Mycorrhiza (2008) 18:205–210
DOI 10.1007/s00572-008-0167-4

SHORT NOTE

Bipolar incompatibility system of an ectomycorrhizal basidiomycete, *Rhizopogon rubescens*

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Received: 13 September 2007 / Accepted: 4 February 2008 / Published online: 5 March 2008

Abstract The mating systems of most ectomycorrhizal fungi have not been elucidated because of two reasons. One is the difficulty of obtaining homokaryotic isolates for mating tests caused by the low germination rate of basidiospores, and another is the difficulty of checking dikaryotization caused by the absence or inconsistent production of clamp connections on heterokaryotic mycelia under laboratory conditions. Basidiospore germination of a few ectomycorrhizal fungi has been induced by living roots of their host plants. Based on this information, we examined methods to obtain homokaryotic isolates of *Rhizopogon rubescens* using its host plant, *Pinus thunbergii*. The basidiospores of *R. rubescens* appeared to germinate well on an agar plate, on which axenic pine seedlings were grown in advance to induce germination, even when the seedlings were removed from the plate at the time of spore inoculation. To enhance the production rate of clamp connections on the heterokaryotic mycelia of *R. rubescens*, the culture medium composition was modified. The pH of the medium was critical for the production of clamp connections, and the optimal pH was higher for the production of clamp connections than for mycelial growth. These findings made it possible to conduct mating tests, and we found that the mating system of *R. rubescens* is bipolar with a multiallelic mating type factor.

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Keywords Bipolar incompatibility · Multiallelic mating type · *Rhizopogon rubescens*

Introduction

Certain species of Ascomycetes and Basidiomycetes produce fruit bodies generally called mushrooms as their sexual reproduction organ. The sexual reproduction of Ascomycetes is fundamentally controlled by a simple mating system represented by a set of symbols such as A and a, or '+' and '−'. On the other hand, the sexual reproduction of higher Basidiomycetes is mostly regulated by two types of heterothallism, one is bipolar incompatibility with one mating factor and another is tetrapolar incompatibility with two unlinked factors A and B (Fincham et al. 1979). The heterothallic fungi have numerous mating types determined by multiple alleles of mating factors which increase intraspecific diversity.

The mating systems of saprotrophic basidiomycetes have been studied widely. By the 1940s, approximately 10% of 230 species of *Hymenomycetes* and *Gasteromycetes* were considered homothallic, 35% heterothallic and bipolar, and 55% heterothallic and tetrapolar (Whitehouse 1949). These mating systems are thought to control the balance between inbreeding and outbreeding of natural populations (Fincham et al. 1979); thus, the study of mating systems is important to understand the population structure, taxonomy, and evolution of fungi. However, the mating systems of most ectomycorrhizal basidiomycetes remain unknown.

There are two challenges to studying of the mating systems of ectomycorrhizal fungi. One is the difficulty of obtaining homokaryotic isolates for mating tests. Basidiospores of saprotrophic basidiomycetes generally germinate well on nutrient agar; however, basidiospores of ectomycorrhizal

fungi do not germinate easily under laboratory conditions, except some species in *Laccaria* (Fries 1977), *Suillus* (Fries et al. 1987; Fries 1988), *Tricholoma*, and a few other genera (Ohta 1986, 1988). Another is the difficulty of checking dikaryotization after crossing homokaryotic isolates due to the absence or inconsistent production of clamp connections on heterokaryotic mycelia cultured on artificial media.

Rhizopogon rubescens (Tul.) Tul. is an ectomycorrhizal basidiomycete with a worldwide distribution (Molina et al. 1999). In Japan, this fungus mainly inhabits the forests near the sea and lake, forming ectomycorrhizae with Japanese black pine, *Pinus thunbergii* Parl.; its fruit body is prized as an edible mushroom called "shouro" in Japanese and traded at a high price in local markets. Our specific objectives were to (1) obtain single spore homokaryotic isolates of *R. rubescens*, (2) optimize growth nutrients in agar culture to stimulate and visualize clamp connections on heterokaryotic mycelia, and (3) determine mating type and incompatibility factors of *R. rubescens*.

Materials and methods

Fungal materials

Heterokaryotic mycelia of *R. rubescens* were isolated from fruit bodies collected in five prefectures on Honshu Island, Japan (Fig. 1), from 1988 to 2005 using GY medium (glucose 20 g, yeast extract 2 g, agar 15 g, distilled water 1,000 ml; unless mentioned in particular, adjusted to pH 5.4 with 1 M HCl). These isolates were stocked at 5°C and subcultured every 2 years until they were used.

To obtain single spore isolates, mature fruit bodies were collected from two *P. thunbergii* forests around Lake Biwa in Shiga Prefecture, Japan, in April 2005 (sites T and site S in Fig. 1). One fruit body whose inner tissue was brown (mature but not overripe) was selected from each site, and a small part of the inner tissue was excised, placed in sterile water, and stirred to produce a basidiospore suspension. The concentration of the spore suspension was determined by counting spores with a Thoma hemacytometer under a light microscope. The heterokaryotic mycelia were also isolated from the same section of each fruit body.

Cultivation of pine seedlings

Because no basidiospore germinated on GY plates, axenic seedlings of *P. thunbergii* were used as the germination inducer, following the methods of Kropp and Fortin (1988). Pine seeds were sterilized with 30% H_2O_2 for 10 min, rinsed twice with sterile water, and then placed on 10 ml slants of G4 medium (glucose 4 g, yeast extract 0.4 g, agar 12 g, distilled water 1,000 ml; adjusted to pH 5.4 with 0.1 M HCl) in test tubes. They were incubated for 14–21 days at 22°C under 1,000 Lux illumination with fluorescent lamps during the day (5:00–19:00). Axenically germinated seedlings with 15–25 mm long roots were transferred to new 15-ml G4 Petri dishes (two seedlings per dish) and incubated under the same conditions until basidiospore inoculation (approximately 2 weeks).

Isolation of homokaryons

The spore suspension was diluted to a concentration of 100–400 spores/ml, and 0.8 ml of the diluted suspension

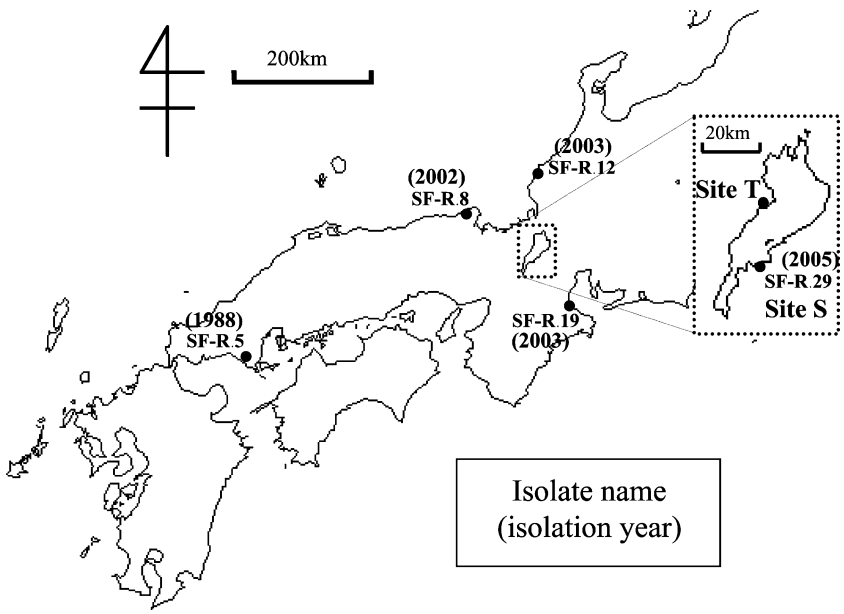

Fig. 1 Locations where the fruit bodies were collected

was poured onto the agar plate immediately after the removal of pine seedlings. A total of 48 plates were used for inoculation of spores from site T and S. The water that was used to suspend the spores was fully absorbed by the medium by the next day because of the desiccation of the plates during the cultivation of pine seedlings.

Mycelial colonies that became visible 2–3 weeks after inoculation were separately transferred to G4 slants in test tubes and further cultured for approximately 3 weeks. After that, they were cultured on GN plates, whose composition will be given later, and were checked for the presence of clamp connections using an inverted microscope; magnification was ×400, and the observation field was 480 μm in diameter. Isolates that did not produce a clamp connection were used as homokaryotic isolates for the mating test.

Improvement of culture medium

The medium composition for increasing mycelial growth of *R. rubescens* was determined by supplementing G4 medium with five nutrients and two nutrient mixture at each three concentrations using $L_{27}(3^{13})$ orthogonal table. A complete 3^7 factorial design of 2,187 treatments was thereby reduced to 27 treatments. The nutrients and their amounts examined (/1,000 ml) were glucose (20, 25, 30 g), ammonium tartrate (0.5, 1, 2 g), KNO_3 (0, 0.1, 0.2 g), KH_2PO_4 (0.1, 0.3, 0.5 g), $MgSO_4{\cdot}7H_2O$ (1, 2, 3 g), $CaCl_2$ (0, 10, 50 mg), a mineral mixture ($FeCl_3{\cdot}6H_2O$ 5 g, acetyl acetone 0.5 ml, $ZnSO_4{\cdot}7H_2O$ 1 g, $NiSO_4{\cdot}6H_2O$ 300 mg, $CuSO_4{\cdot}5H_2O$ 300 mg, $MnSO_4{\cdot}5H_2O$ 300 mg, $CoSO_4{\cdot}7H_2O$ 100 mg; distilled water 1,000 ml; 5, 10, 15 ml), a vitamin mixture (thiamine·HCl 2 g, nicotinic acid 3 mg, biotin 1 mg, pyridoxine·HCl 5 mg, calnitine chloride 0.1 mg, adenine·$H_2SO_4{\cdot}2H_2O$ 3 mg, choline chloride 10 mg; distilled water 1,000 ml; 2, 5, 10 ml), gibberellin A3 (2, 3, 5 mg), and soluble starch (10, 15, 20 g). Citric acid and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were added as pH buffers and citric acid was also used as a chelator of Fe. All reagents were purchased from Wako Pure Chemical Industries Ltd., Osaka, Japan, except for yeast extract "Bacto," which is from Becton, Dickinson and Company, MD, USA. The mineral and vitamin mixtures were designed for the mycelial growth of *R. rubescens* in a previous study (Ohta 1990). In 100-ml flasks, 10 ml each of the medium to be tested was autoclaved at 120°C for 15 min. Fungal inocula were prepared by incubation of SF-Rr5, 12, and 29 in 500-ml flasks, each containing 100 ml GY medium for 42 days at 22°C. The mycelia were washed twice with sterile water and homogenized for 3 s with a homogenizer (HE type, Nihonseiki Co., Tokyo, Japan), and 1 ml of the homogenate was inoculated into a set of 27 flasks for each isolate. They were incubated at 22°C for 16 days. Grown mycelia were decanted on a grass filter, washed with water, dried for 40 h at 65°C in a forced-air oven and then weighed. The modified medium selected in this experiment is referred to as GN medium after this.

Four levels of pH (4.8, 5.1, 5.4, and 5.7) were examined for the media, using 1 M KOH to adjust the pH for GN medium and 1 M HCl for GY medium. Each 15 ml nutrient agar was poured into a plastic plate (9 cm in diameter) and 16 plates (two plates for each pH and two kinds of media) were used for one isolate. For all heterokaryotic isolates cultured on GY medium, one agar plug (5 mm in diameter)/plate was transferred to each treatment plate and incubated for 17 days at 22°C. A total of ten fields were randomly selected on two plates of each pH level and the number of microscopic fields containing a clamp connection was counted using the inverted microscope.

Mating test

Twenty homokaryotic isolates were randomly selected from all the homokaryons of each site. Mycelial agar blocks of two homokaryotic isolates to be tested were placed on a 15-ml GN plate in the Petri dish at 5-mm spacing and incubated at 22°C. The production of clamp connections was observed using the inverted microscope every 2–3 days from 7 to 28 days after inoculation. Clamp connections were found mainly between 14 and 22 days of incubation.

Statistical analysis

We used Statistica ver 98J (StatSoft Japan Inc., Tokyo) for all the statistical analysis. Analysis of variance was used to analyze data of the experiment for determining medium composition. Fisher's exact test was used to determine the optimal pH and medium ($P<0.05$).

Results

Single spore isolation

In contrast to the case where no basidiospores of *R. rubescens* germinated without pine seedlings, the spores germinated well on plates on which pine seedlings had been raised and then removed just before spore inoculation. The germination rates exceeded approximately 40% at the place where the pine roots had been laid down, although accurate rates were not determined because of the very low densities of inoculated spores.

Mycelial colonies were also mainly formed near the pine root, particularly where the basal part (near to the stem) of the root had been laid down. One to 11 colonies were isolated when 320 spores were inoculated to the dish.

Thirty-four isolates from site T (t1–t34) and 64 isolates from site S (s1–s64) were transferred to GN media to check clamp connection. Twenty isolates from site T and 42 isolates from site S had no clamp connections, and they were used as homokaryotic isolates.

Medium for the observation of clamp connections

The heterokaryotic mycelia of SF-Rr5, 19, and 29, isolated from the fruit bodies of *R. rubescens*, did not produce clamp connections on GY medium, which is commonly used for the cultivation of ectomycorrhizal fungi in Japan (Fig. 2). The remaining two isolates, SF-Rr8 and 12, which produced clamp connections on GY medium showed greater mycelial growth. Because it was expected that there was a close relationship between mycelial growth rate and production of clamp connections, the medium composition was modified to increase mycelial growth. Some nutrients did not show significant differences among the concentration levels examined. In those cases, we selected a concentration which gave the highest average dry weight of mycelia. As a result, the medium composition which gave the greatest mycelial growth was glucose 30 g, citric acid 0.2 g, ammonium tartrate 1 g, KNO_3 0.2 g, KH_2PO_4 0.5 g, $MgSO_4.7H_2O$ 1 g, $CaCl_2$ 0.05 g, mineral mixture 15 ml, vitamin mixture 10 ml, gibberellin A3 2 mg, HEPES 2.4 g, yeast extract 0.4 g, soluble starch 20 g, agar 10 g, and distilled water 1,000 ml adjusted to pH 5.7 with 1 M KOH. The improved medium was referred to as GN medium. Most heterokaryotic isolates produced clamp connections on GN medium except for SF-Rr5, which is the oldest among the tested isolates.

Fisher's exact test showed that the total number of microscopic fields containing a clamp connection was significantly greater on GN medium than GY medium ($P<0.05$) at any pH level. The pH of the medium also affected the production of clamp connections. The number of microscopic fields containing a clamp connection was significantly greater at pH 5.7 than pH4.8 on both GY and GN media ($P<0.05$). This result indicates that the optimal pH for the production of clamp connections is around or higher than 5.7, which is markedly higher than that for their mycelial growth (approximately 4.1; Ohta 1990). GN medium at pH 5.7 was best of all the tested media, and was used to test mating reactions of *R. rubescens*.

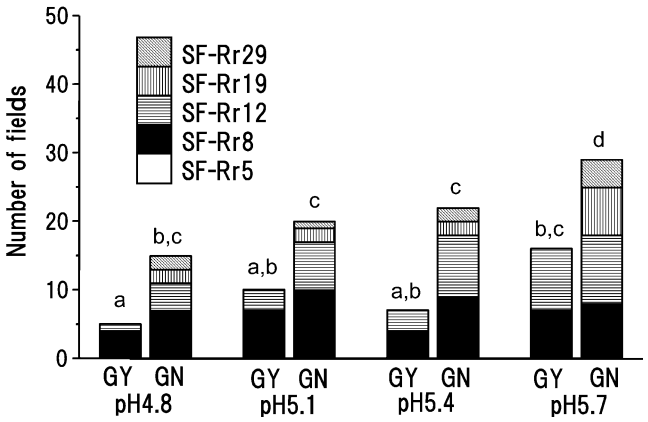

Fig. 2 Number of microscopic fields containing a clamp connection. Observed in a total of ten fields on two culture plates at each pH. *Columns with different letters* indicate significantly different number of fields ($p<0.05$)

Incompatibility of isolates

We base our conclusions on the following assumptions regarding compatibility systems. If a fungus has a homothallic mating system including secondary homothallism, then single spore isolates should not split into groups by mating tests. However, if the fungus is heterothallic with a tetrapolar incompatibility system shown by such factors as a_1b_1, a_1b_2, a_2b_1, and a_2b_2, the isolates will split into four groups because a_1b_1, for instance, mates only with a_2b_2. For site T (Table 1), isolates fell into two distinct groups; group one included t4, t8, t9, t18, t21, t26, t28, t29 and group two included t5, t6, t10, t12, t14, t15, t16, t17, t19, t27, t31, t34. Similarly, for site S (Table 2), the tested isolates also fell into two distinct groups: group one included s1, s2, s3, s4, s5, s6, s7, s8, s9, s11, s12, s13, s19, s23, s26 and group two included s10, s16, s17, s18, s24. These results indicate a heterothallic and bipolar incompatibility system for *R. rubescens*.

Mating factors of bipolar isolates from single fruit body are generally shown as a_1 and a_2. If the mating factor of a fungus was multiallelic, and the isolates from the other fruit bodies had different factors such as a_3 and a_4, then these isolates mate with a_1 and a_2 isolates in all combinations. We confirmed that clamp connections were produced in all combinations when two typical homokaryotic isolates from site T, i.e., t4 and t16, were crossed with isolates s8 and s10 from site S. This result suggests that the mating factor of *R. rubescens* is multiallelic.

Discussion

Although in vitro germination of sexual spores is the first essential step in isolating single spore cultures to determine the fungal mating system, basidiospores of only a few ectomycorrhizal species have been germinated under laboratory conditions. Basidiospores of *Laccaria laccata* (Scop. Ex Fr.) Berk. and Br. are induced to germinate by a living red yeast and the secondary mycelia of *L. laccata*. Activated charcoal also effectively absorbs germination inhibitor(s) in agar medium (Fries 1977, 1983). Using this information, several investigators determined that the mating systems of *L. amethystina* (Hud.) Ck., *L. bicolor*

Table 1 Production of clamp connections by crossing homokaryotic isolates

Site T	t4	t8	t9	t18	t21	t26	t28	t29	t5	t6	t10	t12	t14	t15	t16	t17	t19	t27	t31	t34
t4									(+)	+	+	+	+	+	+	+	+	+	+	+
t8									(+)		+	+	+	+	+	+	+	+	+	+
t9									+	+	+	+	+	+	+	+	+	+	+	+
t18									+	+		+		+	+		+	+	+	+
t21									(+)	+	+	(+)	+	+	+	+	+	+	(+)	+
t26									+	+	+	+	+	+	+	+	+	+	(+)	+
t28									(+)	+	+	+	(+)	+	+	+	+	+	+	+
t29									+	+	+	+	+	+	+	+	+	+	+	+
t5	+	+	+	+	+	+	+	+												
t6	+		+	+	+	+	+	+												
t10	+	+	+		+	+	+	+												
t12	+	+	+	+	+	+	+	+												
t14	+	+	+		+	+	+	+												
t15	+	+	+	+	+	+	+	+												
t16	+	+	+	+	+	+	+	+												
t17	+	+	+		+	+	+	+												
t19	+	+	+	+	+	+	+	+												
t27	+	+	+	(+)	+	+	+	+												
t31	+	(+)	+	(+)	(+)	(+)	+	+												
t34	+	+	+	+	+	+	+	+												

+: Clamp connections were produced on both sides of the crossed colony
(+): Clamp connection was produced either on one side of the crossed colony or on contacted portion of the two isolates

(Maire) Orton and *L. proxima* (Boud.) Pat. were tetrapolar (Fries and Mueller 1984; Kropp and Fortin 1988; Doudrick and Anderson 1989).

The germination of basidiospores in *Suillus* is induced by abietic acid (Fries et al. 1987; Fries 1988). Studies using abietic acid, thus, determined that the mating systems of four *Suillus* species collected in Sweden are bipolar (Fries and Neumann 1990; Fries and Sun 1992; Fries 1994). Enhanced germination of *Rhizopogon luteolus* Fr. and Nord basidiospores was obtained by spreading spores with

Table 2 Production of clamp connections by crossing homokaryotic isolates

Site S	S1	s2	s3	s4	s5	s6	s7	s8	s9	s11	s12	s13	s19	s23	s26	s10	s16	s17	s18	s24
s1																+	+	(+)	+	+
s2																+	+	+	+	+
s3																+	+	(+)	+	+
s4																+	+	+	+	
s5																+	+	+	(+)	+
s6																+	+	+	+	+
s7																+	+	+	+	+
s8																+	+	+	+	+
s9																+	+	+	+	+
s11																+	+	+	+	+
s12																+	+	+	+	+
s13																+	+	+		
s19																+	+	+	(+)	+
s23																+	+	+	(+)	+
s26																+	+	+	+	+
s10	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+					
s16	+	+	+	(+)	+	+	+	+	+	+	+	+	+	+	+					
s17	+	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+					
s18	+	+	(+)	+	(+)	(+)	(+)	+	+	+	+		(+)	+	+					
s24	+	+	+		+	+	+	+	+	+	+		+	+	+					

+ Clamp connections were produced on both sides of the crossed colony
(+) Clamp connection was produced either on one side of the crossed colony or on contacted portion of the two isolates

melted agar on the living roots of host pine seedlings (Theodorou and Bowen 1987). We achieved the germination of basidiospores of *R. rubescens* on agar plates on which pine seedlings were previously grown, even when the seedlings were removed from the plate before spore inoculation. This indicates that germination of *R. rubescens* basidiospores is enhanced by pine root exudates retained in the agar. The exudates also appear to remain active for at least several days until the spores begin to germinate. These findings led to our successful isolation of homokaryons of *R. rubescens* without using complicated procedures.

Determining the mating systems for some basidiomycetes remains difficult, in spite of the success in obtaining single spore isolates. For example, the mating system of *Suillus granulatus* (L. ex Fr.) O. Kuntze isolates from the USA remains undetermined because heterokaryons of *S. granulatus* do not produce clamp connections (Fries and Neumann 1990; Jacobson and Miller 1994). Homokaryons of *S. pungens* were not obtained by Bonello et al. (1998), who concluded that only binucleate, heterokaryotic spores would germinate in vitro under normal conditions (a case of secondary homothallism). Fortunately, in the case of *R. rubescens*, the problem of isolating heterokaryotic cultures derived from binucleate basidiospores can be avoided because basidiospores in *Rhizopogon* are mostly uninucleate (Horton 2006). Further, our modified medium facilitates the observation of clamp connections. Thus, we found that the incompatibility system of *R. rubescens* is bipolar (unifactorial) with a multiallelic mating type factor.

Genetic analysis of mitochondrial DNA in *Rhizopogon* shows that *Rhizopogon* is closely related to *Suillus* (Bruns et al. 1989; Bruns and Szaro 1992). The finding that both *R. rubescens* and *Suillus* species have bipolar mating systems, which are rare relative to tetrapolar systems, supports their close evolutionary relationship.

Acknowledgements We thank Professor Kazumasa Yokoyama, Shiga University, for providing information on the fungus and Mr. Hiromi Fujita, Dr. Yasumi Akamatsu, and Dr. Tomoaki Ishikawa for collecting samples. We also thank Ms. Hatsuko Deno and Ms. Mito Sagawa for helpful assistance.

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