Physiological variation among *Tricholoma matsutake* isolates generated from basidiospores obtained from one basidioma

Akiyoshi Yamada [a, b, c*], Norio Hayakawa [a], Chika Saito [a], Yuka Horimai [a], Hiroki Misawa [a], Takashi Yamanaka [d], Masaki Fukuda [a, c]

[a] Department of Agricultural and Life Science, Graduate School of Science and Technology, Shinshu University 8304, Minami-minowa, Nagano, 399-4598, Japan

[b] Division of Terrestrial Ecosystem, Institute of Mountain Science, Shinshu University, 8304, Minami-minowa, Nagano, 399-4598, Japan

[c] Department of Fungal Symbiotic Science & Resource Utilization Technology, Research Center for Fungal and Microbial Dynamism, Shinshu University, 8304, Minami-minowa, Nagano, 399-4598, Japan

[d] Forestry and Forest Products Research Institute, Matsunosato 1, Tsukuba, Ibaraki, 305-8687, Japan

[*]Corresponding author:

A. Yamada

E-mail address: akiyosh@shinshu-u.ac.jp

Phone: +81-265-77-1631

Fax: +81-265-77-1629

Text: 25 pages; Tables: 2; Figures: 7; Supplementary Tables: 6.

Abstract

Matsutake (*Tricholoma matsutake*) is a commercially valuable edible ectomycorrhizal mushroom. The physiological traits of *T. matsutake* have been previously assessed using mycelial isolates isolated from basidiomata; however, few studies have focused on basidiospores. Here, we report that sibling *T. matsutake* isolates generated from basidiospores on a single basidioma show distinct physiological variation. We first established 145 isolates of *T. matsutake* on modified Norkrans' C (MNC) agar medium and found that their radial growth varied significantly. The mycelial biomasses of nine isolates with different growth rates were reduced on low-carbon and low-nitrogen MNC media. However, the colony diam of one isolate was significantly elevated on low-carbon medium, and the colony diam of two isolates were significantly elevated on low-nitrogen medium. In co-cultures of two or three isolates, commensal and amensal interactions were observed. The physiological variation induced by low carbon and nitrogen levels and the mycelial interactions between sibling isolates imply mechanisms for the genetic and functional characteristics of mycelia of *T. matsutake*.

Key words: Ectomycorrhizal symbiosis, Genet interaction, Soil microbial ecology, Spore dispersal

1. Introduction

Tricholoma matsutake (S. Ito & S. Imai) Singer is a commercially valuable wild edible ectomycorrhizal mushroom known, together with related species, as 'matsutake' (Yamada et al., 2010; Trudell et al., 2017; Vaario et al., 2017). Research on this mushroom species has focused on its ecophysiological traits, resource conservation (e.g., Endo et al., 2015; Narimatsu et al., 2015; Furukawa, Masuno, & Takeuchi, 2016; Nishino et al., 2017), and the establishment of novel techniques for cultivation of mycorrhizal seedlings (e.g., Yamanaka et al., 2014; Kobayashi, Terasaki, & Yamada, 2015; Saito et al., 2018). The annual yield of *T. matsutake* in Japan in recent years has been less than 100 tons, and thus 1,000–2,000 tons of matsutake must be imported annually to meet the demand (Ministry of Agriculture, Forestry, and Fisheries, Japan).

In vitro ectomycorrhization of *T. matsutake* with *Pinus densiflora* has been investigated (Yamada, Maeda, & Ohmasa, 1999; Guerin-Laguette et al. 2000, 2004; Vaario et al., 2000), and a technique to promote formation of shiro structures, the massive visible extraradical mycelia that surround thousands of ectomycorrhizal root tips in the soil mineral layer, has been developed (Yamada, Maeda, Kobayashi, & Murata, 2006; Kobayashi, Terasaki, & Yamada, 2008, 2015). This technique would be enhanced if *T. matsutake* isolates were available as symbionts for the host pine; such isolates could be used in cultivation studies under controlled environmental conditions. However, this requires selection of suitable *T. matsutake* isolates. Several hundred *T. matsutake* isolates have been established and investigated in vitro for mycelium growth (Kawai & Ogawa, 1976; Ohta, 1986, 1988), catabolic enzyme production (Terashita, Kono, Yoshikawa, & Shishiyama, 1995; Kusuda et al., 2003, 2006), and ectomycorrhization (Guerin-Laguette et al., 2004; Yamada et al., 2010; Vaario et al., 2010; Murata et al., 2013; Yamanaka et al. 2014; Saito et al. 2018). The matsutake isolates used in these studies were established from basidiomata harvested from

geographically and vegetationally different areas. However, little is known about the physiological or functional variation in *T. matsutake* isolates established from a single basidioma by basidiospore isolation (Murata et al., 2015). Indeed, this type of physiological variability has been examined in only a few ectomycorrhizal fungi (Kropp & Fortin, 1988; Kawai, Yamahara, & Ohta, 2008). As basidiospore populations obtained from a single basidioma exhibit genetic diversity due to sexual recombination during basidiospore formation, they are used for breeding of saprobic basidiomycetous mushrooms (Miles and Chang, 2004; Moor, Gange, Gange, & Boddy, 2008; Kües, Badalyan, Gieβler, & Dörne, 2016).

Genetic analyses of shiro mycelia of *T. matsutake* at forest sites have revealed the complexity of these structures. Over the course of a decade, a large *T. matsutake* shiro can extend several meters (Hamada 1974; Narimatsu et al., 2015; Furukawa, Masuno, & Takeuchi, 2016). Such shiro can comprise multiple individuals (Murata et al., 2005; Lian, Narimatsu, Nara, & Hogetsu, 2006). This implies the existence of physiological variation within a single shiro of *T. matsutake*, e.g., variability in nitrogen acquisition from the soil, nutritional exchanges with hosts, or associations between neighboring genets. If this were the case, such a shiro structure would benefit from complementarity rather than selection between genets to sustain and develop its mycelial structure during annual changes in conditions in a forest habitat (Loreau & Hector, 2001; Johnson, Martin, Cairney, & Anderson, 2012; Yamada et al., 2014). In addition, di-mon mating between the established dikaryotic shiro-mycelium (parent mycelium) and the descendant basidiospores may increase the genetic diversity within a single shiro structure. Therefore, we evaluated the variation in the carbon and nitrogen nutrition of *T. matsutake* by establishing a genetic line of spore isolates from a single basidioma. We established *T. matsutake* isolates from a single basidioma and assessed their growth characteristics on nutrient media that differed in carbon/nitrogen balance (C/N

balance). C/N balance is one of the primary determinants of fungal growth, survival, and adaptation (Dix & Webstar, 1995; Smith & Read, 2008).

2. Materials and Methods

2.1 Multispore isolation of *Tricholoma matsutake*

A fresh, young *T. matsutake* basidioma commercially harvested from a *P. densiflora* forest in Takagi village, Nagano Prefecture, Japan (35°28'N, 137°55'E), was obtained on Nov 1, 2007 (dry specimen identification code in the laboratory of Applied Mycology, Faculty of Agriculture, Shinshu University: 2071101-01). In the laboratory, the outer surface of the basidioma was cleaned using cotton wool moistened with 70% ethanol. Next, the basidioma was removed from the stipe using a scalpel, and the pileus was prepared on a clean bench. The hymenial veil was removed from the pileus using fine forceps, and gills (ca., 1 × 1 cm) with pileal trama were axenically excised using a sterile scalpel, transferred to a modified Norkrans' C (MNC) agar plate (Yamada & Katsuya, 1995), and incubated upside-down overnight to allow basidiospores to drop onto the lid. Subsequently, the basidiospores were transferred with a microstreaker onto MNC agar plates containing 5 mg/L butyric acid to stimulate spore germination (Ohta, 1988) and incubated at 20 °C in the dark.

Spore germination was checked daily under a dissecting microscope. If spore germination was observed, the colony was transferred to a fresh MNC agar plate to establish a *T. matsutake* spore isolate. The isolates were stored as slants on MNC agar at 4 °C until use. In total, 145 isolates were obtained from one basidioma.

2.2 Selection of established isolates

The 145 isolates stored as slant cultures on MNC agar were inoculated onto MNC agar plates and incubated at 20 °C for 2 mo. Two to three replicates of each isolate were

prepared. Colony size and shape were recorded, and 100 isolates were subcultured on fresh MNC agar plates for 2 mo to measure the mycelial growth rates. The margins of mycelial colonies were punched using a cork borer (7 mm diam), one mycelial plug (four replicates per isolate) was inoculated onto the center of an MNC agar plate (9 cm diam; ca., 20 mL medium volume), then the plates were incubated at 20 °C in the dark for 2 mo. Subsequently, the maximum and minimum diam of each colony were measured, and the averages were calculated. The measurements made on the four replicates per isolate were averaged. Nine isolates were selected for further experiments: three isolates with the largest colony diam, three isolates with intermediate colony diam, and three isolates with small colony diam.

2.3 Effect of C and N levels on mycelial growth

We evaluated the mycelial growth of the nine selected isolates on MNC agar and in liquid medium at 20 °C for 1 mo in the dark. Mycelial colonies grown for 2 mo on MNC agar were punched using a cork borer (7 mm diam), and the mycelial plugs (five replicates per isolate) were inoculated onto the centers of fresh MNC agar plates (9 cm diam; ca., 20 mL of medium). Mycelial plugs (five replicates per isolate) were inoculated into 10 mL MNC liquid medium in a 75-mL wide-mouth glass bottle. After incubation for 1 mo, the maximum and minimum diam of colonies on MNC agar were measured, and the average was calculated. Mycelia were recovered from liquid culture on filter paper and dried at 60 °C for 48 h; then their dry weights were measured using a digital analytical balance (AUW220D, Shimadzu Corp., Kyoto). We calculated colony density (mg/mm^2) from estimates of mycelial dry weight (mg) and colony area (mm^2) that was calculated based on the averaged colony diam (in most cases, colonies were almost circular).

Solid and liquid MNC media with low glucose (2.0 g/L; designed as 0.1×C), low nitrogen (0.05 g ammonium tartrate, 0.05 g yeast extract, 0.023 g casamino acids; designed as 0.1×N), and low glucose and nitrogen (designed as 0.1×CN) were prepared. Our unpublished

studies have shown that better growth (measured as increases in colony diam) in selected *T. matsutake* isolates occurred in the 0.1×CN treatment, which we therefore selected for examining variation between sibling isolates. This treatment has ecological relevance because soil nitrogen levels (g/L) are 10–100 times lower than concentrations in MNC medium (Saito et al., 2018). We used eight of the nine fungal isolates obtained for tests with media containing different concentrations of C and N. Fungal inoculation and incubation were performed as described above.

2.4 Mycelial interactions between sibling isolates

Three isolates, i.e., #52, #121, and #126, were selected from the tested 8 isolates on the different C and/or N reduced MNC media due to their strikingly different mycelial growth patterns each other on those medium conditions. Pairs and trios of isolates #52, #121, and #126 were co-cultured on solid or liquid MNC medium. For the paired co-cultures, two mycelial plugs (7 mm diam) were adjacently inoculated onto the center of an MNC agar plate (9 cm diam containing ca., 20 mL medium) or into MNC liquid medium (10 mL in a 75-mL wide mouth glass bottle). For the trio co-cultures, three mycelial plugs were inoculated in a triangular shape onto the center of an MNC agar plate or into MNC liquid medium. Duplicate or triplicate plugs of the same isolates were established as controls. All co-cultures were incubated at 20 °C for 1 mo in the dark, after which colony radii were measured at the opposite sides of the paired or triplicate plugs. Mycelia were recovered from liquid culture and dried as described above; their dry weights were measured using a digital analytical balance. Five replicates were prepared for all combinations of isolates.

2.5 Data analyses

Colony size was determined as the colony diam in monocultures and as the combined radii of paired or trio colonies in co-cultures (Figs. 2–4).

Statistical calculations were performed with KaleidaGraph ver. 4.5 J software

(Hulinks, Tokyo). Two-way analysis of variance (ANOVA) was used to test for the significant ($P < 0.05$) effects of isolates, medium conditions, and their interactions in each experiment. One-way ANOVA was used to test the significance of differences among mean colony size in the monocultures and co-cultures. Tukey's honestly significant difference (HSD) post hoc test was used for multiple comparisons ($P < 0.05$) betweens pairs of means to evaluate the effects of isolates in a given medium. Dunnett's post hoc test was used for multiple comparisons ($P < 0.05$) between pairs of means to evaluate the effects of medium compositions on a given isolate or pair of isolates. A *t*-test ($P < 0.05$) was used to evaluate the significance of differences between self- and non-self trio cultures. One-way ANOVA was also used to assess the significance of differences in colony dry weights in monocultures and co-cultures. Tukey's HSD or Dunnett's post hoc tests were used ($P < 0.05$) for multiple comparisons according to the objectives.

2.6 Karyotic state of selected isolates

To confirm dikaryotic status of selected isolates, mycelia in MNC liquid medium were visualized by fluorescence microscopy (Axioplan 2 Imaging, Carl Zeiss AG, Jena) using a 100× oil immersion lens (Plan Neofluar) and filter set 01 (488001–9901–000; excitation, BP 365/12; beam splitter, FT 395; emission, LP 397), and photographed (D200, Nikon Imaging Japan, Inc., Tokyo). Hyphae were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Japan, Tokyo). Mycelia cultured in MNC liquid medium for 1 mo were soaked in distilled water, and a small amount of mycelium was mounted on a glass slide in 10 μL of 1 mg/L DAPI solution. The margin of the cover glass was sealed with nail varnish to prevent evaporation of the DAPI solution. After staining for 10–20 min, the mycelia were UV-irradiated and visualized. Around 20 independent fields were observed per slide and several fields were photographed. Frequent paired nuclei were considered indicative of dikaryosis.

2.7 *Genetic diversity of selected* Tricholoma matsutake *isolates*

The sequence variation in the intergenic spacer 1 (IGS1) region of ribosomal DNA (Guerin-Laguette et al., 2002) in the nine isolates was analyzed. DNA extraction, polymerase chain reaction (PCR), and restriction fragment length polymorphism (RFLP) analyses were performed according to Guerin-Laguette et al. (2002) and Endo et al. (2015). To confirm the individuality of the sibling isolates, the long terminal repeat (LTR) marker of *T. matsutake* (Murata et al., 2005) was used; *T. matsutake* isolates AT-0740 and Y1 (=NBRC33036) were used as controls. Isolate AT-0740 was established from a *T. matsutake* basidioma sampled in Nagano Prefecture in 2000. Isolate Y1 was first detected in the *gypsy* group of LTR retroelements (Murata & Yamada, 2000) and its mycorrhizal status in vitro has been previously reported (Yamada, Maeda, & Ohmasa, 1999; Yamada et al., 2010; Murata et al., 2013; Saito et al., 2018).

3. Results

3.1 Selection of *Tricholoma matsutake* isolates grown from spores

Of the 145 isolates stored on MNC slants, 100 recovered their stable mycelial growth on MNC agar plates. After 1 mo, most of them had a mycelial colony diam growth rate on MNC agar within the range 3.1–23.5 mm/mo, with the exception of three isolates (Fig. 1). Isolates with large (#52, #79, and #45), intermediate (#121, #31, and #126), and small (#99, #84, and #111) colony diam were physiologically characterized on MNC agar media containing low C and/or low N levels.

3.2 Growth of *Tricholoma matsutake* isolates at different C and N levels

The nine isolates had significantly different growth rates on normal MNC agar plates (Fig. 2). Although isolate #52 showed the largest colony size, its biomass in liquid medium was the second lowest. By contrast, although the biomass in liquid medium of isolate #121

was twice that of isolate #52, its colony diam on the agar plate was ca., 80% of the colony diam of isolate #52. Thus, the colony density (mg/mm^2) of isolate #121 was 3.07-fold higher than that of isolate #52. As the three growth rate groups (identified by colony diam; Fig. 1) were not significantly different in mycelial biomass growth rate, we did not consider the three groups in the following comparisons.

Eight of the nine isolates were cultured on 0.1×C, 0.1×N, and 0.1×CN MNC agar plates and in liquid media. The mycelial biomass of all isolates in 0.1×C and 0.1×N MNC liquid media was ca., 15 mg dry weight/bottle, compared to 10 mg/bottle in 0.1×CN MNC liquid medium (Fig. 2; Supplementary Tables 1, 2). On 0.1×N MNC agar plates, the colony diam of isolates #52 and #99 significantly increased in comparison with normal MNC plates, while the colony diam of the other six isolates were smaller than or similar to colony diam on normal MNC agar plates. Moreover, on 0.1×CN MNC agar plates the colony diam of four isolates increased. Therefore, the eight isolates were categorized into the following groups based on relative growth on diluted MNC agar plates: group 1 (isolates #31, #45, #111, and #121) formed small-diam colonies on 0.1×C (relative growth rate: 90.6–95.8 %), 0.1×N (relative growth rate: 90.4–100.0 %), and 0.1×CN MNC agar (relative growth rate: 88.2–98.4 %); group 2 (isolates #79 and #126) formed small-diam colonies on 0.1×C (relative growth rate: 74.8–93.5 %) and 0.1×N MNC agar (relative growth rate: 91.9–97.8 %), but slightly larger diam colonies on 0.1×CN MNC agar (relative growth rate: 101–108 %); group 3 (isolate #52) formed conspicuously smaller diam colonies on 0.1×C MNC agar (relative growth rate: 69.1 %), but conspicuously and moderately larger diam colonies on 0.1×N (relative growth rate: 117 %) and 0.1×CN MNC agar (relative growth rate: 109 %), respectively; group 4 (isolate #99) formed conspicuously large-diam colonies on 0.1×C (relative growth rate: 133 %), 0.1×N (relative growth rate: 163 %), and 0.1×CN MNC agar (relative growth rate: 161 %).

3.3 Interactions of *Tricholoma matsutake* isolates at different C and N levels

We examined the interactions among the three isolates #52 (group 3), #121 (group 1), and #126 (group 2) each of which showed different pattern of their mycelial growth in the monoculture experiment at different C and N levels (Fig. 2). Although isolate #99 (group 4) was unique in its mycelial growth pattern in the monoculture experiment, we did not include it in further experiments due to its small colony diam and the difficulty in preparing the inoculum.

On normal MNC agar plates, growth patterns of self-pairs of the three isolates (Fig. 3; Supplementary Tables 3, 4) were similar to those on MNC agar plates in the monoculture experiment (Fig. 2). The mycelial biomass of the isolate #126 self-pair in normal MNC liquid medium (Fig. 3) was larger than the biomass in normal MNC liquid medium in the monoculture experiment (Fig. 2). On the 0.1×C MNC agar plates, all co-cultures of self- and non-self-pairs had small-diam colonies (Fig. 3). By contrast, the combined colony radii of all pairs on the 0.1×N and 0.1×CN MNC agar plates were large (with the exception of the combination of isolates #121 and #126). All co-cultures of self- and non-self-pairs yielded a biomass of around 15 mg irrespective of the C or N level. On MNC agar plates, no distinct demarcation line was observed between colonies of any of the pairs. The colony radius of isolate #52 was significantly increased by co-culture with the other two isolates on 0.1×CN MNC agar plates, and by isolate #126 on the normal MNC agar plates (Table 1). The colony radius of isolate #126 was significantly decreased by co-culture with the other two isolates on 0.1×N MNC agar plates and by isolate #52 on normal MNC agar plates. However, the colony radius of isolate #126 was increased by co-culture with isolate #121 on normal MNC agar plates. The colony radius of isolate #121 was increased by co-culture with isolate #126 on normal MNC agar plates. Therefore, isolates #52 and #126 interacted competitively on normal MNC agar plates; on this medium, isolate #52 had a commensal interaction, and

isolate #126 had an amensal interaction. Isolate #52 also had a commensal interaction with the other two isolates on 0.1×CN MNC agar plates. Isolate #126 had an amensal interaction with the other two isolates on 0.1×N MNC agar plates. No mutualistic interactions occurred in any of the combinations.

Co-cultures of all self- and non-self-trios yielded a biomass of around 20 mg/bottle irrespective of the C or N level (Fig. 4; Supplementary Tables 5, 6). However, colony growth patterns differed between self-trios; colony sizes for non-self-trios were similar to the average radii of the three self-trios. The colony radii of isolates #52 and #126 were increased by co-culture with the other two isolates on normal MNC agar plates, and that of isolate #121 by co-culture with the other two isolates on 0.1×CN MNC agar plates (Table 2). Therefore, isolates #52 and #126 exhibited a commensal interaction on normal MNC agar plates and isolate #121 exhibited a commensal interaction on 0.1×CN MNC agar plates. By contrast, the colony radii of isolates #121 and #126 were reduced by co-culture with the other two isolates on 0.1×C and 0.1×N MNC agar plates (Table 2). Therefore, isolates #121 and #126 had amensal interactions on 0.1×C and 0.1×N MNC agar plates.

3.4 Karyotic status and genets

All nine isolates had paired nuclei (Fig. 5) and were classified as one of three types according to their IGS1 RFLP pattern (Fig. 6). The dominant type-A pattern showed two bands at ca., 310 and 105 bp, and the type-B pattern showed two bands at ca., 205 and 105 bp, following digestion with *Hae*III. The type-C pattern showed three bands that corresponded to type-A and type-B pattern pairs. The nine isolates were distinguishable using an LTR marker (Fig. 7).

Discussion

Sibling isolates of *T. matsutake* varied considerably in carbon and nitrogen metabolism. In addition, the sibling isolates had diverse types of interhyphal interactions, which warrants further investigation.

Intraspecific genetic variation in *T. matsutake*, i.e., growth and interactions with host plants (Vaario et al., 2010; Yamada et al., 2010) and the taxonomic validity of species delineation (Bergius, & Danell, 2000; Guerin-Laguette et al., 2002; Matsushita et al., 2005; Murata et al., 2008), has been investigated previously. However, most studies have analyzed isolates with different geographic origins or from different hosts and habitats. Such isolates likely exhibit marked sequence variation in their nuclear and mitochondrial DNA, leading to diverse phenotypes. By contrast, functional and phenotypic in vitro variation among sibling individuals of *T. matsutake* has rarely been investigated. The basidiomatal productivity of *T. matsutake* differs between genets from any given forest sites (Narimatsu et al., 2015; Furukawa, Masuno, & Takeuchi, 2016), perhaps indicating that different sibling isolates of *T. matsutake* have different interactions with their host plants.

The hyphal growth rates of the selected *T. matsutake* isolates established from a single basidioma were markedly different (Fig. 2). The two-parametric estimation of fungal growth pattern revealed contrasting responses between isolates #52 and #121 on MNC medium: isolate #52 had preferential tip growth of probable leading hyphae (Clémençon, 2012) for distance elongation, but #121 had preferential tip growth of probable exploiting and interacting hyphae for nutrient storage. Thus, isolate #52 likely responded to low levels of nitrogen by promoting the tip growth of leading hyphae to search for nitrogen, despite carbon supply costs over a long distance. In contrast, isolate #121 likely responded to low levels of available nitrogen by promoting tip growth to exploit and interact with hyphae to scavenge nitrogen from the already colonized mycelial area. These contrasting mycelial growth patterns can be explained in part by the ecophysiological traits of *T. matsutake*. As an ectomycorrhizal

symbiont, a deficiency of carbon supply from a host would result in reduced fungal biomass production and colony growth (Smith, & Read, 2008), but nitrogen deficiency around extraradical mycelia would induce ecophysiological variation between individuals (Kuzyakov, & Xu, 2013). This response of *T. matsutake* to nitrogen levels leads to selective pressure between individuals (Loreau and Hector 2001) if the soil nitrogen level is homogeneous, and complementarity if the soil nitrogen level is heterogeneous. Ectomycorrhizal fungi show intraspecific variation in nitrogen metabolism depending on N availability (Sawyer, Chambers, & Cairney, 2003; Guidot, Verner, Debaude, & Marmeisse, 2005; Fransson, Anderson, & Alexander, 2007; Wilkinson et al., 2010). Therefore, sibling populations of *T. matsutake* will respond to variable soil nitrogen levels on the continuum of selection/complementarity between sibling individuals.

Colony radial growth, but not biomass production, significantly differed among co-cultures of self- and non-self-combinations (Figs. 3, 4). In paired cultures of isolates #52 and #121, and #52 and #126 on normal MNC agar plates, combined colony radii were slightly larger than those of the self-pair co-cultures; in co-culture of isolates #121 and #126, the radii were significantly larger than those of self-pair co-cultures ($P < 0.05$; t-test). This was mainly due to the significantly increased radii of colonies of isolate #126 co-cultured with isolate #121 (Table 1), which implies a commensal interaction. A similar phenomenon was observed in trio-culture on normal MNC agar plates. Isolates #52 and #126 had commensal interactions on these plates (Table 2). By contrast, isolate #126 in paired- and trio-cultures on 0.1×N MNC agar plates had amensal interactions (Tables 1, 2). Other commensal and amensal interactions between isolates were not consistent across paired- and trio-culture experiments. The data imply that these interactions facilitate selection of isolates with increased fitness. Our present experiments on isolate interactions were sometimes limited in duration, i.e., 1 mo. Longer incubations may reveal more dramatic outcomes, e.g., isolate #52 might outcompete another

isolate when levels of available nitrogen and carbon are low. We found no mutualism between co-cultured *T. matsutake* isolates in terms of biomass production (Figs. 3, 4), unlike the co-cultures of *Paxillus obscurosporus* studied by Wilkinson et al. (2010).

Putative single mycelial colonies of *T. matsutake* are defined by their fruiting bodies, i.e., so-called fairy rings with a chimeric structure comprising several genets (Murata et al., 2005; Lian, Narimatsu, Nara, & Hogetsu, 2006). As such single mycelial colonies are actually a consortium of genetically different mycelia that expand concentrically for decades (Hamada 1974; Ogawa 1978; Narimatsu et al., 2015; Furukawa, Masuno, & Takeuchi, 2016), inbreeding among siblings (i.e., di-mon or mon-mon mating) will occur annually. This will inevitably result in annual changes in the genetic structure of single mycelial colonies, facilitating their adaptation to changes in forest vegetation and soil conditions. Whether the physiological diversity of sibling isolates of *T. matsutake* occurs during symbiosis with the host plant should be investigated by inoculating spores on an aexenic root system or on an already established root–mycelia system. For this, precise and fine-scale sampling and analyses of ectomycorrhizal root tips are required. As the IGS1 region shows both homozygotic (types A and B) and heterozygotic (type C) RFLP patterns, it can be used to evaluate interbreeding between genets. The LTR marker (Murata et al., 2005) used in this study was able to distinguish all sibling individuals. As *P. densiflora* lacks this marker (Murata *et al*. 2005), it can be used in field work provided that it is not possessed by the microbes surrounding mycorrhizal root tips.

In conclusion, *T. matsutake* isolates established from spores from a single fruiting body exhibited marked differences in physiology. In addition, the isolates showed commensal and amensal interactions on media with different N and C levels. These responses under culture conditions suggest mechanisms by which *T. matsutake* isolates develop complementarity to initiate and grow shiro structures with mosaicism over extended periods

of time. Thus, our findings will facilitate forest-management strategies to conserve, and possibly even expand, the area covered by this important fungal resource.

Disclosure

The authors declare no conflicts of interest. All of the experiments in this study were performed in compliance with the current laws of Japan.

Acknowledgments

This study was supported in part by Grants-in-Aid for Scientific Research (nos. 19380085 and 15H01751) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan, entitled “Technology Development for the Optimal Use of Forest Resources”.

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Figure legends

Fig. 1. Colony diam of *Tricholoma matsutake* mycelia on modified Norkrans' C (MNC) agar plates. Mean colony diam (n = 4) of 100 isolates cultured for 2 mo are shown. Large open circles with numbers indicate the codes of the isolates used in subsequent experiments.

Fig. 2. Colony diam and biomasses of nine *Tricholoma matsutake* isolates in different concentrations of C and N in MNC medium. Values are mean colony diam and biomasses ± standard errors (SEs; n = 5) of nine *T. matsutake* isolates. Broad colored arrows indicate significant increases in colony diam in the low C and/or N media ($P < 0.05$; Dunnett's post hoc test): green indicates low C; red indicates low N, and blue indicates both low C and N. The narrow colored arrow (blue; isolate #52) indicates a significant increase in colony diam in the both low C and N medium ($P < 0.1$; Dunnett's post hoc test).

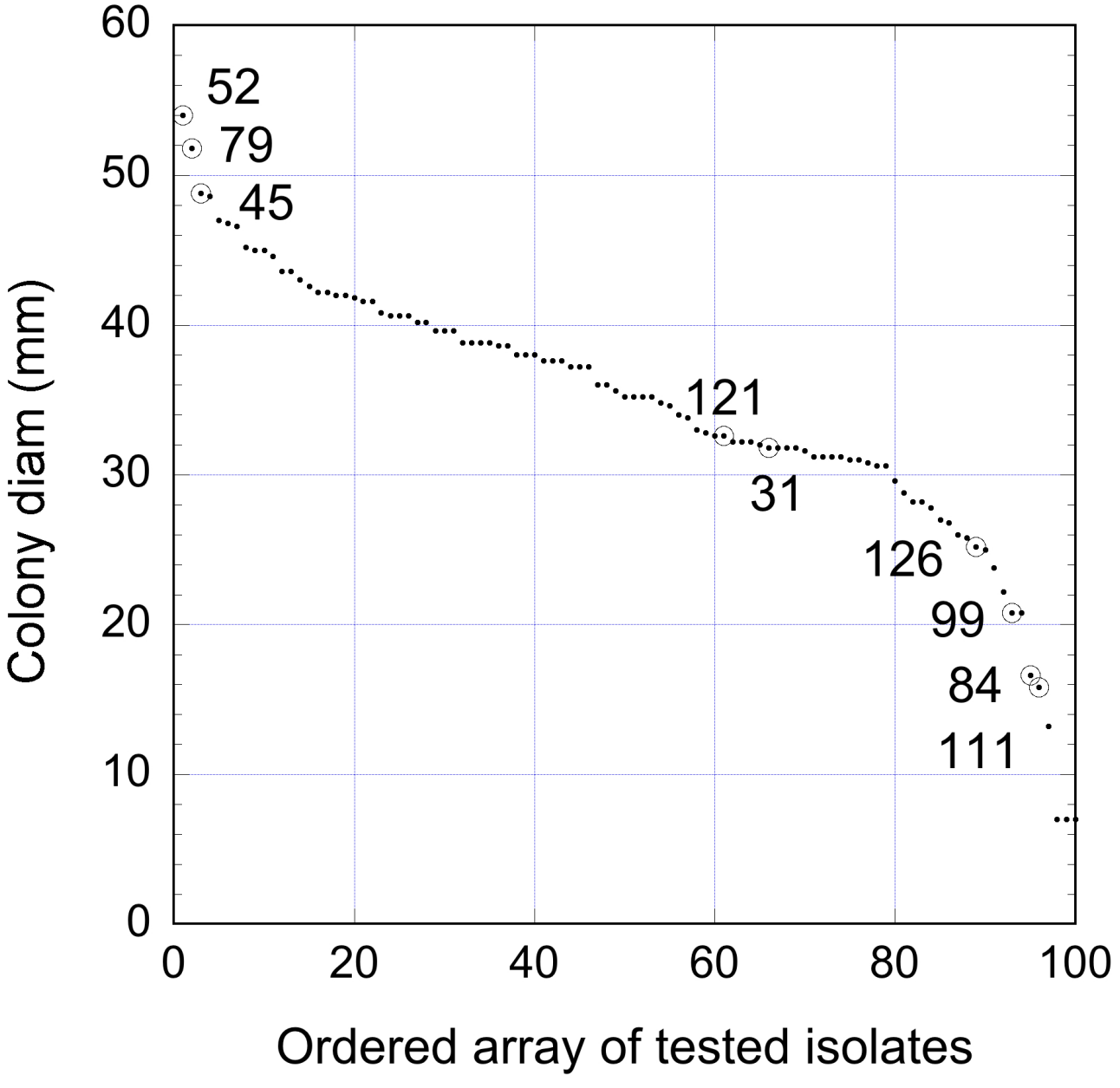
Fig. 3. Combined radii and biomasses of paired colonies in co-cultures of three *Tricholoma matsutake* isolates in different C and N concentrations in MNC medium. Values are mean colony radii and biomasses ± SEs (n = 5) of self-pairs of three isolates and non-self-pairs of two isolates. Broad colored arrows indicate significant increases in the combined paired colony radii in the low N and both low C and N treatments ($P < 0.05$; Dunnett's post hoc test).

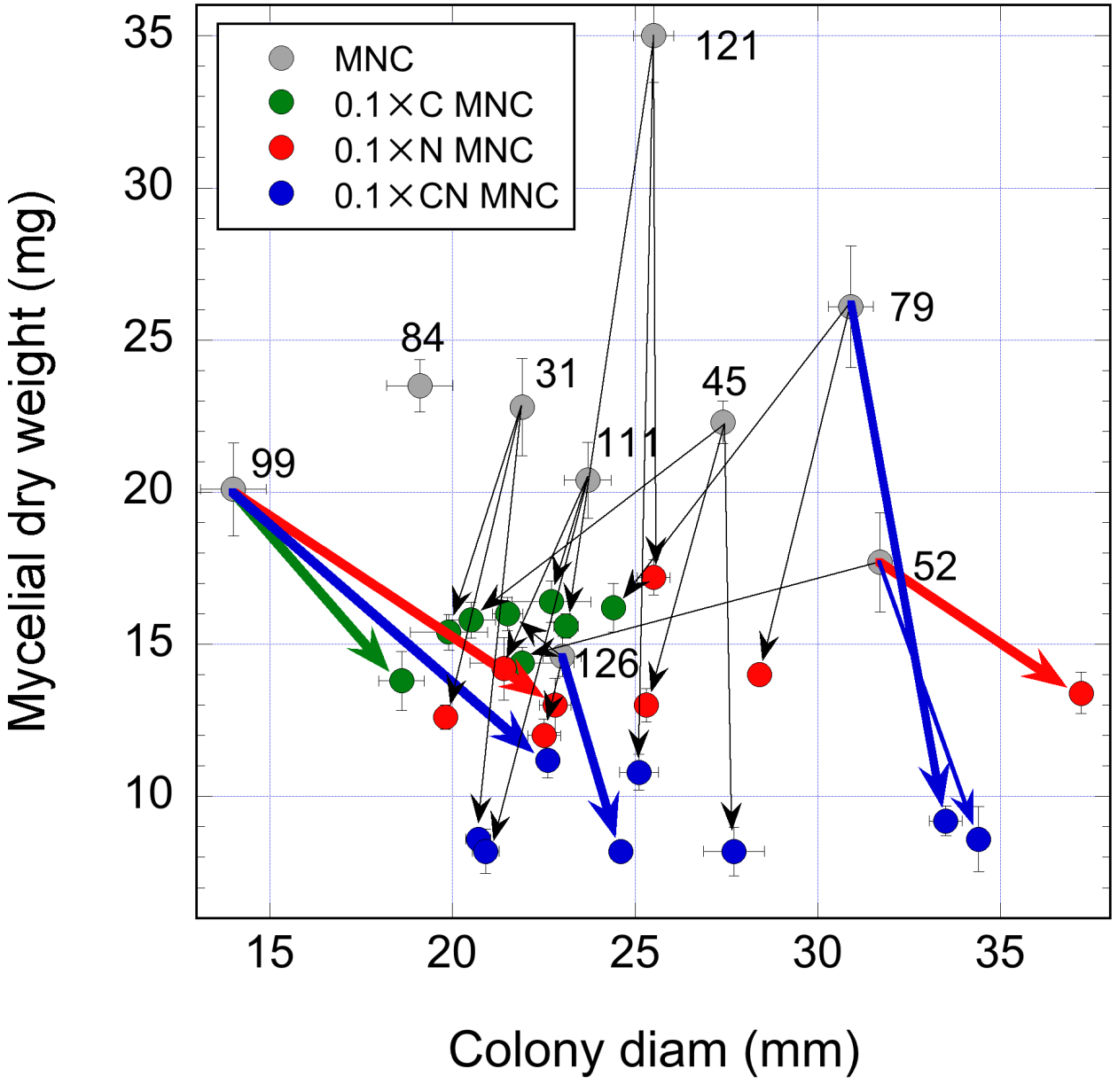
Fig. 4. Combined trio colony radii and biomasses of co-cultures of three *Tricholoma matsutake* isolates in different C and N concentrations in MNC medium. Values are means ± SEs (n = 5). Broad colored arrows indicate significant increases in the combined trio colony radii in the low N and both low C and N treatments ($P < 0.05$; Dunnett's post hoc test).

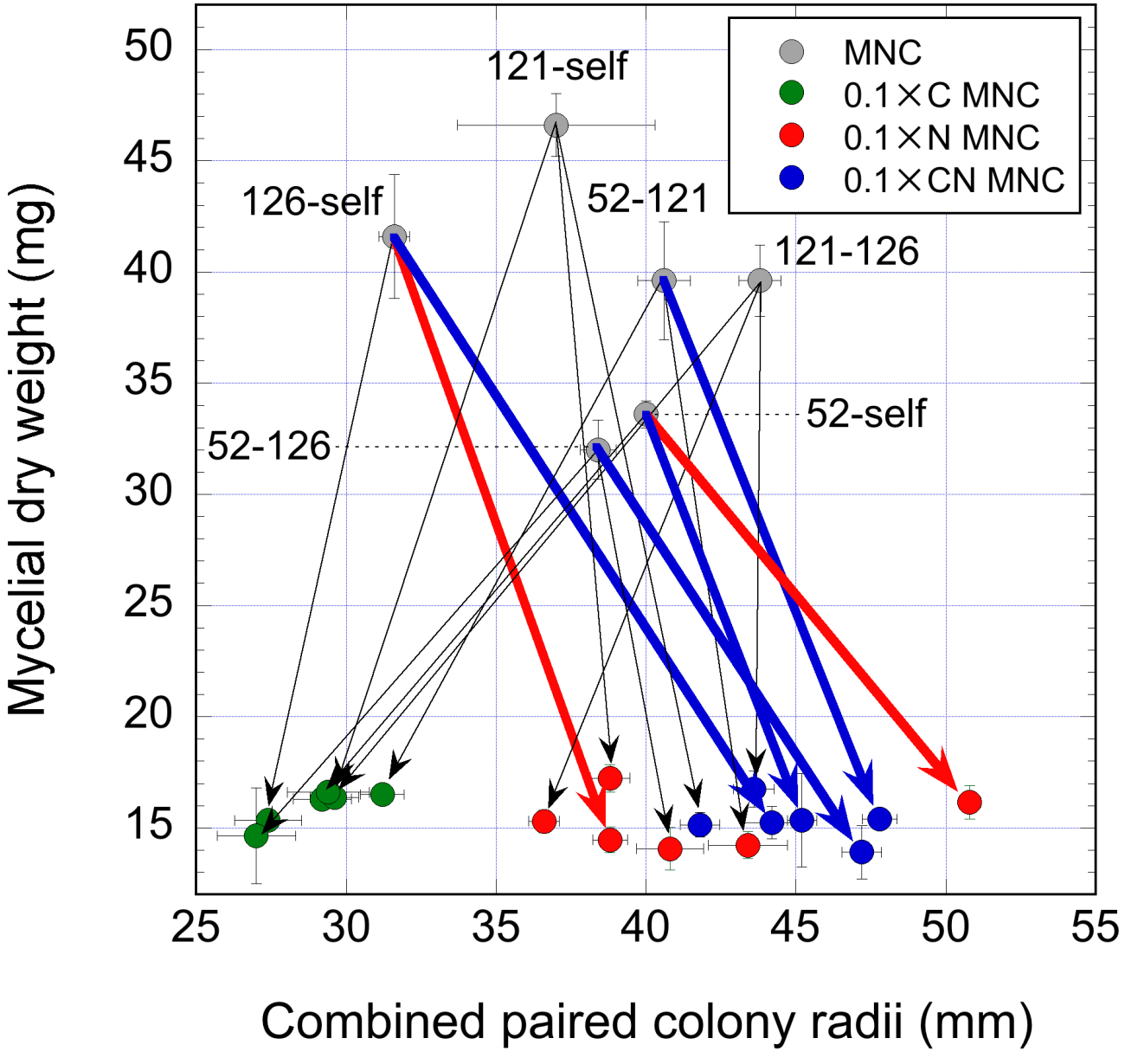
Fig. 5. Karyotic status of *Tricholoma matsutake* isolates. Hypha of isolate #31 with three paired nuclei (arrows) separated by 40–50 μm. *Bar*, 10 μm.

Fig. 6. PCR-RFLP pattern of the IGS1 region. Left- and right-most lanes, DNA size markers (0.1–3.0 kbp); second-left to second-right lanes: PCR amplicons of isolate #126 and isolates #31 to #126 digested by *Hae*III.

Fig. 7. PCR amplicons of the LTR in sibling isolates of *Tricholoma matsutake.* Left: PCR amplicons using the pS1 primer. Left to right: DNA size markers (0.1–3.0 kbp), isolates #31 to #126, isolates AT-0740 and Y1, and negative control (NC).
Right: PCR amplicons using the pS48/pL281 primers. Left to right: isolates #31 to #126, isolates AT-0740 and Y1, negative control (NC), and DNA size markers (0.1–3.0 kbp).


Colony diam (mm)
60
50
40
30
20
10
0
0
20
40
60
80
100
Ordered array of tested isolates
52
79
45
121
31
126
99
84
111


MNC
0.1×C MNC
0.1×N MNC
0.1×CN MNC
121
84
31
45
79
111
99
52
126
35
30
25
20
15
10
15
20
25
30
35
Mycelial dry weight (mg)
Colony diam (mm)


MNC
0.1×C MNC
0.1×N MNC
0.1×CN MNC
121-self
126-self
52-121
121-126
52-self
52-126
Mycelial dry weight (mg)
Combined paired colony radii (mm)
50
45
40
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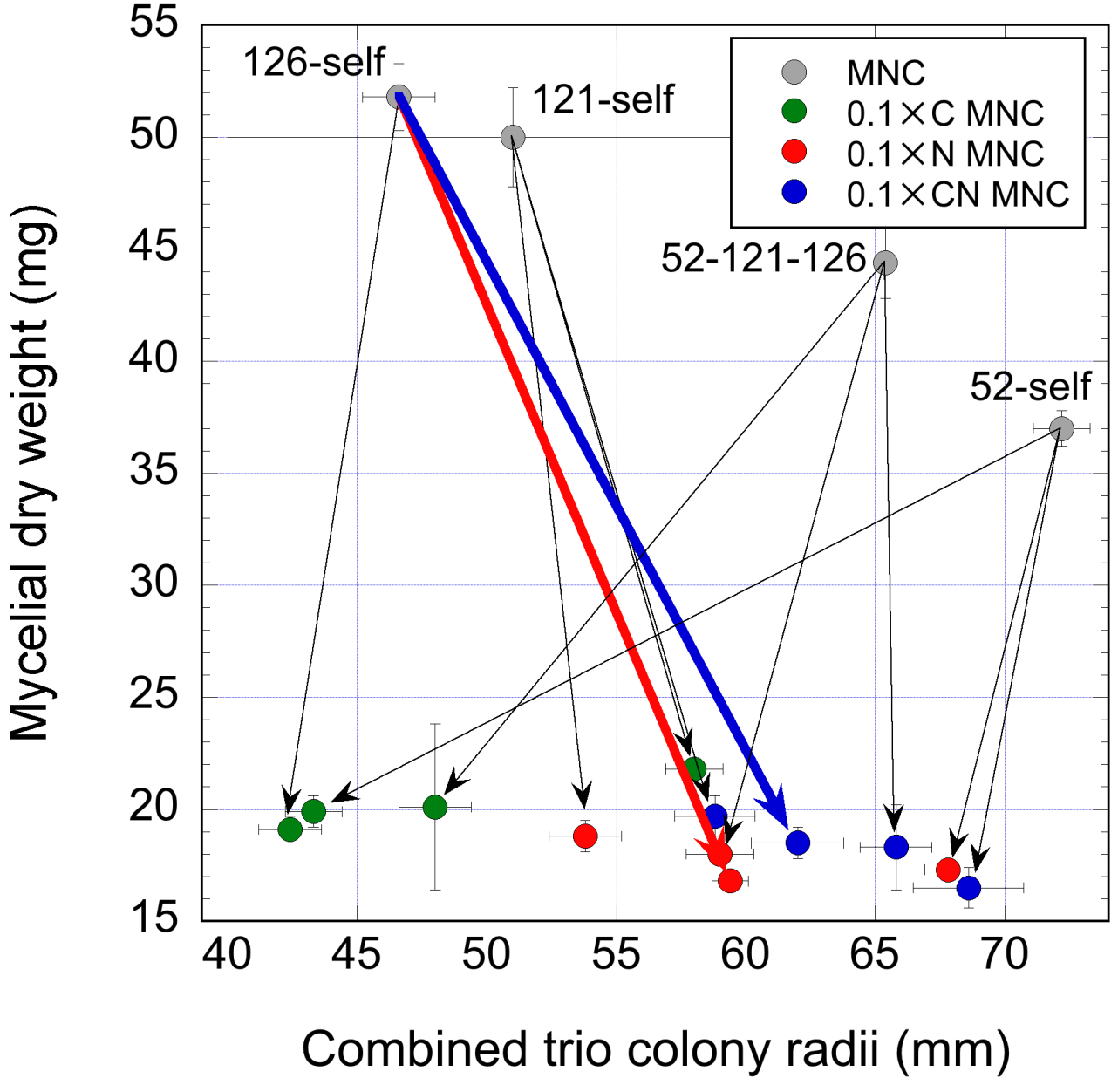
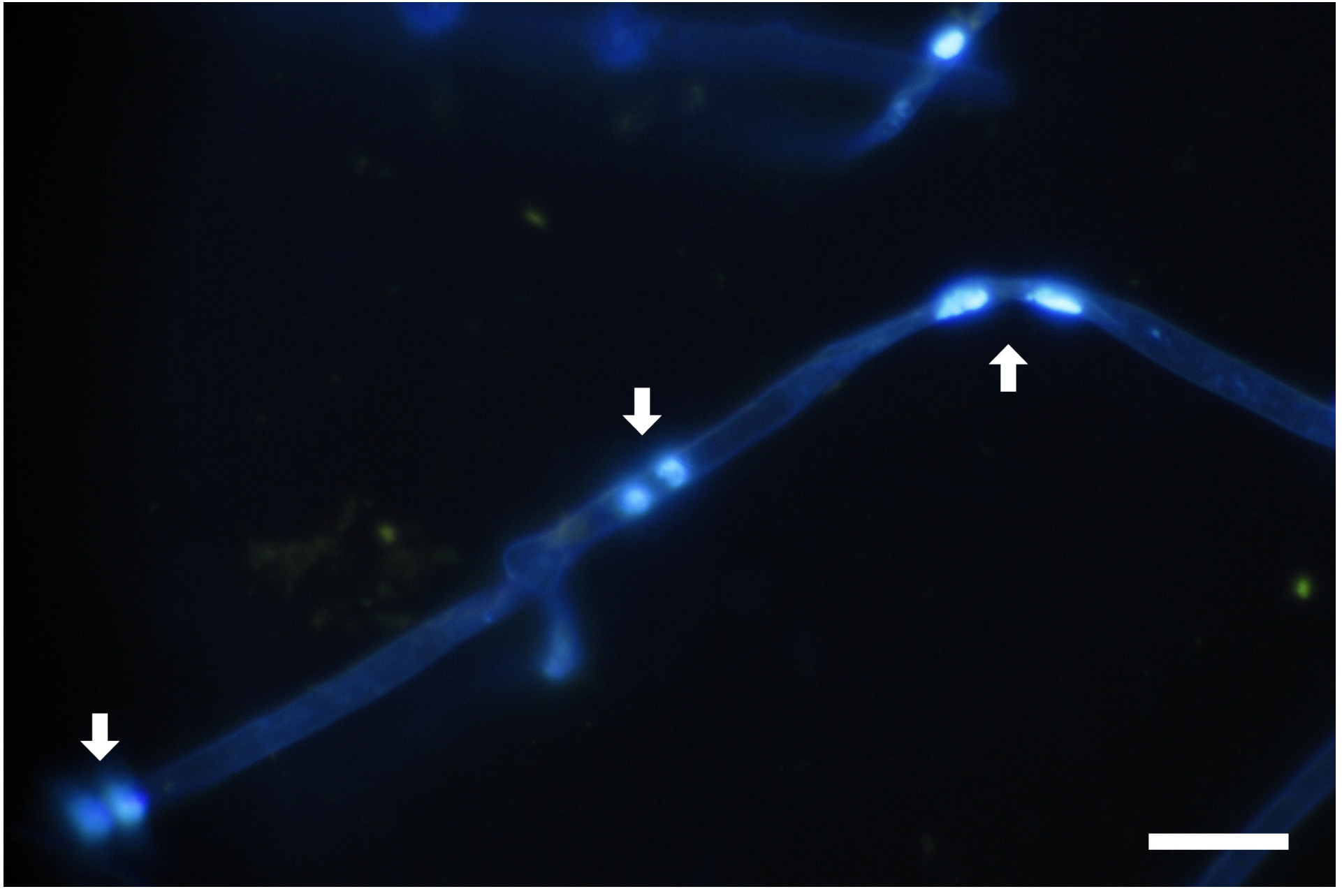
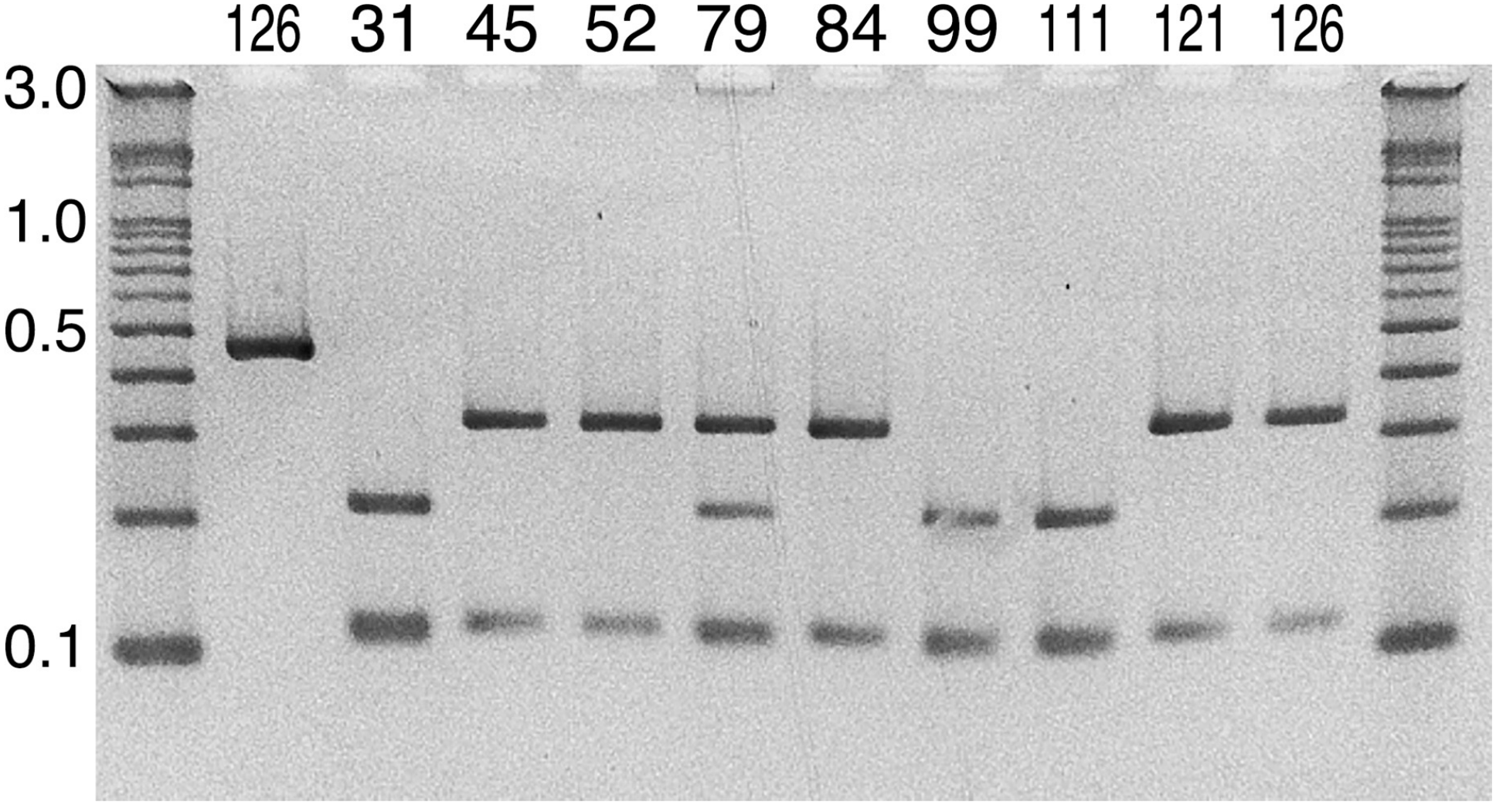
25
20
15
25
30
35
40
45
50
55


126-self
121-self
52-121-126
52-self
MNC
0.1×C MNC
0.1×N MNC
0.1×CN MNC
Mycelial dry weight (mg)
Combined trio colony radii (mm)
55
50
45
40
35
30
25
20
15
40
45
50
55
60
65
70




126
31
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52
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111
121
126
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0.5
0.1

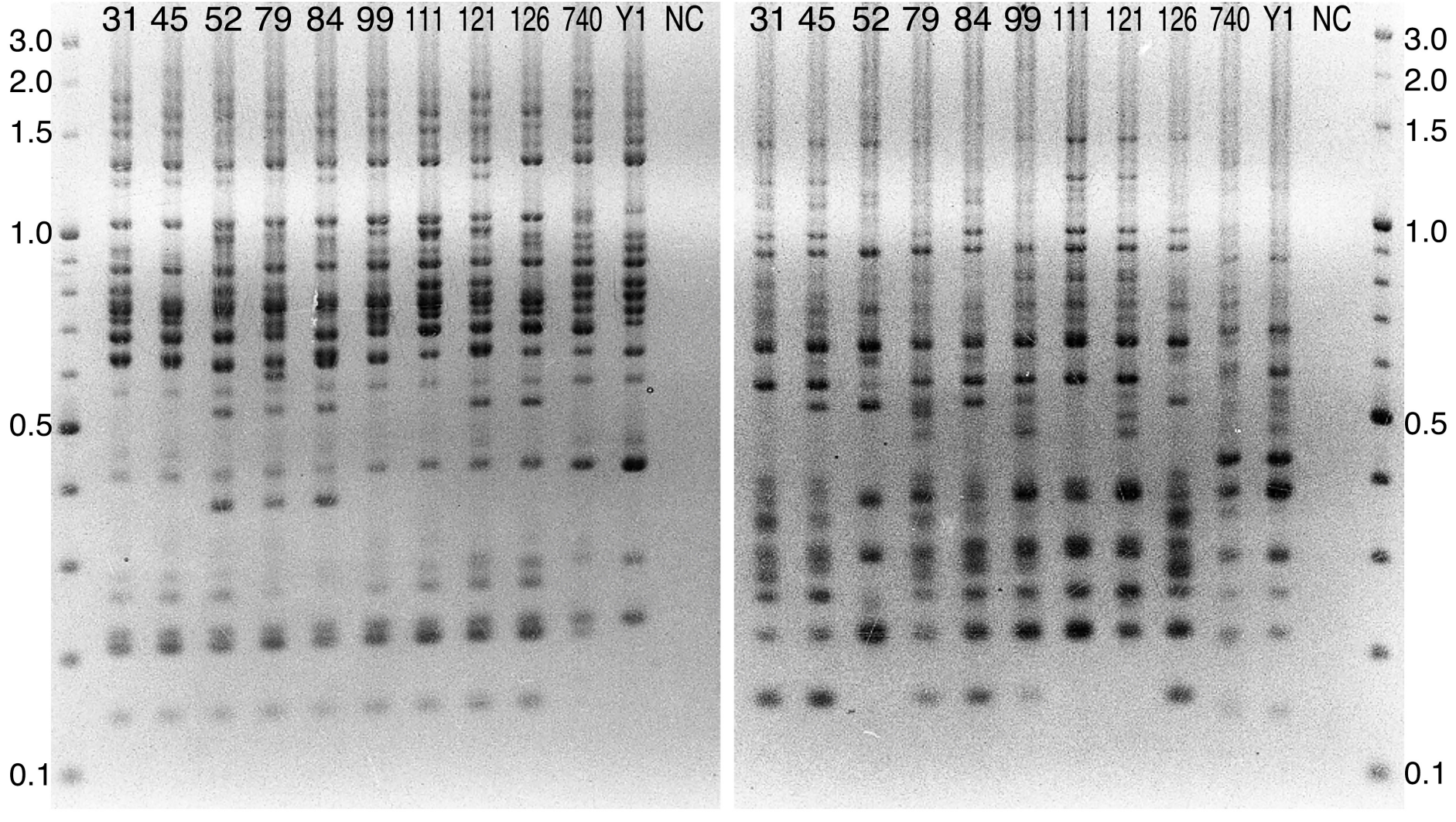
31 45 52 79 84 99 111 121 126 740 Y1 NC
3.0
2.0
1.5
1.0
0.5
0.1
31 45 52 79 84 99 111 121 126 740 Y1 NC
3.0
2.0
1.5
1.0
0.5
0.1

Table 1. Self- and non-self interactions of three *Tricholoma matsutake* isolates after paired inoculations on MNC agar plates with different C and N concentrations.

Paired inoculations of isolates	Colony radius (mm) in different medium compositions							
	Normal MNC		0.1×C MNC		0.1×N MNC		0.1×CN MNC	
(Isolate #52)								
Self	20.4 (0.5)		14.6 (0.4)		25.6 (0.2)		22.4 (0.4)	
Paired with #121	22.6 (0.9)	s*	15.8 (0.5)	ns	24.4 (0.7)	ns	25.6 (0.7)	S
Paired with #126	23.8 (0.7)	S	13.4 (0.8)	ns	23.8 (0.6)	s*	24.8 (0.4)	S
(Isolate #121)								
Self	18.8 (1.7)		14.6 (0.4)		19.4 (0.5)		21.2 (0.6)	
Paired with #52	18.0 (0.0)	ns	15.4 (0.5)	ns	19.0 (0.6)	ns	22.2 (0.2)	ns
Paired with #126	22.2 (0.7)	s*	14.4 (0.5)	ns	19.8 (0.4)	ns	21.4 (0.6)	ns
(Isolate #126)								
Self	16.0 (0.3)		14.4 (0.9)		18.8 (0.2)		22.4 (0.4)	
Paired with #52	14.6 (0.2)	S	13.6 (1.0)	ns	17.0 (0.7)	S	22.4 (0.5)	ns
Paired with #121	21.6 (0.2)	S	15.0 (0.9)	ns	16.8 (0.4)	S	22.2 (0.2)	ns

Mean values are indicated with standard errors in parentheses. S, significant difference from self-pairing (Dunnett's post hoc test; $P < 0.05$). s*, significant difference from self-pairing in t-test ($P < 0.05$) but not in Dunnett's post hoc test. ns, no significant difference in both statistic tests.

Table 2. Self and non-self interactions of three *Tricholoma matsutake* isolates in trio combinations on MNC agar plates with different C and N concentrations

Trio inoculations of isolates	Colony radius (mm) in different medium compositions							
	Normal MNC		0.1×C MNC		0.1×N MNC		0.1×CN MNC	
(Isolate #52)								
Self	24.2 (0.4)		13.5 (0.8)		22.8 (0.4)		22.8 (0.8)	
In trio combination	25.2 (0.2)	S	15.7 (0.9)	ns	23.6 (0.5)	ns	23.2 (0.4)	ns
(Isolate #121)								
Self	18.0 (4.9)		19.8 (0.7)		17.8 (0.5)		19.2 (0.7)	
In trio combination	20.2 (0.6)	ns	16.8 (0.7)	S	18.6 (0.7)	ns	21.6 (0.7)	S
(Isolate #126)								
Self	15.4 (0.5)		13.7 (0.7)		19.8 (0.4)		20.4 (0.7)	
In trio combination	20.0 (0.7)	S	15.5 (0.7)	ns	16.8 (0.2)	S	21.0 (0.6)	ns

Mean values are indicated with standard errors in parentheses. S, significantly different from the radius in self inoculation (t-test; $P < 0.05$). ns, no significant difference.

Supplementary Table 1. Colony diam and biomasses of *Tricholoma matsutake* produced by isolates in different MNC medium compositions.

Isolate code	Normal MNC		0.1×C MNC		0.1×N MNC		0.1×CN MNC	
	Colony diam (mm)	Mycelial dry weight (mg)	Colony diam (mm)	Mycelial dry weight (mg)	Colony diam (mm)	Mycelial dry weight (mg)	Colony diam (mm)	Mycelial dry weight (mg)
#31	21.9 (0.3) C	22.8 (1.6) BC	19.9 (1.1) BC	15.4 (0.6) A	19.8 (0.2) E	12.6 (0.4) B	20.7 (0.3) D	8.6 (0.2) AB
#45	27.4 (0.2) B	22.3 (0.7) BC	20.5 (0.7) BC	15.8 (0.6) A	25.3 (0.3) C	13.0 (0.5) B	27.7 (0.8) B	8.2 (0.8) B
#52	31.7 (0.3) A	17.7 (1.6) CD	21.9 (1.4) ABC	14.4 (0.5) A	37.2 (0.3) A	13.4 (0.7) B	34.4 (0.3) A	8.6 (1.1) AB
#79	30.9 (0.6) A	26.1 (2.0) B	24.4 (0.2) A	16.2 (0.8) A	28.4 (0.2) B	14.0 (0.3) B	33.5 (0.4) A	9.2 (0.5) AB
#84	19.1 (0.9) D	23.5 (0.9) BC	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
#99	14.0 (0.9) E	20.1 (1.5) BCD	18.6 (0.6) C	13.8 (1.0) A	22.8 (0.4) D	13.0 (0.9) B	22.6 (0.1) CD	11.2 (0.6) A
#111	23.7 (0.6) C	20.4 (1.2) BCD	22.7 (1.1) AB	16.4 (0.7) A	21.4 (0.3) D	14.2 (1.0) AB	20.9 (0.4) D	8.2 (0.7) B
#121	25.5 (0.5) BC	35.0 (1.5) A	23.1 (0.3) AB	15.6 (0.4) A	25.5 (0.4) C	17.2 (0.6) A	25.1 (0.5) C	10.8 (0.6) AB
#126	23.0 (0.5) C	14.6 (0.7) D	21.5 (0.4) ABC	16.0 (0.5) A	22.5 (0.4) D	12.0 (0.5) B	24.6 (0.2) C	8.2 (0.2) B

Mean values are indicated with standard errors in parentheses. Different upper case letters in each column indicate statistically significant pairwise differences between means (Tukey's HSD test; $P < 0.05$). n.a., not available. Although we conducted growth test of isolate #84 on low C and/or N conditions of MNC medium, we missed data of 4 culture conditions (colony diam on 0.1×C MNC agar plates, mycelial biomass in 0.1×C, 0.1×N, and 0.1×CN MNC liquid media). Therefore, we did not indicate even available data of two culture conditions (colony diam on 0.1×N and 0.1×CN MNC agar plates).

Supplementary Table 2. Colony diam and biomasses of *Tricholoma matsutake* produced in different MNC medium compositions (data are provided for each single isolate).

Isolate code	Colony diam (mm)							Mycelial dry weight (mg)							
	Normal MNC	0.1×C MNC		0.1×N MNC		0.1×CN MNC		Normal MNC	0.1×C MNC		0.1×N MNC		0.1×CN MNC		
#31	21.9 (0.3)	19.9 (1.1)	s*	19.8 (0.2)	s*	20.7 (0.3)	ns	22.8 (1.6)	15.4 (0.6)	S	12.6 (0.4)	S	8.6 (0.2)	S	
#45	27.4 (0.2)	20.5 (0.7)	S	25.3 (0.3)	S	27.7 (0.8)	ns	22.3 (0.7)	15.8 (0.6)	S	13.0 (0.5)	S	8.2 (0.8)	S	
#52	31.7 (0.3)	21.9 (1.4)	S	37.2 (0.3)	S	34.4 (0.3)	s*	17.7 (1.6)	14.4 (0.5)	ns	13.4 (0.7)	ns	8.6 (1.1)	S	
#79	30.9 (0.6)	24.4 (0.2)	S	28.4 (0.2)	S	33.5 (0.4)	S	26.1 (2.0)	16.2 (0.8)	S	14.0 (0.3)	S	9.2 (0.5)	S	
#84	19.1 (0.9)	n.a.		n.a.		n.a.		23.5 (0.9)	n.a.		n.a.		n.a.		
#99	14.0 (0.9)	18.6 (0.6)	S	22.8 (0.4)	S	22.6 (0.1)	S	20.1 (1.5)	13.8 (1.0)	S	13.0 (0.9)	S	11.2 (0.6)	S	
#111	23.7 (0.6)	22.7 (1.1)	ns	21.4 (0.3)	s*	20.9 (0.4)	S	20.4 (1.2)	16.4 (0.7)	ns	14.2 (1.0)	S	8.2 (0.7)	S	
#121	25.5 (0.5)	23.1 (0.3)	S	25.5 (0.4)	ns	25.1 (0.5)	ns	35.0 (1.5)	15.6 (0.4)	S	17.2 (0.6)	S	10.8 (0.6)	S	
#126	23.0 (0.5)	21.5 (0.4)	s*	22.5 (0.4)	ns	24.6 (0.2)	S	14.6 (0.7)	16.0 (0.5)	ns	12.0 (0.5)	S	8.2 (0.2)	S	

Mean values are indicated with standard errors in parentheses. n.a., not available. S, significant difference from the mean in normal MNC composition medium (Dunnett's multiple comparison test; $P < 0.05$). s*, significant difference at $P < 0.1$, but no significant difference at $P = 0.05$. ns, no significant difference at $P = 0.1$.

Supplementary Table 3. Combined paired colony radii and biomasses of *Tricholoma matsutake* produced by combinations of paired isolates in different MNC medium compositions.

Paired isolate codes	Normal MNC		0.1×C MNC		0.1×N MNC		0.1×CN MNC	
	Combined paired colony radii (mm)	Mycelial dry weight (mg)	Combined paired colony radii (mm)	Mycelial dry weight (mg)	Combined paired colony radii (mm)	Mycelial dry weight (mg)	Combined paired colony radii (mm)	Mycelial dry weight (mg)
#52 self	40.0 (0.3) AB	33.6 (0.6) BC	29.2 (1.0) A	16.3 (0.3) AB	50.8 (0.4) A	16.2 (0.7) AB	45.2 (0.5) ABC	15.3 (2.1) A
#121 self	37.0 (3.3) BC	46.6 (1.4) A	29.6 (0.8) A	16.4 (0.4) AB	38.8 (0.7) C	17.2 (0.6) A	41.8 (0.7) D	15.1 (0.5) A
#126 self	31.6 (0.5) C	41.6 (2.8) AB	27.4 (1.1) A	15.3 (0.2) B	38.8 (0.6) C	14.5 (0.6) AB	44.2 (1.0) BCD	15.2 (0.7) A
#52 and#121	40.6 (0.9) AB	39.6 (2.7) ABC	31.2 (0.7) A	16.5 (0.3) A	43.4 (1.3) B	14.2 (0.6) B	47.8 (0.6) A	15.4 (0.3) A
#52 and #126	38.4 (0.6) AB	32.0 (1.3) C	27.0 (1.3) A	14.6 (2.2) AB	40.8 (1.1) BC	14.1 (0.9) B	47.2 (0.7) AB	13.9 (1.2) A
#121 and #126	43.8 (0.7) A	39.6 (1.6) ABC	29.4 (1.4) A	16.6 (0.3) AB	36.6 (0.5) D	15.3 (0.5) AB	43.6 (0.7) CD	16.7 (0.8) A

Mean values are indicated with standard errors in parentheses. Different upper case letters in each column indicate statistically significant pairwise differences between means (Tukey's HSD test; $P < 0.05$).

Supplementary Table 4. Combined paired colony radii and biomasses of *Tricholoma matsutake* produced in different MNC medium compositions (data are provided for each combination of single and paired isolates).

Paired isolate codes	Combined paired colony radii (mm)							Mycelial dry weight (mg)						
	Normal MNC	0.1×C MNC		0.1×N MNC		0.1×CN MNC		Normal MNC	0.1×C MNC		0.1×N MNC		0.1×CN MNC	
#52 self	40.0 (0.3)	29.2 (1.0)	S	50.8 (0.4)	S	45.2 (0.5)	S	33.6 (0.6)	16.3 (0.3)	S	16.2 (0.7)	S	15.3 (2.1)	S
#121 self	37.0 (3.3)	29.6 (0.8)	S	38.8 (0.7)	ns	41.8 (0.7)	ns	46.6 (1.4)	16.4 (0.4)	S	17.2 (0.6)	S	15.1 (0.5)	S
#126 self	31.6 (0.5)	27.4 (1.1)	S	38.8 (0.6)	S	44.2 (1.0)	S	41.6 (2.8)	15.3 (0.2)	S	14.5 (0.6)	S	15.2 (0.7)	S
#52 and #121	40.6 (0.9)	31.2 (0.7)	S	43.4 (1.3)	ns	47.8 (0.6)	S	39.6 (2.7)	16.5 (0.3)	S	14.2 (0.6)	S	15.4 (0.3)	S
#52 and #126	38.4 (0.6)	27.0 (1.3)	S	40.8 (1.1)	ns	47.2 (0.7)	S	32.0 (1.3)	14.6 (2.2)	S	14.1 (0.9)	S	13.9 (1.2)	S
#121 and #126	43.8 (0.7)	29.4 (1.4)	S	36.6 (0.5)	S	43.6 (0.7)	ns	39.6 (1.6)	16.6 (0.3)	S	15.3 (0.5)	S	16.7 (0.8)	S

Mean values are indicated with standard errors in parentheses. S, significant difference from the mean in normal MNC composition medium (Dunnett's multiple comparison test; $P < 0.05$). ns, no significant difference at $P = 0.05$.

Supplementary Table 5. Combined trio and self colony radii and biomasses of *Tricholoma matsutake* produced by combinations of trio isolates in different MNC medium compositions.

Trio isolate codes	Normal MNC		0.1×C MNC		0.1×N MNC		0.1×CN MNC	
	Combined trio colony radii (mm)	Mycelial dry weight (mg)	Combined trio colony radii (mm)	Mycelial dry weight (mg)	Combined trio colony radii (mm)	Mycelial dry weight (mg)	Combined trio colony radii (mm)	Mycelial dry weight (mg)
#52 self	72.2 (1.1) A	37.0 (0.8) C	43.3 (1.1) BC	19.9 (0.7) A	67.8 (0.9) A	17.3 (0.4) A	68.6 (2.1) A	16.5 (0.9) A
#121 self	51.0 (11.0) BC	50.0 (2.2) AB	58.0 (1.1) A	21.8 (0.2) A	53.8 (1.4) C	18.8 (0.7) A	58.8 (1.6) B	19.7 (0.9) A
#126 self	46.6 (1.4) C	51.8 (1.5) A	42.4 (1.2) C	19.1 (0.6) A	59.4 (0.7) B	16.8 (0.3) A	62.0 (1.8) AB	18.5 (0.7) A
#52, #121 and #126	65.4 (0.4) AB	44.4 (1.6) B	48.0 (1.4) B	20.1 (3.7) A	59.0 (1.3) B	18.0 (0.5) A	65.8 (1.4) AB	18.3 (1.9) A

Mean values are indicated with standard errors in parentheses. Different upper case letters in each column indicate statistically significant pairwise differences between means (Tukey's HSD test; $P < 0.05$).

Supplementary Table 6. Combined trio and self colony radii and biomasses of *Tricholoma matsutake* produced in different MNC medium compositions (data are provided for each single and combined trio cultures).

Trio isolate codes	Colony diameter (mm)						Mycelial dry weight (mg)					
	Normal MNC	0.1×C MNC		0.1×N MNC		0.1×CN MNC	Normal MNC	0.1×C MNC		0.1×N MNC		0.1×CN MNC	
#52 self	72.2 (1.1)	43.3 (1.1)	S	67.8 (0.9)	s*	68.6 (2.1)	ns	37.0 (0.8)	19.9 (0.7)	S	17.3 (0.4)	S	16.5 (0.9)	S
#121 self	51.0 (11.0)	58.0 (1.1)	ns	53.8 (1.4)	ns	58.8 (1.6)	ns	50.0 (2.2)	21.8 (0.2)	S	18.8 (0.7)	S	19.7 (0.9)	S
#126 self	46.6 (1.4)	42.4 (1.2)	ns	59.4 (0.7)	S	62.0 (1.8)	S	51.8 (1.5)	19.1 (0.6)	S	16.8 (0.3)	S	18.5 (0.7)	S
#52, #121 and #126	65.4 (0.4)	48.0 (1.4)	S	59.0 (1.3)	S	65.8 (1.4)	ns	44.4 (1.6)	20.1 (3.7)	S	18.0 (0.5)	S	18.3 (1.9)	S

Mean values are indicated with standard errors in parentheses. S, significant difference from the mean in normal MNC composition medium (Dunnett's multiple comparison test; $P < 0.05$). s*, significant difference at $P < 0.1$, but no significant difference at $P = 0.05$. ns, no significant difference at $P = 0.1$.