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ORIGINAL ARTICLE

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.

Keywords Hypogeous basidiomycota · Edible fungi · Morphology · Phylogeny · Taxonomy · Ectomycorrhizae

Introduction

The genus *Rhizopogon* Fr. & Nordholm (Basidiomycota, Boletales, Rhizopogonaceae) contains approximately 212 species of hypogeous fungi (https://www.Indexfungorum.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

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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, and 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, 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$15–25 kg^{-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).

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

Table 1 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)

(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 1 h. 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 was 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°N,

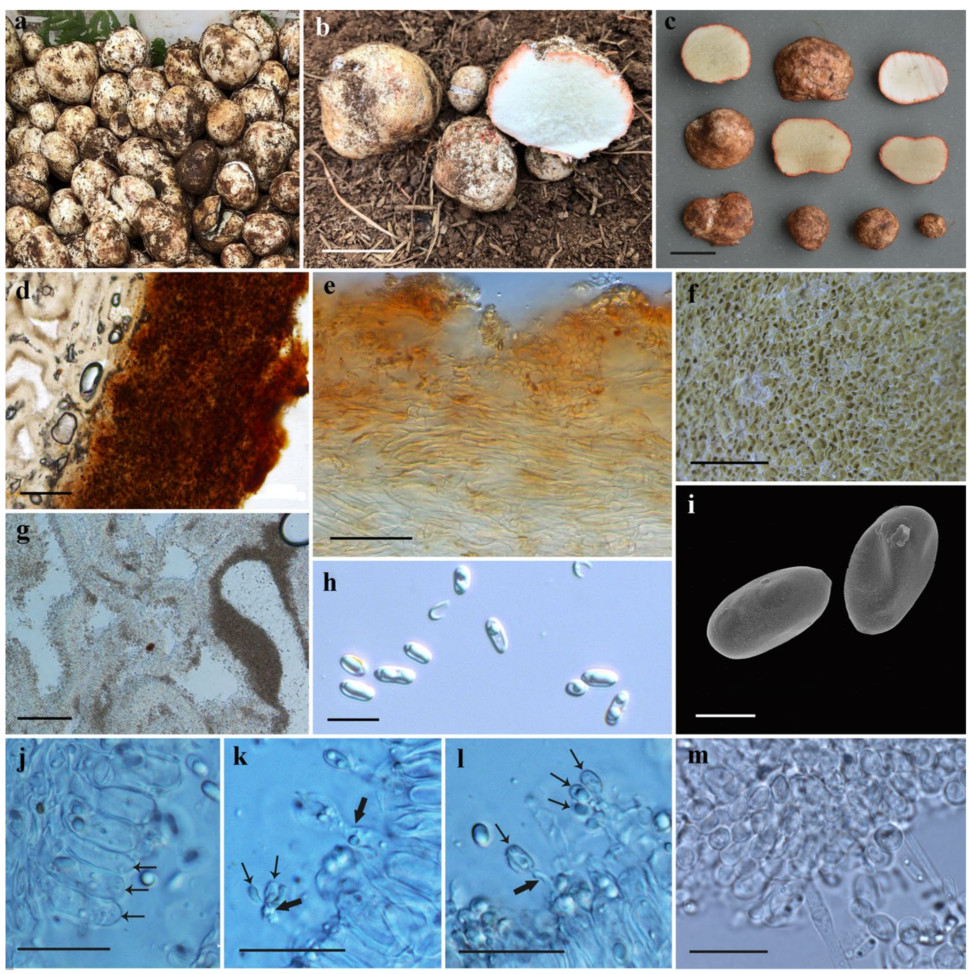

Fig. 1 *Rhizopogon songmaodan*. **a** Basidiomata sold at markets. **b, c** Basidiomata collected from *Pinus armandii* forest. **d, e** Cross section of peridium. **f** Cross section of gleba. **g** Cross section of irregular and sinuous glebal chambers. **h, i** Basidiospores. **j** Clavate to cylindrical 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

102.8839°E, alt. 2710 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 the forest of *Pinus armandii* Franch., 25.3855°N, 102.8846°E, alt. 2642 m, April 13, 2019, razy-237 (HKAS 106768); 25.3918°N, 102.8963°E, alt. 2634 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°N, 102.66346161°E, 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*.

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.

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 analyses 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

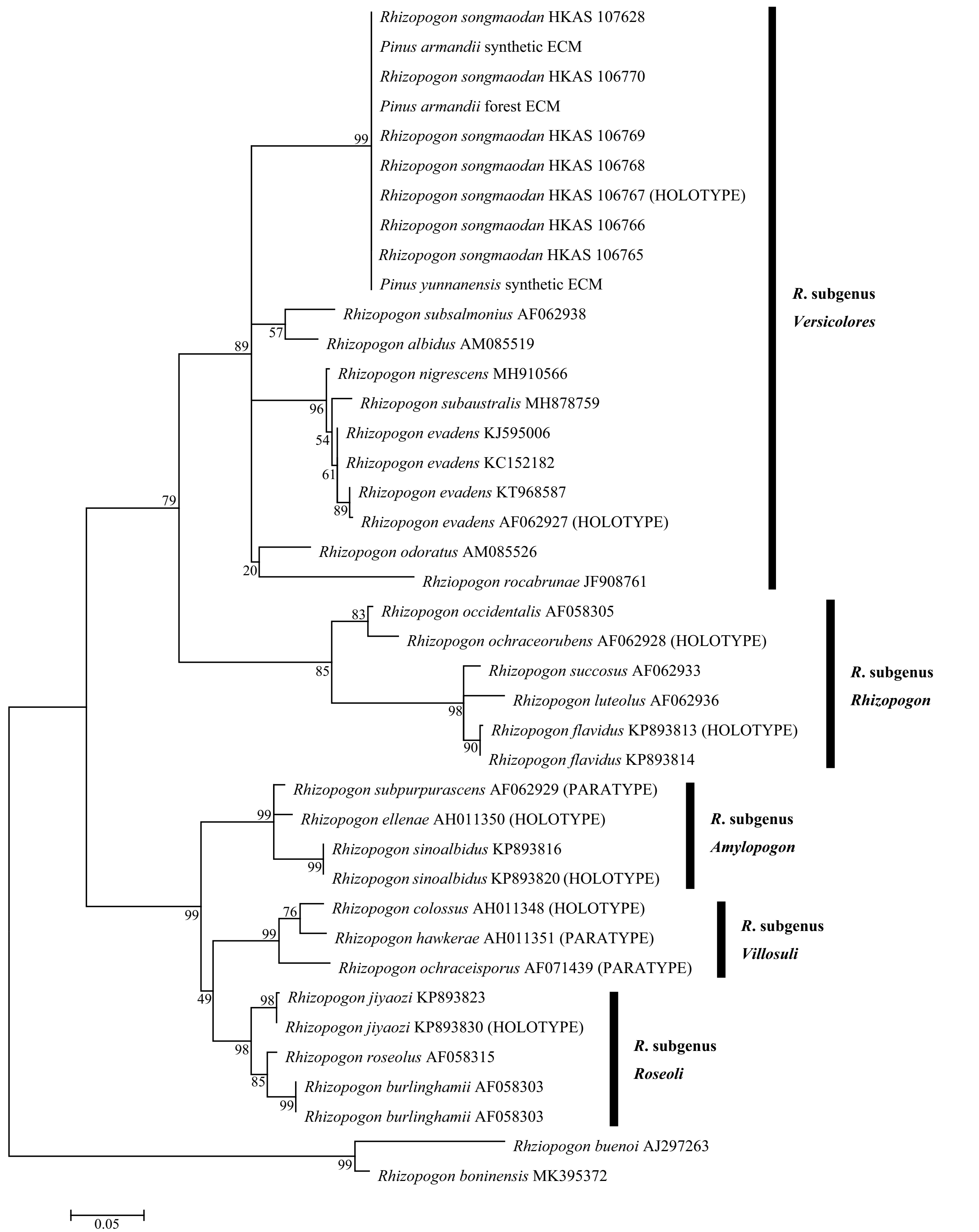

Fig. 2 Phylogenetic relationships among *Rhizopogon songmaodan* basidiomata, ectomycorrhizal root tips (ECM) sampled either in the forest or synthesized with *Pinus yunnanensis* or *P. armandii* in greenhouse and other species included in subgenera of the genus *Rhizopogon* based on their ITS rDNA sequences.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

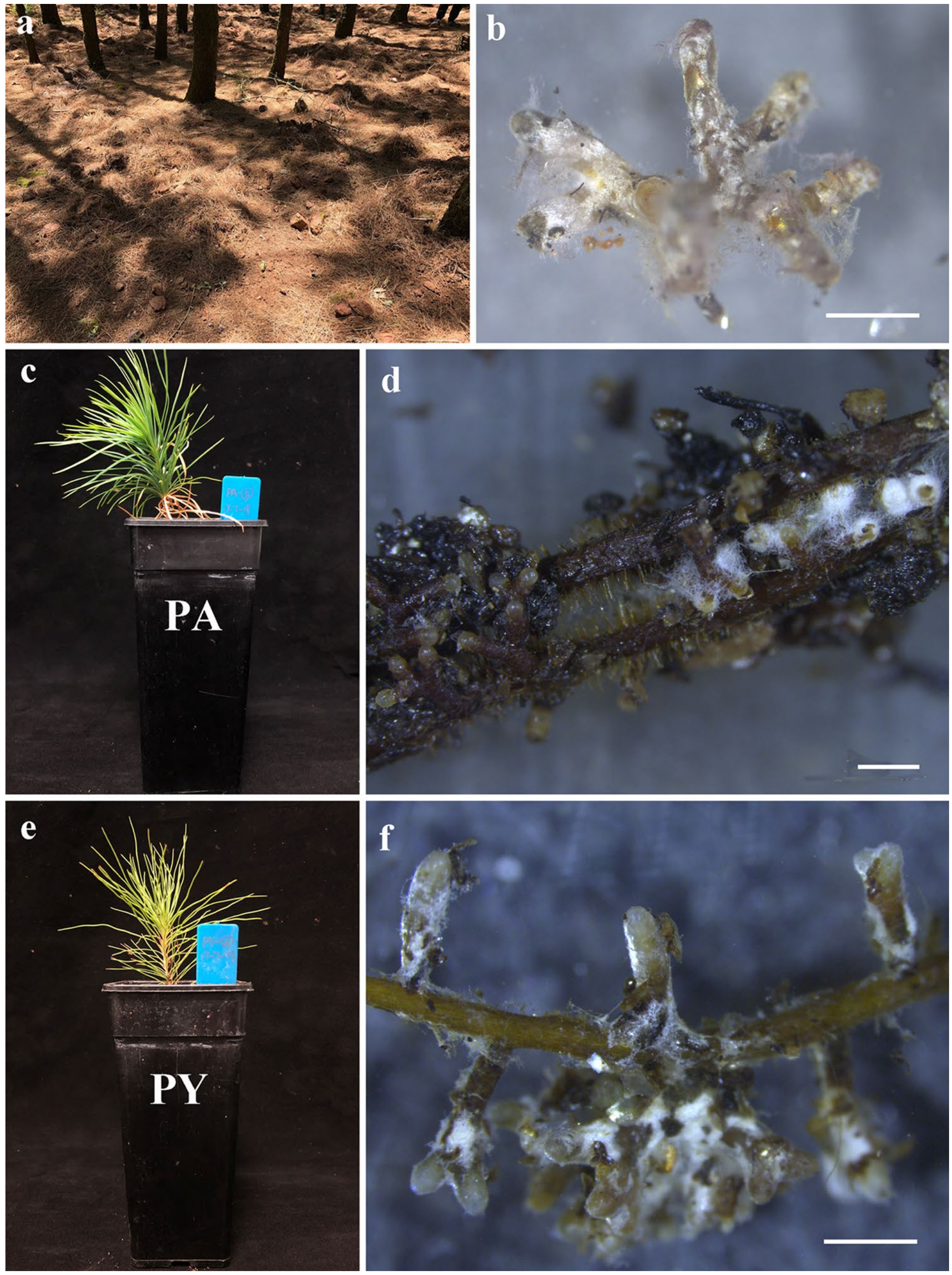

Fig. 3 **a** Habitat of *Rhizopogon songmaodan* in *Pinus armandii* forest. **b** Ectomycorrhizal root tip (ECM) sampled from *P. armandii* forest. **c** Four-month *P. armandii* seedling inoculated with *R. songmaodan* in pot. **d** Synthesized ECM of *P. armandii* with *R. songmaodan* in greenhouse.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

this study, two typical pine species, *Pinus armandii* and *P. yunnanensis* from central Yunnan, were inoculated with spores of *R. songmaodan*, and abundant ectomycorrhizae where produced after 4 months. Compared with 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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