecosystem [22, 23]. Wang and Liu [7] studied systematically the trade of fungi in Yunnan markets and showed that about 81.2% of the WEF species are EMF. Limited by cultivation techniques, mushrooms, especially of EMF have been almost exclusively harvested from the wild [24]. Their high economic value has been driving forest-dependent communities to completely devote their resources to hunting mushrooms for immediate cash thanks to an endless market demand [25,26]. Disorderly digging and hunting, habitat loss, and vegetation deterioration, has caused overexploitation of many species and is threatening the survival of fungal populations and the forests that support them [27-30]. A survey of mushroom markets and natural habitats in Yunnan, to a large extent, will reveal the problems of development and utilization of WEF [31,32].

Based on this scenario, in the present research we studied the areas in Pu'er Prefecture in the southern part of Yunnan province which has the highest diversity of both cultures and fungi. Yu et al. [33] studied the species diversity, use, and threatened status of WEF in two counties of Pu'er Prefecture and found that large-scale commercial harvesting had led to the decline of mushroom production. Pu'er Prefecture has an area of 45,385 km² and its population is 2.4 million. It is located in southwest Yunnan and bordered by Myanmar, Laos and Vietnam. The Tropic of Cancer runs through the middle of Pu'er. It generally belongs to subtropical monsoon climate with lower altitude, diverse topography, rich forest resources and unique ethnic groups, like Hani, Lahu, Wa or Dai.

In this study, we aimed (1) to gather ethnomycological knowledge regarding the fungal species used by ethnic groups in Pu'er; (2) to update the knowledge about fungal species sold in Pu'er markets, especially ectomycorrhizal species; (3) to document the fungal diversity inhabiting Pu'er forests through natural habitats sampling; and (4) to identify the fungal species (sold in markets and collected in the natural habitats) using taxonomical and molecular approaches.

Methods

Study area

Pu'er Prefecture, with a total area of 45,385 square kilometers is the largest prefecture in Yunnan Province. It is located between 22°02' N~24°50' N and 99°09' E~102°19' E, and the Tropic of Cancer runs across the middle of the prefecture. About 62.8% of Pu'er is forested where the main type of vegetation is broad-leaved forest, mixed forest (*Alnus*, *Castanopsis*, *Olea*, *Pinus*, *Quercus*) and *Pinus* forests [34]. Pu'er is one of the most culturally diverse prefectures, with fourteen ethnic groups inhabiting this area. Our investigation was carried out in five nationality autonomous counties (Lancang Lahu, Menglian Dai Lahu Wa, Mojiang Hani, Ning'er Hani Yi, Ximeng Wa) and one homonymous municipality, Pu'er (Figure 1), all located south of the Tropic of Cancer.

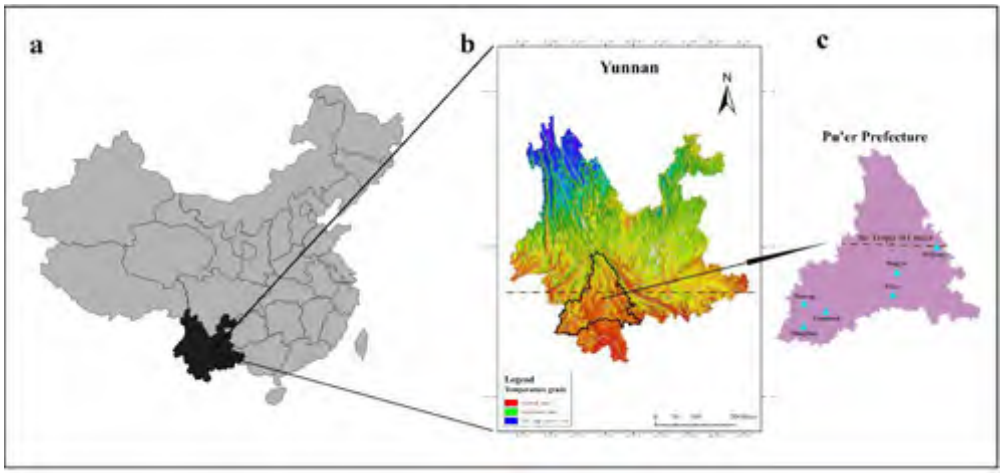

Fig. 1 Location of study sites in six counties, Pu'er Prefecture, Yunnan Province, Southwest China. (a) China; (b) Yunnan Province; (c) Pu'er Prefecture.

Ethnomycological survey

Semi-structured interviews were carried during the mushroom season (July to October) in three consecutive years (2019 to 2021) in established mushroom markets, mobile markets and street-stalls beside county highways or village roads (Figure 2, Table 1). The number and the male−female ratio of vendors in markets, the knowledge, attitude, and practice of human−mushroom interaction including the local names of mushrooms and their local uses (medicine, food, etc.), habitat, seasonality of species, marketability, form of mushrooms used (fresh/dried), methods of preparation for food, and preservation (storage) were also recorded. For illiterate vendors, interviews were carried out mainly in Mandarin Chinese, although local languages were also used with assistance from local guides.

Diversity of wild edible fungi in forests

In order to record the vegetation types associated with the fungal species sold in the markets and to investigate the presence of additional edible fungal species different than those recorded in the markets, WEF were collected from forests nearby the studied markets in Pu'er prefecture. The forest areas were selected according to the information provided by some collectors previously interviewed in the markets. Forests nearby visited markets, reforested areas and a national nature reserve (Table 2) were

investigated. Field work was conducted during the same season as the interviews were carried out using the random line transect method [35]. In order to gather more ethnomycological information regarding WEF, participant observation was performed in some forest areas. We joined some collectors in their daily routine of collecting WEF. While walking with them we recorded some local names of the mushrooms, hours invested in this activity, types of collectors, and habitat ecological information.

Fig. 2 Sampled markets. Two big markets: (a−c) Wuyi Market, Pu'er Municipality; (d, e) Lancang Street Market, Lancang County; (f−i) Some small markets in Mojiang, Ning'er, Ximeng and Menglian counties.

Table 1 The timetable of selling mushrooms, minority and the average number of vendors with different gender in markets in three years

Markets' name	Type of market[1]	Business Hours	Ethnic groups	July		August		September		October	
				Female	Male	Female	Male	Female	Male	Female	Male
Wuyi, Pu'er market	EM	2 p.m−6 p.m	Hani, Yi, Lahu	47	19	172	56	141	46	14	9
Lancang street	EM	7 a.m−12 p.m on Sunday	Lahu, Hani, Yi	44	17	93	25	183	56	55	15
Monjiang market	EM	1 p.m−4 p.m	Hani, Yi	10	2	67	37	15	8	4	1
Ning'er market	MM	2 p.m−5 p.m	Yi, Hani	17	5	49	13	51	27	26	10
Menglian market	MM	7 a.m−11 a.m every five days	Lahu, Dai, Wa	3	2	8	2	23	5	10	4
Ximeng market	MM	4 p.m−8 p.m	Wa, Lahu, Dai	2	1	2	8	41	7	10	3
No name	SS[2]	1pm−5 p.m	×	×	×	×	×	×	×	×	×

Note: [1] Type of markets. EM is established market, MM is mobile market, SS is street-stall.

[2] Street-stalls beside county highways or village roads. We only recorded information about business time because of strong mobility.

Table 2 Description of the sampling sites in natural habitats.

Location	Altitude (m)	Locality	Forest type	Habitat
Pu'er	1450	22°49'13" N, 101°00'12"	Forests nearby markets	Pure pine forests (*Pinus kesiya*)
Municipality	1608	22°60'38"N, 101°09'65"E	The Sun River National Forest Park	Mixed forests (*Pinus*, *Quercus*, *Castanopsis*, *Olea*)
Mojiang County	1595	23°22'48.85"N, 101°41'0.69"E	Forests nearby markets	Mixed forests (*Pinus*, *Quercus*)
	1627	23°44'43.00"N, 101°12'36.1"E	Ecological forest (Kuaifa village)	Pure pine forests (*P. kesiya*)
Ning'er County	1437	23°0'11.35"N, 100°59'47.6"E	Forests nearby markets	Mixed forests (*Pinus*, *Quercus*)
	1537	22'59'50.84"N, 101°0'19.18"E	Ecological forest (Hualiang village)	Pure pine forests (*P. kesiya*)
Lancang County	1350	22°19'51"N, 100°00'34"E	Forests nearby markets	Mixed forests (*Quercus*, *Alnus*, *Pinus*)
	1490	22°35'02"N, 99°58'44"E	Forests nearby markets	Mixed forests (*Quercus*, *Pinus*)
Ximeng County	1128	22°37'14.06"N, 99°35'53.98"E	Forests nearby markets	Mixed forests (*Quercus*, *Alnus*, *Pinus*)
	1497	22°36'10.53"N, 99°35'0.02"E	Forests nearby markets	Mixed forests (*Quercus*, *Alnus*, *Pinus*)
Menglian County	1250	22°16'21.99"N, 99°16'30.06"E	Forests nearby markets	Mixed forests (*Quercus*, *Alnus*, *Pinus*)
	1380	22°16'46.11"N, 99°16'27.97"E	Forests nearby markets	Mixed forests (*Quercus*, *Alnus*, *Pinus*)

Morphological study

Collections purchased from markets and collected from natural habitats were identified through taxonomical and molecular studies. Morpho-anatomical descriptions based on fresh samples were obtained following Largent [36]. A small sample of tissue, mostly hymenophore, was stored in silica gel and/or frozen in Eppendorf's tubes and stored at −20 °C to be used later for molecular analyses. Then, all the samples were dried in a hot air dehydrator at 45 °C for further analyses. All collections were deposited in the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (HKAS). Microscopic characteristics were described from fresh specimens. Dried samples were sectioned with a razor blade by hand, mounted in 5% KOH solution, and then stained with Melzer's reagent. The sections were examined under a compound light microscope (Leica DM2500).

DNA extraction, PCR amplification and sequencing

DNA of samples was extracted using an Aidlab™ kit (Beijing). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified from DNA extracts using the ITS1F/ITS4 primer pair [37, 38]. To amplify the ribosomal large subunit (LSU), the primer combination of LROR and LR5 [39] was used. Each 25 μL PCR mixture consisted of 2.5 μL 10 × PCR buffer (Mg^{2+}), 1.5 μL dNTPs (1 mM), 1 μL BSA (0.1%), 1 μL each primer (5 μM), 1 μL 25−fold diluted DNA extracts (obtained following the manufacturer's instructions), 0.5 μL $MgCl_2$ (25 mM), and 1.5 U Taq DNA polymerase (Takara, Takara Biotechnology, Dalian Co. Ltd, China). The amplifications were performed with the following cycling parameters for ITS: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and with a final

extension at 72 °C for 10 min. The amplifications were performed with the following cycling parameters for LSU: 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1.5 min, and 72 °C for 2 min, and with a final extension at 72 °C for 10 min. Three microliters of each PCR product were run on 1% (w/v) agarose gels and stained with ethidium bromide. The PCR products were purified and sequenced forward and reverse sequences at TsingKe Biological Technology, Kunming, China, using ITS1F/ITS4 and LROR/LR5 primer pairs. Sequences were edited manually using Sequencher™ 4.1.4 (Gene Codes, USA) and queried against the NCBI public database GenBank with the BLASTn algorithm for identification. Sequences generated in this study were deposited in GenBank.

Results

Diversity of wild mushrooms in markets and in the natural habitats

Update and supplement of mushroom species

A total of 623 (HKAS 106765− HKAS 122601) samples were obtained and identified. From those, 110 were collected from markets and 513 from the natural habitats. A total of 310 wild mushroom species, varieties and some undescribed species which are currently under taxonomic study along with ethnomycological catalog information such as scientific names, family names, ecology and edibility were recorded (Table 3), while 310 ITS sequences were deposited in GenBank. Edibility information of most of the mushrooms was gathered directly from sellers and confirmed by taxonomists, professional atlases [40-43] and specialized literature. The 310 recorded species belong to 56 families and 112 genera. Approximately 70% of the species are ectomycorrhizal. Among of them, the 101 species collected in the markets, belong to 22 families and 39 genera, and about 76% of them are EMF. The 283 species collected in the natural habitats, belong to 52 families and 100 genera, and about 70% are EMF.

Table 3 List of the mushroom species observed and acquired in the 3 years of the study at the markets and forests.

Scientific name	Family name	Market	Natural habitat	ECM	Edible part	Voucher No.
Abortiporus biennis (Bull.) Singer	Podoscyphaceae	√			Inedible, wood-decay fungus	HKAS-111766
Acervus globulosus Ekanayaka, Q. Zhao & K.D. Hyde	Pyronemataceae		√		Inedible, too tiny	HKAS-122632
Agaricus heterocystis Heinem. & Gooss.-Font.	Agaricaceae		√		Edible	HKAS-122370
Agaricus luteofibrillosus M.Q. He, Linda J. Chen & R.L. Zhao	Agaricaceae		√		Edible	HKAS-122412
Agaricus sp.	Agaricaceae		√		Unknown	HKAS-122511
Albatrellus sp.	Albatrellaceae		√	√	Edible	HKAS-111880
Amanita albidostipes Y.Y. Cui, Q. Cai & Zhu L. Yang	Amanitaceae		√	√	Toxic	HKAS-124004
Amanita angustilamella (Höhn.)	Amanitaceae		√	√	Unknown	HKAS-123967

Boedijn						
Amanita caojizong Zhu L. Yang, Y.Y. Cui & Q. Cai	Amanitaceae	√	√	√	Edible	HKAS-124005
Amanita cf. *griseofarinosa*	Amanitaceae		√	√	Unknown	HKAS-122658
Amanita citrinoannulata Y.Y. Cui, Q. Cai & Zhu L. Yang	Amanitaceae		√	√	Toxic	HKAS-122410
Amanita elata (Massee) Corner & Bas	Amanitaceae		√	√	Maybe toxic	HKAS-123968
Amanita esculenta Hongo & I. Matsuda	Amanitaceae		√	√	Toxic	HKAS-122372
Amanita eijii Zhu L. Yang	Amanitaceae		√	√	Unknown	HKAS-111744
Amanita fritillaria (Sacc.) Sacc.	Amanitaceae		√	√	Toxic	HKAS-111691
Amanita griseofolia Zhu L. Yang	Amanitaceae		√	√	Edible	HKAS-111779
Amanita levistriata D.T. Jenkins	Amanitaceae		√	√	Toxic	HKAS-111778
Amanita princeps D.T. Jenkins	Amanitaceae		√	√	Toxic	HKAS-122502
Amanita pseudoporphyria Hongo	Amanitaceae		√	√	Toxic	HKAS-111708
Amanita pseudovaginata Hongo	Amanitaceae		√	√	Unknown	HKAS-122692
Amanita rubescens Pers.	Amanitaceae		√	√	Toxic	HKAS-122544
Amanita rubromarginata Har. Takah. Zhu L. Yang	Amanitaceae	√	√	√	Edible	HKAS-122664
Amanita rubrovolvata S. Imai	Amanitaceae		√	√	Toxic	HKAS-122702
Amanita rufoferruginea Hongo	Amanitaceae		√	√	Toxic	HKAS-111723
Amanita sinensis Zhu L. Yang	Amanitaceae	√	√	√	Edible	HKAS-122507
Amanita spissacea S. Imai	Amanitaceae		√	√	Toxic	HKAS-111877
Amanita subglobosa Zhu L. Yang	Amanitaceae		√	√	Maybe toxic	HKAS-122396
Amanita subhemibapha Zhu L. Yang, Y.Y. Cui & Q. Cai	Amanitaceae		√	√	Edible	HKAS-122503
Amanita sychnopyramis Corner & Bas	Amanitaceae		√	√	Toxic	HKAS-122650
Amanita virgineoides Bas	Amanitaceae		√	√	Maybe toxic	HKAS-111833
Amanita yuaniana Zhu L. Yang	Amanitaceae		√	√	Edible	HKAS-122505
Amanita zonata Y.Y. Cui, Qing Cai & Zhu L. Yang	Amanitaceae		√	√	Maybe toxic	HKAS-122624
Amauroderma rugosum (Blume & T. Nees) Torrend	Ganodermataceae	√	√		Medicinal	HKAS-111701
Anamika angustilamellata Zhu L. Yang & Z.W. Ge	Hymenogastraceae		√	√	Maybe toxic	HKAS-111783
Asterophora lycoperdoides (Bull.) Ditmar	Lyophyllaceae		√		Unknown	HKAS-122678
Aureoboletus mirabilis (Murrill)	Boletaceae		√	√	Edible	HKAS-123972

Halling						
Auricularia delicata (Mont. ex Fr.) Henn	Auriculariaceae	√	√		Edible	HKAS-111857
Auricularia fuscosuccinea (Mont.) Henn	Auriculariaceae	√			Edible	HKAS-122598
Blastosporella zonata T.J. Baroni & Franco-Mol.	Lyophyllaceae		√	√	Unknown	HKAS-111854
Boletellus indistinctus G. Wu, Fang Li & Zhu L. Yang	Boletaceae	√	√	√	Edible	HKAS-111749
Boletus sp1.	Boletaceae		√	√	Unknown	HKAS-111715
Boletus sp2.	Boletaceae		√	√	Unknown	HKAS-111794
Boletus sp3.	Boletaceae		√	√	Unknown	HKAS-122405
Boletus aereus Bull.	Boletaceae	√		√	Edible	HKAS-124009
Boletus auripes Peck	Boletaceae	√		√	Edible	HKAS-111826
Boletus bainiugan Dentinger	Boletaceae	√		√	Edible	HKAS-111821
Boletus monilifer B. Feng, Y.Y. Cui, J.P. Xu & Zhu L. Yang	Boletaceae		√	√	Edible	HKAS-111704
Boletus reticulatus Schaeff.	Boletaceae	√	√	√	Edible	HKAS-122381
Boletus subvelutipes Peck	Boletaceae	√		√	Edible	HKAS-111756
Boletus violaceofuscus W.F. Chiu	Boletaceae		√	√	Edible	HKAS-123966
Bondarzewia berkeleyi (Fr.) Bondartsev & Singer	Bondarzewiaceae		√		Unknown	HKAS-122722
Butyriboletus peckii (Frost) Kuan Zhao & Zhu L. Yang	Boletaceae	√		√	Edible, but sour or bitter	HKAS-111872
Butyriboletus huangnianlaii N.K. Zeng, H. Chai & Zhi Q. Liang	Boletaceae	√		√	Edible	HKAS-111755
Caloboletus yunnanensis Kuan Zhao & Zhu L. Yang	Boletaceae		√	√	Edible	HKAS-122727
Cantharellus albovenosus Buyck, Antonín & Ryoo	Hydnaceae	√	√	√	Edible	HKAS-123957
Cantharellus amethysteus (Quél.) Sacc	Hydnaceae	√	√	√	Edible	HKAS-111841
Cantharellus appalachiensis R.H. Petersen	Hydnaceae	√	√	√	Edible	HKAS-123956
Cantharellus cibarius Fr.	Hydnaceae	√	√	√	Edible	HKAS-123958
Cantharellus cinnabarinus (Schwein.) Schwein	Hydnaceae	√	√	√	Edible	HKAS-111815
Cantharellus sp1.	Hydnaceae		√	√	Edible	HKAS-111824
Cantharellus sp2.	Hydnaceae		√	√	Edible	HKAS-124011
Cantharellus tabernensis Feib. & Cibula	Hydnaceae		√	√	Edible	HKAS-111856
Cantharellus yunnanensis W.F.	Hydnaceae	√	√	√	Edible	HKAS-123959

Chiu						
Cantharellus vaginatus S.C. Shao, X.F. Tian & P.G. Liu	Hydnaceae	√	√	√	Edible	HKAS-111852
Ceriporiopsis semisupina C.L. Zhao, B.K. Cui & Y.C. Dai	Meruliaceae		√		Unknown	HKAS-111855
Cerrena zonata (Berk.) H.S.Yuan	Cerrenaceae		√		Unknown	HKAS-122586
Clarkeinda trachodes (Berk.) Singer	Agaricaceae		√		Toxic	HKAS-122723
Clavaria zollingeri Lév.	Clavariaceae		√		Inedible, contains lectin	HKAS-111865
Clavulina alpina Franchi & M. Marchetti	Hydnaceae		√	√	Edible	HKAS-122671
Clavulina cristata (Holmsk.) J. Schröt.	Hydnaceae		√	√	Edible	HKAS-111850
Clavulina flava (Holmsk.) J. Schröt.	Hydnaceae		√	√	Maybe edible	HKAS-122481
Clavulina rugosa (Bull.) J. Schröt.	Hydnaceae		√	√	Edible	HKAS-111717
Clavulina sp.	Hydnaceae		√	√	Maybe edible	HKAS-122494
Clavulinopsis fusiformis (Sowerby) Corner	Clavariaceae		√		Edible	HKAS-122627
Clitopilus chalybescens T.J. Baroni & Desjardin	Entolomataceae		√		Unknown	HKAS-111784
Clitopilus sinoapalus S.P. Jian & Zhu L. Yang	Entolomataceae		√		Unknown	HKAS-122631
Clitopilus sp.	Entolomataceae		√		Unknown	HKAS-122655
Collybiopsis fibrosipes (Berk. & M.A. Curtis) R.H. Petersen	Marasmiaceae		√		Unknown	HKAS-122635
Coltricia crassa Y.C. Dai	Marasmiaceae		√		Inedible, dry and tough	HKAS-122441
Coltricia weii Y.C. Dai	Hymenochaetaceae		√	√	Inedible, dry and tough	HKAS-122593
Cordyceps militaris (L.) Fr.	Cordycipitaceae		√		Medicinal	HKAS-111869
Cordyceps nutans Pat.	Cordycipitaceae		√		Medicinal	HKAS-122491
Cortinarius aff. *torvus*	Cortinariaceae		√	√	Unknown	HKAS-122452
Cortinarius albocyaneus Fr.	Cortinariaceae		√	√	Unknown	HKAS-111851
Cortinarius alpinus Boud.	Cortinariaceae		√	√	Unknown	HKAS-122660
Cortinarius boulderensis A.H. Sm.	Cortinariaceae		√	√	Unknown	HKAS-122445
Cortinarius caesiifolius A.H. Sm.	Cortinariaceae		√	√	Unknown	HKAS-122446
Cortinarius cotoneus Fr.	Cortinariaceae		√	√	Edible	HKAS-122455
Cortinarius croceus (Schaeff.) Gray	Cortinariaceae		√	√	Unknown	HKAS-122559
Cortinarius fulvo-ochrascens Rob. Henry	Cortinariaceae		√	√	Unknown	HKAS-122657

Cortinarius picoides Soop.	Cortinariaceae		√	√	Edible	HKAS-111713
Cortinarius purpurascens Fr.	Cortinariaceae		√	√	Edible	HKAS-122529
Cortinarius sp.	Cortinariaceae		√	√	Unknown	HKAS-111771
Cortinarius tenuipes (Hongo) Hongo	Cortinariaceae	√		√	Edible	HKAS-122467
Cortinarius trivialis J.E. Lange	Cortinariaceae		√	√	Unknown	HKAS-111789
Cortinarius valgus Fr.	Cortinariaceae		√	√	Unknown	HKAS-111836
Cortinarius vinaceobrunneus Ammirati, Beug, Liimat., Niskanen & O. Ceska	Cortinariaceae		√	√	Unknown	HKAS-122626
Craterellus aureus Berk. & M.A. Curtis	Hydnaceae	√		√	Edible	HKAS-123973
Craterellus cornucopioides (L.) Pers.	Hydnaceae	√	√	√	Edible	HKAS-111827
Craterellus luteus T.H. Li & X.R. Zhong	Hydnaceae		√	√	Edible	HKAS-111759
Craterellus parvogriseus U. Singh, K. Das & Buyck	Hydnaceae		√	√	Edible	HKAS-122486
Craterellus sp.	Hydnaceae	√	√	√	Edible	HKAS-122643
Craterellus tubaeformis (Fr.) Quél.	Hydnaceae		√	√	Edible	HKAS-111843
Crocinoboletus laetissimus (Hongo) N.K. Zeng, Zhu L. Yang & G. Wu	Boletaceae	√	√	√	Edible	HKAS-122417
Crocinoboletus sp.	Boletaceae	√	√	√	Edible	HKAS-111764
Cyptotrama asprata (Berk.) Redhead & Ginns	Physalacriaceae		√		Unknown	HKAS-122721
Entocybe trachyospora (Largent) Largent, T.J. Baroni & V. Hofst.	Entolomataceae		√		Maybe toxic	HKAS-122647
Entoloma omiense (Hongo) E. Horak	Entolomataceae		√		Toxic	HKAS-111709
Entoloma petchii E. Horak	Entolomataceae		√		Maybe toxic	HKAS-122493
Entoloma praegracile Xiao L. He & T.H. Li	Entolomataceae		√		Maybe toxic	HKAS-111787
Entoloma subsinuatum Murrill	Entolomataceae		√		Maybe toxic	HKAS-122542
Entoloma sp.	Entolomataceae		√		Unknown	HKAS-111834
Fistulina hepatica (Schaeff.) With.	Fistulinaceae		√		Edible, but acidic and slightly bitter	HKAS-111775
Fistulina sp.	Fistulinaceae		√		Unknown	HKAS-111893
Fistulina subhepatica B.K. Cui & J. Song	Fistulinaceae		√		Unknown	HKAS-122466
Fomitopsis pinicola (Sw.) P. Karst.	Fomitopsidaceae		√		Medicinal	HKAS-111896

Ganoderma lingzhi Sheng H. Wu, Y. Cao & Y.C. Dai	Polyporaceae	√	√		Medicinal	HKAS-111736
Geastrum velutinum Morgan	Geastraceae		√	√	Unknown	HKAS-111879
Gerronema xanthophyllum (Bres.) Norvell, Redhead & Ammirati	Marasmiaceae		√		Unknown	HKAS-122652
Gloeophyllum sepiarium (Wulfen) P. Karst.	Gloeophyllaceae		√		Medicinal	HKAS-122703
Gomphus orientalis R.H. Petersen & M. Zang	Gomphaceae	√		√	Edible	HKAS-111823
Gymnopilus penetrans (Fr.) Murrill	Hymenogastraceae		√		Toxic	HKAS-122710
Gymnopus dryophilus (Bull.) Murrill	Omphalotaceae		√		Edible, but not worthwhile because of thin flesh and tough stem	HKAS-122640
Gymnopus subnudus (Ellis ex Peck) Halling	Omphalotaceae		√		Unknown	HKAS-122729
Gyrodon sp.	Paxillaceae		√		Unknown	HKAS-122638
Gyroporus longicystidiatus Nagas. & Hongo	Gyroporaceae		√		Edible	HKAS-122449
Harrya chromipes (Frost) Halling, Nuhn, Osmundson & Manfr. Binder	Boletaceae	√		√	Edible	HKAS-123979
Hebeloma angustilamellatum (Zhu L. Yang & Z.W. Ge) B.J. Rees	Hymenogastraceae		√	√	Unknown	HKAS-122492
Hebeloma crustuliniforme (Bull.) Quél.	Hymenogastraceae		√	√	Toxic	HKAS-122681
Hebeloma parvisporum Sparre Pedersen, Læssøe, Beker & U. Eberh.	Hymenogastraceae	√	√	√	Edible	HKAS-111767
Heimioporus conicus N.K. Zeng & Zhu L. Yang	Boletaceae		√	√	Toxic	HKAS-122685
Heimioporus japonicus (Hongo) E. Horak	Boletaceae	√	√	√	Toxic, but sold in market	HKAS-111748
Heinemannomyces splendidissimus Watling	Agaricaceae		√	√	Unknown	HKAS-111897
Hourangia nigropunctata (W.F. Chiu) Xue T. Zhu & Zhu L. Yang	Boletaceae		√	√	Maybe toxic	HKAS-111700
Hydnum albidum Peck	Hydnaceae		√	√	Edible	HKAS-111707
Hydnum berkeleyanum K. Das, Hembrom, A. Baghela & Vizzin	Hydnaceae	√	√	√	Edible	HKAS-122362
Hydnum repandum K. Das,	Hydnaceae	√	√	√	Edible	HKAS-111770

Hembrom, A. Baghela & Vizzini						
Hydnum rufescens pers.	Hydnaceae	√	√	√	Edible	HKAS-122528
Hydnum sp.	Hydnaceae	√		√	Edible	HKAS-111800
Hygrocybe cantharellus (Schwein.) Murrill	Hygrophoraceae		√		Edible, but not worthwhile. Because it is too tiny	HKAS-124010
Hygrocybe coccineocrenata (P.D. Orton) M.M. Moser	Hygrophoraceae		√		Unknown	HKAS-124006
Hygrocybe conica var. conica	Hygrophoraceae		√		Maybe toxic	HKAS-111878
Hygrocybe cuspidate (Peck) Murrill	Hygrophoraceae	√	√		Unknown	HKAS-124008
Hymenochaete subferruginea Bres. & Syd.	Hymenochaetaceae		√		Unknown	HKAS-122472
Hymenopellis orientalis (R.H. Petersen & Nagas.) R.H. Petersen	Physalacriaceae		√		Edible	HKAS-111710
Hypomyces chlorinigenus Rogerson & Samuels	Hypocreaceae		√		Inedible, parasitic fungus	HKAS-122599
Hypomyces chrysospermus Tul. & C. Tul.	Hypocreaceae		√		Inedible, parasitic fungus	HKAS-122567
Hypomyces perniciosus Magnus	Hypocreaceae		√		Inedible, parasitic fungus	HKAS-111690
Hypomyces pseudolactifluorum F.M. Yu, Q. Zhao & K.D. Hyde	Hypocreaceae		√		Inedible, parasitic fungus	HKAS-122679
Inocybe sp.	Inocybaceae		√	√	Unknown	HKAS-123963
Laccaria amethystina Cooke	Hydnangiaceae		√	√	Edible	HKAS-122734
Laccaria aurantia Popa, Rexer, Donges, Zhu L. Yang & G. Kost	Hydnangiaceae		√	√	Edible	HKAS-122365
Laccaria laccata (Scop.) Cooke	Hydnangiaceae	√	√	√	Edible	HKAS-111743
Laccaria moshuijun Popa & Zhu Liang Yang	Hydnangiaceae		√	√	Edible	HKAS-122719
Laccaria vinaceoavellanea Hongo	Hydnangiaceae	√	√	√	Edible	HKAS-111721
Laccaria yunnanensis Popa, Rexer, Donges, Zhu L. Yang & G. Kost	Hydnangiaceae	√	√	√	Edible	HKAS-123996
Lactarius acerrimus Britzelm.	Russulaceae		√	√	Edible, but not tasty	HKAS-111712
Lactarius aff. subplinthogalus	Russulaceae	√	√	√	Edible	HKAS-111825
Lactarius akahatsu Nobuj. Tanaka	Russulaceae	√	√	√	Edible	HKAS-122497
Lactarius austrotorminosus H.T. Le & Verbeken	Russulaceae		√	√	Edible	HKAS-122639
Lactarius cinnamomeus W.F. Chiu	Russulaceae		√	√	Edible	HKAS-122463
Lactarius conglutinatus X.H.	Russulaceae		√	√	Toxic	HKAS-111697

Wang						
Lactarius formosus H.T. Le & Verbeken	Russulaceae		√	√	Unknown	HKAS-111772
Lactarius glabrigracilis Wisitr. & Nuytinck	Russulaceae		√	√	Unknown	HKAS-111699
Lactarius gracilis Hongo	Russulaceae		√	√	Unknown	HKAS-111829
Lactarius hatsudake Nobuj. Tanaka	Russulaceae	√	√	√	Edible	HKAS-111725
Lactarius hirtipes J.Z. Ying	Russulaceae		√	√	Toxic	HKAS-122708
Lactarius purpureus R. Heim	Russulaceae		√	√	Edible, but not tasty	HKAS-111745
Lactarius rubrobrunneus H.T. Le & Nuytinck	Russulaceae	√	√	√	Edible	HKAS-111805
Lactarius sp.	Russulaceae		√	√	Edible	HKAS-122654
Lactifluus aff. *tropicosinicus*	Russulaceae		√	√	Edible	HKAS-122728
Lactifluus ambicystidiatus X.H. Wang	Russulaceae		√	√	Maybe inedible, bitter and spicy	HKAS-122435
Lactifluus dwaliensis (K. Das, J.R. Sharma & Verbeken) K. Das	Russulaceae	√		√	Edible	HKAS-111781
Lactifluus gerardii (Peck) Kuntze	Russulaceae	√	√	√	Edible	HKAS-122402
Lactifluus hygrophoroides (Berk. & M.A. Curtis) Kuntze	Russulaceae	√	√	√	Edible	HKAS-123965
Lactifluus leae (D. Stubbe & Verbeken) Verbeken	Russulaceae		√	√	Edible	HKAS-111695
Lactifluus pilosus (Verbeken, H.T. Le & Lumyong) Verbeken	Russulaceae	√	√	√	Edible	HKAS-111859
Lactifluus pinguis (Van de Putte & Verbeken) Van de Putte	Russulaceae	√	√	√	Edible	HKAS-122422
Lactarius piperatus (L.) Pers.	Russulaceae	√	√	√	Edible	HKAS-111795
Lactifluus pseudoluteopus (X.H. Wang & Verbeken) X.H. Wang	Russulaceae		√	√	Maybe toxic	HKAS-122349
Lactifluus rugatus (Kühner & Romagn.) Verbeken	Russulaceae	√	√	√	Edible	HKAS-111848
Lactifluus subpruinosus X.H. Wang	Russulaceae		√	√	Edible	HKAS-122371
Lactifluus volemus (Fr.) Kuntze	Russulaceae	√	√	√	Edible	HKAS-122387
Lanmaoa pallidorosea (Both) Raspé & Vadthanarat	Boletaceae	√		√	Edible	HKAS-123971
Lauriomyces heliocephalus (V. Rao & de Hoog) R.F. Castañeda & W.B. Kendr.	Lauriomycetaceae		√		Inedible, pathogenic fungus	HKAS-111894
Leccinellum quercophilum M.	Boletaceae		√	√	Edible	HKAS-122418

Kuo						
Leccinum rugosiceps (Peck) Singer	Boletaceae		√	√	Edible	HKAS-122386
Lentinula edodes (Berk.) Pegler	Omphalotaceae	√			Edible	HKAS-111768
Lentinus squarrosulus Mont.	Omphalotaceae	√	√		Edible	HKAS-111758
Leotia atrovirens Pers.	Leotiaceae		√		Unknown	HKAS-111847
Leotia lubrica (Scop.) Pers.	Leotiaceae		√		Edible, but tasteless	HKAS-111791
Lyophyllum fumosum (Pers.) P.D. Orton	Lyophyllaceae	√		√	Edible	HKAS-111813
Lyophyllum rhopalopodium Clémençon	Lyophyllaceae		√	√	Unknown	HKAS-111793
Macowanites chlorinosmus A.H. Sm. & Trappe	Russulaceae		√		Unknown	HKAS-122489
Macrocybe gigantea (Massee) Pegler & Lodge	Callistosporiaceae	√			Edible	HKAS-122496
Macrolepiota velosa Vellinga & Zhu L. Yang	Garicaceae		√		Unknown	HKAS-122634
Marasmius sp.	Marasmiaceae		√		Unknown	HKAS-111705
Marasmius pseudopurpureostriatus Wannathes, Desjardin & Lumyong	Marasmiaceae	√	√		Edible, but not worthwhile because of small size and thin flesh	HKAS-123994
Microporus xanthopus (Fr.) Kuntze	Polyporaceae		√		Inedible, leathery flesh	HKAS-111716
Micropsalliota furfuracea R.L. Zhao, Desjardin, Soytong & K.D. Hyde	Agaricaceae		√		Toxic	HKAS-122485
Micropsalliota globocystis Heinem.	Agaricaceae		√		Unknown	HKAS-111724
Nigroporus vinosus (Berk.) Murrill	Steccherinaceae		√		Inedible, wood-decay fungus	HKAS-111839
Neoboletus multipunctatus N.K. Zeng, H. Chai & S. Jiang	Boletaceae		√	√	Unknown	HKAS-111883
Ophiocordyceps nutans (Pat.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	Ophiocordycipitaceae	√	√		Medicinal	HKAS-122621
Ophiocordyceps oxycephala (Penz. & Sacc.) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora	Ophiocordycipitaceae	√	√		Medicinal	HKAS-123960
Panellus pusillus (Pers. ex Lév.) Burds. & O.K. Mill.	Mycenaceae		√		Inedible, maybe medicinal	HKAS-122667
Panus tigrinus (Bull.) Singer	Polyporaceae	√			Edible	HKAS-123984
Paxillus involutus (Batsch) Fr.	Paxillaceae		√	√	Toxic	HKAS-122442

Phaeocollybia pseudofestiva A.H. Sm.	Hymenogastraceae		√	√	Unknown	HKAS-111858
Phaeocollybia ratticauda E. Horak	Hymenogastraceae		√	√	Unknown	HKAS-111769
Phaeocollybia redheadii Norvell	Hymenogastraceae		√	√	Unknown	HKAS-111780
Phaeolus schweinitzii (Fr.) Pat.	Fomitopsidaceae		√		Inedible, too tough	HKAS-122400
Pholiota multicingulata E. Horak	Strophariaceae		√	√	Maybe toxic	HKAS-122568
Phylloporus luxiensis M. Zang	Boletaceae		√	√	Edible	HKAS-111881
Phylloporus rubiginosus M.A. Neves & Halling	Boletaceae		√	√	Unknown	HKAS-122582
Pisolithus tinctorius (Mont.) E. Fisch.	Sclerodermataceae		√	√	Medicinal	HKAS-123964
Pluteus septocystidiatus Ševčíková, Antonín & Borov.	Pluteaceae		√		Unknown	HKAS-111864
*Podoscypha involuta (*Klotzsch ex Fr.) Imazeki	Podoscyphaceae		√		Unknown	HKAS-111782
Polyporus cuticulatus Y.C. Dai, Jing Si & Schigel	Polyporaceae	√	√		Edible	HKAS-111809
Pulveroboletus icterinus (Pat. & C.F. Baker) Watling	Boletaceae		√	√	Toxic, maybe medicinal	HKAS-111741
Pulveroboletus subrufus N.K. Zeng & Zhu L. Yang	Boletaceae		√	√	Toxic	HKAS-122514
Ramaria asiatica (R.H. Petersen & M. Zang) R.H. Petersen	Gomphaceae		√	√	Edible	HKAS-123983
Ramaria cartilaginea Marr & D.E. Stuntz	Gomphaceae	√	√	√	Edible	HKAS-123998
Ramaria cyanocephala (Berk. & M.A. Curtis) Corner	Gomphaceae		√	√	Maybe toxic	HKAS-122630
Ramaria fennica (P. Karst.) Ricken	Gomphaceae		√	√	Edible, but bitter	HKAS-111790
Ramaria flava (Schaeff.) Quél.	Gomphaceae		√	√	Edible, but little bitter	HKAS-111706
Ramaria pallida (Schaeff.) Ricken	Gomphaceae	√	√	√	Edible	HKAS-123982
Ramaria sanguinipes R.H. Petersen & M. Zang	Gomphaceae		√	√	Edible	HKAS-111746
Ramaria sp.	Gomphaceae	√		√	Edible	HKAS-111774
Ramaria thindii K. Das, Hembrom, A. Parihar & A. Ghosh	Gomphaceae		√	√	Edible	HKAS-122425
Ramaria vinosimaculans Marr & D.E. Stuntz	Gomphaceae		√	√	Edible	HKAS-111785

Retiboletus fuscus (Hongo) N.K. Zeng & Zhu L. Yang	Boletaceae		√	√	Edible	HKAS-122545
Retiboletus sinensis N.K. Zeng & Zhu L. Yang	Boletaceae		√	√	Edible	HKAS-122610
Retiboletus sp.	Boletaceae		√	√	Unknown	HKAS-122552
Rhizocybe alba Y.X. Ding & E.J. Tian	Agaricales		√		Maybe toxic	HKAS-122720
Rhizopogon songmaodan R. Wang & Fu Q. Yu	Rhizopogonaceae	√		√	Edible	HKAS-123980
Rubroboletus esculentus Kuan Zhao, H.M. Shao & Zhu L. Yang	Boletaceae	√		√	Edible	HKAS-124003
Rugiboletus extremiorientalis (Lj.N. Vassiljeva) G. Wu & Zhu L. Yang	Boletaceae	√		√	Edible	HKAS-123978
Russula adusta (Pers.) Fr.	Russulaceae		√	√	Edible	HKAS-122583
Russula amarissima Romagn. & E.-J. Gilbert	Russulaceae	√		√	Edible	HKAS-111737
Russula cerea (Soehner) J.M. Vida	Russulaceae		√	√	Unknown	HKAS-122509
Russula compacta Frost	Russulaceae	√	√	√	Edible	HKAS-111734
Russula crustosa Peck	Russulaceae		√	√	Edible	HKAS-122506
Russula cyanoxantha (Schaeff.) Fr.	Russulaceae	√	√	√	Edible	HKAS-122577
Russula delica Fr.	Russulaceae	√	√	√	Edible	HKAS-123987
Russula densifolia Secr. ex Gillet	Russulaceae		√	√	Edible	HKAS-122430
Russula dissimulans Shaffer	Russulaceae		√	√	Edible	HKAS-122628
Russula flavida Frost ex Peck	Russulaceae		√	√	Edible	HKAS-122512
Russula foetens Pers.	Russulaceae		√	√	Toxic	HKAS-111702
Russula griseocarnosa X.H. Wang, Zhu L. Yang & Knudsen	Russulaceae	√	√	√	Edible	HKAS-122424
Russula lakhanpalii A. Ghosh, K. Das & R.P. Bhatt	Russulaceae		√	√	Unknown	HKAS-122622
Russula lilacea Quél.	Russulaceae	√	√	√	Edible	HKAS-111853
Russula nigricans Fr.	Russulaceae	√	√	√	Edible	HKAS-123961
Russula purpureogracilis F. Hampe, Looney & Manz	Russulaceae		√	√	Unknown	HKAS-111722
Russula rosea Pers.	Russulaceae	√	√	√	Edible, but some consider it inedible	HKAS-122342
Russula senecis S. Imai	Russulaceae	√	√	√	Edible	HKAS-122352
Russula sp.	Russulaceae		√	√	Unknown	HKAS-122376
Russula sororia (Fr.) Romell	Russulaceae		√	√	Edible	HKAS-122487
Russula substriata J. Wang,	Russulaceae		√	√	Unknown	HKAS-122625

X.H. Wang, Buyck & T. Bau						
Russula virescens (Schaeff.) Fr.	Russulaceae	√	√	√	Edible	HKAS-122384
Russula viridicinnamomea F. Yuan & Y. Song	Russulaceae		√	√	Edible	HKAS-122524
Russula vinosa Lindblad	Russulaceae	√	√	√	Edible	HKAS-122380
Sarcoporia polyspora P. Karst.	Sarcoporiaceae		√		Inedible, woody-decay fungus	HKAS-122725
Schizophyllum commune Fr.	Schizophyllaceae	√			Edible and medicinal	HKAS-123962
Scleroderma flavidum Ellis & Everh	Sclerodermataceae		√	√	Toxic	HKAS-122469
Scleroderma sinnamariense Mont.	Sclerodermataceae		√	√	Toxic	HKAS-111718
Scleroderma yunnanense Y. Wang	Sclerodermataceae	√	√	√	Edible	HKAS-111786
Scleroderma sp.	Sclerodermataceae		√	√	Unknown	HKAS-111776
Sparassis sp.	Sparassidaceae		√		Unknown	HKAS-122536
Stereopsis radicans (Berk.) D.A. Reid	Stereopsidaceae		√	√	Unknown	HKAS-111876
Strobilomyces confusus Singer	Boletaceae		√	√	Edible	HKAS-122534
Strobilomyces latirimosus J.Z. Ying	Boletaceae		√	√	Edible	HKAS-122520
Strobilomyces seminudus Hongo	Boletaceae		√	√	Edible	HKAS-111720
Stropharia rugosoannulata Farl. ex Muriil	Strophariaceae		√		Edible	HKAS-122474
Sulzbacheromyces yunnanensis D. Liu, Li S. Wang & Goffinet	Lepidostromataceae		√		Unknown	HKAS-122355
Suillellus sp.	Boletaceae		√	√	Unknown	HKAS-111890
Suillellus subvelutipes (Peck) Murrill	Boletaceae		√	√	Maybe toxic	HKAS-111754
Suillus bovinus (L.) Roussel	Suillaceae		√	√	Edible	HKAS-111891
Suillus luteus (L.) Roussel	Suillaceae		√	√	Toxic	HKAS-111788
Suillus placidus (Bonord.) Singer	Suillaceae		√	√	Toxic	HKAS-122590
Tapinella panuoides (Fr.) E.-J. Gilbert	Tapinellaceae		√		Toxic	HKAS-122726
Termiticola sp.	Agaricaceae		√		Unknown	HKAS-111738
Termitomyces albiceps S.C. He	Lyophyllaceae	√	√		Edible	HKAS-111703
Termitomyces aurantiacus (R.Heim) R. Heim	Lyophyllaceae	√	√		Edible	HKAS-122633
Termitomyces clypeatus R. Heim	Lyophyllaceae	√	√		Edible	HKAS-123988
Termitomyces eurrhizus (Berk.) R. Heim	Lyophyllaceae	√	√		Edible	HKAS-124007

Termitomyces heimii Natarajan	Lyophyllaceae	√			Edible	HKAS-123975
Termitomyces fuliginosus R. Heim	Lyophyllaceae	√	√		Edible	HKAS-111732
Termitomyces microcarpus (Berk. & Broome) R. Heim	Lyophyllaceae	√	√		Edible	HKAS-111735
Termitomyces sp.1	Lyophyllaceae	√			Edible	HKAS-122510
Termitomyces sp.2	Lyophyllaceae	√			Edible	HKAS-122623
Termitomyces striatus (Beeli) R. Heim	Lyophyllaceae	√	√		Edible	HKAS-124012
Thelephora ganbajun M. Zang	Thelephoraceae	√	√	√	Edible	HKAS-111698
Thelephora regularis Schwein	Thelephoraceae	√	√	√	Edible	HKAS-111874
Thelephora sikkimensis K. Das, Hembrom & Kuhar	Thelephoraceae		√	√	Unknown	HKAS-122715
Thelephora sp.	Thelephoraceae	√	√	√	Edible	HKAS-111830
Thelephora vialis Schwein.	Thelephoraceae	√	√	√	Edible	HKAS-122373
Trichaptum abietinum (Pers. ex J.F. Gmel.) Ryvarden	Hymenochaetales		√		Inedible, leathery flesh	HKAS-122706
Tricholoma albobrunneum (Pers.) P. Kumm.	Tricholomataceae		√	√	Toxic	HKAS-122501
Tricholoma equestre (L.) P. Kumm.	Tricholomataceae	√	√	√	Toxic, but sold in market	HKAS-111762
Tricholoma fulvocastaneum Hongo	Tricholomataceae	√		√	Edible	HKAS-106954
Tricholoma olivaceum Reschke, Popa, Zhu L. Yang & G. Kost	Tricholomataceae		√	√	Unknown	HKAS-122580
Tricholoma saponaceum (Fr.) P. Kumm.	Tricholomataceae	√	√	√	Mild toxic, but sold in market	HKAS-111763
Trogia infundibuliformis Berk. & Broome	Marasmiaceae		√		Edible	HKAS-122453
Turbinellus floccosus (Schwein.) Earle ex Giachini & Castellano	Gomphaceae	√	√	√	Edible	HKAS-122519
Tylopilus balloui (Peck) Singer	Boletaceae		√	√	Toxic	HKAS-122578
Tylopilus neofelleus Hongo	Boletaceae	√	√	√	Toxic, but sold in market	HKAS-123985
Tylopilus vinosobrunneus Hongo	Boletaceae		√	√	Toxic	HKAS-111693
Xylaria brevipes Sacc. & Fairm.	Xylariaceae		√		Medicinal	HKAS-122468

In the markets, 91 species are edible and about 80% are EMF. A few new species which have only been published in recent years [44-47] were found in markets. And some previously described species were revised or classified in other section or genus by molecular phylogenetic study [48-50]. Furthermore, four species from markets are medicinal, two of which, *Ophiocordyceps nutans* (Pat.) G.H. Sung, J.M. Sung, Hywel-

Jones & Spatafora and *O. oxycephala*, are mainly distributed in tropical and subtropical broad-leaved forests. It is interesting that four species which have been reported to cause gastroenteritis type poisoning, including *Heimioporus japonicus* (Hongo) E. Horak and *Tylopilus neofelleus* Hongo (in July) were sold in large quantities in Pu'er market, and *Tricholoma equestre* (L.) P. Kumm. (Agust to October) was mixed with a few *Tricholoma saponaceum* (Fr.) P. Kumm. in some small stalls. Some specimens, of one inedible species, *Abortiporus biennis* (Bull.) Singer, were recorded to be sold in a few markets as *Thelephora ganbajun* M. Zang. Similarly, *Hygrocybe cuspidata* (Peck) Murrill with unknown toxicity was sold occasionally in some stalls maybe because for some people it is *Cantharellus*-looking. Therefore, the accurate taxonomic status of these apparently toxic species has to be carefully checked, in order to determine if they correspond to new taxa or if the ecotypes in the area are non-toxic species. Most commercial mushrooms are common species in all markets (Figure 3 a-i). Six sampled markets shared 49 mushroom species, while 12 unique species were only sold in Pu'er market and 9 unique species were only sold in Lancang market (Figure 3j).

Fig. 3 Popular species sold in the studied markets. (a) *Lactifluus volemus*; (b) *Termitomyces* spp.; (c) *Russula griseocarnosa*; (d) *Scleroderma yunnanense*; (e) *Craterellus cornucopioides*; (f) *Lactifluus piperatus*; (g) *Ramaria* spp.; (h) *Boletus* spp.; (i) *Laccaria laccata*; (j) Flower plot diagram showing shared and unique edible wild mushroom species in the sampled markets.

The forest areas selected for the natural habitats work (according to information gathered from some collectors) were within 15 kilometers of the markets. Due to its protected status, the Ecological Conservation Forests and the Sun River National Forest Park are less visited by gatherers or recreational visitors. A total of 283 species were recorded and collected from natural habitats, which include 129 edible species, accounting for about 84% EMF, 15 inedible species, 11 medicinal species, 53 poisonous species and 75 species with unknown edibility. Moreover, 23 species are undescribed and are currently under taxonomic study (Figure 4).

Fig. 4 Typical edible wild mushrooms and their natural habitats. (a-c) Sampled vegetation types: (a) *Pinus kesiya* forest; (b) Coniferous and broad-leaved forest mixed forest; (c) Broad-leaved forest. (d-i) Representative abundant mushroom species: (d) *Ramaria* sp.; (e) *Cantharellus cinnabarinus*; (f) *Lactifluus piperatus*; (g) *Craterellus cornucopioides*; (h) *Amanita caojizong*; (i) *Laccaria yunnanensis*. (j-l) Some undescribed fungi: (j) *Cortinarius* sp.; (k) *Phaeocollybia* sp.; (l) *Ramaria* sp.

Local preference and acceptability of WEF species

A total of 74 species were recorded in both markets and natural habitats. *Amanita cajizong* Zhu L. Yang, Y.Y. Cui & Q. Cai, *Cantharellus cinnabarinus* (Schwein.) Schwein, *Craterellus cornucopioides* (L.) Pers., *Laccaria yunnanensis* Popa, Rexer, Donges, Zhu L. Yang & G. Kost, *Lactifluus piperatus* (L.) Pers., *Lactifluus volemus* (Fr.) Kuntze and *Ramaria* spp. were popular in markets and easy to find in natural habitats in mushroom season (Figure 5). The most frequently bought wild mushrooms belonged mainly to Boletaceae (16 species), Hydnaceae (14 species), Lyophyllaceae (11 species) and Russulaceae (23 species). The families Amanitaceae (26 species), Boletaceae (32 species), Cortinariaceae (16 species), Hydnaceae (24 species), Hydnangiaceae (6 species), Lyophyllaceae (11 species), and Russulaceae (50 species) were common in natural habitats and forests. Mushroom species and amount showed a high correspondence between markets and the natural habitats on different months (Figure 5). Preference of WEF for locals was mostly related to their availability in the forests.

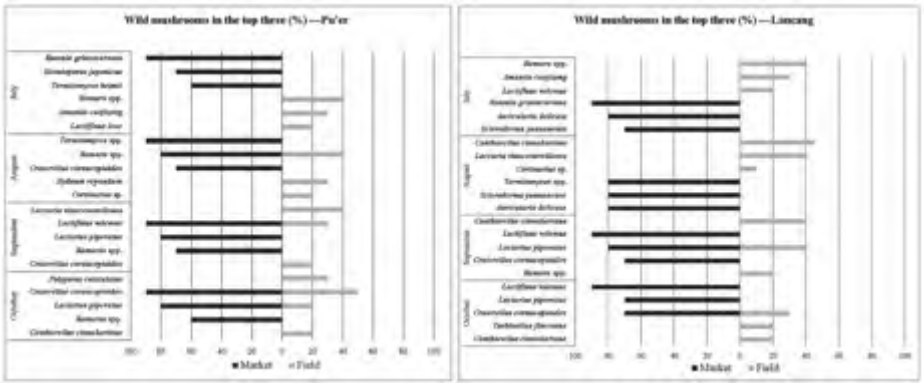

Fig. 5 Comparison of the most abundant edible wild mushrooms sampled in markets (black bars) and natural habitats (gray bars) of Pu'er Municipality and Lancang County during July to October. Numbers of black bars represent percentages of stalls sold the most abundant edible wild mushrooms to total stalls; numbers of gray bars represent percentages of numbers of collected the most abundant edible wild mushrooms to total collected mushrooms.

Ethnomycological data

Type of markets and constitution of vendors

A total of 11 markets were visited during this study. As illustrated in the table 1, three markets were established markets, 3 markets were mobile markets and 5 street-stalls without names. Different markets have different sale time to sell mushrooms according to the local people's different lifestyles. The highest number of vendors in allmarkets was recorded in August and September. The vendors in the mobile markets and in the street-stalls were usually low-income people, who travel usually by foot from the natural collection areas to the selling points.

Almost all vendors were able to speak Mandarin in Wuyi market of Pu'er City although most of vendors belong to ethnic groups, like Hani, Yi and Lahu people. This is the largest market in Pu'er, and up to 200 vendors, including gatherers, two−way merchants (those who buy mushrooms from gatherers directly in natural habitats) and brokers (those who buy mushrooms from gatherers or to two−way merchants), sold mushrooms in August and September (Figure 2a, b, c). Most of the valuable mushrooms are usually sold at higher prices to large markets or restaurants of Kunming (the capital of Yunnan province) by brokers. Vendor's main age group was between 35 and 55, and most of them were able to receive contactless payments through their mobile phones.

In the markets of Mojiang and Ning'er Counties, the number of vendors reached 100 in August or September. The Yi and Hani people are the main ethnic groups who inhabit these two counties. In recent years, local governments have paid great attention to the development of WEF resources marketing, and more mushroom markets have been established. Vendors in these markets were gatherers, merchants and some brokers, and the main age group was between 20 and 45. A small group of aged vendors (60+) spoke southwest Mandarin and could not use mobile phone apps to receive payments for the mushroom sale.

Lancang Street Market (Figure 2 d, e) had mostly Lahu and Hani people. The villagers in the surrounding towns bring a variety of products to Lancang Street Market

on Sunday every week. Vendor's main age group was between 40 and 65, 48% of which could not speak Mandarin, only Lahu language and southwest Mandarin. In addition, most of aged vendors accepted cash only. Nearly all vendors in Lancang Street Market were gatherers, and most of them usually sell mushrooms along with vegetables, fruits or local products, so seller mobility in this market was not strong within market time.

Menglian and Ximeng Counties are not far from Lancang County. The Lahu, Dai, and Wa people are the main ethnic groups in these two counties. Vendors here spoke Lahu, Dai and Wa languages and southwest Mandarin. Compared with other markets, fewer vendors sold mushrooms. However, some vendors said that many buyers from Lancang County or Pu'er Municipality came here to buy mushrooms to process or dry and then resell them, so many vendors collect mushrooms and sell them directly to wholesale buyers.

Mushroom species from five street-stalls which have 1 to 15 vendors by county highways or village roads were also recorded. These vendors come from nearby villages and most of them were aged people. They don't have transportation to go to markets to sell mushrooms, so they usually sell them on the side of the road after collecting them. As a consequence only very fresh mushrooms were recorded (Figure 2 g, i).

Gender of vendors

The male to female ratio of vendors showed that women outnumber men in markets. Female vendors were involved in every stage of mushroom utilization from collection to processing and marketing.

Mushroom prices in three years

The prices of popular mushrooms were similar in the six studied counties, and the price of each species did not fluctuate much over the three years (Table 4), but a large fluctuation occurred throughout the season due to the available abundance and and quality of wild mushrooms. Overall, the prices of popular mushrooms, *Russula griseocarnosa* X.H. Wang, Zhu L. Yang & Knudsen, *Termitomyces* spp. (e.g., *T. globulus*, *T. striatus*) and *Thelephora ganbajun* were higher than those of other mushroom species. *Schizophyllum commune* Fr. was sold in a few stalls in each market, and its price was be up to 200 yuan/kg. In each market, vendors carefully placed mushrooms on green banana leaves or in plastic bags, baskets or plates (Figure 6) with a certain weight (generally 0.5 kg or 1 kg), which due to the arrangement always looked beautiful and clean.

Table 4 Sale prices of frequently bought mushrooms as recorded in 2019 to 2021

Species	Year 2019	Year 2020	Year 2021
Boletus spp. (porcini group)	30−90 yuan/kg	20−75 yuan/kg	30−85 yuan/kg
Craterellus cornucopioides	20−70 yuan/kg	20−60 yuan/kg	20−70 yuan/kg
Laccaria spp.	20−40 yuan/kg	15−40 yuan/kg	15−40 yuan/kg
Lactifluus piperatus	10−30 yuan/kg	10−30 yuan/kg	10−30 yuan/kg
Lactifluus volemus	30−90 yuan/kg	25−90 yuan/kg	30−90 yuan/kg
Ramaria spp.	20−40 yuan/kg	20−40 yuan/kg	10−40 yuan/kg
Russula griseocarnosa	45−120 yuan/kg	50−120 yuan/kg	40−130 yuan/kg
Russula virescens	30−80 yuan/kg	30−90 yuan/kg	30−80 yuan/kg

Scleroderma yunnanense	15−40 yuan/kg	15−40 yuan/kg	15−40 yuan/kg
Termitomyces spp.	30−160 yuan/kg	30−160 yuan/kg	25−160 yuan/kg
Thelephora ganbajun	90−180 yuan/kg	95−180 yuan/kg	90−195 yuan/kg

Fig. 6 In each market, vendors place mushrooms on green banana leaves, plastic bags, baskets or cans with a defined weight (usually 0.5kg or 1 kg), which facilitates the selling process.

Twenty percent of vendors in markets were randomly selected as respondents to answer the semi-structured interviews. Except for brokers, most collectors are farmers who grow tea and other crops or raise hogs and cattle. During the mushroom season, they usually collect wild mushrooms in the mountains near their homes and sell them for an extra income (3000−6000 yuan per family per year, approximately equivalent to USD$450 to 900) for their families.

The use and preparation methods of WEF

The main use of wild mushrooms is for food, and a few are medicinal species used to make medicinal liquor (Figure 7, Table 5). The most common cooking preparation way among local people was to fry the mushrooms with fermented bamboo shoots or other local vegetables. *Lactifluus piperatus,* which has a spicy taste is considered to be a perfect match for sour pickles. *Tylopilus neofelleus*, is an interesting species consider toxic by some local people, however other people enjoy its bitter flavor. They found a cooking method to remove toxic components, which was by drying slices of the mushroom and then deep frying them. For species of Boletaceae, local people had a common understanding of adding more garlic and cooking them for more than 30 minutes. Likewise, local people soak peeled *Scleroderma yunnanense* Y. Wang fruiting bodies or slices in water or saline water before cooking to remove some components to avoid any gastrointestinal upset.

Local people stored mushrooms by drying, pickling and frying, but they enjoy more to eat fresh mushrooms. Some dry mushrooms, like *Boletus* spp. (porcini), *Russula griseocarnosa*, *Russula virescens* (Schaeff.) Fr. and *Ramaria* spp. were usually sold to people from other cities.

Fig. 7 Preparation way of wild fungi. (a) *Termitomyces* soup; (b) Stir-fried *Cantharellus cinnabarinus*; (c) Hot pot with Boletaceae, *Lactarius*, *Lyophyllum*, *Russula* and some artificial cultivated mushrooms.

Table 5 Local preferred preparations and storage methods for edible mushrooms

Species	Preparation	Note	Storage
Amanita caojizong, *A. sinensis*	Make soup, stir-fry with little garlic	–	–
Boletaceae	Fried with garlic and chili (dry chili or fresh chili)	Cooking time must be longer	Slice and dry Fry and soak in oil
Cantharellus spp.	Stir-fried with little garlic	–	Dry
Craterellus cornucopioides	Stir-fry with garlic and chili	Cooking time is short to keep its crisp mouthfeel	–
Lactifluus piperatus	Chop mushrooms, then fry with garlic, dry chili and sour bamboo shoots or pickles	–	–
Lactifluus volemus	Fry with garlic, chili and meat	–	–
Ramaria spp.	Fry with garlic, dry chili and sour bamboo shoots or pickles	–	Dry
Russula griseocarnosa	Cook with chicken soup	–	Dry
Russula virescens	Stir-fry with garlic and fresh chili Cook with meat soup	–	Dry
Scleroderma yunnanense	Slice, fry with garlic and chili	Peel and soak in water before cooking to reduce bitter taste	Slice and pickle with salt
Termitomyces spp.	Make soup Fried mushroom oil	–	Fry and soak in oil
Thelephora ganbajun	Fried with garlic, chili and bacon	–	–
Tylopilus neofelleus	Dry, slice and deep fry	–	–

Traditional recognition methods of WEF

The rich variety of mushroom species gathered by local people demonstrates that they have a rich traditional knowledge. Local mushroom names demonstrate a particular taxonomic knowledge. According to the color, shape, taste, texture, habitat and some

special features of mushrooms or even local legends, interesting and vivid names have been given to mushrooms and people are able to make a local classification system for mushrooms (Table 6). Sometimes, mushrooms have more than one name, like *Scleroderma yunnanense* is named "bubble with horse skin" in most areas of Pu'er because of its shape and texture, but Lahu people call it "soil fruits" because of its habitat. *Lactifluus rugatus* (Schaeff.) Fr. is named "milk cap mushroom" because of the fluid it produces, and the names "monkey mushroom" (local monkeys are yellow) and "sweet yellow mushroom" come from its pileus color and taste. Experienced gatherers have a more impressive knowledge. Such as valuable *Russula griseocarnosa* could be distinguished from other similar or poisonous species by its thick pileus, light-grey context and solid stipe (they usually squeeze the stipe). *Amanita caojizong* and poisonous *Amanita pseudoporphyria* Hongo are locally distinguished by the stipe shape and smell. The knowledge of selecting mushrooms has usually passed from generation to generation. Moreover, some collectors have their own mental maps to find specific places where mushrooms, especially valuable ones appear every year, and the information is usually kept within their family to avoid the collection by other people, which would affect their family´s income.

Table 6 Interesting local name of popular commercial mushrooms in markets.

Species	**Local name (in Chinese)**	**Local name (in English)**	**Origin of name**
Amanita sinensis	麻母鸡	Pock chicken	Color and pulverulent to flocculent squamules
Amanita caojizong	鸭蛋菌	Duck's egg mushroom	Smooth and rounded pileus
	露水鸡堎	Dew termite mushroom	Fruiting time and termite mushroom shape
Boletus spp.	牛肝菌/羊肝菌	Beef/ lamb liver mushroom	Plump flesh
	见手青	Turn to green when hands touch	Indigo color reaction after injury
Cantharellus spp.	鸡油菌	Chicken fat mushroom	Fruiting body's color
Cortinarius tenuipes	黄栎窝	Nest of yellow mushroom under the oak	Color, habitat and cluster
Craterellus spp.	喇叭菌	Trumpet mushroom	The shape of fruiting body
Hydnum spp.	羊腮巴	Goat's cheek	Soft spines
Laccaria spp.	鸡屁眼菌	Chicken ass mushroom	Shape of pileus
Lactifluus piperatus	辣菌	Spicy mushroom	Spicy taste
Lactifluus volemus	奶浆菌	Milk cap mushroom	Milk flowing out when cut
Panus tigrinus	八担柴	Eight loads of wood	Tough texture, need a lot of wood to cook
Ramaria spp.	刷把菌	Brush mushroom	Multiple-branch
Russula griseocarnosa	大红菌	Red mushroom	The color of fruiting body
Russula nigricans	火炭菌	Charcoal mushroom	The color of fruiting body
Russula virescens	青头菌	Green head mushroom	The color of fruiting body
Scleroderma yunnanense	马皮泡	Horse skin bubble	Shape of fruiting body
Thelephora spp.	干巴菌	Jerky mushroom	Chewy flesh
Tricholoma equestre	荞面菌	Buckwheat mushroom	Fruiting body's color
Tylopilus neofelleus	苦马肝	Bitter horse liver	Bitter taste and plump flesh

Discussion

A total of 310 wild mushroom species, varieties and some undescribed species were collected from markets and natural areas. Approximately 70% of the species were ectomycorrhizal. In the markets from the 91 edible species about 80% were EMF. With the development of transportation infrastructures, Pu'er has become one of the main supply centers of WEF for central Yunnan, and WEF processing industries are becoming large-scale. Yu et al. [33] surveyed markets in Pu'er from 2002 to 2009 and reported a sharp decline of WEF production of 43 species, such as *Lactifluus volemus*, *Russula griseocarnosa*, *Termitomyces* spp. and *Thelephora ganbajun* which were considered important in Yunnan. In our study, interviews with vendors showed that production of these species had declined even more in recent years and they had to travel farther to collect them. However, we also found that some mushrooms, that were not so common then, are now popular in Pu'er area, such as *Cantharellus cinnabarinus*, *Laccaria laccata* and *Boletus edulis* [33]. These species have a high market value and high production in the sites sampled in our study. This change might have due to the growing mycological knowledge of Pu'er people. The increase of mushroom species could reduce the pressure of collection of valuable species to some extent. But local people still act cautiously and even refuse eating some edible mushrooms that have only recently become mainstream edibles. In our study, 57 good edible species that we found in nature were not sold in markets. Very tasty species as *Amanita subhemibapha* Zhu L. Yang, Y.Y. Cui & Q. Cai, *Boletus violaceofuscus* W.F. Chiu and *Laccaria amethystina* Cooke have beautiful color and good production in the forests, but they were not recorded in the markets maybe due to the fact that they are preferred for self-consumption rather than commercialization. The utilization of WEF resources in Pu'er still has great potential to be developed.

A total of 11 markets from one municipality and 5 counties were visited during this study. Sales activities of wild mushrooms can be carried out uniformly in established markets, while local government strengthen the sales supervision of markets to make the sale of wild mushrooms more standardized and reduce the probability of wild mushroom poisoning. In each market, the male to female ratio of vendors showed that women outnumber men. It seems that in many regions of the world women are often the main collectors [51-53]. But women usually collected mushrooms closest to their houses, while men go farther to collect. Therefore, men usually have developed a more profound knowledge on the biology, ecology, and phenology of mushrooms and were able to identify them, even at a species level. In addition, the age of collectors was mainly between 45-65 years old and only few young people were involved in mushroom collecting or selling. Traditional knowledge is being lost through economic change, modernization, urbanization and even formal education. Therefore, further research on ethnomycology is urgent to preserve the current knowledge before their lost forever.

Despite the fact that open air markets in southeast Asia are relevant reservoirs of biocultural diversity in southeast Asia, they have been largely understudied. As far as useful mycological resources concerns, it has been shown that these markets are

additionally an important source of traditional knowledge due to the fact that frequently the sellers are the current gatherers, recipients of ancestral mycological knowledge. Some areas in different parts of southwest Asia have shown to harbour a great biodiversity of edible mushrooms. For example, in the markets of Luang Prabang in north Central Laos, 54 species of fungi have been reported to be sold [54]. In this area a large number of rare species of *Russula,* some probably new to science, are commercialized in local markets. Some of the species reported from markets of this region of Laos were also recorded to be sold in the Puer's studied markets in our work including: *Amanita princeps*, *Auricularia delicata*, *Boletus reticulatus*, *Lactifluus pinguis*, *Lentinula edodes*, *Lentinus squarrosulus*, *Macrocybe gigantea*, *Russula delica*, *R. virescens*, *Schizophyllum commune*, *Termitomyces fuliginosus*, *T. eurhizus*, *T. heimii* and *T. microcarpus* [54]. Recently, a monograph of the useful fungi of Northern Laos, including edible and medicinal species has been published [55]. There being also a large number of species reported in this monograph with those sold in the markets of Pu'er in China. These include members of the genera *Amanita, Auricularia, Boletus Cantharellus, Craterellus, Lactarius, Lactifluus, Lentinus, Lentinula, Lyophyllum, Ramaria, Russula, Thelephora, Termitomyces, Tricholoma* and *Tylopilu*s, most of which are EMF. The situation of the ethnomycological understudy of open-air markets selling wild mushrooms is not exclusive of Southeast Asia, but it is a global issue. For example in Tanzania, 128 edible wild mushrooms are commercialized in 31 traditional markets. Among them the genera with the highest diversity were *Termitomyces, Cantharellus* and *Russula* with 21, 17 and 9 species, respectively [56]. In Mexico, in one single market located in the central part of the country, called Ozumba, 60 species of WEF were reported to be sold. In this market, with 411 stands selling WEF mainly during July and August, 90% of the vendors were women, and 64% were between 40 to 60 years old [52]. In southeastern Poland, 30 edible wild mushrooms were recorded to be sold [57]. A similar number of species, have been recorded to be sold in western Black Sea region in Turkey, where 33 edible wild mushrooms are commercialized in 70 local markets [58]. In other areas, smaller number of species have been recorded to be sold in open-air markets, for example in Armenia located in Western Asia, only 12 edible wild species of mushrooms have been reported to be commercialized [59]. In fact, in general the open-air markets constitute cultural treasures, which should receive more attention in order to increase the knowledge and cosmovision related with the use of wild mushrooms as a paramount local source of food around the globe.

Conclusion

We recorded a wealth of ethnomycological knowledge through interviews and collected abundant wild mushroom samples from local markets and forests in three consecutive years. Mushroom harvesting is a challenging activity that requires a deep local environmental knowledge to achieve success. Local mushroom collectors in Pu'er have rich experience with the habitats where their WEF proliferate, their fruiting time and species identification which comes mainly from the previous generation, as well as special cooking and preservation methods. There are established markets, mobile

markets and street-stalls for selling mushrooms in Pu'er area. In markets, men usually develop a more profound knowledge on WEF than woman, although the number of female vendors is larger than that of male vendors. Our current study provides useful documentation, which contributes to preserving ethnomycological knowledge in Pu'er Prefecture. In addition, the diversity of species of wild fungi, especially ectomycorrhizal fungi, in markets and natural areas have been updated and supplemented which helps us to recognize mushroom species accurately and detect valuable species. Local preference and acceptance of more mushroom species of WEF may reduce the pressure to collect traditional choice species. However, the rational management of WEF species with high yield in natural areas and the collection and use of ectomycorrhizal fungi germplasm resources for cultivation will benefit the sustainable utilization of local WEF. Finally, it is necessary to continue the research of ethnomycology in order to preserve existing knowledge, since knowledge of fungi remains mainly among the elderly population.

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Not applicable

Consent for publication
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Not applicable

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Competing interests
The authors declare that they have no competing interests

Authors' contributions
RW: conducted the investigation and experiment, analyzed the data, and prepared the manuscript. MH: conducted the investigation and edited the manuscript. WJX: conducted the investigation and experiment. PZ: conducted the investigation. JPM and CC: guided in study plan and revised the manuscript. FQY: supervised the research and edited the manuscript. All authors contributed to the article and approved.

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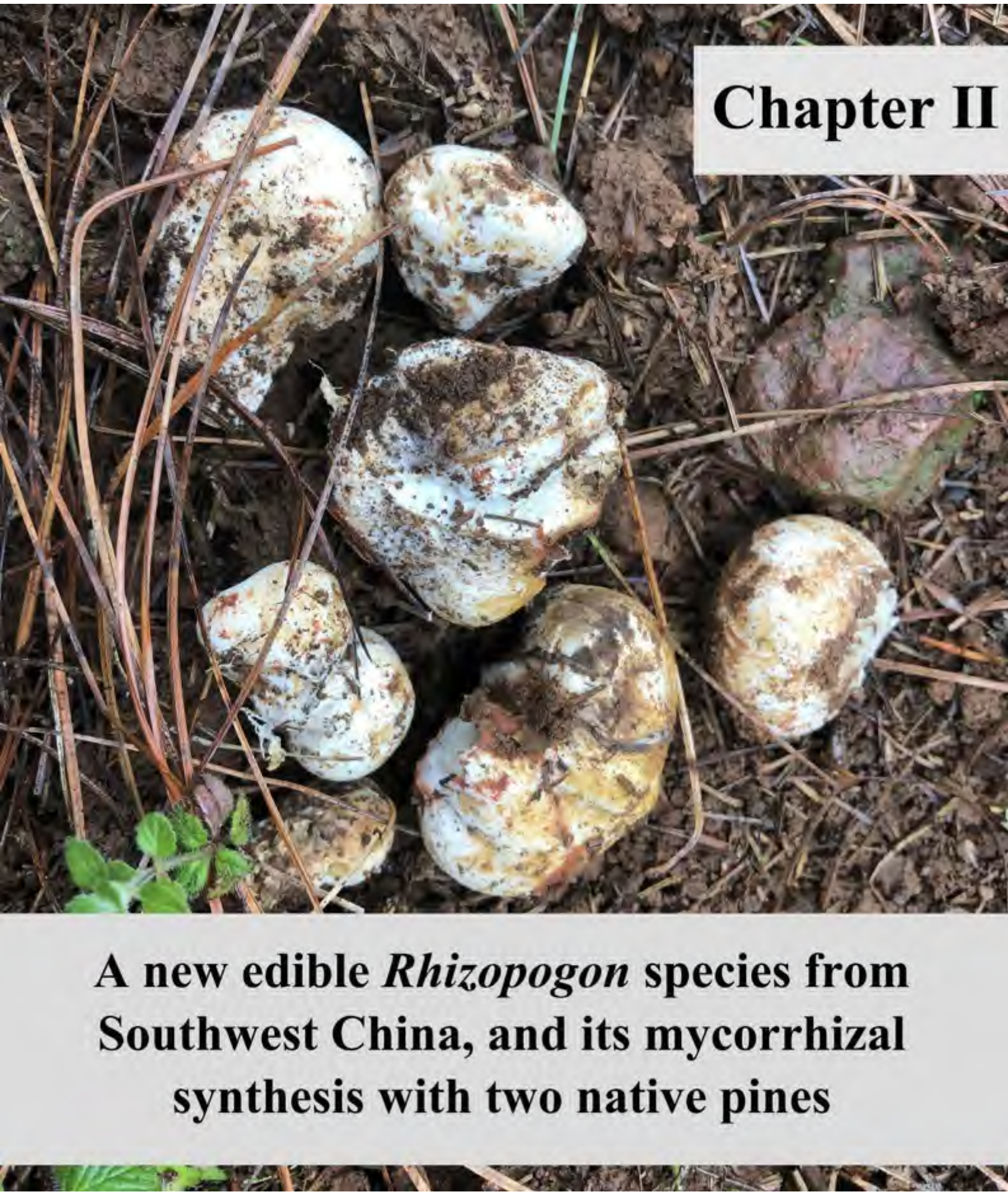

Chapter II

A new edible *Rhizopogon* species from Southwest China, and its mycorrhizal synthesis with two native pines

A new edible *Rhizopogon* species from Southwest China, and its mycorrhizal synthesis with two native pines

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Abstract

A new *Rhizopogon* species associated with *Pinus* was discovered at local wild mushroom markets and *Pinus armandii* forests from March to July in Southwest China where it is considered a delicacy. Based on morphological and molecular phylogenetic analyses, the collections were described as *Rhizopogon songmaodan* sp. nov. belonging to the subgenus *Versicolores*. The new species described here increases the current number of *Rhizopogon* species known in China to ten. *R. songmaodan* establishes ectomycorrhizal associations with *P. armandii* which was confirmed by comparing rDNA ITS sequences from basidiomata and ectomycorrhizal root tips. Mycorrhizal synthesis via spore inoculation between *R. songmaodan* and two native pine species, *Pinus armandii* and *P. yunnanensis* was successfully carried out in a greenhouse study. The ease of *R. songmaodan* inoculation onto pine species, and the high market demand of its sporocarps, could make *R. songmaodan* a good candidate for cultivation in Southwest China.

Key words Hypogeous basidiomycota; Edible fungi; Morphology; Phylogeny; Taxonomy; Ectomycorrhizae

Introduction

The genus *Rhizopogon* Fr. & Nordholm (Basidiomycota, Boletales, Rhizopogonaceae) contains approximately 212 species of hypogeous fungi (http://www.Index fungorum.org/names/names.asp). Because of the difficulty in finding hypogeous sporocarps, up to now only nine *Rhizopogon* species have been reported from China (Liu 1985; Tao and Chang 1988; Yu and Liu 2005; Dai and Yang 2008; Dai et al. 2010;

Shao et al. 2013; Li et al. 2016). Fungi in the genus *Rhizopogon* establish ectomycorrhizal associations with Pinaceae, specifically *Pinus* and *Pseudotsuga* and play an important ecological role in these forest ecosystems (Molina et al. 1999).

All *Rhizopogon* species form "truffle-like" hypogeous basidiomata (Smith et al. 1966). They are popular edible fungi at wild mushroom markets in Southwest China, especially Yunnan and Sichuan provinces, with different local names, such as "jiyaozi" (chicken's kidney), "bugujun" (cuckoo mushroom) and "songmaodan" (egg in pine needles). *R. jiyaozi* L. Li & Shu H. Li is the most popular edible species of *Rhizopogon* in Yunnan and Sichuan (Li et al. 2016) where it grows and is marketed from August to October each year. It was misidentified as *R. roseolus* in the past (Yu and Liu 2005), but described as a novel species by Li et al. (2016) from Yunnan and Sichuan provinces based on morphological and molecular phylogenetic analyses. In Kunming region, Yunnan Province, one species of *Rhizopogon* with white to tawny peridium is popular and appears in restaurants and local markets in early March. Local people call it "songmaodan" because it is egg-like and covered by pine needles. From March to July, 2019 and in May, 2020, several *R. songmaodan* fruiting bodies were purchased in local mushroom markets and collected in forests of *Pinus armandii* in Yunnan and Sichuan provinces. Morphological and molecular phylogenetic analyses showed the collections belonged to a new species that had not been scientifically described before. It was named as *Rhizopogon songmaodan* sp. nov., belonging to the subgenus *Versicolores*. Its relationships with other *Rhizopogon* species were discussed.

Different species of *Rhizopogon* including *R. flavidus, R. jiyaozi, R. luteolus R. piceus, R. roseolus, R. rubescens* and *R. sinoalbidus* have been reported to be consumed in China, Nepal, Russia, Turkey, Uruguay (Boa 2004; Li et al. 2016; Wang et al. 2020). However, the cultivation of *Rhizopogon* species worldwide has rarely been reported, with some initial success of *R. roseolus* in Japan and New Zealand (Yamada et al. 2001; Wang et al. 2002; Wang et al. 2012). In China, *Rhizopogon* is regarded as a promising species for afforestation. The mycorrhizal synthesis of *R. luteous*, *R. roseolus* and *R. superiorensis* with Chinese indigenous pines has been studied, but the mycorrhizal colonization rate obtained in those studies was rather limited (Li 2013, Shao 2013; Wu 2006). As the earliest wild edible mushroom, *R. songmaodan* is sold for US$15−25kg^{-1} early in the season, which makes it an attractive candidate for cultivation. Such cultivation would depend on the ability to mass inoculate seedlings in forest nurseries, successfully outplant inoculated seedlings into forest settings, and maintain the ectomycorrhizal symbiosis with *R. songmaodan* until sporocarps are formed. The goal of this study is to examine the feasibility of inoculating seedlings of two *Pinus* species, *Pinus armandii* and *P. yunnanensis* (the main forest and economic trees in Yunnan province) with spores of *R. songmaodan* in the greenhouse. If successful, the procedure could be scaled up to inoculate several hundreds of seedlings for a follow up field experiment as part of a commercialization process.

Material and method

Morphological examination

In 2019, three collections of *Rhizopogon songmaodan* sporocarp (HKAS 106765, 106766, 106769) were purchased from markets in Songming county and Aziying village near Kunming city, Yunnan Province. Three collections (HKAS 106767, 106768, 106770) from a native *Pinus armandii* forest at Aiziying village were obtained by raking needles or soil gently. In 2020, one collection (HKAS 107628) was obtained from *P. armandii* forest in Duge village, Huidong county, Sichuan Province. All collections were deposited in the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (HKAS). Microscopic characteristics were described from fresh specimens. Dried samples were sectioned with a razor blade by hand, mounted in 5% KOH solution, and then stained with Melzer's reagent. The sections were examined under a compound light microscope (Leica DM2500).

One collection of ectomycorrhiza was also collected from *P. armandii* forest at Aziying village in 2019. Three soil cores (10 × 12 cm with a depth of 10 cm) beneath *R. songmaodan* fruiting bodies were collected following Agerer (1991) and Gardes and Bruns (1996). From each soil sample, fine roots were separated under the stereomicroscope (Leica S8AP0) in order to select, photograph, and characterize morphotypes macroscopically (Agerer 1987–2002).

DNA extraction, PCR amplification and sequencing

DNA of basidiomata and ectomycorrhizal (ECM) tips were extracted using Aidlab™ kit (Beijing). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified from DNA extracts using the ITS1F/ITS4 primer pair (White et al. 1990; Gardes and Bruns 1993). Each 25 μL PCR mixture consisted of 2.5 μL 10 × PCR buffer (Mg^{2+}), 1.5 μL dNTPs (1 mM), 1 μL BSA (0.1%), 1 μL each primer (5 μM), 1 μL 25−fold diluted DNA extracts (obtained following the manufacturer's instruction), 0.5 μL $MgCl_2$ (25 mM), and 1.5 U Taq DNA polymerase (Takara, Takara Biotechnology, Dalian Co. Ltd, China). The amplifications were performed with the following cycling parameters: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and with a final extension at 72 °C for 10 min. Three microliters of each PCR product were run on 1% (w/v) agarose gels and stained with ethidium bromide. The PCR products were purified and sequenced forward and reverse sequences at TsingKe Biological Technology, Kunming, China, using ITS1F and ITS4 primers. Sequences were edited manually using Sequencher™ 4.1.4 (Gene Codes, USA) and queried against the NCBI public database GenBank with the BLASTn algorithm for identification. Sequences generated in this study have been deposited in GenBank (Table. 1).

Table1 Materials used for molecular analyses

Species name	Voucher	Origin	GenBank No. of ITS	References
R. songmaodan	Natural ECM of *Pinus armandii*	Yunnan, China	MN846303	This study
R. songmaodan	Synthesized ECM of *Pinus armandii*	Yunnan, China	MN846304	This study

R. songmaodan	Synthesized ECM of *Pinus armandii*	Yunnan, China	MN846305	This study
R. albidus A. H. Smith	AHS 69642	USA	AM085519	From GenBank
R. boninensis S. Ito & S. Imai	KPM-NC 26928	Japan	MK395372	From GenBank
R. buenoi Calonge & M.P. Martín	47676	Spain	AJ297263	From GenBank
R. burlinghamii A. H. Smith	JMT 17882	California, USA	AF058303	Grubisha et al. (2002)
R. colossus A. H. Smith	AHS 49480	Oregon, USA	AH071441	Grubisha et al. (2002)
R. ellenae A. H. Smith	AHS 66137	Idaho, USA	AH071445	Grubisha et al. (2002)
R. evadens A. H. Smith	AHS 65484	Oregon, USA	AF062927	Grubisha et al. (2002)
R. evadens A. H. Smith	OSC 62146	USA	KT968587	From GenBank
R. evadens A. H. Smith	GO-2009-323	Mexico	KC152182	From GenBank
R. evadens A. H. Smith	GO-2009-164	Mexico	KJ595006	From GenBank
R. hawkerae A. H. Smith	AHS 68417	Washington, USA	AH071447	Grubisha et al. (2002)
R. jiyaozi Lin Li & Shu H. Li	YAASL2335	China	KP893823	Li et al (2016)
R. jiyaozi Lin Li & Shu H. Li	YAASL2929	China	KP893830	Li et al (2016)
R. flavidus Lin Li & Shu H. Li	YAASL2957	China	KP893813	Li et al (2016)
R. flavidus Lin Li & Shu H. Li	YAASL2956	China	KP893814	Li et al (2016)
R. luteolus Fr	JMT 22516	Uppsala, Sweden	AF062936	Grubisha et al. (2002)
R. nigrescens Coker & Couch	NAMA 2015-326	USA	MH910566	From GenBank
R. ochraceorubens A. H. Smith	AHS 59643	Idaho, USA	AF062928	Grubisha et al. (2002)
R. occidentalis Zeller & Dodge	JMT 17564	Oregon, USA	AF058305	Grubisha et al. (2002)
R. ochraceisporus A. H. Smith	AHS 65963	Idaho, USA	AF071439	Grubisha et al. (2002)
R. odoratus A. H. Smith	AHS 71319	USA	AM085526	From GenBank
R. rocabrunae M.P. Martín	17067	Spain	JF908761	From GenBank
R. roseolus Corda	isolate T1PK2	Latvia	JX907816	Klavina et al. (2013)
R. sinoalbidus Lin Li & Shu H. Li	YAASL2944	China	KP893816	Li et al (2016)
R. sinoalbidus Lin Li & Shu H. Li	YAASL2949	China	KP893820	Li et al (2016)
R. songmaodan	HKAS 106767 (Holotype)	Yunnan, China	MN655983	This study
R. songmaodan	HKAS 106766	Yunnan, China	MN655982	This study
R. songmaodan	HKAS 106765	Yunnan, China	MN655981	This study
R. songmaodan	HKAS 106768	Yunnan, China	MN655984	This study

R. songmaodan	HKAS 106769	Yunnan, China	MN655986	This study
R. songmaodan	HKAS 106770	Yunnan, China	MN655985	This study
R. songmaodan	HKAS 107628	Sichuan, China	MT821479	This study
R. subaustralis A. H. Smith	MES-798	USA	MH878759	From GenBank
R. subsalmonius A. H. Smith	JMT 17218	Oregon, USA	AF062938	Grubisha et al. (2002)
R. subpurpurascens A. H. Smith	AHS 65669	Idaho, USA	AF062929	Grubisha et al. (2002)
R. succosus A. H. Smith	JMT 19321	West Virginia, USA	AF062933	Grubisha et al. (2002)
R. vulgaris (Vitt.) M. Lange	JMT19154	USA	AF062934	Grubisha et al. (2002)

Phylogenetic analyses

To infer the phylogenetic placement of *Rhizopogon songmaodan* within the genus *Rhizopogon*, ITS sequences retrieved from the *Rhizopogon* sequence dataset of Grubisha, Trappe, Molina, and Spatafora (2002), GenBank and obtained by us in this study are listed in Table 1. A total of 40 ITS sequences including 10 newly generated in this study and 30 retrieved from GenBank formed the dataset. *R. buenoi* (AJ29726) and *R. boninensis* (MK395372) were chosen as the outgroup.

Sequences were edited and assembled using Sequencher™ 4.1.4 (Gene Codes, USA). Alignment of nucleotide sequences was performed by MAFFT v.6.8 (Katoh et al. 2005). Sequences were adjusted manually using BioEdit 7.0.9. Maximum likelihood (ML) phylogenetic analyses were performed using MEGA 6.0 (Tamura et al. 2013) with 500 bootstrap replicates following the ML heuristic method nearest neighbor interchange (NNI). Bootstrap values (BS) ≥ 60% was considered significant.

Mycorrhizal synthesis with pine

Seeds of *Pinus armandii* and *P. yunnanensis* were obtained from Dounan flower market, Kunming, Yunnan. They were surface sterilized with 30 % hydrogen peroxide during 5 min for *P. yunnanensis* and 10 min for *P. armandii*, and then rinsed thoroughly in distilled water. They were germinated on a mixture of perlite and peat (1: 1, V: V), previously, autoclaved at 121 °C for one hour. Substrate for inoculation was made of vermiculite: peat: perlite (3: 2: 1, V: V) (Huang, unpublished). Spore slurry was made from dried basidiomata which were soaked in distilled water for 24 h at 4 °C and then homogenized with a blender. Seedlings were rinsed in tap water to remove remaining substrate and roots were kept moistened until inoculation. Five seedlings each of *P. armandii* and *P. yunnanensis* were inoculated with *R. songmaodan* spore slurry. Each seedling tree received 4.78×10^8 spores. Ten milliliters of spore slurry were distributed with a pipette around the third upper zone of the root system of each seedling. Control, uninoculated seedlings received 10 mL of distilled water. The inoculated seedlings were kept in 420 mL square plastic containers (“olive pots”, Daltons Ltd., New Zealand) and

grown under natural light in a greenhouse on the Kunming Institute of Botany (KIB) campus for four months (Shi 2004). All containers were arranged in groups rather than using a randomized arrangement. Each group consisted of a given pine species inoculated or not with *R. songmaodan*: 10 seedlings distributed in two rows (one inoculated, one uninoculated) of five seedlings. While this arrangement is generally not a good practice (Hurlbert 1984), it was felt that practical necessity outweighed possible spatial trends, since conditions within the area of only≈0.15 m^2 where seedlings grew were fairly homogeneous (Wang 2019). Seedlings were watered to reach field capacity with tap water three times a week.

Results

Taxonomy

Rhizopogon songmaodan R. Wang & Fu Q. Yu **sp. nov.** Fig. 1
MycoBank MB 833899
Etymology: From Chinese *songmaodan* referring to the local name of the fungus (song=pine; mao=litter; dan=egg), which describes the habitat host tree and the egg-like shape of the mushrooms.
Type: CHINA, Yunnan Province, Kunming, Aziying Village, in forest of *Pinus armandii* Franch., 25.3866 ºN, 102.8839 ºE, alt. 2,710 m, April 13, 2019, razy-236 (HKAS 106767).

Description: Basidiomata subglobose to irregular, slightly soft to rubbery, 1–5 cm × 1–4 cm globose, White when fresh, becoming reddish, then yellowish to brown when bruised. Odor not distinctive. White rhizomorphs were not abundant, mainly concentrated on the base of sporocarp. Peridium up to 230 μm, with yellowish-ocher pigments, becoming rose-pink when cut, made of hyaline interwoven hyphae (3-8 μm wide), septate and unbranched. Gleba white when immature, becoming yellowish to yellowish brown at maturity; chambers small, numerous, labyrinthine; consisted of interwoven hyaline hyphae, finally gelatinized. Basidiospores narrowly oblong to ovoid, smooth, 5.3−8.7 (−9.3) × 2.7−3.3 μm, non-amyloid in Melzer's reagent. Basidia 4-spored, 6.4−14.5 × 2.7−4 μm, cylindric to clavate. Brachybasidioles 12−22.7 × 3.3−7.3 μm, ellipsoid to clavate. Trama plate is composed of laxly interwoven hyphae, 3.3-6 μm diam., highly gelatinous-refractive, septate, thin-walled and not branched.

Chemical reactions, yellowish brown becoming dark brown immediately with 5 % KOH on dried peridium.

Habit, Habitat: on soil under trees of pine.

Distribution: Currently known from Yunnan and Sichuan provinces, Southwest China.

Additional specimen examined: CHINA, Yunnan Province: Kunming City, Aziying Village, in forest of *Pinus armandii* Franch., 25.3855 ºN, 102.8846 ºE, alt. 2,642 m, April 13, 2019, razy-237 (HKAS 106768); 25.3918 ºN, 102.8963 ºE, alt. 2,634 m, July 6, 2019, razy-252 (HKAS 106770). CHINA, Sichuan Province: Liangshan Yi Autonomous Prefecture, Huidong County, Duge Village, in forest of *Pinus armandii*

Franch., 26.72640815 ºN 102.66346161 ºE, alt. 2338 m, May, 25, 2020, rsc-315 (HKAS 107628).

Other collections: razy-234 (HKAS 106765), rsm-235 (HKAS 106766) and razy-306 (HKAS 106769) were purchased from markets, Aziying Village, Songming County, Kunming City, Yunnan Province, CHINA in 2019.

Ectomycorrhiza PA-1 (GenBank Acc. No.: MN846303) was collected from *Pinus armandii* forest at Aziying village in 2019. Ectomycorrhiza PA-2 (GenBank Acc. No.: MN846304) and PY (GenBank Acc. No.: MN846305) were synthesized in a greenhouse in KIB.

Phylogenetic analyses

ITS sequences from seven *Rhizopogon* basidiomata sampled in the market and in nearby *P. armandii* forests and those of ECM roots from *P. armandii* forests collected in the same natural forest or synthesized in greenhouse were identical (nucleotide similarity=100%). All samples of *R. songmaodan* are grouped together to form a monophyletic group. This clade was placed in the subgenus *Versicolores* (Fig. 2) and was clearly separated from other groups. The phylogenetic analysis shows that *R. songmaodan* is distinct from any of the previously known species in the genus *Rhizopogon*.

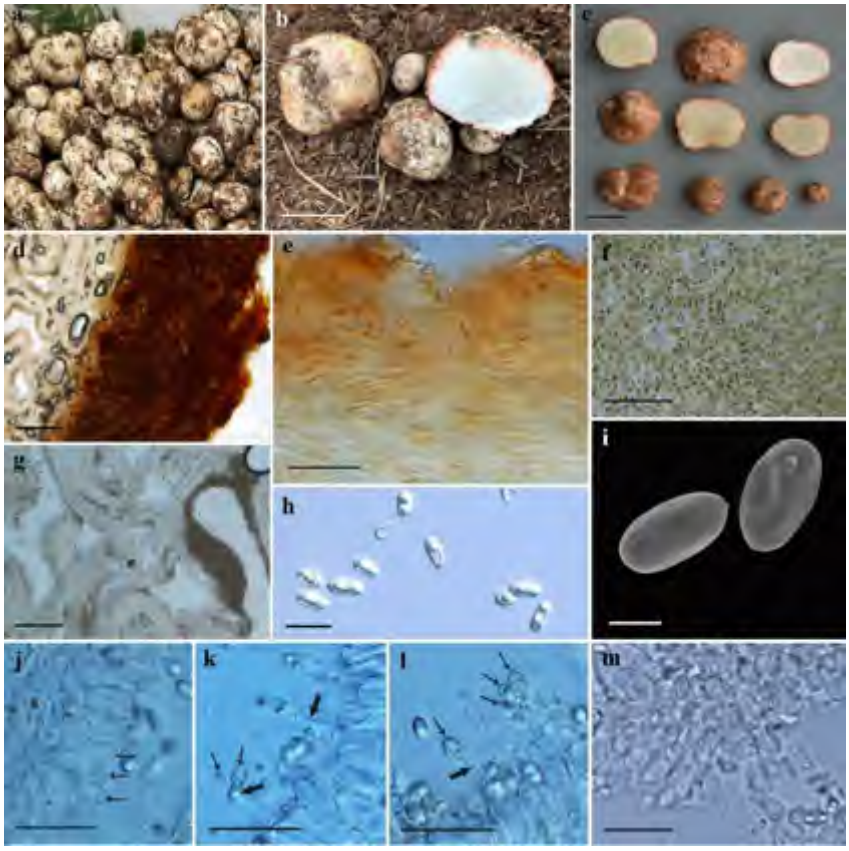

Fig. 1 *Rhizopogon songmaodan*. **a** Basidiomata sold at markets. **b, c** Basidiomata collected from *Pinus armandii* forest. **d, e** Cross section of peridium. **f** Cross section of gleba. **g** Cross section of

irregular and sinuous glebal chambers. **h, i** Basidiospores. **j** Clavate to cylindrical brachybasidioles (arrow). **k, l** Basidia (thick arrow) and spores (thin arrow). **m** Hymenium showing cystidia. Bars **b, c** 2 cm; **d, e** 50 µm; **f** 2 mm; **g** 100 µm; **h** 10 µm; **i** 3 µm and **j-m** 20 µm.

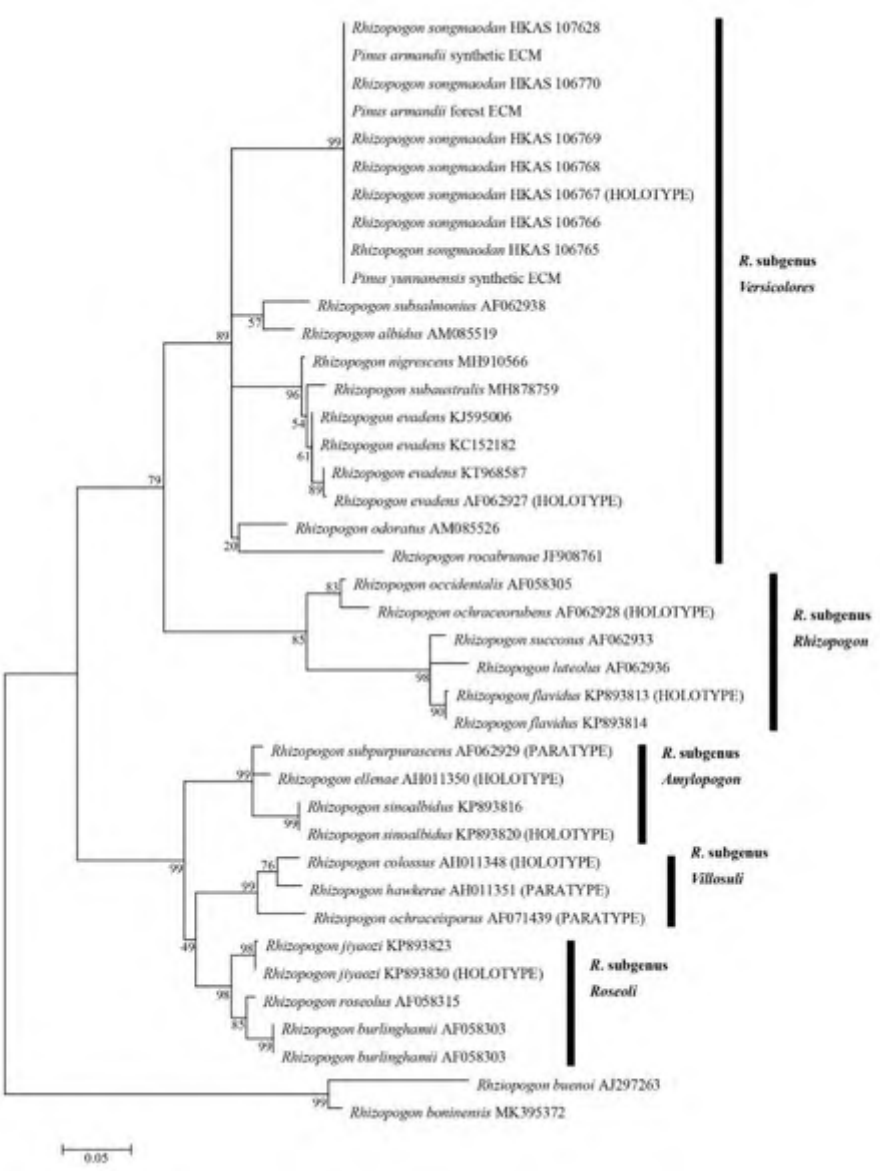

Fig. 2 Phylogenetic relationships among *Rhizopogon songmaodan* basidiomata, ectomycorrhizal root tips (ECM) sampled either in the forest or synthesized with *Pinus yunnanensis* or *P. armandii* in greenhouse and other species included in subgenera of the genus *Rhizopogon* based on their ITS rDNA sequences. The topologies of maximum likelihood (ML) is shown along with bootstrap values≥60 %

Ectomycorrhizae

The ECM root tips from *P. armandii* forest (Fig. 3) were whitish when young and brown with age. Four months after inoculation in the greenhouse (November 2019), combinations of *R. songmaodan* with *P. armandii* and those with *P. yunnanensis*

formed ectomycorrhizae on all of the five replicates of the inoculated seedlings, respectively (Fig. 3d, f). No ectomycorrhiza was detected in any of the control seedlings. The mycorrhizae were monopodial or dichotomously branched and complex, coralloid. Rhizomorphs were abundant, white, cotton-wool like with abundant external mycelium. The mantle surface was woolly with abundant emanating hyphae. The synthesized mycorrhizae were morphologically (Fig. 3b) and molecularly (Fig. 2) identical to those sampled in the natural environments.

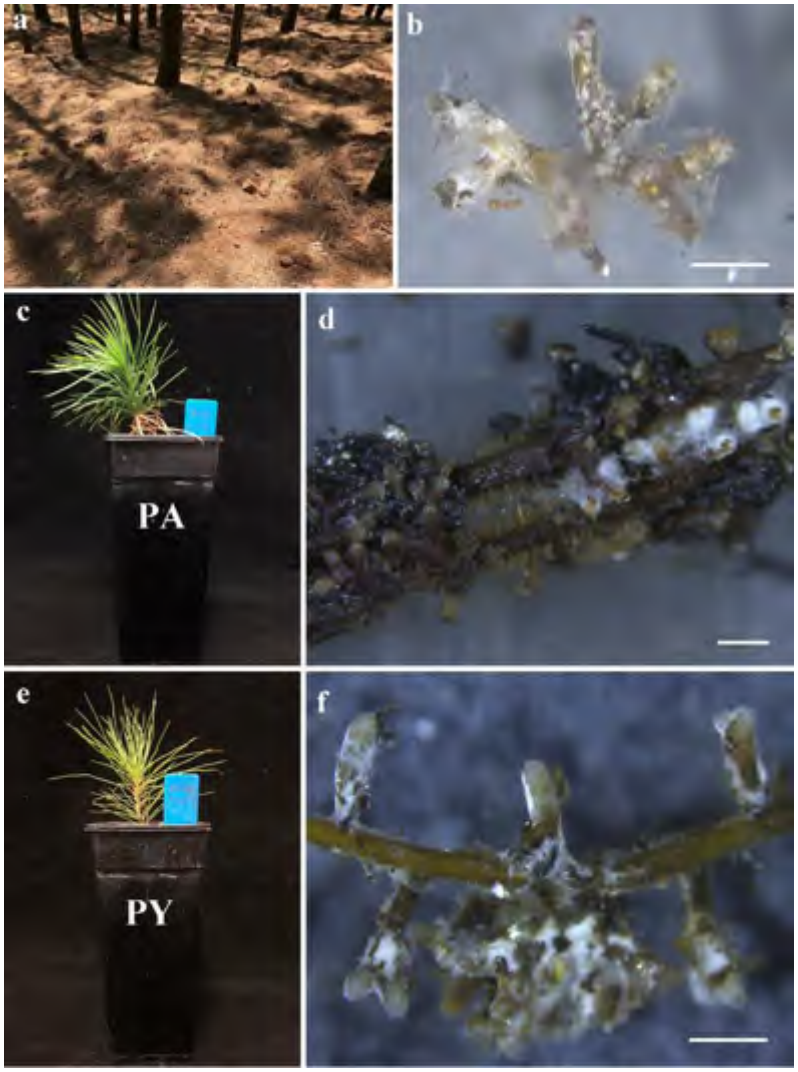

Fig. 3 a Habitat of *Rhizopogon songmaodan* in *Pinus armandii* forest. **b** Ectomycorrhizal root tip (ECM) sampled from *P. armandii* forest. **c** Four-month *P. armandii* seedling inoculated with *R. songmaodan* in pot. **d** Synthesized ECM of *P. armandii* with *R. songmaodan* in greenhouse. **e** Four-month *P. yunnanensis* seedling inoculated with *R. songmaodan* in pot. **f** Synthesized ECM of *Pinus yunnanensis* with *R. songmaodan*. Bars=1 mm

Discussion

Rhizopogon songmaodan is the tenth *Rhizopogon* species reported from China. The genus *Rhizopogon* comprises five subgenera, *Amylopogon*, *Rhizopogon*, *Roseoli*,

Versicolores, and *Villosuli* based on morphological and DNA sequence analyses (Grubisha 2001; Grubisha et al. 2002). Morphological and molecular analysis show that *R. songmaodan* belongs to the subgenus *Versicolores,* which is characterized by a peridium of interwoven hyphae and lack of yellow staining in the peridium (Grubisha et al. 2002). Morphologically *R. songmaodan* is similar to *R. evadens* in having white rhizomorphs and becoming reddish when basidiomata are cut. However, *R. songmaodan* has thinner peridium and phylogenetic analysis showed that *R. songmaodan* is grouped in a different subclade than *R. evadens* (Fig. 2). *R. jiyaozi* is the most popular edible *Rhizopogon* species in Yunnan and Sichuan Provinces. Morphologically *R. jiyaozi* and *R. songmaodan* are similar in having white and discolored basidiocarps. However, *R. jiyaozi* belongs to the subgenus *Roseoli* and is different from *R. songmaodan* by presenting yellow staining in the peridium. Additionally, *R. jiyaozi* produces basidiocarps between August and October each year, later than *R. songmaodan*.

In Japan and New Zealand, basidiomata of *R. roseolus* were produced from mycorrhized seedlings in plantations and its cultivation has the potential to be developed at a commercial scale (Wang et al, 2012). In China, the mycorrhizae of a few *Rhizopogon* species were synthesized by mycelium inoculation and showed the potential of mycorrhizal seedlings in afforestation (Shao 2013; Li 2013). In this study, two typical pine species, *Pinus armandii* and *P. yunnanensis* from central Yunnan, were inoculated with spores of *R. songmaodan*, and abundant ectomycorrhizae were produced after four months. Compared to inoculation with mycelium, the production of mycorrhizal seedlings from spore inoculum is more efficient and inexpensive, and it is recommended when producing seedlings on large scales. Considering the commercial and ecological value of *R. songmaodan*, and the inoculation efficiency (100% mycorrhizal colonization rate), it could be a good candidate for cultivation in Southwest China. However, further work on mycorrhizal synthesis and trial plantations are needed to turn the cultivation of this species into a powerful rural development tool capable of generating income for farmers in less favored rural areas.

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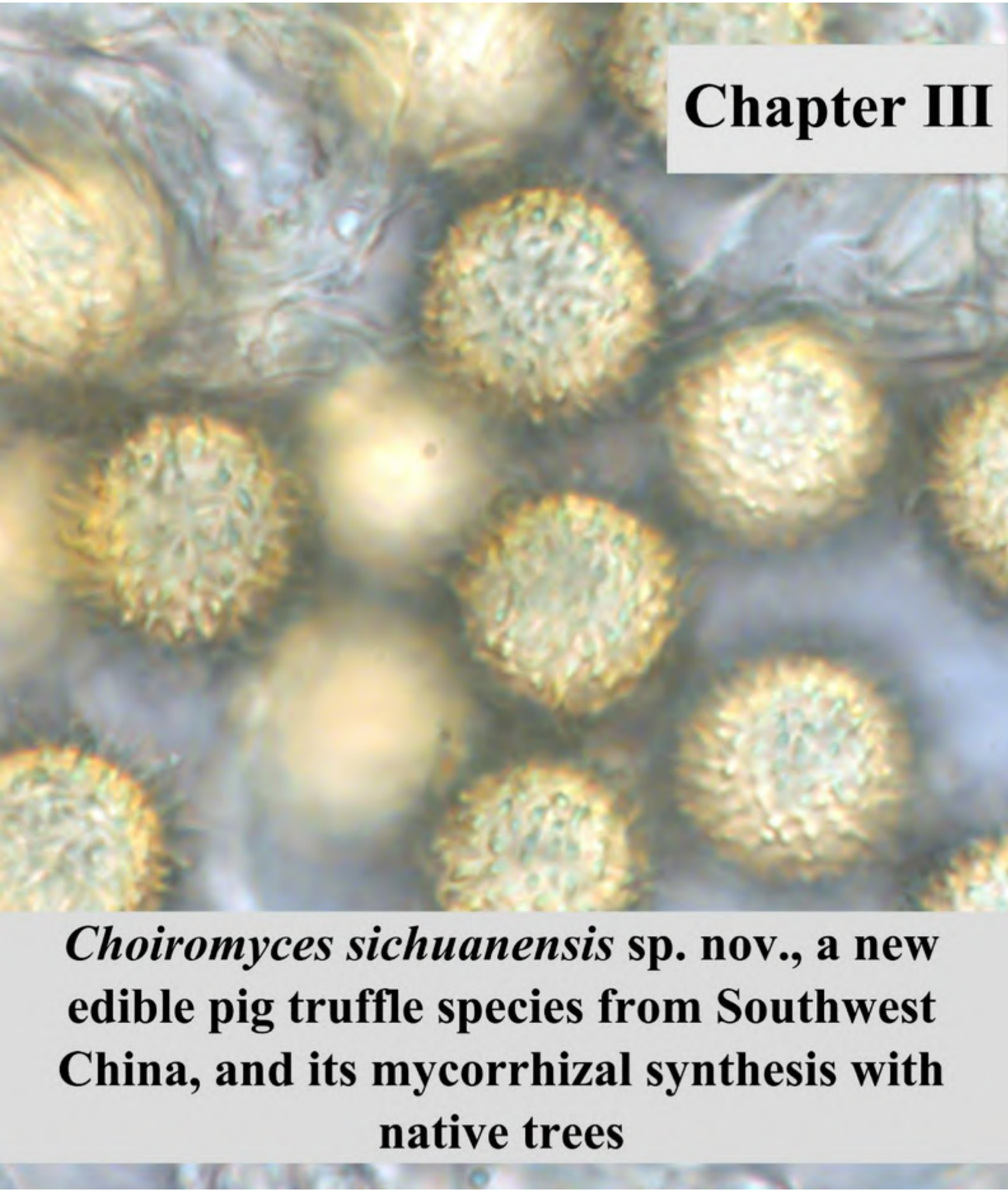

Chapter III

Choiromyces sichuanensis sp. nov., a new edible pig truffle species from Southwest China, and its mycorrhizal synthesis with native trees

Choiromyces sichuanensis sp. nov., a new edible pig truffle species from Southwest China, and its mycorrhizal synthesis with native trees

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Abstract

A new *Choiromyces* species was discovered at local wild mushroom markets in Songpan County, Sichuan, southwest China where it has been considered as a white truffle. Based on both morphological and molecular phylogenetic analyses, the collection was described as *Choiromyces sichuanensis* sp. nov.. The new described species increases the current number of *Choiromyces* species to three in China. In addition, the mycorrhizal synthesis via spore inoculation between *C. sichuanensis* and *Pinus armandii* or two *Picea* species of *Pi. likiangensis* and *Pi. crassifolia* was performed in a greenhouse. Both morphoanatomical and molecular analyses evidenced well-developed mycorrhization between *C. sichuanensis* and *P. armandii*, but not in *Picea* seedlings. Our current study provides fundamental data about the species diversity and mycorrhizal research of this genus for further studies. In addition, a successfully artificial mycorrhization between *C. sichuanensis* and selected tree species, irrespective of *Pinus* genus or other plant species, would develop an effective pathway to produce both *C. sichuanensis* mushrooms and timbers under either planted or natural forests.

Key words Ectomycorrhizae; Edible pig truffle; Hypogeous fungi; Morphology; Phylogeny

Introduction

The genus *Choiromyces* Vittad. (Tuberaceae, Pezizales, Ascomycotina) with *C. meandriformis* Vittad. as the type species, was first described in 1843 (Vittadini 1831).

At present a total of 35 *Choiromyces* species are listed in the Index Fungorum online database (https://www.Index fungo rum. org/names /names.asp). Sixteen out of them are now reclassified and transferred to other families and genera including Pezizaceae, Pyronemataceae and Rhizopogonaceae. Although widely distributed in Asia, Europe and North America (Pegler et al. 1993; Moreno et al. 2012; Chen at al. 2016; Yuan et al. 2021), the species diversity of *Choiromyces* is low and only 8 species have been categorized (*C. alveolatus, C. cerebriformis, C. cookei*, *C. ellipsosporus*, *C. helanshanensis, C. meandriformis*, *C. tetrasporus* and *C. venosus*) (Trappe 1975; Pegler et al. 1993; Kirk et al. 2008; Moreno et al. 2012; Chen at al. 2016; Yuan et al. 2021). Among them, *C. venosus* is commonly considered conspecific with *C. meandriformis* (McNeill et al. 2012; Chen et al. 2016), while *C. cerebriformis* and *C. helanshanensis* are sporadically reported from China (Chen at al. 2016; Yuan et al. 2021).

Choiromyces species has been a delicacy in Europe (Weden et al. 2009). It is hypogeous, subglobose or irregular, whitish color, fragrant ascomata and solid gleba, which is often mistaken for a species of the genus *Tuber*. For example, *C. meandriformis* was once sold as *T. magnatum* Picco. at a highly prized mushroom in the European markets (Moreno et al. 2012). Interestingly, a similar situation also occurred in 2020 in the local fresh mushroom markets of Sonpan county, Sichuan, southwest China where "white truffle-like" mushrooms were sold as white truffles at a comparatively high price of \$80−100 USD kg^{-1}. Our morphological and molecular phylogenetic analyses showed that these market collections belonging to a new species that had not been previously classified. Herein we described these collections as *Choiromyces sichuanensis* sp. nov., and its relationships with other *Choiromyces* species were discussed.

By enquiring local collectors and identifying plant leaves on the fruiting bodies, we assumed that those mushroom samples grow under a *Picea* dominated forest at altitude of ~2,000 m. *Choiromyces* species has been described to be able to establish ectomycorrhizal associations in the field with Rosaceae (*Dryas*), Salicaceae (*Salix*), Pinaceae (*Abies*, *Picea*, *Pinus*) and thus play an importantly ecological role in these forest ecosystems (Maia et al. 1996; Chen et al. 2016; Yuan et al. 2021). However, no information is available on how such an ectomycorrhization could be constructed under laboratory environments. In this study, using spores of *C. sichuanensis* sp. nov., we artificially examined their mycorrhizal symbiosis of three selected tree species including *Pinus armandii* Franch, an economically planted tree species that also widely naturally distributed at high altitude in southwest China (Zhang et al. 2018), and two other important afforestation tree species, *Picea likiangensis* (Franch.) Pritz. and *Pi. crassifolia* Kom., which have wide distributions in southwest China and northwest China, respectively. A successful mycorrhizal synthesis between *C. sichuanensis* and selected tree species provides the foremost pathway to produce both *C. sichuanensis* mushrooms and timbers under either planted or natural forests.

Materials and methods

Morphological studies

Fresh specimens were purchased from markets in Songpan County, Tibetan Qiang

Autonomous Prefecture of Ngawa, Sichuan, China. Gross morphology was described based on the fresh ascomata, and microscopic examination was later conducted using dry material according to Yang and Zhang (2003). Hand-cut sections were mounted in a 5% (w/v) KOH solution and examined under a light microscope (Leica DM2500, Leica Microsystems, Wetzlar, Germany). For the evaluation of the range of spore size, 80 ascospores were measured from the specimen. For the scanning electron microscopy (SEM) observation, spores were scraped from the dried gleba onto double-sided tape, and then directly mounted on a SEM stub, coated with gold-palladium, examined and photographed using a JSM-5600LV SEM (JEOL, Tokyo, Japan). The specimen has been deposited at Yunnan Agricultural University, Kunming, Yunnan, China.

Molecular and Phylogenetic analyses

Total DNA was extracted from pieces of dried ascomata using a modified CTAB procedure (Gardes and Bruns 1993). The ITS region of nuclear ribosomal DNA (nrDNA) was amplified using primers ITS1F and ITS4 (White et al. 1990, Gardes and Bruns 1993). Polymerase chain reactions (PCR) were performed using the following procedure: 25 μL of PCR reaction solution contained 1 μL DNA, 1 μL (5 μM) of each primer, 2.5 μL 10 × buffer (Mg^{2+} dNTP (1 mM), 0.5 μL BSA (0.1%), 0.5 μL $MgCl_2$, 1 U of Taq DNA polymerase (Takara Taq, Takara Biotechnology, Dalian, China). PCR reactions were run as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 1 min and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products were sent to Tsingke Biotechnology Co., Ltd. (Beijing, China) for sequencing.

Complete ITS sequences data for 55 taxa were obtained, including 3 new collections and other sequences down loaded from the GenBank database according to previous studies (Table 1). *Dingleya* spp. (JF300131, HM485334, JQ925627 and JQ925628) were selected as the outgroup (Fig. 1). Dataset was aligned using MAFFT v.7.0 (Katoh and Standley 2013) and then manually edited with BioEdit v.7.0.9 as needed (Hall 1999). The phylogenetic relationships of taxa were inferred using Maximum Likelihood (ML) (Stamatakis 2006) and Bayesian Inference (BI) (Ronquist and Huelsenbeck 2003).

Table 1 Sources of specimens and GenBank accession numbers for sequences used in this study

Species name	Voucher number	Origin	ITS	References
C. alveolatus	MES97	USA	HM485332	Bonito *et al*. (2010)
C. alveolatus	Trappe 17497	USA	EU669384	Unpublished
C. alveolatus	22830	Israel	AF501258	Ferdman *et al.* (2005)
C. alveolatus	HS2886	USA	HM485333	Bonito *et al*. (2010)
C. cerebriformis	YAAS 8890 Holotype	Diqing, China	MW209701	Yuan et al. (2021)
C. cerebriformis	YAAS TJ16-2	Yunnan, China	MT672014	Yuan et al. (2021)
C. cerebriformis	YAAS TJ16-1	Yunnan, China	MT672013	Yuan et al. (2021)
C. helanshanensis	YAAS L3063	China	MT672012	Yuan et al. (2021)
C. helanshanensis	YAAS L3062	China	MT672011	Yuan et al. (2021)
C. helanshanensis	YAAS L3051	China	MT672010	Yuan et al. (2021)
C. helanshanensis	HKAS 80639	Inner Mongolia, China	KP019351	Chen *et al*. (2016)

C. helanshanensis	HKAS 80647	Inner Mongolia, China	KP019350	Chen *et al*. (2016)
C. helanshanensis	HKAS80631	Inner Mongolia, China	KP019349	Chen *et al*. (2016)
C. helanshanensis	HKAS80642	Inner Mongolia, China	KP019348	Chen *et al*. (2016)
C. helanshanensis	HKAS80646	Inner Mongolia, China	KP019347	Chen *et al*. (2016)
C. helanshanensis	HKAS80634 Holotype	Inner Mongolia, China	KP019346	Chen *et al*. (2016)
C. helanshanensis	HKAS80645	Inner Mongolia, China	KP019345	Chen *et al*. (2016)
C. helanshanensis	HKAS80638	Inner Mongolia, China	KP019344	Chen *et al*. (2016)
C. helanshanensis	HKAS80636	Inner Mongolia, China	KP019343	Chen *et al*. (2016)
C. helanshanensis	HMAS83766	Heilongjiang, China	KU531609	Unpublished
C. helanshanensis	HKAS80641	Inner Mongolia, China	KU531606	Unpublished
C. magnusii	AH11894	Spain	JF300144	Moreno *et al*. (2012)
C. magnusii	AH19770	Spain	JF300143	Moreno *et al*. (2012)
C. meandriformis	K(M):171388	United Kingdom	MZ159438	Unpublished
C. meandriformis	K(M)53644	England	EU784185	Brock *et al*. (2009)
C. meandriformis	K(M)135393	England	EU784184	Brock *et al*. (2009)
C. sichuanensis	**YNAU003 Holotype**	**Sichuan, China**	**MW380902**	**This study**
C. sichuanensis	**YNAU004**	**Sichuan, China**	**OK585070**	**This study**
C. sichuanensis	**YNAU0022**	**Sichuan, China**	**OM417587**	**This study**
C. sichuanensis	**Synthesized ECM of *Pinus armandii***	**Sichuan, China**	**ON113253**	**This study**
C. venosus	AH38904	Romania	JF300147	Moreno *et al*. (2012)
C. venosus	AH38915	Italy	JF300146	Moreno *et al*. (2012)
C. venosus	AH38935	UK	JF300145	Moreno *et al*. (2012)
Choiromyces sp.	AR1291	Canada	JX630965	Timling *et al*. (2012)
Choiromyces sp.	AR1293	Canada	JX630960	Timling *et al*. (2012)
Choiromyces sp.	AR1647	Canada	JX630948	Timling *et al*. (2012)
Choiromyces sp.	HV_D3_8	USA	JX630713	Timling *et al*. (2012)
Choiromyces sp.	PP_S3_3_287_1	Canada	JX630610	Timling *et al*. (2012)
Choiromyces sp.	FB_D3_5_188_1	USA	JX630491	Timling *et al*. (2012)
Choiromyces sp.	FB_D3_1_184a_4	USA	JX630489	Timling *et al*. (2012)
Choiromyces sp.	JLF1765	USA	MH220314	Unpublished
Choiromyces sp.	58-351	USA	MH038084	Hart et al. 2018
Uncultured *Choiromyces*	NX1-4299	Ningxia, China	LC622741	Unpublished
Uncultured *Choiromyces*	NX2-148	Ningxia, China	LC623368	Unpublished
Uncultured *Choiromyces*	t24	Xinjiang, China	MF142389	Unpublished
Uncultured *Choiromyces*	NX1-4464	Ningxia, China	LC622777	Unpublished
Uncultured *Choiromyces*	NX1-4377	Ningxia, China	LC622757	Unpublished
Uncultured *Choiromyces*	NX1-4472	Ningxia, China	LC622778	Unpublished
Uncultured *Choiromyces*	NX2-137	Ningxia, China	LC623365	Unpublished
Uncultured *Choiromyces*	3232A6	USA	KF617408	Taylor et al. 2014
Uncultured *Choiromyces*	AR699	Canada	JX630936	Timling et al. (2012)
Uncultured *Choiromyces*	TK21_OTU183	USA	EF434127	Taylor et al. 2007
Dingleya sp.	JT31036	Australia	JQ925628	Bonito *et al*. (2013)
Dingleya sp.	JT31575	Australia	HM485334	Bonito *et al*. (2010)

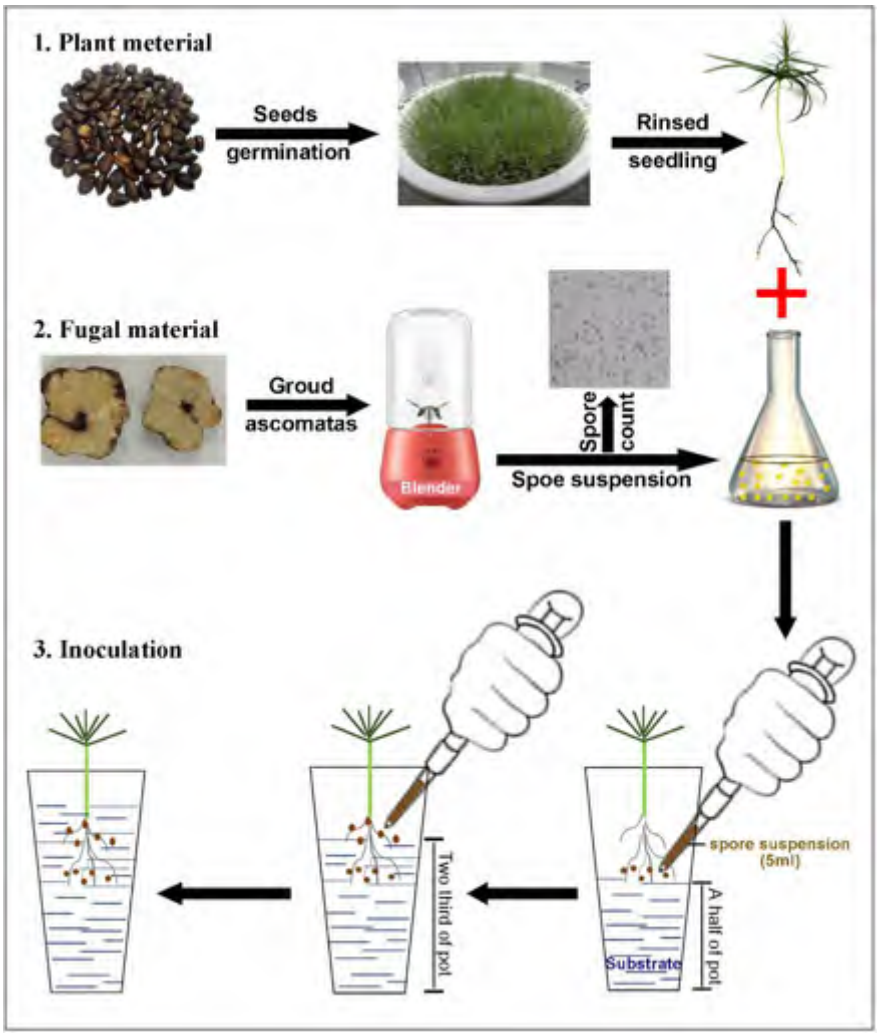
Dingleya sp.	JT27686	Australia	JQ925627	Bonito *et al*. (2013)
Dingleya sp.	AH37860	Australia	JF300131	Moreno *et al*. (2012)

Fig. 1. RAxML tree based on ITS sequences of *Choiromyces sichuanensis* and related species. Bootstrap (BS) values derived from Maximum Likelihood (ML) analysis (≥ 70%) and Posterior Probabilities (PPs) from Bayesian Inference (≥ 0.90) are shown above or beneath the branches at nodes. New sequences are in colored bold font.

Mycorrhizal synthesis with plants (Suppl. Fig. 1)

Seeds of *Pinus armandii* were purchased from the Ciba market, Kunming, Yunnan. Seeds of *Picea likiangensis* and *Pi. crassifolia* were obtained from the Southwest Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences. 30% hydrogen peroxide was used for surface-sterilization of seeds for 10 min, and then rinsed thoroughly in distilled water. They were germinated on a mixture of perlite and peat (1:1, V:V), which was autoclaved at 121 °C for one hour. Substrate for inoculation was made of vermiculite: peat: perlite (3: 2: 1, V:V:V) (Huang 2021). Dry sliced ascomatas were soaked in distilled water for 24 h at 4 °C and then homogenized with a blender. Three-month-old seedlings were rinsed with tap water to remove remaining substrate, and roots were kept moistened until inoculation. Five seedlings of

each tree species were inoculated with *C. sichuanensis* spore slurry. Each seedling received 8.0×10^6 spores. Ten milliliters of spore slurry were distributed with a pipette around the third upper zone of the root system. Non-*C. sichuanensis* inoculated control seedlings received 10 mL of distilled water. Seedlings were kept in 420 mL square plastic containers ("olive pots", Daltons Ltd., New Zealand) and grown under natural light for six months in a greenhouse on the Kunming Institute of Botany (KIB) campus. The containers with or without *C. sichuanensis* inoculation were grown in groups, rather than a randomized arrangement. Each group consisted of a given pine/*Picea* species inoculated or not with *C. sichuanensis*: 10 seedlings distributed in two rows (one inoculated, one uninoculated) of five seedlings. While this arrangement is generally not a good practice (Hurlbert 1984), it was felt that practical necessity outweighed possible spatial trends, since conditions within the area of only≈0.45 m^2 where seedlings grew were fairly homogeneous (Wang 2019). Seedlings were watered with tap water three times a week.



Supplementary Figure 1 The procedure of spore inoculation.

Morphological observations of ectomycorrhizae (ECM)

Each seedling was carefully removed from the container to identified the macro-

morphological and anatomical characters of *C. sichuanensis* ECM using a stereomicroscope (Leica S8AP0, Leica Microsytems, Wetzlar, Germany) and a compound light microscope (Leica DM2500, Leica Microsytems, Wetzlar, Germany) following the methods of Agerer (2006) and Giraud (1988). Photographs were captured using the Leica Application Suite. Cross- and longitudinal-sections were made by a freezing microtome (Leica CM3050S). The description of morphological characters of ECM focused on external aspect and color, outer mantle cells and emanating hyphae.

Molecular identification of ectomycorrhizae

For each seedling, genomic DNA of 10 pooled mycorrhizal tips displaying morphology characteristic of *C. sichuanensis* was extracted using Aidlab™ kit (Beijing). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified from DNA extracts using the ITS1F/ITS4 primer pair (White et al., 1990; Gardes and Bruns, 1993). The PCR procedure of ECM was the same as the above-mentioned PCR procedure for ascomata. Mycorrhizal sequences generated in this study have been deposited in GenBank (See Results).

Results

Taxonomy

Choiromyces sichuanensis S. P. Wan, R. Wang & F. Q. Yu, **sp. nov.** (Fig. 2).

MycoBank no.: MB843523

Type: CHINA, Sichuan Province, in humic soil under *Picea asperata* forest, 29 October 2020, wsp971 (Holotype, YNAU003, GenBank Acc. No.: ITS = MW380902).

Etymology: sichuanensis, referring to the location of the type collection.

Discription: Ascomata 2–9 cm in diam, hypogeous, subglobose or tuberiform, knobby with deep lobes, surface smooth, white, pale yellow to reddish-brown with dark gullies when fresh (Fig. 2a), then becoming brown when dry. Peridium 37–567 mm thick, not clearly differentiated, composed of light brown (outer) or hyaline (inner) hyphae, 0.7–10 μm at septa in diam (Fig. 2b, c). Gleba solid, milky white, with a slightly brown and marbled with white veins, of interwoven thin-walled hyphae. Odor strong, pleasant. Asci hyaline, clavate to saccate, 8-spored, (35.0–)50.0–88.0(–103.5) × (31.0–)34.0–65.0(–67.0) μm (N = 30). Ascospores spherical, pale yellow-brown at maturity, (16.7–)17.0–21.4(–24.6) μm in diam (N = 80), irregularly covered with curved blunt spines up to 4 μm (Fig. 2 d–f).

Habitat and distribution: The specimens were found and collected from a humus soil under a *Picea* sp. forest in October. Additional specimens examined: CHINA, Sichuan Province: *Picea* sp., 29 Oct. 2020, wsp 971-1 (YNAU0022, GenBank Acc. No.: ITS = OM417587); wsp 971-2 (YNAU004, GenBank Acc. No.: ITS = OK585070).

Remarks: *Choiromyces sichuanensis* is characterized by hypogeous, glabrous, white, pale yellow or brown, irregularly knobby, much lobed ascomata, and globose spores ornamented with conspicuous, straight or slightly curved blunt spines. This species resembles the true “white” truffle of *Tuber panzhihuanense* in appearance (large, whitish, irregularly knobby ascomata and whitish gleba with sinuous veins when young). However, *C. sichuanensis* differs from *T. panzhihuanense* and any other white

Tuber species in its globose spores ornamented with conspicuous spines. In fact, *C. sichuanensis* can easily be distinguished from other two phylogenetically related Chinese *Choiromyces* species based on the ascosporal ornamentation with long blunt spines (up to 4 μm) whereas spines' height was<3 μm in Chinese *C. helanshanensis* (2–2.5 μm), *C. cerebriformis* (2–3 μm) and southern African *C. echinulatus* (<2 μm) (Ferdman et al. 2005, Chen et al. 2016, Yuan et al. 2021). In addition, the spores of *C. meandriformis* are ornamented with free and "hollow" rods with truncated tips (with apical depressions) (Moreno et al. 2012). *Choiromyces venosus* is commonly considered conspecific with *C. meandriformis* (Chen et al. 2016). Besides, spores' ornamentation of *C. magnusii* looks pitted to verrucose-reticulated with a minutely-meshed reticulum, and spores of the American species of *C. alveolatus* are ornamented small pores, which are clearly different from those of *C. sichuanensis* (Moreno et al. 2012).

Phylogenetic analyses

The final ITS alignment included 56 sequences (specimens and ECM), which contained 665 aligned sites. The Bayesian analysis yielded similar trees as the parsimony analysis; thus, only the phylogenetic tree established from the parsimony analysis was presented (Fig. 2). All analyzed *Choiromyces* species formed a monophyletic group with 100% bootstrap support. Three *Choiromyces* specimens and one *Choiromyces*-like mycorrhiza from this study independently clustered as a well-supported subclade (PP = 1.0, BP = 100) and were clearly distinct from the known Chinese *C. helanshanensis* and *C. cerebriformis*.

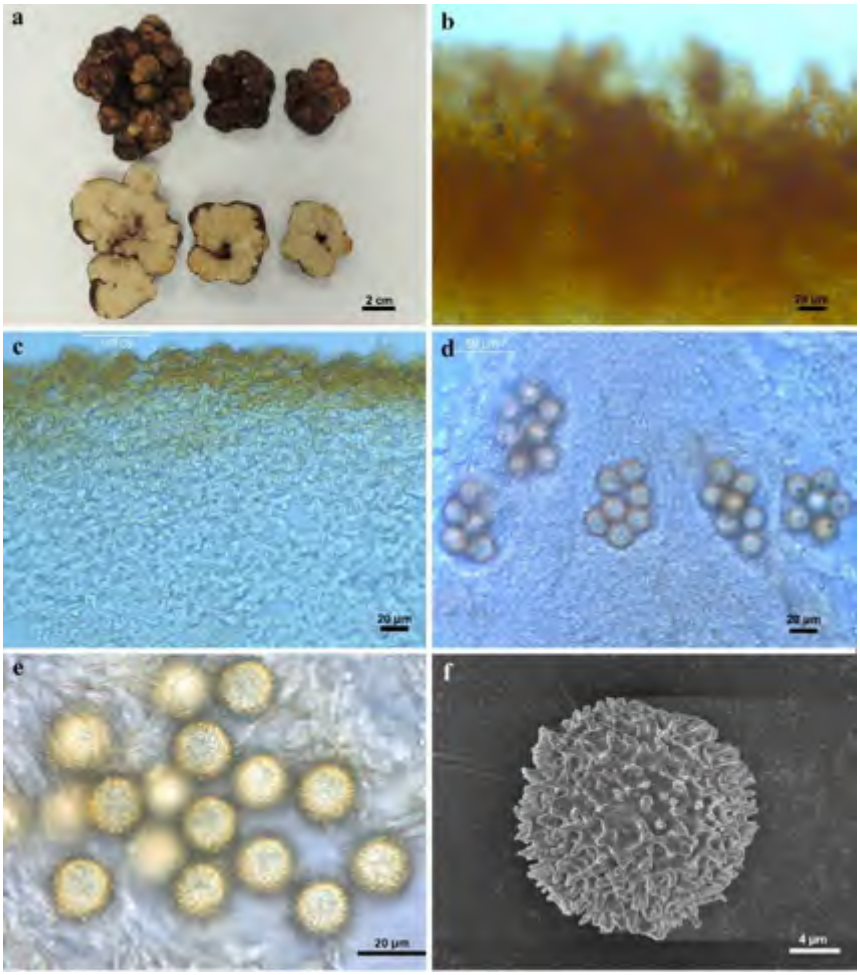


Fig. 2. *Choiromyces sichuanensis* (YNAU003, Type) **a** Ascoma. **b** Dermatocystidia. **c** Peridium section. **d, e** Light micrograph (LM) of ascospores. **f** SEM image of an ascospore.

Ectomycorrhizae

After inoculation in the greenhouse for six months (September 2021 to March 2022), ECM was formed between *C. sichuanensis* and all inoculated *P. armandii* seedlings (Fig. 3a-c). No ECM was detected in any of *Picea* or control seedlings (Fig. 4 b, d). ECM from *P. armandii* roots were monopodial or dichotomously branched and complex, coralloid, yellowish-brown-ochre. ECM systems were up to 4 mm long, 5.6 mm wide. Mantles were smooth or slightly woolly. Emanating hyphae were long, sample or branched, septate, rounded tips, up to 3.0–4.8 μm diam at the base and frequent on young ECM. Anatomical characteristics of ECM were shown in Fig. 3 d-g. Mantle 18–45 μm thick, 4–9 layers. Hartig net was palmetii and single hyphal rows. The outer mantle surface was irregular tree branch-like. ECM of other fungal species were not detected in any of the inoculated or control seedlings.

The ITS sequence of ECM of *C. sichuanensis* (mycor-YANU004, accession number ON113253) has been deposited in GenBank. The Bayesian analysis showed *C. sichuanensis*-like mycorrhiza clustered with three *Choiromyces* specimens from this study as a well-supported subclade (PP = 1.0, BP = 100) (Fig. 1).

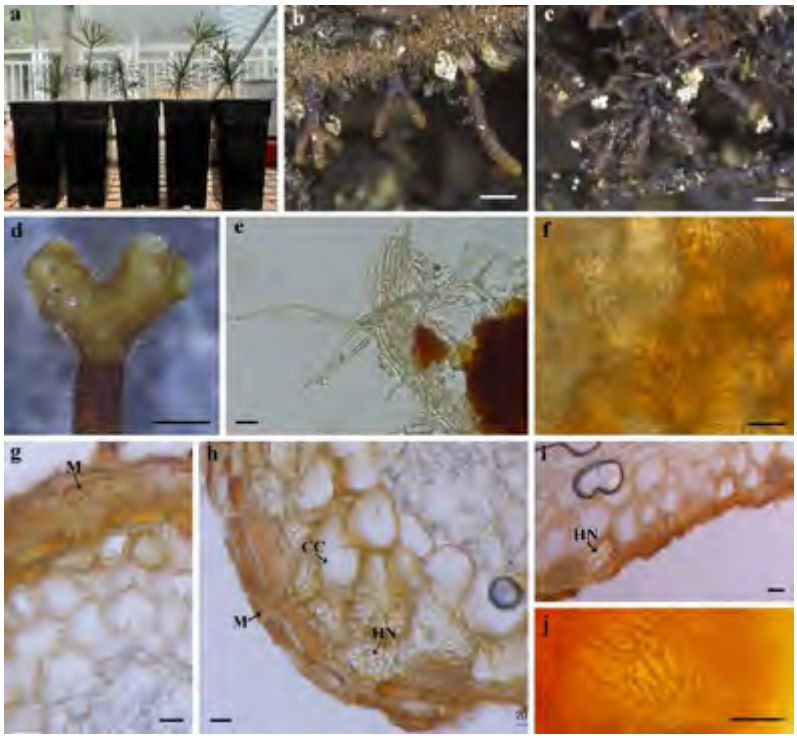


Fig. 3. a Six-month-old *Pinus armandii* seedlings inoculated with *Choiromyces sichuanensis* in pots. **b–d** Ectomycorrhizal tips after six-month inoculation (*bars* = 1 mm). **e** Septate, and see-through hyphae emanating from the outer mantle layer (*bar* = 20μm). **f** Plan view of the outer mantle (*bar* = 20μm). **g, h** Cross-section showing the mantle (M), cortical cells (CC), and Hartig net (HN) (*bar* = 20μm). **i** Tangential section showing Hartig net (HN) (*bar* = 20μm). **j** Irregular tree branch–like pattern formed by epidermoid cells (*bar* = 10μm)

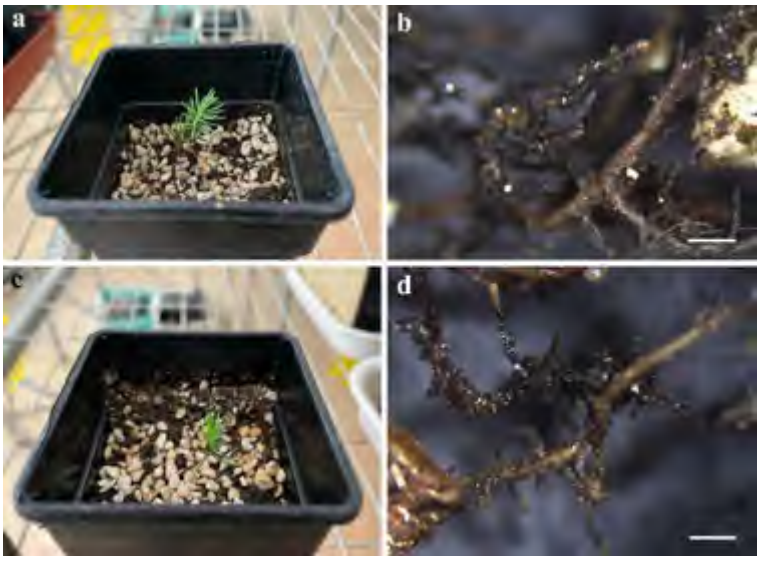


Fig. 4 a Six-month-old *Picea likiangensis* seedling inoculated with *Choiromyces sichuanensis* in pot. **b** Root tips of *Pi. likiangensis* after six-month inoculation (*bars* = 1 mm). **c** Six-month-old *Pi. crassifolia* seedling inoculated with *C. sichuanensis* in pot. **d** Root tips of *Pi. crassifolia* after six-month inoculation (*bars* = 1 mm).

Discussion

The present morphological and molecular results confirmed that at least three species of *C. cerebriformis*, *C. helanshanensis* and *C. sichuanensis* were within the genus *Choiromyces* in China. Morphologically, ascosporal ornamentation in *C. sichuanensis* had longer blunt spine than that in two other Chinese *Choiromyces* species. Phylogenetic analyses based on ITS sequences indicated that the *Choiromyces* Chinese specimens formed an independent clade (Fig. 1, Clade 1) with high Bayesian Posterior Probability and were easily distinguishable from *C. meandriformis*. Therefore, these morphological and molecular results provided strong support for C. *sichuanensis* as a new species (100% and 98% PP). In China, little is known about hypogeous fungi other than the genus *Tuber*. However, the abundance and diversity of *Tuber* species in southwest China indicate a potential biodiversity of other hypogeous fungi in this region. In this study, we confirmed an independent species within the genus *Choiromyces* and provided fundamental data about the diversity of species for future studies.

The ECM between C. *sichuanensis* and *Pinus armandii* was well developed, while no such an ECM formation was observed on *Picea* roots. Both morphological descriptions and molecular identification fully confirmed the real identities of mycorrhization between C. *sichuanensis* and *Pinus armandii* as the ITS sequences of the synthesized mycorrhizae were identical to the original ascomata used for inoculation. This result is the first report on the synthesized mycorrhizae between pine plants with a commercial pig truffle of *C. sichuanensis.* Both morphological and molecular evidence in this study could provide reliable references for future studies of other *Choiromyces* species. In contrast, we had not observed ECM formation either from *Picea likiangensis* or *Pi. crassifolia*, although *C. sichuanensis* samples might come from a *Picea* dominated forest, which has to be confirmed in nature.

Mai et al (2012) showed that *C. meandriformis* positively affected the development of *Taxus wallichiana* Zucc, indicating the ecological importance of *Choiromyces*. In this study, we had not shown an effect of *C. sichuanensis* on the development of host trees. Thus further studies are required to better understand the relationships between *C. sichuanensis* and other host trees, no matter whether they are belong to the *Pinus* genus or other ectomycorrhizal plant species. The expected results could provide management strategies for developing mushroom cultivation under planted and natural forests.

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Author contribution

RW conducted the experiment, analyzed the data, and prepared the manuscript; SPW

conducted the experiment, analyzed the data and prepared the manuscript; JY contributed to plant materials; FQY supervised the research and edited the manuscript. All authors contributed to the article and approved.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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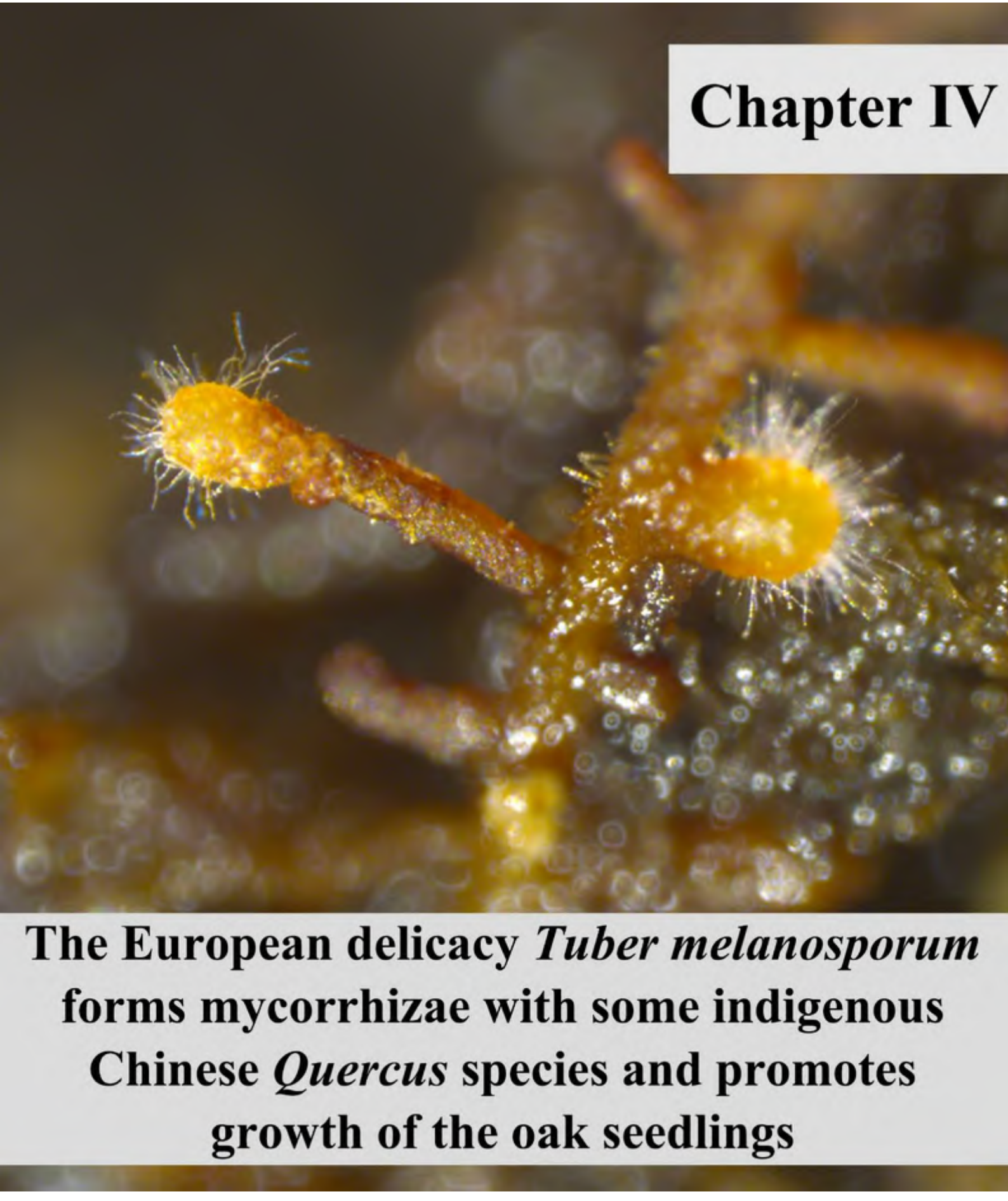

Chapter IV

The European delicacy *Tuber melanosporum* forms mycorrhizae with some indigenous Chinese *Quercus* species and promotes growth of the oak seedlings

The European delicacy *Tuber melanosporum* forms mycorrhizae with some indigenous Chinese Quercus species and promotes growth of the oak seedlings

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Abstract

We aimed to test whether *Tuber melanosporum* and native Chinese oak species could form stable mycorrhizal symbioses. Six oak species were all either inoculated or not, with spores of the Périgord black truffle in the greenhouse. Ectomycorrhizal development was monitored for up to 32 months. Seedling growth was assessed 2 years after inoculation. From 6 months after inoculation, *Tuber melanosporum* ectomycorrhizas were successfully produced on five *Quercus* species endemic to China, as shown by morphological, anatomical, and molecular analyses. *Quercus mongolica* and *Q. longispica* showed high receptivity to mycorrhization by *T. melanosporum*. The symbioses obtained with these two species and with *Quercus senescens* were stable for at least 32 months. Averaged over all three oak species, mycorrhization by *T. melanosporum* significantly enhanced canopy diameter, number of leaves, and mean leaf dimension. In spring 2019, mycorrhization by *T. melanosporum* accelerated budbreak in *Q. mongolica*. *Quercus fabrei* and *Q. variabilis* formed ectomycorrhizae up to 9 months after inoculation but seedlings died 3 months later, probably because of damage by grazing insects. *Quercus pseudosemecarpifolia* failed to form ectomycorrhizae. Results suggest that *T. melanosporum*–mycorrhized *Q. mongolica* and *Q. longispica* seedlings could be tested for ascocarp production and increased performance in the field.

Keywords *Tuber melanosporum*. *Quercus* spp.. Mycorrhizal synthesis. China. Host

plant growth

Introduction

The Périgord black truffle, *Tuber melanosporum* Vittad. (Ascomycota, Pezizales), also known as "the black diamond of French cuisine," is highly appreciated because of its special taste and aroma (Mello et al. 2006). It is probably the most economically important truffle in Europe (Donnini et al. 2013) and its commercial value is much higher than those of other black truffle species. *Tuber melanosporum* grows naturally in symbiosis with several oak species and hazelnut trees in Mediterranean conditions, in Spain, France, and Italy (Reyna and Garcia-Barreda 2008, Culleré et al. 2017). The cultivation of *T. melanosporum* has been studied from the early 1970s (Fassi and Fontana 1967). Chevalier and Grente (1978) demonstrated the commercial potential of truffle mycorrhizal seedlings. The delicacy is now cultivated worldwide by establishing truffières with seedlings, mostly European *Quercus* species or *Corylus avellana* L., mycorrhized in the nursery by *T. melanosporum* (Bonet et al. 2006, Murat 2015, Martin-Santafe et al. 2014, Reyna and Garcia-Barreda 2014, Morcillo et al. 2015). This nursery mycorrhizal technology has allowed the cultivation of the black truffle to be generalized and has enabled the development and growth of the truffle industry worldwide (Murat 2015).

Tuber melanosporum is not naturally present in China, but this country has great potential for both the cultivation and the marketing of gourmet truffles, given its wide range of climates and soils, its strong forestry sector (Wu 1980, Ni et al. 1998), its large population, and the strong interest of consumers for edible fungi cultivation since ancient times (Jia et al. 2018). So far, more than 50 *Tuber* spp. have been found in China (Chen 2007; Chen et al. 2011; Wan 2017). The first cultivated ascocarps were harvested from a *Tuber formosanum* plantation in Taiwan in 1997 (Hu et al. 2005). *Tuber indicum* Cook and Massee, *Tuber himalayense* Zhang Minter, and *Tuber sinens*e Tao and Liu, the black truffles originating from Asia, have become well-known edible fungi internationally (Murat et al. 2008). Despite their high similarity with *T. melanosporum*, these black truffles are considered by some to be inferior in taste and aroma (Paolocci et al. 1997; Zhang et al. 2005, Culleré et al. 2013). However, it is also recognized that the perception of inferior organoleptic properties was in part due to the predominance of immature ascocarps of Chinese truffles available in the markets (Liu et al. 2011). Indeed, many Chinese farmers hunt truffle early in the season without the assistance of dogs (Wang Yun, pers. comm.). To introduce the Périgord black truffle in China, mycorrhizae were synthesized successfully by inoculating *T. melanosporum* onto different Chinese indigenous trees, including *Castanopsis hystrix* (Chen 2002), *Pinus yunnanensis* (Lin et al. 2013), and *P. armandii* (Su et al. 2017). Although the persistence of introduced mycorrhizae of *T. melanosporum* has been observed in Sichuan and Guizhou in plantations of exotic and indigenous host trees, no truffle has yet been produced in those plantations which are currently 4 to 15 years old (Wang Yun, pers. comm.).

All truffles live in mycorrhizal symbiosis with the roots of suitable host plants.

Tuber-associated host trees may have economical value as timber (pine, oak), food (pecan, hazelnut), and in fuel production (poplar, oak) (Del Lungo et al. 2006; Benucci et al. 2012). They also have an important ecological value in nature as major reforestation species. More than 60 *Quercus* spp. are naturally distributed in China (Wu and Bao 2008). Over 20 deciduous species dominate forests in temperate and warm temperate zones (Wu and Bao 2008). Faber's oak *Quercus fabrei* Hance, producing edible acorns (Min et al. 2008), and Mongolian oak *Quercus mongolica* Fisch. (Shen et al. 2014) grow from the south to the north of China as excellent landscaping species (Zhou 1992; Wu and Bao 2008). More than 20 species of sclerophyllous evergreen broad-leaf oaks are distributed in northwest Yunnan, western Sichuan and Southeast Tibet (Jin and Qu 1981). Most of these species grow in regions with poor soil or adverse climates, including *Quercus franchetii* Skan. and *Quercus baronii* Skan., which are both present in dry-hot valleys, as they have the ability to tolerate heat and drought. Species in *Quercus* sect. Heterobalanus are adapted to a cold and dry environment and dominate in the Hengduan Mountain regions (Zhou et al. 2003; Meng et al. 2017).

The careful selection of tree host species is important to the successful cultivation of truffles (Palazón and Barriuso 2007). In our study, we attempted to inoculate six species of *Quercus* indigenous to China with *T. melanosporum* spore inoculum. Three of these species are deciduous, i.e., the Chinese cork oak *Quercus variabilis* Blume (Lei et al. 2013), *Q. fabrei*, and *Q. mongolica*, and three are evergreen species of *Quercus* sect. Heterobalanus, i.e., *Quercus longispica* (Hand.-Mazz.) A. Camus, *Quercus senescens* Hand.-Mazz., and *Quercus pseudosemecarpifolia* A. Camus. These species were retained because they have a well-developed root system and can adapt to different habitats and altitudes (Editorial Committee of the Flora of China of Chinese Academy of Sciences 1990; Zhou 1993). All species are common and important in different areas. *Quercus mongolica*, for example, is a timber species. Its leaves contain 12.4% of proteins and can be used to feed silkworms. Its seeds contain 47.4% of starch and can be used to make wine or fodder. In Chinese traditional medicine, its bark is used to treat diarrhea and dysentery (Editorial Committee of the Flora of China of Chinese Academy of Sciences, 1990).

We aimed to acclimatize the mycorrhizae obtained in glasshouse conditions and assess which oak species could establish a persistent mycorrhizal symbiosis with *T. melanosporum* and, therefore, be potential candidates for cultivating this high-value truffle species in China in the future. We included control, uninoculated seedlings, as a reference for mycorrhiza development and as a means to analyze the effects of mycorrhization on host plant growth and phenology.

Materials and methods

Plant origin and truffle inoculation

The origin of seeds is given in Table 1. Seeds of *Q. fabrei* and *Q. variabilis* were collected from the Kunming Botanical Garden in winter 2014. Seeds were soaked in tap water for 1 day, with an initial water temperature of 55 °C (Mao et al. 2013). Soaked

seeds were sterilized in sodium hypochlorite (2% available chlorine) for 2 h, then thoroughly rinsed in tap water. Seeds were sown in February 2015 in a sterilized mixture of perlite and vermiculite (50:50, v:v). Sterile, agargrown, sprouted seeds of *Q. mongolica*, *Q. longispica*, *Q. senescens*, and *Q. pseudosemecarpifolia* were obtained from the Southwest Germplasm Bank of Wild Species in early November 2015. They were transplanted into a sterilized mix of perlite and vermiculite (50:50, v:v). For both seeds and germinated seedlings, the perlite:vermiculite mixture was held in large plastic crates lined with a cotton mesh.

Ascocarps of *T. melanosporum* were sourced from Canterbury, New Zealand (Tewnion truffière https://blacktruffles.co.nz/), confirmed true to species by morphological identification (Riousset et al. 2001), then sliced and dried at room temperature for over 72 h (using an air dehumidifier) and stored in plastic bags at 4 °C until use except during the 12-h transport from New Zealand to China. Substrate for inoculation was made of peat (Jiffy, The Netherlands), pumice (Fuyuan, Kunming), pine bark (Mu-mu Biology, Zhejiang), and lime (Kunming) in the following proportions (9:9:2:2, v:v), respectively. This substrate previously produced mycorrhizae of *T. indicum*, *Tuber formosanum*, and *Tuber lijiangense* with several host plants such as *Populus yunnanensis* and *Quercus wutaishanica* (Huang, unpublished). It was also successfully used to produce over 4000 mycorrhizal seedlings including combinations such as *T. indicum* × *Quercus fabrei* or *Quercus variabilis* (Yu et al. unpublished). Substrate was sterilized by autoclaving in 10-L bags for 1.5 h at 121 °C. The final pH of the substrate was adjusted to 8.0 by adding CaCO3 and MgCO3, 0.6 g and 0.3 g/L, respectively. Dried ascocarps were soaked in non-sterile distilled water for 24 h at 4 °C, then blended for 2– 3 min with distilled water (1 g dry truffle/10 mL). Each seedling was rinsed in tap water to remove remaining substrate and roots were kept moistened until inoculation. Ten milliliters of spore slurry (containing the equivalent of 5 g of fresh truffle or 15×10^7 spores per seedling) was distributed with a 5-mL pipette around the third upper zone of the root system of each seedling. Control, uninoculated seedlings received 10 mL of distilled water. The inoculation of seedlings took place in 688-mL square pots (13.2 × 6.4 × 9.1 cm, S9, Xinguanghe horticulture, Zhejiang) using the substrate described above.

Two separate inoculation series were made. Five seedlings each of *Q. fabrei* and *Q. variabilis* were inoculated with *T. melanosporum* using this method on 3 December 2015. In addition, five seedlings each of *Q. mongolica*, *Q. longispica*, *Q. senescens*, and *Q. pseudosemecarpifolia* were inoculated with *T. melanosporum* on 18 July 2016 using the same method. All seedlings were 10 months old at inoculation.

Seedlings were grown at the Kunming Institute of Botany (KIB) under natural light (e.g., 169 μmol^{-2} s^{-1} inside in June) in a 28.5-m^2 recent glasshouse, fitted with roof panels that can be opened and with an extractor fan cooling system with a maximal midday temperature of 30 °C in summer months. Seedlings were watered to reach field capacity with tap water three times a week. Seedlings were maintained for 6 to 32 months after inoculation (some seedlings died during the course of the study). Pots were arranged in groups rather than using a randomized arrangement. Each group consisted of a given oak species inoculated or not with *T. melanosporum*: 10 seedlings distributed

in two rows (one inoculated, one uninoculated) of five seedlings. While this arrangement is generally not a good practice (Hurlbert 1984), it was felt that practical necessity outweighed possible spatial trends, since conditions within the area of only ≈ 0.6 m2 where seedlings grew were fairly homogeneous. Three months after inoculation, 2.5 mL of slow-release Osmocote® fertilizer (No 5, Lily's gardening, Shanghai, N:P:K/14:13:13) was added per container. Nine months following the inoculation of *Q. fabrei* and *Q. variabilis* seedlings, grazing insects were found and Shiqi insecticide solution was applied to all seedlings of all species: 30 mL three times at ≈ 6-week intervals (active substance 50% acetamiprid, Guoguang, Sichuan province; 1 g per 2.5 L as per the manufacturer's instructions). Therefore, seedlings inoculated in July 2016 received the insecticide treatment 7 months earlier than the other two deciduous species. In addition, 15 sticky fly traps (25 × 15 cm Jida, Shanghai) were placed among the seedlings at the same time.

Table 1 Geographic origin, altitude and date of seed collection

Quercus (*Q.*) species	Origin	Altitude (m)	Seed collection date
Q. fabrei	Kunming Botanical Garden, Kunming, Yunnan	1956	October 2014
Q. variabilis	Kunming Botanical Garden, Kunming, Yunnan	1956	October 2014
Q. mongolica	Beijing Botanical Garden, Beijing	42	September 2015
Q. longispica	Tiger Leaping Gorge and Haba Snow Mountain, Shangri-La, Yunnan	1900-3000	October 2015
Q. senescens	Da Shao village, Kunming, Yunnan	1943	October 2015
Q. pseudosemecarpifolia	Da Shao village, Kunming, Yunnan	1943	October 2015

Morphological observations of ectomycorrhizae

Six, 9, 12, and 24 months after inoculation, all seedlings of each oak species were monitored for mycorrhiza formation. Both inoculated and uninoculated seedlings of *Q. mongolica*, *Q. longispica*, and *Q. senescens* were checked again for mycorrhization 32 months after inoculation. Each seedling was carefully removed from the container and its root system analyzed minimizing the physical disturbance to the mycorrhizae: only the superficial substrate that was not bound to the mycorrhizal roots could fall during that process. Seedlings were carefully repotted in the same containers straight after the examination. The macro-morphological and anatomical characters of *T. melanosporum* ectomycorrhizae were identified using a stereomicroscope (Leica S8AP0) and compound light microscope (Leica DM2500) following the methods of Agerer (2006) and Giraud (1988). Photographs were captured using the Leica Application Suite. Cross- and longitudinal sections were made by a freezing microtome (Leica CM3050S). The identification of *T. melanosporum* ectomycorrhizae based on morphological characters is reliable when all the key parameters are present, i.e., correct external aspect and color, puzzlelike outer mantle cells, and emanating hyphae being light brown in color, loosely arranged and branching at right angles (Giraud 1988; Guerin-Laguette et al. 2013). However, since ectomycorrhizae of *T. indicum*, widely present in Yunnan, share characteristics similar to those of *T. melanosporum*, a DNA sequence analysis

was required to confirm that the mycorrhizae observed belong to *T. melanosporum*.

Molecular identification of ectomycorrhizae

For each seedling, genomic DNA of three to 10 pooled mycorrhizal tips displaying morphology characteristic of *T. melanosporum* was extracted using Aidlab™ kit (Beijing). The internal transcribed spacer (ITS) region of the ribosomal DNA was amplified from DNA extracts using the ITS1F/ITS4 primer pair (White et al., 1990; Gardes and Bruns, 1993). Each 25-µL PCR mixture consisted of 2.5 µL 10× PCR buffer (Mg^{2+}), 1.5 µL dNTPs (1 mM), 1 µL BSA (0.1%), 1 µL each primer (5 µM), 1 µL 25-fold diluted DNA extracts (obtained following the manufacturer's instruction), 0.5 µL $MgCl^2$ (25 mM), and 1.5 U Taq DNA polymerase (Takara, Takara Biotechnology, Dalian Co. Ltd, China). The amplifications were performed with the following cycling parameters: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and with a final extension at 72 °C for 10 min. Three microliters of each PCR product were run on 1% (w/v) agarose gels and stained with ethidium bromide. The PCR products were purified and sequenced both ways at TsingKe Biological Technology, Kunming, China, using ITS1F and ITS4 primers. Sequences were edited manually using Sequencher™ 4.1.4 (Gene Codes, USA) and queried against the NCBI public database GenBank with the BLASTn algorithm for identification. Mycorrhiza sequences generated in this study have been deposited in GenBank (see the "Results" section).

In addition, mycorrhiza DNA extracts of *Q. mongolica*, *Q. longispica*, and *Q. senescens* were amplified using the multiplex primers developed by Paolocci et al. (1999).

Assessment of host plant growth and statistical analyses

Two years after inoculation, the effects of mycorrhization on plant growth were photographed for *Q. longispica*, *Q. mongolica*, and *Q. senescens*. The numbers of leaves attached to the stem, including any dead ones at the bottom of the crown, were counted for each seedling. The following measurements were made on each seedling using a digital caliper (Shengong, Shanghai): (1) the largest diameter of the canopy, i.e., the maximal distance between the apices of opposite leaves and (2) the length and widest diameter of the limb of each leaf, including dead leaves, to estimate the leaf area, assuming that limbs were approximately of an ellipsoid shape. Three variables were analyzed: canopy diameter, numbers of leaves (both one measurement per pot), and mean leaf dimension (leaf length+leaf width)/2, with one measurement per leaf for each pot. For each variable, tree species and mycorrhizal treatment (*T. melanosporum*–inoculated or control) plus their interaction were assessed. For leaf dimension, approximate area, (π × length × width)/4 and its square root were explored, but the results were very similar to those for mean leaf dimension. Canopy diameter was analyzed by analysis of variance, and numbers of leaves with a Poisson generalized linear model with a logarithmic link (McCullagh and Nelder 1989). Leaf dimension

was analyzed using a mixed model analysis, fitted with REML (Payne et al. 2017b), with pot as a random effect. All analyses were carried out with Genstat (Payne et al. 2017a). In spring 2019, 32 months after inoculation, the growth of the three oak species was photographed again.

Results

Mycorrhization, morphological, and anatomical traits of ectomycorrhizae

3 December 2015 inoculation

Six months after inoculation (June 2016), combinations of *T. melanosporum* × *Q. variabilis* and *Q. fabrei* formed ectomycorrhizae on two and one seedlings out of the five replicate inoculated seedlings, respectively (Suppl. Fig. 1 and Table 2). Seven fresh, single, mycorrhiza tips were found on each of the two *Q. variabilis* seedlings (Suppl. Fig. 1b). Mycorrhizae, with branches, were frequently found on the *Q. fabrei* seedling (Suppl. Fig. 1e-f). Mycorrhizal systems were monopodial with lateral, mostly simple ramifications (Suppl. Fig. 1b, e-f). Simple ectomycorrhizae were straight and club-shaped, yellowish-brown-ochre. The mantle surface was smooth to hairy or slightly woolly, with abundant emanating hyphae (Suppl. Fig. 1c, e-f, h). Emanating hyphae were long, wandering, hyaline, often branched, finely septate, never tapered at the apex, and patchily distributed. The outer mantle surface was jigsaw puzzle–like. Ectomycorrhizae of other fungal species were not detected in any of the inoculated or control seedlings.

Nine months after inoculation (September 2016), grazing damage was observed both on mycorrhizae and roots (Suppl. Fig. 1j-k). Symptoms such as yellow-burnt leaves and a weak root system (Suppl. Fig. 1i) were apparent on inoculated and control trees. Four *Q. fabrei* (three inoculated and one control) and six *Q. variabilis* (four inoculated and two control) seedlings died; all had weak root systems. Several live fungus gnat larvae were found in the substrate (Suppl. Fig. 1l-m) and many flies, i.e., the corresponding adult forms, were caught on the sticky traps in the greenhouse. Only one mycorrhized seedling had survived for each oak species (Table 2). A few fresh *T. melanosporum* mycorrhizae were still present on these surviving seedlings (data not shown).

Twelve months after inoculation (December 2016), all trees died displaying fragile root systems (e.g., breaking easily) and dead mycorrhizae (Table 2).

Supplementary Figure 1 a Seedlings of *Quercus variabilis* (*bar*=3.8 cm). **b-c** Ectomycorrhizae of *Tuber melanosporum* formed with *Q. variabilis*. **b** Young mycorrhizal tips (*bar*=0.5 mm). **c** *T. melanosporum*-characteristic epidermoid cells structured in an irregular jigsaw puzzle-like pattern with thin-walled, septate and see-through emanating hyphae (*bar*=20 μm). **d** Seedlings of *Quercus fabrei* (*bar*= 1.8 cm). **e-h** Ectomycorrhizae of *T. melanosporum* formed with *Q. fabrei*. **e-f** Ectomycorrhizae with abundant emanating hyphae (f, arrow) (*bars*=0.5 mm). **g** Epidermoid cells structured in an irregular jigsaw puzzle-like pattern (*bar*=20 μm). **h** Branched, septated, thin-walled and see-through emanating hyphae (*bar*=20 μm). **i-m** Damage caused by fungus gnat larvae. **i** Weak *Q. fabrei* seedling with withered leaves and fragile roots, 9 months after inoculation (*bar*= 3 cm). **j** Damaged root showing vascular tissue (arrow) (*bar*= 0.5 mm). **k** Damaged ectomycorrhizae (*bar*=0.5 mm) **l-m** Fungus gnat larvae (*bars*=0.5 mm).

Table 2 Number of surviving (Surv.) and mycorrhizal (Myc.) *Quercus* seedlings at four assessments (months) following inoculation. Control means uninoculated seedlings.

Trees	6 months		9 months		12 months		24 months	
	Surv.	Myc.	Surv.	Myc.	Surv.	Myc.	Surv.	Myc.
December 2015 inoculation								
QF	5/5	2/5	2/5	1/2	0/5	N/A	N/A	N/A
QF-control	5/5	0/5	4/5	0/4	0/5	N/A	N/A	N/A
QV	5/5	2/5	1/5	1/1	0/5	N/A	N/A	N/A
QV-control	5/5	0/5	3/5	0/3	0/5	N/A	N/A	N/A
July 2016 inoculation								
QL	5/5	5/5	5/5	5/5	3/5	3/3	3/5	3/3

QL-control	5/5	0/5	5/5	0/5	4/5	0/4	4/5	0/4
QM	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
QM-control	5/5	0/5	5/5	0/5	5/5	0/5	5/5	0/5
QS	5/5	1/5	5/5	2/5	2/5	2/2	2/5	2/2
QS-control	5/5	0/5	5/5	0/5	3/5	0/3	3/5	0/3
QP	4/5	0/4	2/5	0/2	2/5	0/2	2/5	0/2
QP-control	4/5	0/4	3/5	0/3	2/5	0/2	2/5	0/2

QF, *Quercus fabrei*; QV, *Quercus variabilis*; QM, *Quercus mongolica*; QL, *Quercus longispica*; QP, *Quercus pseudosemecarpifolia*; QS, *Quercus senescens*; N/A, not applicable

18 July 2016 inoculation

Six months after inoculation (January 2017), combinations of *T. melanosporum* × *Q. longispica* or *Q. mongolica* formed ectomycorrhizae on all replicate inoculated seedlings (Table 2 and Figs. 1 and 2), while mycorrhizae of *T. melanosporum* × *Q. senescens* were found on only one inoculated seedling (Table 2, Suppl. Fig. 2). Mycorrhizal systems and morphological characteristics formed by *T. melanosporum* and these three oak species were similar to those observed with *Q. variabilis* and *Q. fabrei*. Cross- and longitudinal sections of *T. melanosporum* mycorrhizae with *Q. longispica*, *Q. mongolica*, or *Q. senescens* displayed a characteristic distribution of the Hartig net (Figs. 1f, g, i and 2g, h, j, k and Suppl. Fig. 2e-g, j-k). One inoculated and one control *Q. pseudosemecarpifolia* seedlings had died. Ectomycorrhizae were absent from all inoculated *Q. pseudosemecarpifolia* seedlings. For all oak species, no ectomycorrhizae of other fungal species were detected in any of the inoculated or control seedlings. A live fungus gnat larva was found on one inoculated tree of *Q. mongolica*.

Nine months after inoculation (April 2017), the same results were observed except that a further *Q. senescens* seedling showed *T. melanosporum* mycorrhizae (Table 2) and a further three *Q. pseudosemecarpifolia* seedlings (two inoculated and one control) had died. Dead trees had withered leaves and fragile roots.

Twelve months after inoculation (July 2017), five *Q. senescens* seedlings had died (three inoculated and two control) while one more control *Q. pseudosemecarpifolia* seedling also had died. Twenty-four months after inoculation, fresh and abundant *T. melanosporum* mycorrhizae were still present on all replicate seedlings of *Q. mongolica*, *Q. longispica*, and on two seedlings of *Q. senescens* (data not shown). Finally, 32 months after inoculation, the mycorrhization persisted on all replicate seedlings of these three species and well-developed, branched mycorrhizae forming large clusters were observed on both *Q. longispica* and *Q. mongolica* (Figs. 1m, n and 2m, n). No contaminant mycorrhizal species were detected except for traces of Hebeloma-like mycorrhizae on one inoculated seedling of *Q. longispica* and the presence of *Sphaerosporella brunnea* mycorrhizae on two uninoculated seedlings of *Q. senescens* (data not shown).

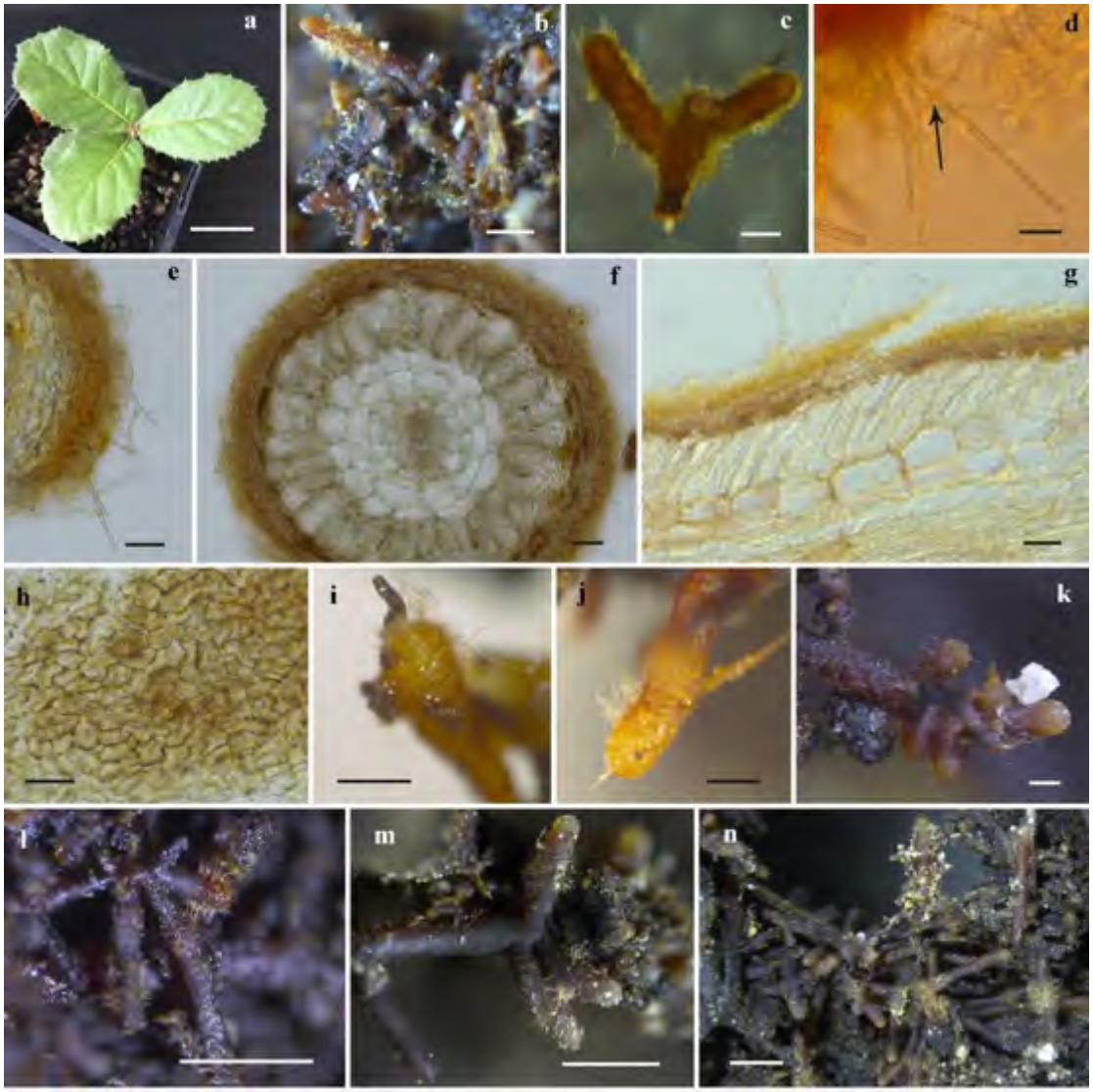

Fig. 1 Seedling of *Quercus longispica* (*bar*= 3 cm). **b-l** Macro-morphological and anatomical characters of artificially synthesized ectomycorrhizae of *Tuber melanosporum* on *Q. longispica*. **b-c** Ectomycorrhizae with abundant emanating hyphae 6 months after inoculation (*bars*=0.5 and 0.2 mm, respectively). **d** Branched (arrow), septate and see-through hyphae emanating from the outer mantle layer, whole tip mounted in water (*bar*= 20 µm). **e-f** Cross-mycorrhiza sections showing branched emanating hyphae (e) and a well-developed mantle and Hartig net around elongated cortical cells (f) (*bars*= 20 µm). **g** Longitudinal mycorrhiza section showing mantle, elongated cortical cells, Hartig net and emanating hyphae (*bar*=20 µm) **h** Irregular jigsaw puzzle-like pattern formed by epidermoid cells (*bar*=20 µm). **i-j** Ectomycorrhizae 12 months after inoculation with emanating hyphae (*bars*=0.2 mm). **k-l** Ectomycorrhizae 24 months after inoculation with emanating hyphae (*bars*=0.2 and 1 mm, respectively). **m-n** Ectomycorrhizae 32 months after inoculation including clusters (**n**) (*bars* = 1 mm).

Molecular identification of mycorrhizae

ITS sequences obtained from all *T. melanosporum*–like mycorrhizae obtained with each oak species confirmed their identity, showing maximum 100% identity with a *T.*

melanosporum sequence (KM659870.1) from GenBank. The sequences of *T. melanosporum* ectomycorrhizae obtained in this study with *Q. mongolica* (mwr-3, accession number MF590056), *Q. longispica* (mwr-1, MF590054), and *Q. senescens* (mwr-2, MF590055) were deposited in GenBank. The mycorrhiza DNA extracts of *Q. mongolica*, *Q. longispica*, and *Q. senescens* produced a *T. melanosporum*–specific 440 bp fragment only (data not shown).

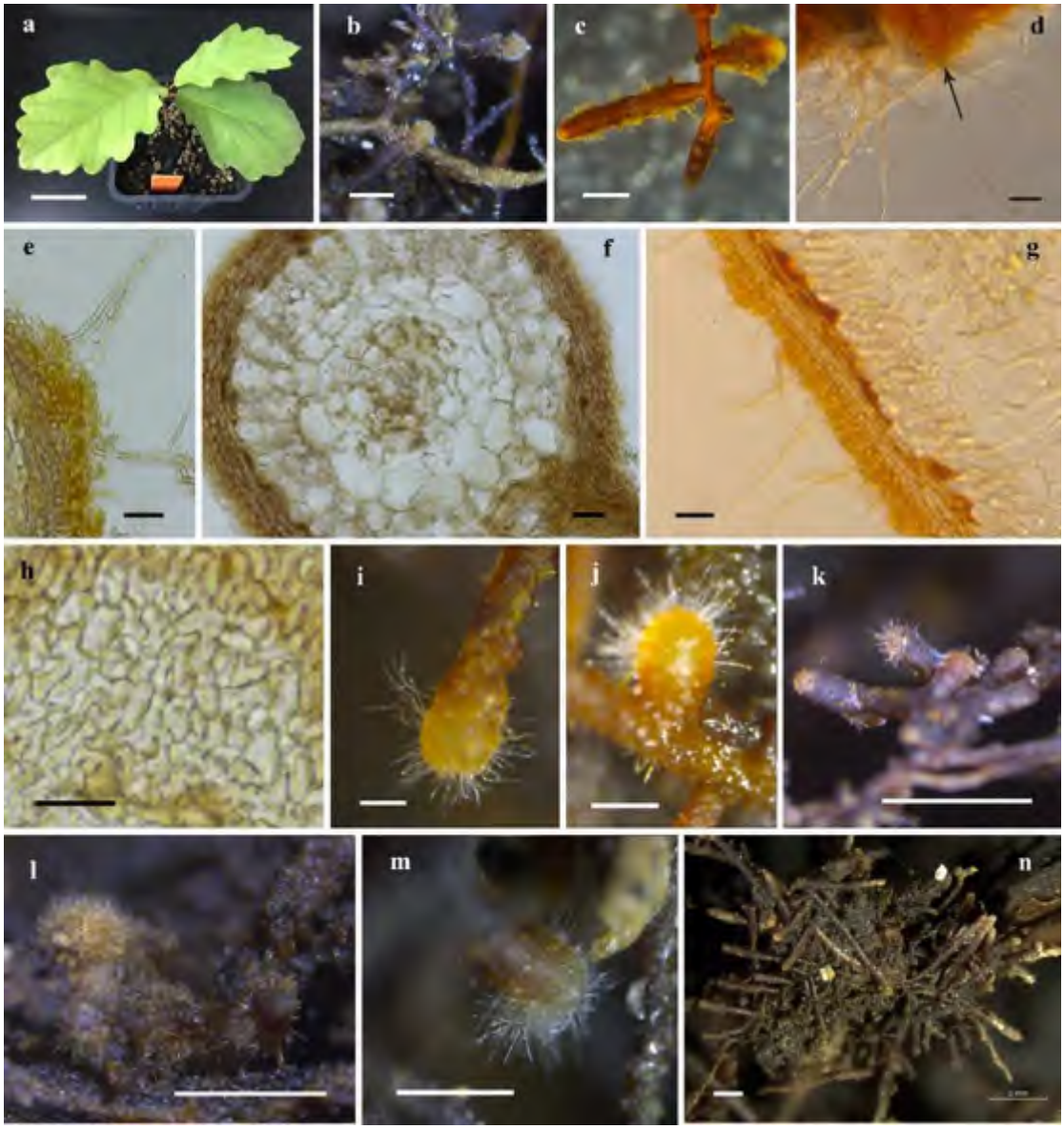

Fig. 2 a Seedling of *Quercus mongolica* (*bar*= 3 cm). **b-l** Macro-morphological and anatomical characters of artificially synthesized ectomycorrhizae of *Tuber melanosporum* on *Q. mongolica*. **b-c** Ectomycorrhizae with abundant emanating hyphae 6 months after inoculation (*bars*=0.5 mm). **d** Branched (arrow), thin-walled, septate and see-through emanating hyphae, mounted tip (*bars*=20 μm). **e-f** Cross-ectomycorrhiza sections showing emanating hyphae (e) and a well-developed mantle (e-f) and Hartig net around elongated cortical cells (f) (*bars*= 20 μm). **g** Longitudinal ectomycorrhizal section showing emanating hyphae, mantle and Hartig net around elongated cortical cells (*bar*=20 μm). **h** Jigsaw puzzle of the inner mantle surface (*bar*=20 μm). **i-j**

Ectomycorrhizae 12 months after inoculation with emanating hyphae (*bars*=0.2 mm). **k-l** Ectomycorrhizae 24 months after inoculation with emanating hyphae (*bars*=1 mm). **m-n** Ectomycorrhizae 32 months after inoculation including clusters (**n**) (bars = 0.5 and 1 mm, respectively).

Supplementary Figure 2 a Seedling of *Quercus senescens* (*bar*= 1.2 cm). **b-l** Macro-morphological and anatomical characters of artificially synthesized ectomycorrhizae of *Tuber melanosporum* on *Q. senescens.* **b-c** Ectomycorrhizae with abundant emanating hyphae 6 months after inoculation (*bars*=0.5 mm). **d** Branched (arrow), thin-walled, septate and see-through emanating hyphae, mounted tip (*bars*=20 µm). **e-f** Cross-ectomycorrhiza sections showing mantle (e-f), emanating hyphae (e) and Hartig net around elongated cortical cells (f) (*bars*= 20 µm). **g** Longitudinal ectomycorrhiza section showing mantle and Hartig net around elongated cortical cells (*bar*=20 µm). **h** Jigsaw puzzle-like pattern formed by epidermoid cells (*bars*=20 µm). **i-j** Ectomycorrhizae 12 months after inoculation with emanating hyphae (arrow, i) (*bar*=0.3 mm). **k-l** Ectomycorrhizae 24 months after inoculation with emanating hyphae (*bars*=0.5 and 0.3 mm, respectively).

Effect of mycorrhization on host plant growth and phenology

Two years after inoculation, the growth of plants of *Q. longispica*, *Q. mongolica*, and *Q. senescens* mycorrhizal with *T. melanosporum* was greater than those of uninoculated seedlings of the corresponding species (Fig. 3). At that time, none of the Quercus seedlings, inoculated or not with *T. melanosporum*, was contaminated by any ectomycorrhizal fungi (data not shown).

Taking the mean over all three oak species, canopy diameter, numbers of leaves, and mean leaf dimension were larger by about 46 mm, 2.3 leaves, and 10 mm, respectively, with *T. melanosporum* than with uninoculated control seedlings ($p < 0.001$, $p = 0.017$, $p < 0.001$ respectively, for the mycorrhiza main effect; Table 3, Fig. 3a, b, d, e, g, h). This change between *T. melanosporum*–inoculated and control seedlings was fairly similar for all species ($p = 0.245$; $p = 0.614$; $p = 0.072$ for the species by mycorrhizal treatment interaction for diameter, leaf numbers, and dimension, respectively). These results clearly demonstrate a beneficial effect of *T. melanosporum* on the growth of these three oak species. On average, numbers of leaves were similar for the three species ($p = 0.319$ for the species main effect), but both canopy diameter and leaf dimension varied with species ($p < 0.001$ for both) and were higher for *Q. mongolica* (means of 110 and 43.6 respectively) than for either of *Q. senescens* (81; 24.1) or *Q. longispica* (73; 25.8).

In March 2019, the growth-stimulating effect of the mycorrhization by *T. melanosporum* on the two evergreen species was still conspicuous (Fig. 3c, f). We further observed that the buds of *Q. mongolica* seedlings mycorrhized by *T. melanosporum* broke considerably earlier than those of the control uninoculated plants (Fig. 3i, j).

Table 3 Plant growth assessment: mean of each variable analysed (95% confidence limits).

Variable	Species	Seedlings mycorrhized by *Tuber melanosporum*	Uninoculated control seedlings
Canopy diameter (mm)	*Quercus longispica*	92.0 (73.6,110.4)	57.5 (41.6,73.4)
	Quercus mongolica	141.6 (127.4,155.8)	83.6 (69.4,97.8)
	Quercus senescens	101.0 (78.5,123.5)	64.3 (46.0,82.7)
No of leaves	*Quercus longispica*	5.7 (3.4,9.5)	4.5 (2.7,7.4)
	Quercus mongolica	5.8 (3.9,8.6)	3.6 (2.2,5.9)
	Quercus senescens	9.5 (5.8,15.5)	4.7 (2.6,8.2)
Leaf dimension (mm)	*Quercus longispica*	30.1 (23.9,36.2)	21.6 (15.5,27.6)
	Quercus mongolica	52.4 (47.7,57.2)	34.8 (29.6,40.1)
	Quercus senescens	26.5 (19.5,33.4)	21.9 (15.5,28.3)

Fig. 3 a-h Growth stimulation of seedlings of three Chinese *Quercus* species mycorrhized by *Tuber melanosporum* versus the growth of uninoculated control seedlings of the same species. Clear background, two years after inoculation; black background, 2 years and 8 months after inoculation. M, seedlings mycorrhized by *T. melanosporum*; C, control, uninoculated seedlings; in top views, M seedlings are on the left, C seedlings on the right. **a-c** *Quercus longispica*. **d-f** *Quercus senescens*. **g-h** *Quercus mongolica*. **i-j** Accelerated budbreak of the mycorrhizal *Q. mongolica* seedlings (top rows) in comparison with uninoculated seedlings (bottom rows) 2 years and 8 months after inoculation (spring, March 2019). Note the leaves of the control seedlings just about to emerge (i, arrow).

Discussion

The mycorrhizae obtained in this study between *T. melanosporum* and five Chinese oak species were well developed and displayed similar shape and color to those obtained

on Mediterranean *Quercus* spp., the natural hosts of the Périgord black truffle (Guerin-Laguette et al. 2013). External characteristics include a brown-red mantle with abundant emanating hyphae. Mounted mycorrhizal tips revealed the typical jigsaw puzzle pattern of the mantle cells (Giraud 1988), while cross-sections revealed an "epidermal" Hartig net, i.e., confined to the external layer of elongated cortical cells, as expected in the majority of angiosperms (Smith and Read 2008). Altogether, these observations confirmed the formation of fully developed ectomycorrhizae of *T. melanosporum*. None of the uninoculated plants produced mycorrhizae except for a few seedlings showing traces of non-Tuber species 32 months after inoculation. These observations strongly suggest that the mycorrhizae obtained were of *T. melanosporum* only. Their identity was further confirmed using ITS sequence comparison. As expected, a multiplex PCR failed to detect T. indicum on three mycorrhiza samples tested.

Although fully developed mycorrhizae were obtained on each of these five oak species, the efficiency and intensity of mycorrhization varied among them. In this study, *Q. mongolica* and *Q. longispica* were more receptive to *T. melanosporum* colonization than the other three species, since all inoculated plants were mycorrhized 6 months after inoculation (Table 2). In comparison, fewer than half the seedlings of *Q. fabrei*, *Q. variabilis*, and *Q. senescens* formed ectomycorrhizae with *T. melanosporum* within the same period, while none of *Q. pseudosemecarpifolia* seedlings did (Table 2). Of all species, *Q. mongolica* appeared to be the most promising species since, in addition to its receptivity to *T. melanosporum* mycorrhization, all seedlings, including controls, were still alive after 24 months (Table 2, Fig. 3). More work is required to confirm the variations seen in the receptivity of these oak species to mycorrhization by *T. melanosporum*. In a separate experiment, we have successfully inoculated *Quercus ilex*, i.e., one of *T. melanosporum* natural hosts, with the same method and the same substrate (Huang, unpublished), suggesting that the experimental conditions were suitable to *T. melanosporum* mycorrhization. Further work would need to focus on optimizing the substrates adapted to each tree species. An initial high rate of mycorrhization of seedlings is critical to contribute to the survival and development of the inoculated truffle over other soil-resident ectomycorrhizal fungal competitors (Kennedy 2010) and therefore to achieve successful truffle cultivation. More work is required to understand the mycorrhization of these indigenous Chinese oak species by *T. melanosporum*. It is worth noting here that *T. indicum* can form ectomycorrhizae with European and North American tree species (Murat et al. 2008; Bonito et al. 2011).

To our knowledge, this is the fourth study to describe a positive effect of the mycorrhization by *T. melanosporum* on the shoot growth of broad-leaf plants under greenhouse conditions. Domínguez et al. (2008) reported a significant increase in height, shoot, and root dry weights of *Quercus petraea* seedlings that were, however, only predominantly mycorrhized by *T. melanosporum*, i.e., with a slight colonization by nursery-contaminating mycorrhizal fungi. In the present work, 2 years after inoculation, no other ectomycorrhizal species were detected on any of the seedlings, control or inoculated. Martin et al. (2010, Supplementary Information section 1 and Supplementary Fig. 3) also reported that the inoculation of three *Cistus* species (Giovannetti and Fontana 1982) and of *Quercus pubescens* with *T. melanosporum* led

to an increased shoot growth. Therefore, our results constitute the third evidence that such a beneficial growth effect on broad-leaf plants was due to *T. melanosporum* only. Other works on *T. melanosporum*–inoculated broad-leaf plants showed enhanced physiological functions, e.g., enhanced root biomass, root conductance per unit leaf area, and an increased diameter of the stem of *Q. ilex* seedlings barely detectable visually (Nardini et al. 2000). Furthermore, in Nardini et al.'s (2000) study, the effect of *T. melanosporum* on *Q. ilex* height was negative. A positive effect on height, shoot, and root dry weights of *T. melanosporum* on conifers, i.e., *Pinus halepensis*, has however previously been recorded (Domínguez et al. 2008, 2012). Besides *T. melanosporum*, other *Tuber* spp. effects on host plant growth in the nursery have been observed, mainly on root biomass by *T. brumale* (Álvarez-Lafuente et al. 2018) and *Tuber* spp. (Bencivenga and Venanzi 1990) and on root physiology by *T. indicum* (Zhang et al. 2019). Finally, the shoot-stimulating effect of *T. melanosporum* mycorrhization has previously been reported in field conditions for two oak species, *Q. ilex* and *Q. faginea* (Domínguez et al. 2006). The present study is a further confirmation that, at least in the early stage of the mycorrhizal association, *T. melanosporum* behaves like a typical ectomycorrhizal fungus, enhancing the growth of its host plants. Our results suggest that *T. melanosporum* could also improve the performance of out-planted mycorrhizal seedlings, a very valuable feature for afforestation programs.

The accelerated budbreak of mycorrhized versus uninoculated *Q. mongolica* seedlings is an unprecedented observation. Splivallo et al. (2009) have shown that truffles can regulate plant root morphogenesis via the production of auxin and ethylene. Our study strongly suggests that mycorrhization by *T. melanosporum* can similarly modify the endogenous hormonal balance of the host plant, triggering budbreak in the frost-free, stable environment of a glasshouse. Accelerated budbreak would in turn benefit *T. melanosporum* growth. In future work, we propose to use *Q. mongolica* × *T. melanosporum* to measure, under various environmental conditions, the physiological changes induced in the host plant by the mycorrhization.

On *Q. mongolica* and *Q. longispica*, the mycorrhization persisted and developed up to 32 months after inoculation, further suggesting that both species could be suitable host trees for *T. melanosporum* cultivation in China. *Quercus mongolica* is a widespread deciduous tree species and a crucial timber species in North of China that can tolerate drought and soil infertility (Shen et al. 2014). An abundant ectomycorrhizal fungi composition was revealed in *Q. mongolica* stands, including *Tuber* spp. (Wang 2013; He et al. 2016), but the symbiotic relationship with Tuber spp. was not studied. Our trial demonstrated that *Q. mongolica* can form ectomycorrhizae with *T. melanosporum*, while benefiting from the association at the nursery stage. This opens the possibility of conducting field trials to attempt cultivation of *T. melanosporum* with *Q. mongolica* in northern China and monitor its potential competition with native ectomycorrhizal fungi, including other Tuber species. *Tuber melanosporum* is however known to be often outcompeted by other Tuber species in Europe (Molinier et al. 2013).

Quercus longispica, *Q. senescens*, and *Q. pseudosemecarpifolia* are endemic evergreen species in Hengduan Mountains and belonged to *Quercus* sect. *Heterobalanus*. The external morphology and ecological suitability of *Quercus* sect.

Heterobalanus are similar to those of *Q. ilex* L., a natural host tree of *T. melanosporum* distributed in Mediterranean regions (Zhou 1992). Truffles (e.g., *T. oligospermum* Trappe) were found in forests made of oak from sect. *Heterobalanus* in China (Xu 1999). Ectomycorrhizae of *T. melanosporum* were synthesized successfully for the first time in this study on *Q. longispica* and *Q. senescens*, but the rate of mycorrhization of *Q. senescens* was slow and low, i.e., only two trees out of five formed ectomycorrhizae after 9 months, in comparison with *Q. longispica* whose five replicate seedlings were mycorrhized after 6 months. *Quercus longispica* and *Q. senescens* can tolerate seasonal drought and cold, and have important value as reforestation species in their distribution range (Ma 2006). Our study further demonstrated that the growth of both species could benefit from controlled mycorrhization by *T. melanosporum*. While further work is required to optimize the mycorrhization of *Q. senescens*, both oak species appear to be worth considering for Périgord black truffle cultivation projects in high-altitude areas of China.

This study added five new species, for the first time within *Quercus* in China, to the long list of non-traditional trees that have been demonstrated to host *T. melanosporum* under nursery conditions: *Carya illinoinensis* (Marozzi et al. 2017), *Nothofagus* spp. (Pérez et al. 2007), *Castanopsis hystrix* (Chen 2002), and *Pinus* spp., i.e., *P. armandii* (Su et al. 2017), *P. nigra* (Domínguez-Nuñez et al. 2015), *P. yunnanensis* (Lin et al. 2013), and *P. halepensis* (Domínguez et al. 2012). In the wild, even the rockrose *Cistus laurifolius* was shown to host mycorrhizae and produce ascocarps of *T. melanosporum* (García-Montero et al. 2007). The present study strongly suggests that other economically or environmentally important tree species in China, suited to particular geo-climatic conditions, could be similarly tested for their ability to form mycorrhizae with the Périgord black truffle in the nursery. Species receptive to *T. melanosporum* could be considered for truffle production in plantations or as "carriers" of inocula for forestry applications (Domínguez et al. 2012, García-Montero et al. 2007). Culleré et al. (2017) demonstrated that host tree can influence the aroma of *T. melanosporum* truffles by increasing their concentrations of isoamyl alcohol and 3-ethyl-5-methylphenol. Therefore, testing and comparing different host tree species for their receptivity to mycorrhization by *T. melanosporum*, their growth responses, and their potential to produce aromatic ascomata could have an important economic impact.

More work is required to understand whether insect grazing prevented the mycorrhization to fully establish on some of the species studied. The morphology of the larvae seen on the oak seedlings (Suppl. Fig. 1l-m) was identical to those found on pines grown in the same nursery and identified as *Bradysia impatiens* (Wang et al. 2019), a common pest in greenhouses, known to damage mushroom's mycelium or sporocarps (Cloyd, 2015; Ye et al., 2017). Greenhouse management techniques reducing insect populations (Cloyd 2015) are required to ensure the large-scale production of truffle-mycorrhized seedlings in the future and to further assess the potential of *Q. variabilis*, *Q. fabrei*, and *Q. senescens* as potential host trees for *T. melanosporum* cultivation in China.

In conclusion, our work demonstrated, for the first time, the successful controlled mycorrhization of five indigenous Chinese oak species by *T. melanosporum* and the

positive effects of this fungus on the growth of three of these species in the nursery, 24 months after inoculation. The mycorrhization continued to develop, including the formation of clusters, up to 32 months after inoculation. The stimulating growth effect was still clearly visible on the two evergreen species while *T. melanosporum* markedly accelerated budbreak in *Q. mongolica*. Two species, *Q. mongolica* and *Q. longispica*, appear to be promising symbionts for gourmet truffle cultivation projects in China, while potentially contributing to afforestation and ecosystem conservation. However, more work is required to determine if these two host species could undergo large-scale nursery production. In addition to the optimization of greenhouse conditions, the monitoring of high-quality mycorrhizal seedlings planted in the field is needed to assess (1) the persistence and development of synthesized mycorrhizae and (2) the production potential of *T. melanosporum* associated with Chinese host trees. The next step of this research is to establish trial plantations with these oak species.

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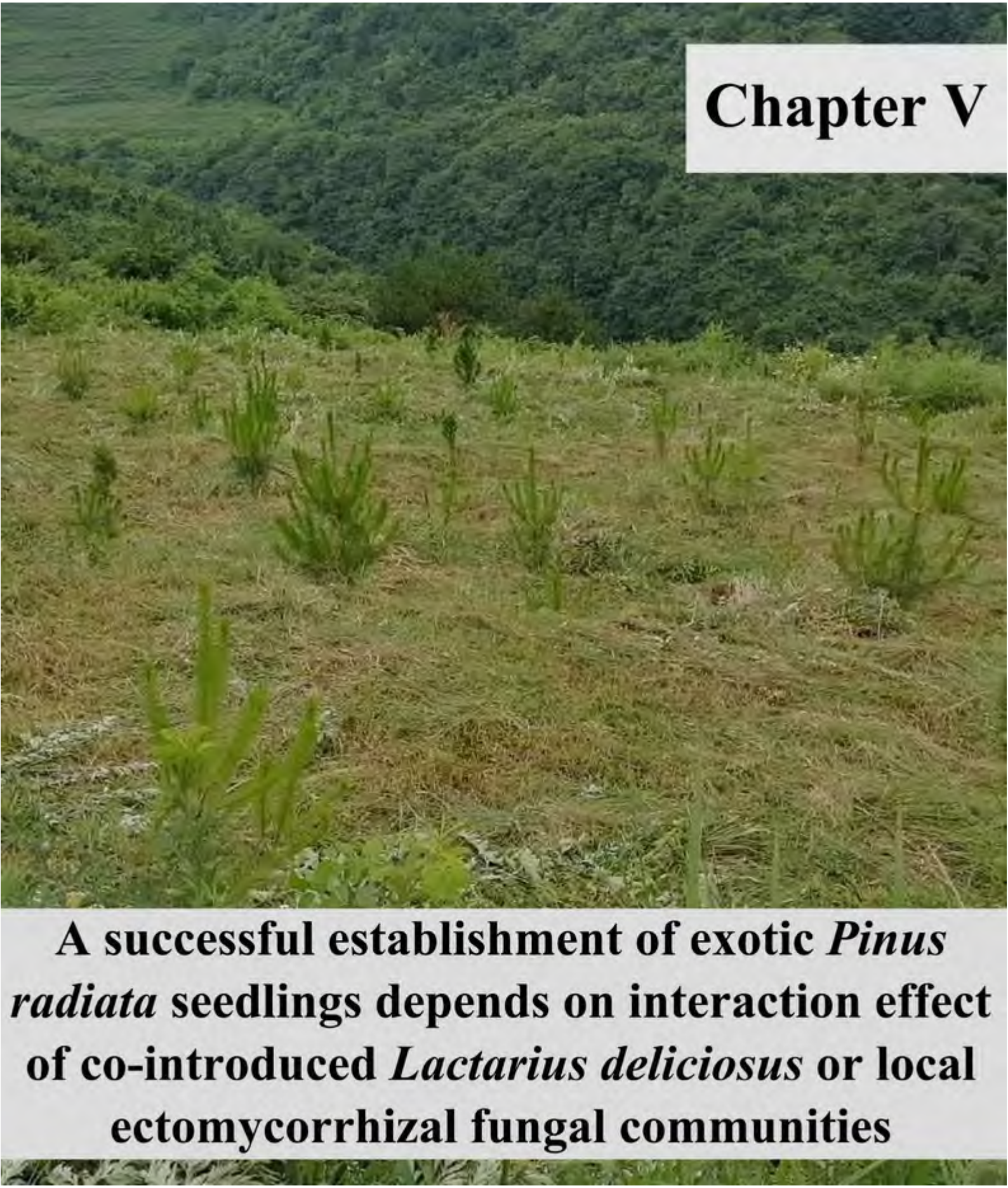

Chapter V

A successful establishment of exotic *Pinus radiata* seedlings depends on interaction effect of co-introduced *Lactarius deliciosus* or local ectomycorrhizal fungal communities

A successful establishment of exotic *Pinus radiata* seedlings depends on its interaction with co-introduced *Lactarius deliciosus* or local ectomycorrhizal fungal communities

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ABSTRACT

An introduction of exotic or non-native trees may fail due to lack of suitable fungal partners. We planted exotic *Pinus radiata* in a new habitat in Xifeng, Guizhou Southwest China. Strategies to introduce *P. radiata* seedlings either colonized with an ectomycorrhizal fungus (EcMF), *Lactarius deliciosus*, or expect them to form familiar/new associations with local EcMF in a new habitat were studied to know how *P. radiata* could be successfully established over a period of 2.5 years. Plant height and needle nutrient acquisition, persistence of co-introduced *L. deliciosus* and fungal community composition in rhizosphere soil and root tips were analyzed. In addition, a greenhouse bioassay experiment of local soil to assess the differences in the EcMF community between exotic and native pine seedlings was also conducted. The current results demonstrated that either co-introduced *L. deliciosus* or associations with local EcMF could both help *P. radiata* establish in Xifeng. The co-introduced *L. deliciosus* might be naturalized with *P. radiata* in the new area. Host identity had no effect on fungal composition since exotic *P. radiata* and native *P. massoniana* recruited similar local fungal communities, but *L. deliciosus* pre-colonization significantly altered the mycorrhizosphere fungal composition. Cosmopolitan species *Suillus placidus* with high relative abundance was found in rhizosphere soil and root tips in the plantation. In addition, the greenhouse bioassay experiment further proved that a local EcMF, *Suillus* sp., contributed relatively higher extracellular enzymes by forming ectomycorrhizas with *P. radiata*, and there was no alteration of the fungal community caused by *P. radiata* during early establishment. Our study showed that exotic *P. radiata* could be a suitable tree capable to get established successfully in Xifeng either by interaction with

co-introduced *L. deliciosus* or with local EcMF, while more attention should be paid to local EcMF community changes induced by the introduced *L. deliciosus*.

Keywords: exotic *Pinus radiata*; co-introduced *Lactarius deliciosus*; local ectomycorrhizal fungi; *Suillus*; extracellular enzyme

INTRODUCTION

A number of exotic tree species from the genera *Pinus*, *Eucalyptus* and *Populus* have been introduced to China since the 1980's, which have become the dominant tree species in timber plantations (Song, 1983; Wang et al., 2006; Chen et al., 2018; Farooq et al., 2021). Among them, pine species are widely distributed in the northern hemisphere and play an important role in afforestation, due to their easy propagation, strong adaptability, short growth cycle and high-quality wood (Song, 1983; Richardson et al., 2000; Wang et al., 2006). In nature, pine trees form obligate symbioses with a variety of ectomycorrhizal fungi (EcMF) which provide plants with mineral nutrients, water, or defense against pathogens, whereas plants supply EcMF with energy-rich carbon compounds in return (Allen, 1991; Liang et al., 2002; Smith and Read, 2008).

A successful establishment of exotic pines in non-native areas is dependent on various factors, such as pathway of introduction, soil properties and local climate (Keane and Crawley, 2002; Pysek et al., 2011, 2015; Dodet et al., 2012; Dickie et al., 2017). Besides, some biotic factors like pathogens, predators and suitable mutualists also play important roles in naturalization of introduced plants (Keane and Crawley, 2002; Pringle et al., 2009; Jurkien et al., 2020; Moyano et al., 2020; Ning et al., 2020). Ectomycorrhiza, the root tips colonized by EcMF, produce a variety of extracellular enzymes including oxidase and hydrolase to mobilize soil organic matter (Courty et al., 2005). As a consequence, EcMF communities largely affect ecosystem processes, such as carbon or nutrient cycling (Chapela et al., 2001; Read and Perez-Moreno, 2003; Clemmensen et al., 2014).

Initial planting of pines failed in many parts of the southern hemisphere (Marx, 1991; Pringle et al., 2009) due to the lack of suitable EcMF. It has been long recognized that the absence of co-evolved EcMF in soils is a major obstacle to successful establishment of introduced plants (Mikola, 1970; Poynton, 1979; Richardson et al., 2000; Pringle et al., 2009, Nunez and Dickie, 2014). In general, there could be three strategies for exotic plants to overcome detrimental loss of mutualistic symbionts: (1) interaction with EcMF co-introduced from the plant's native habitats, (2) familiar association with EcMF present in both native and new habitats, and (3) new association with EcMF in a fresh habitat (Dickie et al., 2010 and 2017).

By analyzing 42 independent global-scale datasets of EcMF communities associated with Pinaceae and *Eucalyptus* spp. in the introduction areas, Vlk et al (2020a) showed that successful introduction of exotic Pinaceae relied on co-introduced EcMF, especially in areas where Pinaceae did not naturally exist. Co-introduced EcMF may preadapt to new areas as early successional fungi and then help exotic plants successfully establish in new areas (Vlk et al., 2020 b). Many exotic tree species have

been frequently transported to new areas as seedlings along with soil rather than specific inoculum from their native range, but the identities and distributions of EcMF co-introduced with Pinaceae species are poorly known (Mikola, 1970; Dickie et al., 2010; Hynson et al., 2013). Hayward et al (2015) reported that the co-introduction of an EcMF was sufficient to successfully introduce *Pinus contorta* outside its natural distribution range or promote expansion of its natural range. Ectomycorrhizal plants usually host highly diverse EcMF communities on their root system in their native range, thus whether a successful introduction is related to a few unique co-introduced EcMF remains questionable. On the other hand, co-introduced EcMF species may be replaced by local EcMF species or displace local EcMF (Richardson et al., 2000; Dell et al., 2002; Vellinga et al., 2009). Therefore, the ecology of an introduced EcMF at new sites needs to be studied during tree restoration.

Vlk et al (2020a) summarized that familiar or new associations in new sites with native EcMF prevailed among exotic Pinaceae introduced to regions where other Pinaceae species occur naturally. Selection of high-efficiency partners in the local areas might be another important strategy for exotic pine establishment. Exotic plants may recruit different EcMF communities because of their phylogenetic distance (Trocha et al., 2012; Nguyen et al., 2016; Ning et al., 2021). In a bioassay experiment with natural soil cores from pine forests, Ning et al (2020, 2021) documented that host identity was a key factor determining EcMF community assembled in plant early establishment and the shifts of EcMF community composition might happen to exotic pines for selecting beneficial mutualists against less beneficial counterparts (Hortal et al., 2017; Ning et al., 2021). In addition, the interaction between exotic pine species and local EcMF resulted in higher capacity for organic nitrogen and phosphorus utilization than that of native pine species, which might indicate a host-specific advantage of plant-mycorrhiza symbiosis for nutrient uptake and cycling. A better understanding of such nutritional effects could provide insights into the mechanism of exotic plant establishment during ecosystem restoration.

Pinus radiata D. Don is a native species to the central coast of California (U.S.A). It has the lowest mortality and best growth rate during early establishment where other forest species are difficult to get established (Bi et al., 2003). Since 1990's, *P. radiata* has become the main planted tree species in New Zealand. One plantation of *P. radiata* from New Zealand has been established in Guizhou province, southwest of China since 2018. Mycorrhizal seedlings were obtained with a *Lactarius deliciosus* culture originally from fruiting bodies in New Zealand's *P. radiata* plantations (Wang et al., 2002, Guerin- Laguette et al., 2014, Wang et al., 2019). There was no record of *Lactarius deliciosus* fruiting bodies in the area before the Xifeng plantation establishment. In May 2018, mycorrhizal and non-mycorrhizal seedlings of exotic *P. radiata*, and non-mycorrhizal seedlings of native *P. massoniana* Lamb. were respectively planted in Xifeng County, Guizhou Province. Fruiting bodies of *L. deliciosus* have been observed after two and half years in the plantation with the mycorrhizal, not with the non-mycorrhizal seedlings (**Figure 1E, F)**.

The primary purpose for establishing plantation was to introduce radiata pines, reuse wasteland and might realize *L. deliciosus* cultivation. In this present study, the

following studies in the plantation were conducted: (a) seedlings' height was measured every six months, (b) nutrient concentration in needles was analyzed after fruiting, (c) identification of the composition and diversity of the EcMF communities was analyzed through high-throughput sequencing and (d) a greenhouse bioassay experiment was conducted by planting exotic *P. radiata* and native *P. massoniana* seedlings in soils collected from Xifeng and activity of extracellular enzymes related to C, N, and P acquisition were assayed in ectomycorrhizal root tips.

FIGURE 1 Plantation in Xifeng and mushroom production in plantation in November 2020. (**A**) Study site (yellow line). (**B**) Trees in the plantation 2.5 years after planting, including *Pinus radiata* + *Lactarius deliciosus* (PR+LD), *P. radiata* (PR) and *P. massoniana* (PM). (**C**) Ectomycorrhizas of PR+LD (blue arrows showing ECM tips). (**D**) *Suillus* colonization to roots of PR+LD (yellow arrow). (**E**) *Lactarius deliciosus* fruiting bodies (yellow circle) occurred about one meter from the PR+LD tree. (**F**) *L. deliciosus* fruiting bodies from four PR+LD trees. (**G**) *Suillus* sp. fruiting bodies occurred near the PR tree.

Based on the above-mentioned analysis, the following questions were addressed: (1) Are there differences in growth effect and nutrient acquisition between seedlings? (2) Are there differences in diversity and relative abundance of fungal communities in rhizosphere soil of co-introduced *P. radiata* with or without *L. deliciosus*? (3) What is the difference in their EcMF communities recruited by exotic *P. radiata* and native *P. massoniana* in their early establishment under greenhouse and field plantation? (4) Are there distinctive relationships in ectomycorrhizal enzyme activity and nutrient content of tissue between native and exotic pine seedlings? Answers to the above-mentioned questions would improve our understanding on how an introduced EcMF *L. deliciosus* or local EcMF could help an exotic *P. radiata* to be successfully established in a new habitat.

MATERIAL AND METHOD

Study Sites

The plantation (0.67 ha, **Figure 1A, B**) is located in Xifeng County (27° 21′ N, 106°41′ E, alt. 1213 m above the sea level), Guizhou province, southwest China, where the annual rainfall is ~1,200 mm. The plantation area was an abandoned farmland where shrubs and weeds (dominated by *Pyracantha* sp. and Fabaceae) had grown for more than five years. The native *Pinus massoniana* (PM) forests are 1,000 m from the plantation. Soil physico-chemical properties were tested before plantation (**Table 1**). *Pinus radiata-Lactarius deliciosus* (PR+LD) seedlings were obtained in the greenhouse in October 2017 according to the method of Wang et al (2019). Before plantation establishment, all seedlings were checked morphologically under a stereomicroscope (Leica S8AP0) to confirm mycorrhization. Ectomycorrhizal root tips from PR+LD seedlings, root tips from PR and PM seedlings were randomly selected for DNA extraction and subsequent Illumina MisSeq sequencing analysis (see **Supplementary Method 1** for fungal community sequencing, **Supplementary Figure 1**). In May 2018, a total of 34 PR+LD, 10 PR and 24 PM seedlings were randomly planted in the Xifeng plantation (**Figure 1B**) in a 4 × 4 grid. In November 2020, LD fruiting bodies were collected from plantation (**Figure 1E, F**). SSR results confirmed that fruiting bodies were from co-introduced LD isolate (Tang and Wang, unpublished).

TABLE 1 Soil physico-chemical characteristics in the Xifeng plantation

Location	pH	C (g kg^{-1})	N (g kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	Ca (mg kg^{-1})
Xifeng (*n*=10)	6.30±0.10	15.80±3.30	1.45±0.20	243.50±38.90	9362.00±426.40	1458.00±128.00

Method S1: ECM fungal communication DNA extraction and ITS sequencing

Fungal DNA was extracted from 0.2 g of mycorrhizal root tips, root tips and mycorrhizospheric and rhizospheric soil using Powersoil™ DNA isolation kits (MoBio, San Diego, CA, USA) following manufacturer's instructions for maximum DNA yield. The quality and quantity of the DNA extracts were checked using a spectrophotometer (Nanodrop, PeqLab, Germany). The ITS1 region was amplified using the forward primer ITS5 (5'- GGAAGTAAAAGTCGTAACAAGG-3') and the reverse primer ITS2 (5'- GCTGCGTTCTTCATCGATGC-3'). Purified amplicons were pooled in equimolar concentrations and pair-end sequenced on an Illumina MisSeq platform, Novaseq-

PE250 (Personalbio®, Shanghai, China). The raw reads were analyzed using QIIME2 software (version 1.9.1, http://qiime2.org/) to trim off the low-quality reads, adaptors, barcodes, and primers. Sequences were clustered into operational taxonomic units (OTUs) by setting a 97 % similarity (Edgar 2010). The UNITE SH database v8.0 (http://unite.ut.ee/repository.php, Kõljalg et al., 2013) was used in chimera checking and OTU clustering. The Bray-Curtis distance-based dissimilarity distance, Simpson and Chao1 diversity index, principal coordinate analysis (PCoA) and a Venn diagram with shared and unique OTUs were performed on the Genescloud platform of Personalbio® to evaluate the fungal community differences between different samples. Raw sequence data have been deposited in the NCBI Sequence Read Archive database under the bioproject identifier PRJNA792310 and PRJNA 795335.

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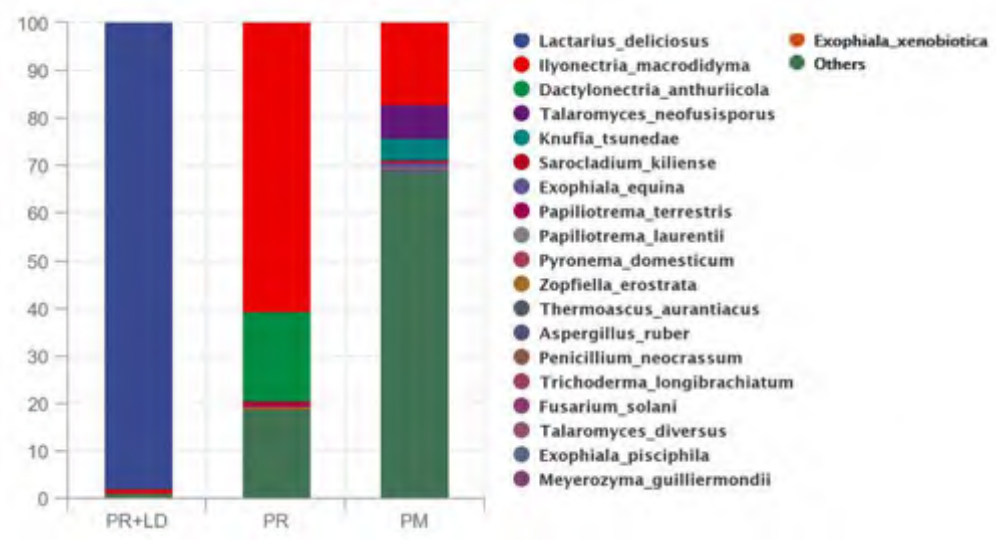

FIGURE S1 Relative abundance of fungal community composition at the general level in root tips of *Pinus radiata* + *Lactarius deliciosus* (PR+LD), *P. radiata* (PR) and *P. massoniana* (PM) seedlings before plantation establishment.

Sampling Procedure in Plantation

Seedlings' height was measured every six months from November 2018 to May 2021 (T1~T6). The survival rate of seedlings was also recorded one year after planting. In March 2021, five plants of each tree species were randomly selected and soil layer was lightly excavated at 0.5 m and 1.0 m from the trunk to observe mycorrhizae or fine roots. Soil samples were collected with a sterilized stainless-steel spoon within 1.0 cm around mycorrhizae or roots. The corresponding mycorrhizae or roots were cut with sterile scissor and soil attached to the mycorrhizae or roots was gently shaken off and

then collected. All samples were stored in sterile centrifuge tubes and put in foam boxes with ice packs, then brought back to the laboratory as soon as possible. Mycorrhizal tips and roots were gently washed with sterile water and then collected after the removal of soil debris. All samples were then used for Illumina MisSeq sequencing (see details in **Supplementary Method 1**). The raw sequence data have been deposited in the NCBI Sequence Read Archive database under the bioproject identifier PRJNA 795335.

Healthy mature pine needles were sampled, and their fresh and dry weight (48h at 65 °C) were measured. The dried needles were then ground into fine powder and the content of carbon (C) and nitrogen (N) were determined by a Vario MAX CN instrument (Elementary Analyse system GmbH, Germany). Total phosphorus (P), potassium (K), calcium (Ca), iron (Fe) and manganese (Mn) were determined with an inductively coupled plasma atomic-emission spectrometer (IRIS Advantage-ER; Thermo Jarrell Ash Corporation, Franklin, MA, USA).

Bioassay Experiment in Greenhouse

(a) Soil collection from plantation

Using a steel soil auger (10 cm diam, ~12 cm deep), we collected twenty soil cores in wastelands next to the Xifeng plantation, put them in sealed ziplock bags and transported with ice to laboratory (Ning et al., 2020). Soils from ten of the cores were placed into 688-mL plastic pots (top diameter 9.1cm, bottom diameter 6.4cm, height 13.2cm, S9, Xinguanghe horticulture, Zhejiang) which were pre-sterilized with sodium hypochlorite (2% available chlorine), while the other ten soil cores were autoclaved for 3h at 121°C to be used as control. The soil was sown with either PR seeds (Proseed, New Zealand) or native PM seeds (Guizhou, Duyun City). Before sowing, seeds were surface sterilized with 30% hydrogen peroxide for 10 min (PR seeds) or 5 min (PM seeds), then soaked in sterile water and stored at 4 ℃ for 3 weeks (Ning, et al., 2020). After germination, 8 seedlings were kept in each pot. Plants were grown in a greenhouse at Kunming Institute of Botany (KIB) under natural light (e.g., 169 μmol^{-2} s^{-1} inside the greenhouse in June in a randomized block design for pot arrangements).

(b) Mycorrhizae formation and identification

After three and six months of seed germination (June and September 2021), three pine seedlings were harvested and then gently washed with tap water. Number of root tips and root tip area were estimated using WinRhizo (Regent Instruments Canada Inc., Quebec, Canada). Types of root tips of ectomycorrhiza (ECM) were identified from morphological, anatomical (Agerer 1987–2002, 1991) and molecular characteristics (Karen et al., 1997). The sorted homogeneous ECM morpho-anatomical types were counted. ECM colonization was calculated as the number of ECM root tips divided by the total number of root tips which include ECM root tips and non-ECM root tips (Gehring and Whitham, 1994).

(c) Extracellular enzyme activity of ECM

The activity of Cellobiohydrolase (C1, 3.2.1.91), β-Glucosidase (GC, 3.2.1.21), β-Glucuronidase (GD, 3.2.1.31), β-Xylosidase (X, 3.2.1.37), laccase (LAC, 1.10.3.2), N-Acetylglucosaminidase (NAG, 3.2.1.14), Leucine amino peptidase (LEU, 3.4.11.1) and Acid phosphatase (ACP, EC 3.1.3.2) were assessed with ectomycorrhizal tips in 96-

well filter plates (Pritsch et al., 2011; Ning et al., 2020).

(d) Plant harvest and tissue C, N and P measurements

Three pine seedlings per pot were sampled and separated into shoot and root, dried for 48 h at 60 ℃ and then their biomasses were weighed. Next, dried tissues were ground into fine powder, and root and shoot C, N and P were analyzed.

Data Analyses

Data (means ± SE, $n = 6$) from the plantation were statistically analyzed by *R* software (version 3.2.3) (R Core Team 2015). One-way analysis of variance for independent samples was performed to analyze significant scientific differences. Pearson correlation analysis (for all analyzed samples, $n = 12$) was used to examine the correlations between the relative abundance of EcMF OTUs and measured needle nutrient concentrations. Statistical significance for Pearson correlation was determined by pairwise two-sided comparisons. Fungal alpha-diversity was estimated by richness (Chao 1) and diversity (Simpson) indexes. Patterns in ECM fungal community composition were analyzed and visualized with Principal Coordinates analysis (PCoA) based on Bray-Curtis distance at the OTU level. PERMANOVA analysis with python's scikit-bio package on Bray-Curtis dissimilarity matrices was performed to examine variation in composition of EcMF with respect to co-introduced LD and host interactions. The shared or unique fungal OTU numbers (among soil or roots of different hosts) were calculated based on the OTU abundance matrix using (nVennR) package in R software. A significance α level of 0.05 was applied.

The relative contribution of each EcMF group to community enzyme activity in each pot was calculated using the following formula: Activity of EcMF group/ total activity of EcMF community per seedling. Pearson correlation analysis was performed to examine the correlations between the enzyme activity and plant tissue parameters of biomass production and nutrient concentrations.

RESULTS

Growth Effect of Trees and Needle Nutrient Concentration Responses

We did not find any significant difference ($P>0.05$) in plant height increment between PR+LD and PR, but both were significantly higher than PM ($P<0.05$) (**Table 2**, **Supplementary Figure 2**). Inoculation of PR trees showed no significant effects on needle C, N, P, K, Ca, Fe, Mn concentrations, either (**Table 3**). Needle C ($P<0.01$), P ($P<0.05$) and Fe ($P<0.05$) concentrations of PM were significantly higher than that of PR or PR+LD, but no significant difference in N, K, Ca, Mn concentrations and water content. In addition, one year after planting, the survival rate was highest in PR+LD (94.1%), next was PR (90%) and lowest was PM (58.3%).

TABLE 2 Average plant height (cm) of trees in the Xifeng plantation every six months.

	Nov-2018	May-2019	Nov-2019	May-2020	Nov-2020	May-2021
PR+LD (n=6)	45.80±12.07 a	76.80±21.32 a	103.80±23.94 a	140.00±26.84 a	162.80±32.17 a	203.4±45.97 a
PR (n=6)	33.2±7.76 a	56.6±10.76 a	85.4±9.94 a	120.6±17.98 a	147.2±30.89 a	171±41.31 a
PM (n=6)	12.2±1.30 b	32.3±11.17 b	40.2±8.98 b	81.4±15.95 b	91.4±16.82 b	117.8±12.64 b

Note: Differences in lowercase letters after the same column of data indicate significant differences.

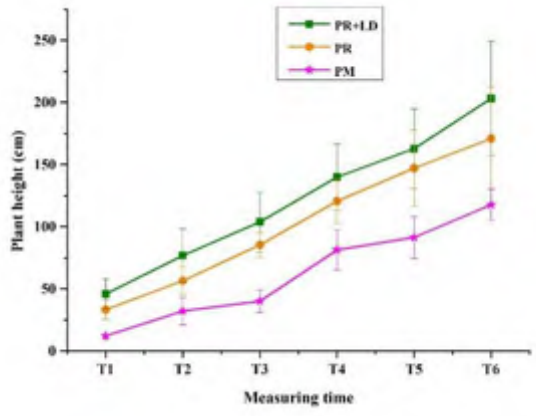

FIGURE S2 Trees' average height in every six months in Xifeng plantation. T1: Nov 2018, T2: May 2019, T3: Nov 2019, T4: May 2020, T5: Nov 2020, T6: May 2021

TABLE 3 Needle nutrient concentrations of trees in the Xifeng plantation. Data represents mean with the standard error in parentheses

	PR+LD (*n*=6)	PR (*n*=6)	PM (*n*=6)
Water (%)	53.43 (2.01)	54.05 (2.99)	52.14 (3.09)
C (%)	49.72 (0.41) b	49.32 (0.41) b	50.22 (0.48) a
N (%)	1.42 (0.14)	1.36 (0.11)	1.56 (0.27)
Total P (mg g^{-1})	1.12 (0.05) b	1.02 (0.19) b	1.20 (0.08) a
Total K (mg g^{-1})	5.16 (0.82)	4.14 (1.39)	3.8 (0.77)
Total Ca (mg g^{-1})	2.11 (0.95)	2.28 (0.99)	1.38 (0.39)
Total Fe (mg g^{-1})	0.25 (0.07) b	0.28 (0.033) b	0.34 (0.03) a
Total Mn (mg g^{-1})	0.23 (0.03)	0.25 (0.05)	0.32 (0.15)

Note: Differences in lowercase letters after the same column of data indicate significant differences.

Fungal Community in Mycorrhizosphere soil, Rhizosphere Soil and Roots

(1) PR+LD and PR

In mycorrhizosphere and rhizosphere soil, fungal community analysis indicated significant differences in β-diversity (dissimilarity distance, **Figure 2B**) but not in Chao1 and Simpson index (α-diversity, **Figure 2A**), between PR+LD and PR trees in the Xifeng plantation. These two groups were clearly defined by the PCoA (**Figure 2D**). About 17.8% OTUs of 3857 total OTUs were shared by both trees (**Figure 3C**). Fungal species with relative abundance in the top 20 included EcMF, plant pathogens, saprophytic and endophytic fungal species (**Figure 2E**). *Suillus* genus from EcMF, was common and dominated in soil (20.4% of PR+LD, 27.4% of PR). Among of the genus, *Suillus placidus* has the highest relative abundance and significantly higher than other *Suillus* species. Based on Species Hypotheses (SH) in the UNITE database (https://unite.ut.ee), OTU_3575 (*Suillus placidus*) was classified as cosmopolitan species and usually formed ectomycorrhizas with *Pinus* (**Supplementary Table 1**). Total 86 detected OTUs from mycorrhizosphere and rhizosphere soil were EcMF

species, but all of them were detected with low relative abundance except *Suillus* spp. (**Figure 4**).

TABLE S1 List of EcMF detected from mycorrhizosphere of *Pinus radiata* (PR+LD) and rhizosphere of *P. radiata* (PR) and *P. massoniana* (PM) in the Xifeng plantation, along with the presumed distribution and hosts.

Speices	OTU_ID	Species hypothesis ID taxa	Host tree	Sampling area	Host trees
Amanita pseudovaginata	OTU_9129	SH1611351.08FU	PR+LD, PR, PM	China, Pakistan	No information
Geastrum triplex	OTU_640	SH1555997.08FU	PM	Canada, Japan, Korea	No information
Hygrocybe nigrescens	OTU_1179	SH1512923.08FU	PR+LD, PR, PM	Canada	No information
Lyophyllum decastes	OTU_2848	SH1588949.08FU	PR	Asia, Europe, North America, South America, Oceania	No information
Russula brevipes	OTU _12341	SH1509932.08FU	PR+LD	China, Japan, Korea	No information
Russula cerolens	OTU_12131	SH1569811.08FU	PR, PM	China	No information
Russula compacta	OTU_7715	SH1546768.08FU	PM	Asia, Oceania	*Cymbidium*, *Lecanorchi Pinus*
Russula cyanoxantha	OTU_13484	SH1567120.08FU	PM, PR	Asia, Europe, South America	*Carya*, *Corylus*, *Fagus*, *Picea*, *Pinus*, *Quercus*
Russula nauseosa	OTU_3422	SH1625349.08FU	PR+LD, PR, PM	Asia, Europe, North America, South America	*Alnus*, *Pinus*, *Pseudotsuga*, *Larix*
Russula sp1	OTU_3845	SH1569729.08FU	PM	Asia	*Cephalanthera*
Russula sp2	OTU_7861	SH1633484.08FU	PR	China, Thailand	*Aphyllorchis*
Russula vesca	OTU _1657	SH1633421.08FU	PR+LD, PR	Asia, Europe, North America, Oceania	*Lecanorchis*, *Fagus*, *Pinus*, *Quercus*
Scleroderma albidum	OTU_9625	SH1544059.08FU	PR+LD	Africa, Asia, Europe, North America, Oceania	*Eucalyptus*, *Salix*
Scleroderma sp.	OTU_11552	SH1526180.08FU	PM	Asia, Europe, Oceania	*Castanopsis*, *Larix*, *Pinus*, *Populus*, *Quercus*
Sistotrema brinkmannii	OTU _4850	SH1514167.08FU	PR	Africa, Antarctica, Asia, North America, South America, Oceania	*Pinus*
Sistotrema coronilla	OTU _10356	SH1506095.08FU	PR+LD	Europe, North America, Oceania	*Quercus*
Suillus collinitus	OTU _5689	SH1555181.08FU	PR+LD, PM	Africa, Asia, Europe, Oceania	*Pinus*
Suillus placidus	OTU _3575	SH1555178.08FU	PR+LD, PR, PM	Africa, Asia, Europe, North America, Oceania	*Pinus*
Suillus luteus	OTU_9078	SH1555172.08FU	PR, PM	Asia, Europe, North America, South America, Oceania	*Pinus*

Tomentella pilosa	OTU_4969	SH1528407.08FU	PR	Asia, Europe, North America	*Castanea*, *Cistus*, *Fagus*, *Picea*, *Populus*, *Quercus*, *Tilia*
Tuber indicum	OTU_14388	SH1563426.08FU	PR, PM	China, Spain	*Quercus*
Tuber pseudohimalayense	OTU_4250	SH1514135.08FU	PM	China	No information

In soil of PR+LD, *Lactarius deliciosus* (32.4%) was dominating but showed no significant difference with the relative abundance of *S. placidus* (P = 0.548). The Pearson correlation analysis revealed significant correlations between the relative abundance of *L. deliciosus* in mycorrhizosphere soil and nitrogen acquisition of needles of PR (r = 0.89, P = 0.018, n = 12). In ectomycorrhizal tips of PR+LD, fungal community analysis indicated that LD was dominating (94.5%) in tips, while 5% of *S. placidus* (OTU_3575) was also colonizing tips. In root tips of PR, the relative abundance of *S. placidus* (OTU_3575) and *S. pseudobrevipes* (OTU_9078) were significantly greater than that of other EcMF.

(2) PR and PM

Fungal community analysis indicated no significant differences in α-diversity and β-diversity (**Figure 3A, B, D**) between PR and PM. A total of 3922 OTUs were displayed and about 17.2 % OTUs were shared by both trees (**Figure 3C**). *Suillus* genus was common in soil (28.2%) (**Figure 3E**) and root tips (33.5%) of PM, which included *Suillus* OTU_3575, OTU_5689 and OTU_9078. In root tips of PR, *Suillus* included OTU_3575 and OTU_9078. The Pearson correlation analysis revealed significant correlations between the relative abundance of *Suillus* and manganese acquisition of needles in PM (r = 0.89, P = 0.044, n = 12). A total of the 84 of the detected OTUs from the rhizosphere soil were EcMF species, but all of them were detected with low relative abundance except those in the genus *Suillus* (**Figure 4**).

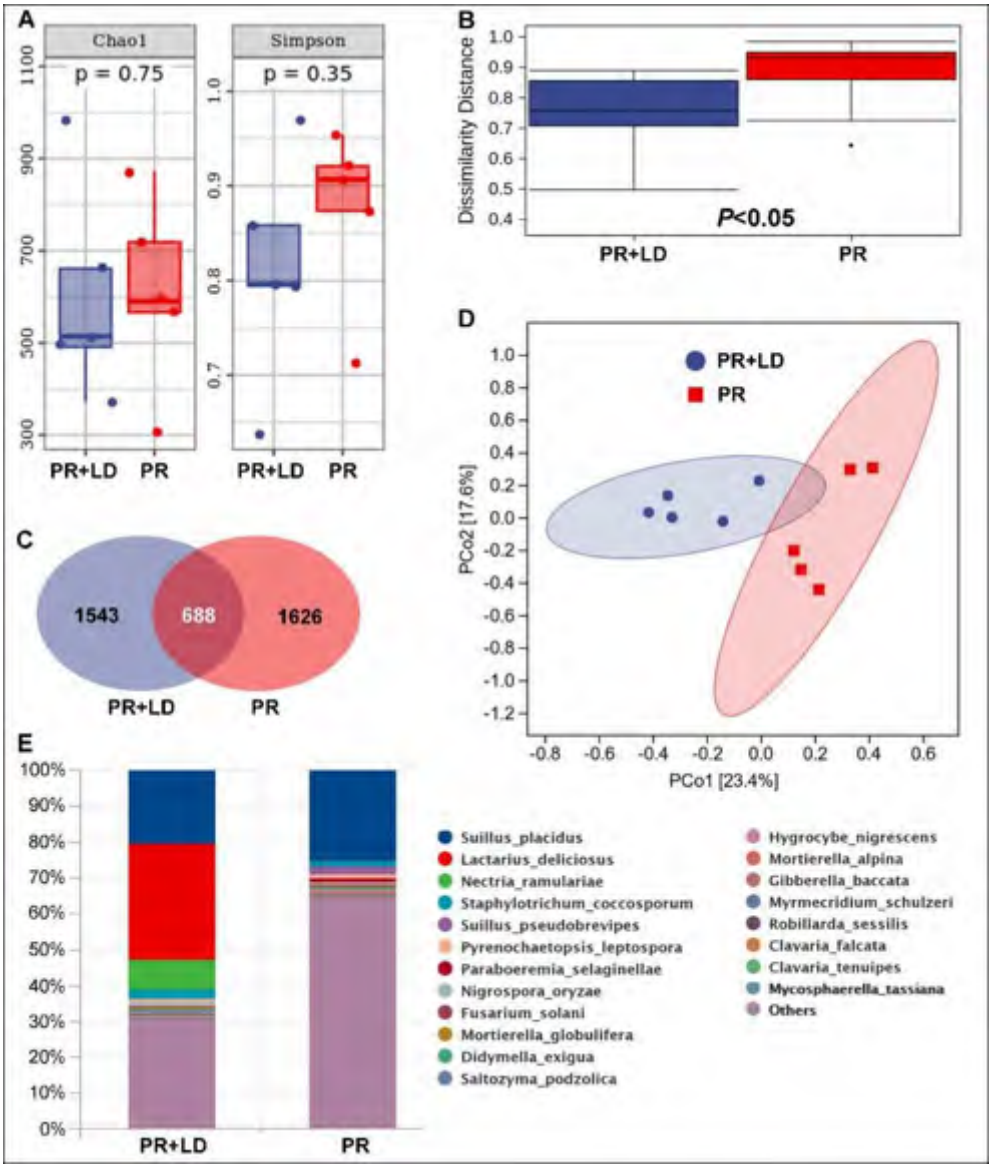

FIGURE 2 Mycorrhizosphere and rhizosphere fungal community of PR+LD and PR trees in the Xifeng plantation. (**A**) Chao1 and Simpson indexes. (**B**) Dissimilarity distance. (**C**) Venn figure showing shared and unique operational taxonomic units (OTUs) between samples of two trees. (**D**) Principal Coordinate analysis (PCoA). (**E**) Relative abundance of fungal composition at the species level.

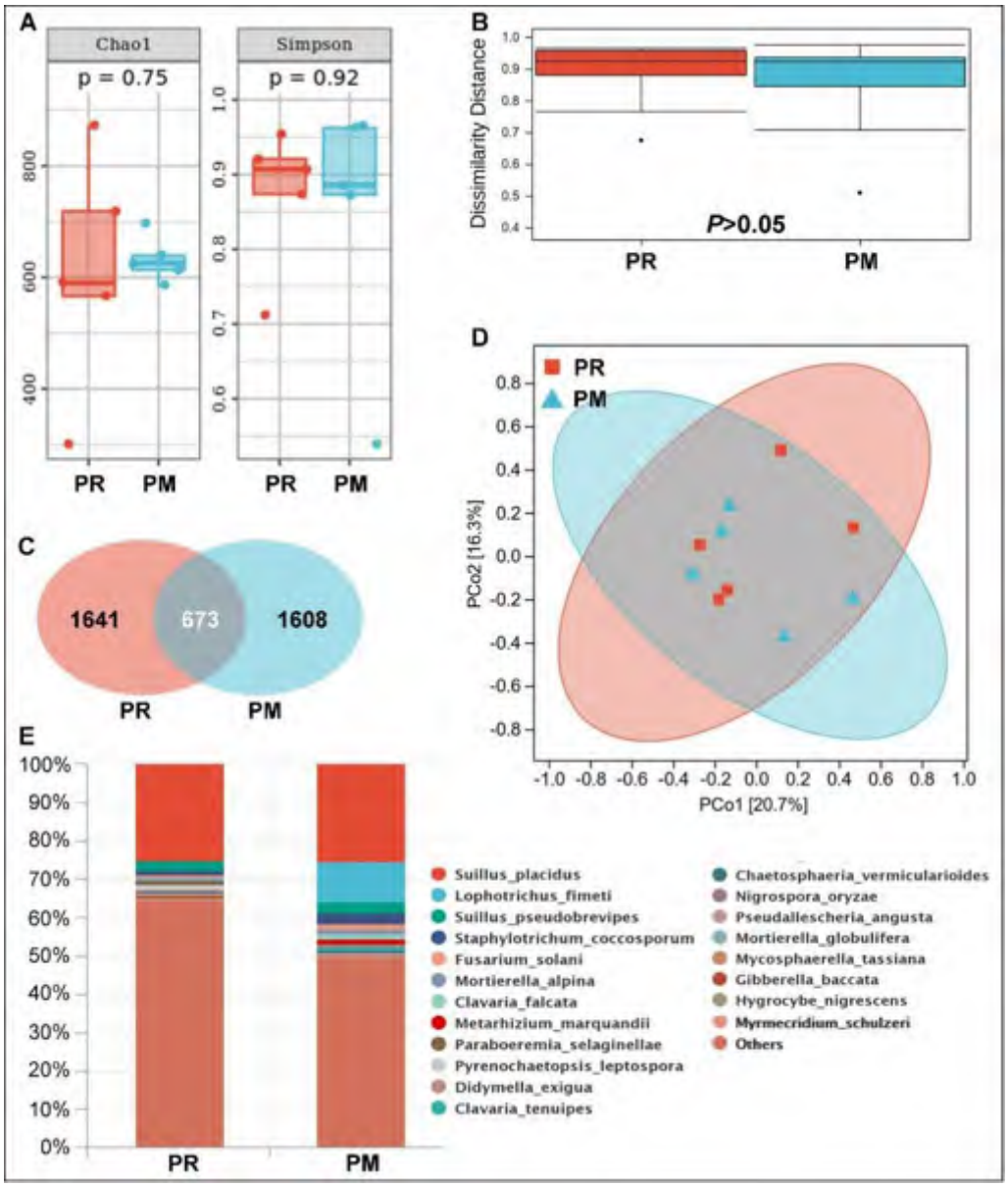

FIGURE 3 Rhizosphere fungal community of PR and PM trees in the Xifeng plantation. (**A**) Chao1 and Simpson indexes. (**B**) Dissimilarity distance. (**C**) Venn figure showing shared and unique operational taxonomic units (OTUs) between samples of two trees. (**D**) Principal Coordinate analysis (PCoA). (**E**) Relative abundance of fungal composition at the species level.

EcMF Community Composition in Early Establishment

PR and PM seeds germinated in the soil from Xifeng plantation after three months and only two PM seedlings formed one type mycorrhizae, and it was identified as *Suillus* sp. by molecular identification. After six months, PR and PM formed *Suillus*-like mycorrhizae (**Figure 5A, B**). The mycorrhizae samples were also identified as *Suillus* sp. (GenBank No: OM131553 and OM131554). Illumina sequencing detected OTUs as *Suillus placidus* (OTU_1487 and OTU_4508) (**Supplementary Table 2**). EcMF colonization rates of the two hosts showed no significant difference (**Table 4**).

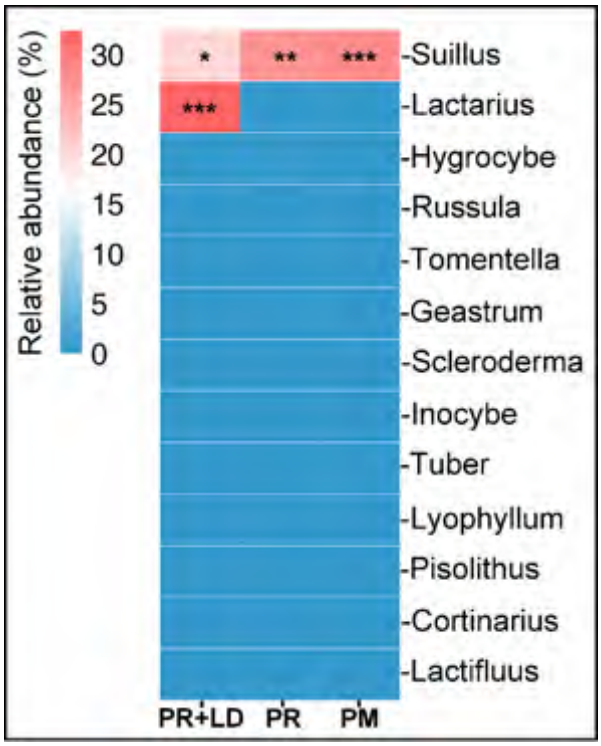

FIGURE 4 Heatmap of the relative abundance of ectomycorrhizal fungi OTUs at genus from mycorrhizosphere and rhizosphere soil of PR+LD, PR and PM (agglomerated to genus). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

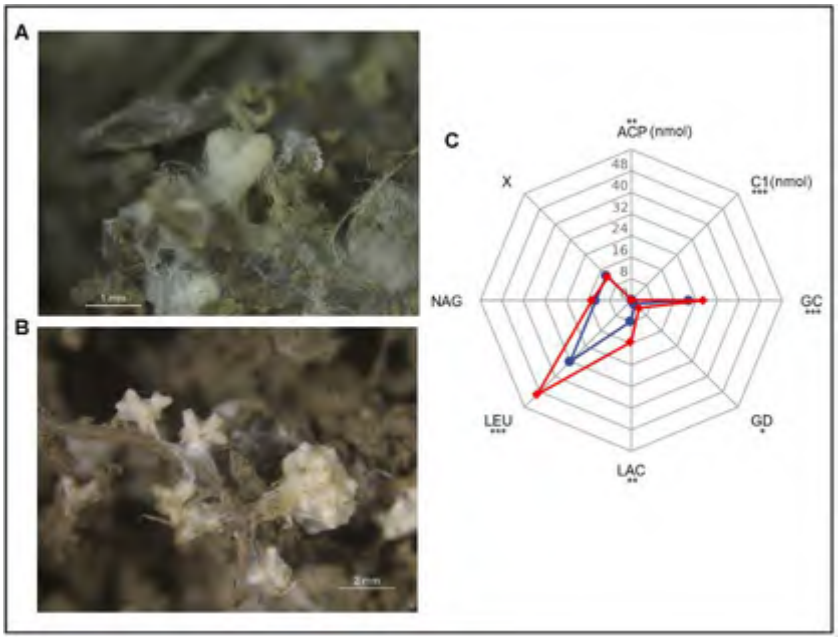

FIGURE 5 Bioassay experiment in greenhouse (**A**) Mycorrhizal root tips of PR. (**B**) Mycorrhizal root tips of PM. (**C**) Enzyme activity (pmol tip^{-1} min^{-1}/ C1 and ACP: nmol tip^{-1} min^{-1}) profiles of mycorrhizal root tips. PR seedlings-red line, PM seedlings-blue line. Abbreviations: C1-cellobiohydrolase, GC-β-glucosidase, GD-β-glucuronidase, LAC-laccase, X-β-xylosidase, LEU-leucine aminopeptidase, NAG-N-acetylglucosaminidase, ACP-acid phosphatase. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE S2 OTUs shared by single root tips in identification and Illumina sequencing data in the greenhouse bioassay experiment.

Species	Best-fit of Illumina OTU	Best-fit of GenBank	SH	Species origin
Suillus placidus	OTU_1487	MN258691	SH1555178.08FU	China
Suillus placidus	OTU_4508	KU721182	SH1555181.08FU	China

TABLE 4 Mean levels of seedling vigor status in greenhouse bioassay experiment. Data represents mean with the standard error in parentheses

	PR + *Suillus* (*n* = 15)	PM + *Suillus* (*n* = 15)
Shoot biomass	0.48 (0.014)	0.19 (0.006)
Root biomass	0.34 (0.004)	0.106 (0.009)
Root tip number	243 (7.52)	229.20 (11.69)
ECM colonization (%)	25.40 (3.26)	28.94 (1.89)
C (mg g^{-1})	489.78 (13.51)	469.19 (4.38)
N (mg g^{-1})	9.27 (0.51)	12.54 (0.79) *
P (mg g^{-1})	7.20 (0.50)	8.20 (0.70)

Asterisks indicate t-tests with significance levels at $P < 0.05$*, $P < 0.01$**, $P < 0.001$***

Exoenzyme Activities of Ectomycorrhiza

Eight enzyme activities of tips indicated a clear separation of enzymatic functions associated with the two hosts (**Figure 5C**, **Supplementary Table 3**). *Suillus* sp. associated with PR seedlings had relatively higher C1, GC, GD, LAC, LEU and ACP activities than those of PM seedlings. There was no significant differences of X and NAG activities between the two hosts. Total enzyme activity of PR was significantly greater than that of PM (P<0.001).

The Effect of Exoenzyme Activities on Seedling Nutrient Acquisition

Extracellular enzyme activities showed significant effects on seedling biomass and tissue nutrient concentrations (**Supplementary Table 4**). The Pearson correlation analysis revealed a positive correlation between LAC, NAG and ACP activity and shoot biomass of PR seedlings, and between LEU activity and root biomass of PR seedlings. P content of PR tissue was associated with GD enzyme activity. For PM seedlings, X enzyme activity had a positive correlation with C content.

TABLE S3 Eight enzyme activities of mycorrhizal root tips of two hosts in the greenhouse bioassay experiment.

	PR + *Suillus* (*n*=15)	PM + *Suillus* (*n*=15)
C1[1] (nmol mm^{-2} min^{-1})	0.19 (0.00045) ***	0.16 (0.0001)
GC (pmol mm^{-2} min^{-1})	26.95 (0.009) ***	21.19 (0.93)
GD (pmol mm^{-2} min^{-1})	4.03 (1.22) *	1.88 (0.1)
LAC (pmol mm^{-2} min^{-1})	15.43 (1.77) **	7.72 (0.02)
X (pmol mm^{-2} min^{-1})	12.2 (0.076)	13.02 (0.72)
LEU (pmol mm^{-2} min^{-1})	49.41 (1.86) ***	32.06 (0.9)
NAG (pmol mm^{-2} min^{-1})	14.59 (1.06)	13.08 (0.59)
ACP (nmol mm^{-2} min^{-1})	0.37 (0.02) **	0.3 (0.02)

[1]C1−cellobiohydrolase, GC−β-glucosidase, GD–β-glucuronidase, LAC–laccase, X–β-xylosidase, LEU–leucine aminopeptidase, NAG–N-acetylglucosaminidase, ACP – acid phosphatase, *P* = significance, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE S4 The Pearson correlation analysis between eight enzymes and seedling vigor status (*Pinus radiata* seedling N = 15, *P. massoniana* seedling N = 15. *P* = significance (<0.05 in bold).

Host	Variable	C1	GC	GD	LAC	X	LEU	NAG	ACP
PR	Shoot biomass	-0.542	0.041	-0.592	0.523	0.294	0.192	0.643	0.631
		***P*=0.037**	*P*=0.885	***P*=0.02**	***P*=0.045**	*P*=0.288	*P*=0.439	***P*=0.01**	***P*=0.012**
	Root biomass	-0.347	-0.207	-0.107	0.215	0.435	0.622	0.007	0.007
		P=0.205	*P*=0.46	*P*=0.705	*P*=0.441	*P*=0.105	***P*=0.013**	*P*=0.979	*P*=0.982
	C	0.264	0.007	0.044	-0.009	-0.197	-0.065	-0.207	-0.146
		P=0.342	*P*=0.98	*P*=0.876	*P*=0.976	*P*=0.483	*P*=0.817	*P*=0.459	*P*=0.603
	N	0.352	0.286	0.201	-0.265	-0.390	-0.583	-0.069	-0.023
		P=0.198	*P*=0.301	*P*=0.472	*P*=0.339	*P*=0.151	***P*=0.022**	*P*=0.808	*P*=0.934
	P	0.221	0.194	0.53	-0.306	-0.274	-0.417	0.137	0.148
		P=0.429	P=0.489	***P*=0.042**	*P*=0.267	*P*=0.322	*P*=0.122	*P*=0.628	*P*=0.599
PM	Shoot biomass	-0.037	-0.274	-0.415	-0.449	-0.23	-0.067	0.117	0.083
		P=0.896	*P*=0.324	*P*=0.124	*P*=0.093	*P*=0.41	*P*=0.812	*P*=0.677	*P*=0.769
	Root biomass	-0.037	0.108	0.186	0.131	0.149	0.461	-0.129	0.234
		P=0.997	*P*=0.702	*P*=0.507	*P*=0.643	*P*=0.595	*P*=0.084	*P*=0.646	*P*=0.401
	C	-0.172	0.209	0.374	-0.078	0.586	-0.041	-0.594	-0.254
		P=0.54	*P*=0.454	*P*=0.17	*P*=0.782	***P*=0.022**	*P*=0.886	***P*=0.019**	*P*=0.361
	N	0.209	0.013	-0.209	-0.015	0.243	-0.04	-0.08	0.247
		P=0.455	*P*=0.964	*P*=0.456	*P*=0.956	*P*=0.382	*P*=0.887	*P*=0.777	*P*=0.375
	P	-0.388	0.26	0.334	-0.364	-0.088	0.017	-0.067	0.096
		P=0.154	*P*=0.35	*P*=0.224	*P*=0.182	*P*=0.755	*P*=0.952	*P*=0.811	*P*=0.733

DISCUSSION

In this study, we found no effects of co-introduced *Lactarius deliciosus* on plant growth and nutrient acquisition of exotic PR in the Xifeng plantation. LD had also no effects on rhizosphere fungal richness or diversity, but significantly altered its fungal composition. Compared with the native PM pines in the plantation, exotic PR pines showed no difference in rhizosphere and root fungal communities but had higher total EcMF enzymatic activity than PM pines. All plant species were well colonized by native EcMF *Suillus* sp., and co-introduced LD was not replaced by local EcMF for the moment.

Adopted Strategy for Establishment of Exotic *Pinus radiata* in New Site

In the plantation, co-introduced LD dominated in the mycorrhizosphere of PR+LD trees and high relative abundance of LD had positive correlation with nitrogen acquisition of needles of PR. In addition, it was easy to find LD ectomycorrhizas around PR+LD trees (**Figure 1C**) and LD fruiting bodies were collected in 2020 (13 fruiting bodies) and 2021 (11 fruiting bodies). Whilst a EcMF *Suillus* sp. formed familiar association with exotic PR and the mycorrhizas contributed relatively higher extracellular enzyme than

PM-*Suillus* mycorrhiza. No significant difference on plant growth and nutrient acquisition between PR+LD and PR indicated that either co-introduced LD or familiar association with local EcMF help PR establish.

Vlk et al (2020 a) proposed that the share of strategies that alien Pinaceae species adopted to establish EcMF partnership in new areas differed greatly among biogeographic regions. Exotic Pinaceae were almost exclusively associated with co-introduced EcMF in areas without native Pinaceae (e.g., Africa, Oceania and South America) whereas familiar or novel association with native EcMF prevailed in regions where other Pinaceae family members naturally occur. In Asia, only very few co-introduced EcMF associated with exotic Pinaceae are found in new areas where other Pinaceae species naturally occur. In our study, the Xifeng plantation is about one kilometer away from native PM forests and it was used for planting crops for a long time. The soil spore banks may be limited, but we still found *Suillus* spp. with high relative abundance in soil, which is likely to come from extensive and long-lived soil spore banks of resistant propagules or native PM forests by spore dispersal (Peay et al., 2011).

Persistence of *Lactarius deliciosus* in a New Site

LD persisted in mycorrhizosphere soil with high relative abundance and LD fruiting bodies have been producing for two years in the Xifeng plantation. An introduced species has the potential to reach any of four stages: transport, establishment, spread, and impact (Lockwood et al., 2007). Vellinga et al (2009) grouped fungal examples from the literature into five different outcomes: (1) fail to establish; (2) be replaced by local fungi; (3) persist with introduced trees but fail to grow with local hosts; (4) persist with introduced trees and spread to local hosts; or (5) fail to persist with introduced trees but nonetheless spread to local hosts. Our study so far demonstrated that LD may persist with introduced PR and naturalize outside the native range and we didn't find LD grow with local PM trees for the moment.

Interaction Effects Between Local EcMF Communities and Hosts

We found that *Suillus* spp. with high relative abundance was detected in the mycorrhizosphere and rhizosphere soil in the Xifeng plantation. Fruiting bodies of *Suillus* were collected around PR trees (**Figure 1G**). *Suillus* or *Rhizopogon* species showed host-specificity to the Pinaceae (Ashkannejhad and Horton, 2006; Collier and Bidartondo, 2009; Nunez et al., 2009) and were among the earliest colonizers of isolated seedlings (Peay et al., 2012). In the greenhouse experiment, PR and PM seedlings were both well-colonized by *Suillus* spp. after 6-months. *Suillus* on PR showed relatively higher activity with cellulases, hemicellulases, β-glucosidase, P- and N- containing organic compounds and oxidative enzyme, which had significant positive correlation with shoot biomass, root biomass and phosphorus content of PR.

Ning et al (2021) proposed that caution should be taken in the use of exotic hosts in afforestation process because of the potential shift of assembled EcMF community under exotic trees may alter below ground functional capabilities and symbiosis-mediated successional trajectories. However, we found the same EcMF community assemble patterns in our exotic and native pine hosts in the early stage: both PR and PM seedlings were well-colonized by *Suillus* sp.. And in plantation, fungal and bacterial

richness, diversity and composition in rhizosphere or roots were not different between PR and PM (**Supplementary Figure 3 a, b**). In our experiment we could not detect any shift of the local EcMF community and host identity had no significant effect on EcMF communities. Here we suggest that PR is a suitable pine species for restoration of degraded lands in Xifeng.

Peay et al (2011) proposed that in a noninvasive context, suilloid fungi often act as early successional species able to colonize pine seedlings, but they may be later displaced by late-successional fungi. Our current study was based on a 3-year old plantation, so long-term monitoring is needed for better understanding the persistence of *Lactarius* and possible shifts of soil EcMF communities.

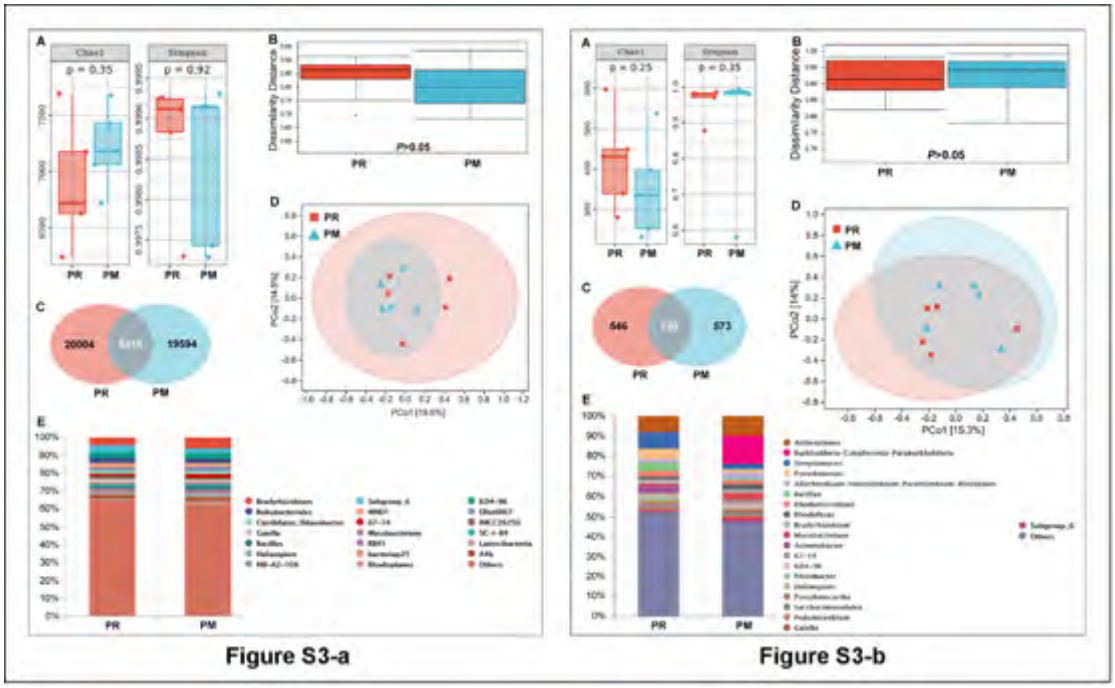

FIGURE S3 a Rhizosphere bacterial community of PR and PM trees in the Xifeng plantation. **b** Bacterial community of roots of PR and PM trees in the Xifeng plantation. (**A**) Chao1 and Simpson indexes. (**B**) Dissimilarity distance. (**C**) Venn figure showing shared and unique operational taxonomic units (OTUs) between samples of two trees. (**D**) Principal Coordinate analysis (PCoA). (**E**) Relative abundance of fungal composition at the genera level.

Co-introduced *Lactarius* Altered Mycorrhizosphere Fungal Communities

Last but not least, co-introduced LD significantly altered the mycorrhizosphere fungal composition in the Xifeng plantation. Extraradical mycelium expansion of inoculated LD in the soil may compete for nutrients and produce secondary metabolites that affect microbe community composition of the mycorrhizosphere (Fransson 2014). Composition of EcMF communities may largely affect ecosystem processes, such as carbon (Chapela et al., 2001) or nutrient (Read et al., 2003, Clemmensen et al., 2014) cycling. It is still needed to further verify whether the changes in fungal community composition influence nutrient cycling in local soils and nutrient uptake of exotic PR as well as LD's capacity to expand into the native forests becoming an invasive species.

CONCLUSION

This study analyzed the interaction effect of EcMF, including co-introduced *Lactarius deliciosus* and local EcMF communities, on the establishment of exotic *Pinus radiata* seedlings in Guizhou, southwest China. The present results revealed that introduced *P. radiata* seedlings could be successfully established in this new habitat by co-introducing *L. deliciosus* or by letting them form familiar associations with local EcMF. Co-introduced *L. deliciosus* persisted in the Xifeng plantation after 3 years and was not replaced by local EcMF. Some cosmopolitan species from *Suillus* genus are common in rhizosphere soil and root tips. An EcMF, *Suillus* sp. in the local habitat formed familiar association with *P. radiata* and we did not observe any shift of EcMF during early establishment of *P. radiata*. Host identity had no effect on fungal community composition since *P. radiata* and native *P. massoniana* recruited similar EcMF communities. But *L. deliciosus* pre-colonization significantly altered the mycorrhizosphere fungal composition. Our study suggests that it is possible to establish exotic *P. radiata* plantations in Xifeng and provides insights into a better understanding of the interaction effects between co-introduced/local EcMF and an exotic pine species. Moreover, a long-term study should be conducted to monitor the local EcMF community changes induced by the introduced *L. deliciosus*.

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AUTHOR CONTRIBUTIONS

RW: conducted the experiment, analyzed the data, and prepared he manuscript. YLW: conducted the experiment, analyzed the data and contributed the manuscript. AGL: contributed to fungal materials and revised the manuscript. PZ: collected experimental materials and conducted the experiment. CC: guided in the experiment plan and revised the manuscript. FQY: supervised the research and edited the manuscript. All authors contributed to the article and approved.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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GENERAL DISCUSSION

The presented thesis aims to (i) accomplish ethnomycological study on wild mushrooms and ectomycorrhizal fungi diversity, (ii) study on new commercial ectomycorrhizal fungal species by taxonomic research and mycorrhizal synthesis, (iii) test *Tuber melanosporum* and native Chinese oak species forming stable mycorrhizal symbioses and (iv) provide insights into plantation establishment by analyzing the interaction effect of ectomycorrhizal fungi, including co-introduced *Lactarius deliciosus* and local ectomycorrhizal fungal communities, on the establishment of exotic *Pinus radiata* seedlings.

A total of eleven markets and surrounding natural habitats were visited for three years in chapter I. Ethnic groups are the major population in markets and different markets have different sale time to sell mushrooms according to the local people's different lifestyles. We recorded a wealth of ethnomycological knowledge through interviews and collected abundant wild mushroom samples from local markets and forests in three consecutive years. Mushroom harvesting is a challenging activity that requires a deep local environmental knowledge to achieve success. The knowledge of selecting mushrooms has usually passed from generation to generation. Sales activities of wild mushrooms can be carried out uniformly in established markets, while local government strengthen the sales supervision of markets to make the sale of wild mushrooms more standardized and reduce the probability of wild mushroom poisoning. In each market, the male to female ratio of vendors showed that women outnumber men. It seems that in many regions of the world women are often the main collectors (Cui et al., 2018; Wang et al., 2015; Zhang et al., 2013). But women usually collected mushrooms closest to their houses, while men go farther to collect. Therefore, men usually have developed a more profound knowledge on the biology, ecology, and phenology of mushrooms and were able to identify them, even at a species level. In addition, the age of collectors was mainly between 45-65 years old and only few young people were involved in mushroom collecting or selling. Traditional knowledge is being lost through economic change, modernization, urbanization and even formal education. Therefore, further research on ethnomycology is urgent to preserve the current knowledge before their lost forever. A total of 101 species collected in the markets, belong to 22 families and 39 genera, and about 76% of them are ectomycorrhizal fungi. The 283 species collected in the natural habitats, belong to 52 families and 100 genera, and about 70% are ectomycorrhizal fungi. Mushroom species and amount showed overlap between markets and the natural habitats on different months. Preference of wild edible mushrooms for locals was mostly related to their availability in the forests. Yu et al (2003) surveyed markets in Pu'er from 2002 to 2009 and reported a sharp decline of WEF production of 43 species, such as *Lactifluus volemus*, *Russula griseocarnosa*, *Termitomyces* spp. and *Thelephora ganbajun* which were considered as important in Yunnan. In our study, interviews with vendors showed that production of these species had declined even more in recent years and they had to travel farther to collect them. In our study, 57 good edible species that we found in

nature were not sold in markets. Very tasty species as *Amanita subhemibapha*, *Boletus violaceofuscus* and *Laccaria amethystina* have beautiful color and good production in the forests, but they were not recorded in the markets maybe due to the fact that they are preferred for self-consumption rather than commercialization. The utilization of WEF resources in Pu'er still has great potential to be developed.

Some species collected from markets and fields are undescribed species according to taxonomical and molecular results in our study. Discovery of more new valued mushroom species will contribute to the sustainable use of WEF resources. In chapter II and III, two new commercial ectomycorrhizal delicacies were identified by morphological and molecular phylogenetic analyses. One species was described as *Rhizopogon songmaodan* sp. nov. belonging to the subgenus *Versicolores* based on morphological and molecular phylogenetic analyses. *R. songmaodan* is the tenth *Rhizopogon* species reported from China. Morphologically *R. songmaodan* is similar to *R. evadens* in having white rhizomorphs and becoming reddish when basidiomata are cut. However, *R. songmaodan* has thinner peridium and phylogenetic analysis showed that *R. songmaodan* is grouped in a different subclade than *R. evadens*. *R. jiyaozi* is the most popular edible *Rhizopogon* species in Yunnan and Sichuan Provinces (Li et al., 2016). Morphologically *R. jiyaozi* and *R. songmaodan* are similar in having white and discolored basidiocarps. However, *R. jiyaozi* belongs to the subgenus *Roseoli* and is different from *R. songmaodan* by presenting yellow staining in the peridium. Additionally, *R. jiyaozi* produces basidiocarps between August and October each year, later than *R. songmaodan*. In China, the mycorrhizae of a few *Rhizopogon* species were synthesized by mycelium inoculation and showed the potential of mycorrhizal seedlings in afforestation (Shao 2013; Li 2013). In this study, two typical pine species, *Pinus armandii* and *P. yunnanensis* from central Yunnan, were inoculated with spores of *R. songmaodan*, and abundant ectomycorrhizae were produced after four months. Considering the commercial and ecological value of *R. songmaodan*, and the inoculation efficiency (100% mycorrhizal colonization rate), it could be a good candidate for cultivation in Southwest China. However, further work on mycorrhizal synthesis and trial plantations are needed to turn the cultivation of this species into a powerful rural development tool capable of generating income for farmers in less favored rural areas.

Another delicacy was described as *Choiromyces sichuanensis* sp. nov. in chapter III. The present morphological and molecular results confirmed that at least three species of *Choiromyces cerebriformis*, *C. helanshanensis* and *C. sichuanensis* were within the genus *Choiromyces* in China. Morphologically, ascosporal ornamentation in *C. sichuanensis* had longer blunt spine than that in two other Chinese *Choiromyces* species. Phylogenetic analyses based on ITS sequences indicated that the *Choiromyces* Chinese specimens formed an independent clade with high Bayesian Posterior Probability and were easily distinguishable from *C. meandriformis*. Therefore, these morphological and molecular results provided strong support for C. *sichuanensis* as a new species (100% and 98% PP). In China, little is known about hypogeous fungi other than the genus

Tuber. However, the abundance and diversity of *Tuber* species in southwest China indicate a potential biodiversity of other hypogeous fungi in this region. The ectomycorrhiza between C. *sichuanensis* and *Pinus armandii* was well developed, while no such an ectomycorrhiza formation was observed on *Picea* roots. Both morphological descriptions and molecular identification fully confirmed the real identities of mycorrhization between C. *sichuanensis* and *P. armandii* as the ITS sequences of the synthesized mycorrhizae were identical to the original ascomata used for inoculation. This result is the first report on the synthesized mycorrhizae between pine plants with a commercial pig truffle of *C. sichuanensis.* We had not observed ECM formation either from *Picea likiangensis* or *Pi. crassifolia*, although *C. sichuanensis* samples might come from a *Picea* dominated forest, which has to be confirmed in nature. In addition, further studies are required to better understand the relationships between *C. sichuanensis* and other host trees, no matter whether they are belonging to the *Pinus* genus or other ectomycorrhizal plant species. The expected results could provide management strategies for developing mushroom cultivation under planted and natural forests.

The important step in ectomycorrhizal fungal cultivation is the production of host seedlings well colonized by the target species (Guerin-Laguette 2021). While partner selection in the mycorrhizal symbiosis is thought to be a key factor stabilizing the mutualism (Werner 2014). In chapter IV, the mycorrhizae obtained between *T. melanosporum* and five Chinese oak species were well developed and displayed similar shape and color to those obtained on Mediterranean *Quercus* spp., the natural hosts of the Périgord black truffle (Guerin-Laguette et al. 2013). External characteristics include a brown-red mantle with abundant emanating hyphae. Mounted mycorrhizal tips revealed the typical jigsaw puzzle pattern of the mantle cells (Giraud 1988), while cross-sections revealed an “epidermal” Hartig net, i.e., confined to the external layer of elongated cortical cells, as expected in the majority of angiosperms (Smith and Read 2008). Altogether, these observations confirmed the formation of fully developed ectomycorrhizae of *T. melanosporum*. None of the uninoculated plants produced mycorrhizae except for a few seedlings showing traces of non-*Tuber* species 32 months after inoculation. These observations strongly suggest that the mycorrhizae obtained were of *T. melanosporum* only. Their identity was further confirmed using ITS sequence comparison. To our knowledge, this is the fourth study to describe a positive effect of the mycorrhization by *T. melanosporum* on the shoot growth of broad-leaf plants under greenhouse conditions. Domínguez et al. (2008) reported a significant increase in height, shoot, and root dry weights of *Quercus petraea* seedlings that were, however, only predominantly mycorrhized by *T. melanosporum*, i.e., with a slight colonization by nursery-contaminating mycorrhizal fungi. In the present work, 2 years after inoculation, no other ectomycorrhizal species were detected on any of the seedlings, control or inoculated. Our results constitute the third evidence that such a beneficial growth effect on broad-leaf plants was due to *T. melanosporum* only. The present study is a further confirmation that, at least in the early stage of the mycorrhizal association, *T. melanosporum* behaves like a typical ectomycorrhizal fungus, enhancing the growth of

its host plants. In addition, the underlying eco-physiological mechanisms of *T. melanosporum*-promoted *Q. mongolica* growth was studied 37 months after inoculation (Wang et al., 2021). The study results strongly suggest that mycorrhization by *T. melanosporum* can similarly modify the endogenous hormonal balance of the host plant, triggering budbreak in the frost-free, stable environment of a glasshouse. *T. melanosporum* ECM colonization may regulate carbon economy and rhizosphere bacterial communities of *Q. mongolica* seedlings grown in a previously sterilized peat-based substrate, to promote plant growth and nutrient cycling. *T. melanosporum* could improve the performance of out-planted mycorrhizal seedlings will be a very valuable feature for future afforestation programs.

It should be noted that our mycorrhizal synthesis experiments are small studies in greenhouse. Field trials should be undertaken to further explain the relationship between selected inoculant fungus, soil microbe and host trees. Last chapter provided insights into establishment of exotic pine plantation. In chapter V, co-introduced *Lactarius deliciosus* dominated in mycorrhizosphere of *Pinus radiata* trees and high relative abundance of *L. deliciosus* had positive correlation with nitrogen acquisition of needles of *P. radiata*. Ectomycorrhizas and fruiting bodies of *L. deliciosus* were found in plantation, which indicated *L. deliciosus* could not be replaced by local ectomycorrhizal fungi for the moment. However, we found no effects of co-introduced *Lactarius* on plant growth and nutrient acquisition of exotic *P. radiata* in plantation. Through high-throughput sequencing, we found that *Suillus plasidus* with high relative abundance was detected from mycorrhizosphere and rhizosphere soil in the plantation. Fruiting bodies of *Suillus plasidus* were collected around *P. radiata* trees. Such as *Suillus* or *Rhizopogon* species showed host-specificity to the Pinaceae (Ashkannejhad and Horton, 2006; Collier and Bidartondo, 2009; Nunez et al., 2009) and were among the earliest colonizers of isolated seedlings (Peay et al., 2012). Familiar or new association with local ectomycorrhizal fungi also helped *P. radiata* establish in a new area. The greenhouse bioassay experiment further proved that a local EcMF, *Suillus* sp. from soil, contributed relatively higher extracellular enzyme by forming ectomycorrhizas with *P. radiata*., and there was no fungal community shuffling by *P. radiata* during early establishment. Our study allows establishment of exotic *P. radiata* in Xifeng and provides insights into a better understanding of interaction effects between co-introduced/local EcMF and exotic pine. Moreover, a long-term study should be conducted since our study was based on a 3-year young plantation and local EcMF community changes by the introduced *L. deliciosus*.

FINAL CONCLUSIONS

- Pu'er Prefecture is rich in local mycological knowledge and fungi diversity. However, it is necessary to continue the research of ethnomycology in order to preserve existing knowledge, since knowledge of fungi remains mainly among the elderly population. And the rational management of WEF species with high yield in natural areas and the collection and use of ectomycorrhizal fungi germplasm resources for cultivation will benefit the sustainable utilization of local wild edible fungi.

- *Rhizopogon songmaodan* sp. nov. is the tenth *Rhizopogon* species reported from China.

- Abundant ectomycorrhizae were produced from *Pinus armandii* after four months by inoculating with spores of *R. songmaodan*.

- *Choiromyces sichuanensis* sp. nov. is the third species of *Choiromyces* described in China.

- Both morphoanatomical and molecular analyses evidenced well-developed mycorrhization between *C. sichuanensis* and *P. armandii*, but not in *Picea* seedlings.

- From 6 months after inoculation, *Tuber melanosporum* ectomycorrhizas were successfully produced on five *Quercus* species endemic to China, as shown by morphological, anatomical, and molecular analyses.

- *Quercus mongolica* and *Q. longispica* showed high receptivity to mycorrhization by *T. melanosporum*.

- *T. melanosporum* markedly accelerated budbreak in *Q. mongolica*.

- *Pinus radiata* seedlings could be successfully established in new habitat by colonizing with *Lactarius deliciosus* or forming familiar associations with local ectomycorrhizal fungi.

- Co-introduced *L. deliciosus* could persist in the plantation and not be replaced by local ectomycorrhizal fungi.

- Local ectomycorrhizal fungi, *Suillus* sp. in the local habitat formed new association with *P. radiata* and no shift of ectomycorrhizal fungi during early establishment of *P. radiata*.

- Host identity had no effect on fungal community composition since *P. radiata* and

native *P. massoniana* recruited similar ectomycorrhizal fungal communities. But *L. deliciosus* pre-colonization significantly altered the mycorrhizosphere fungal composition.

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