

Tricholoma matsutake in a natural *Pinus densiflora* forest: correspondence between above- and below-ground genets, association with multiple host trees and alteration of existing ectomycorrhizal communities

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Summary

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- *Tricholoma matsutake* (matsutake) is an ectomycorrhizal (ECM) fungus that produces economically important mushrooms in Japan. Here, we use microsatellite markers to identify genets of matsutake sporocarps and below-ground ECM tips, as well as associated host genotypes of *Pinus densiflora*.
- We also studied ECM fungal community structure inside, beneath and outside the matsutake fairy rings, using morphological and internal transcribed spacer (ITS) polymorphism analysis.
- Based on sporocarp samples, one to four genets were found within each fairy ring, and no genetic differentiation among six sites was detected. Matsutake ECM tips were only found beneath fairy rings and corresponded with the genotypes of the above-ground sporocarps. We detected nine below-ground matsutake genets, all of which colonized multiple pine trees (three to seven trees per genet). The ECM fungal community beneath fairy rings was species-poor and significantly differed from those inside and outside the fairy rings.
- We conclude that matsutake genets occasionally establish from basidiospores and expand on the root systems of multiple host trees. Although matsutake mycelia suppress other ECM fungi during expansion, most of them may recover after the passage of the fairy rings.

Key words: common mycorrhizal networks, ectomycorrhizal (ECM) community, fungal population, genetic diversity, genetic structure, matsutake (*Tricholoma matsutake*), microsatellite markers.

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Introduction

Tricholoma matsutake is an ectomycorrhizal (ECM) basidiomycete that occurs primarily within Japanese red pine (*Pinus densiflora*) forests in Japan. This species forms underground mycelial aggregations termed ‘shiros’, which usually form a circular arrangement of sporocarps known as fruiting fairy rings (Ogawa, 1975; Ogawa *et al.*, 1978). Matsutake sporocarps are

economically important, edible mushrooms in Japan. Recently, the annual harvest of matsutake has decreased markedly because *P. densiflora* forests have declined as a result of pine wilt disease caused by the pinewood nematode (*Bursaphelenchus xylophilus*), in addition to deforestation and modern forestry management practices that are not suitable to matsutake propagation (Wang *et al.*, 1997; Gill *et al.*, 2000). To satisfy the domestic demand, approximately 3000 tons of these mushrooms are imported

annually, predominantly from Korea, China, North America and North Africa (Wang *et al.*, 1997; Gill *et al.*, 2000).

Efforts have been made to establish an artificial cultivation system that would satisfy the year-round demand for high-quality mushrooms (Ogawa *et al.*, 1978; Tominaga, 1978; Yamada *et al.*, 1999; Vaario *et al.*, 2000). Unfortunately, this has not yet been successful. The maintenance and increase in natural matsutake production in pine forests may be another solution. Knowledge of the natural reproduction of this fungus is therefore indispensable for successful management to protect shiros and regenerate abundant matsutake sporocarps in pine forests.

The reproductive characteristics of ECM fungi in nature have been reported for many species (Dahlberg & Stenlid, 1994, 1995; Gherbi *et al.*, 1999; Zhou *et al.*, 1999; Bergemann & Miller, 2002; Hirose *et al.*, 2004; Kretzer *et al.*, 2005; Wu *et al.*, 2005). In natural forest ecosystems, ECM fungi exist as a complex of mycelia in ECM roots, extraradical mycelia in the soil, and above-ground sporocarps, and they expand their populations by dispersal of basidiospores, fragmented mycelia, mitotic sporulation, and sclerotial and extraradical mycelial extension (Dahlberg & Stenlid, 1995). It is necessary to examine both the above-ground sexual (sporocarps) and below-ground vegetative structures (mycorrhizas and mycelia in the soil) to elucidate ECM fungal reproduction (Guidot *et al.*, 2001; Horton & Bruns, 2001; Zhou *et al.*, 2001b; Kretzer *et al.*, 2003; Hirose *et al.*, 2004).

Population genetic analyses are quite effective in ecological investigations of fungal reproduction. Conventional methods such as somatic incompatibility tests have been employed (Fries, 1987; Dahlberg & Stenlid, 1994) but more recently, dominant molecular markers, such as intersimple sequence repeat (ISSR), amplified fragment length polymorphic (AFLP) and inter-retrotransposon amplified polymorphism (IRAP) markers, have been used as effective tools for identifying ECM fungal genet structures (Anderson *et al.*, 1998; Zhou *et al.*, 1999; Redecker *et al.*, 2001; Murata *et al.*, 2005). Although dominant markers generally do not provide convincing information on population genetics as well as information on the below-ground conditions, they work well in ECM fungal genet identification among sporocarps because of their high levels of polymorphism. More recently, microsatellite or simple sequence repeat (SSR) markers, which are characterized by hypervariability, high reproducibility and codominance, have been used as the most powerful markers to investigate population genetics in ECM species (Zhou *et al.*, 2001a; Kretzer *et al.*, 2003, 2005; Wu *et al.*, 2005). Generally, SSR markers are highly species-specific to ECM fungal species or host plants and amplify only the target species DNA from the ECM template, which is a mixture of plant and fungal DNA (Zhou *et al.*, 2001a; Kretzer *et al.*, 2003; Saari *et al.*, 2005; Wu *et al.*, 2005).

In natural ecosystems, individual ECM fungal species coexist with many other ECM fungal species, interacting with each

other in various ways. Although matsutake also interacts with other surrounding ECM fungal species, the ECM fungal communities around matsutake shiros remain unknown. Information about such communities would be valuable in elucidating the ecological interactions between matsutake and other ECM fungi during shiro development; moreover, such information would be indispensable for practical management of sporocarp production for this species in natural pine forests.

Here, we used SSR analysis to reveal reproductive characteristics of matsutake, including the genet structure within each fruiting fairy ring, the relationship between above- and below-ground genets, and the relationship between matsutake genets and genotypes of its host plant, *P. densiflora*. We also examined below-ground ECM communities beneath, inside and outside fruiting fairy rings by internal transcribed spacer (ITS) region polymorphism analysis.

Materials and Methods

Study area

This study was conducted at seven long-term *Tricholoma matsutake* (S. Ito et Imai) research sites in a pine forest of the Iwate Prefectural Forestry Technology Center near Morioka, north-eastern Japan (39°56' N, 141°14' E; Fig. 1). The study area is located at an altitude between 360 and 380 m above sea level. The mean annual temperature and precipitation are 9.3°C and 1145 mm, respectively. Vegetation in the study area was dominated by *P. densiflora* Sieb. et Zucc. of c. 85 year

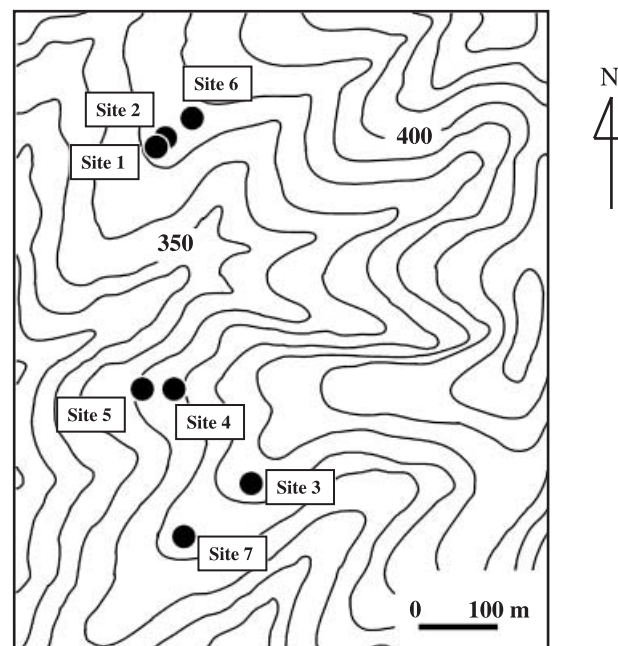


Fig. 1 Map of the study area (seven long-term *Tricholoma matsutake* research sites in a pine forest of the Iwate Prefectural Forestry Technology Center near Morioka, north-eastern Japan).

of age that originated from natural regeneration. Another ECM host species, *Quercus mongolica*, was also distributed throughout the study sites. However, this species did not appear to be colonized by matsutake and was not investigated in this study. The seven *T. matsutake* research sites were separated from each other by 5–500 m. Between 1994 and 2003, the average number of annually developed matsutake sporocarps within the seven research sites was 138 (Narimatsu, 2004).

Sporocarp sampling

Fruiting of matsutake occurred from early September until the end of October. Site 4 was artificially disturbed and was therefore excluded from comparisons of genetic differentiation of matsutake sporocarps among sites. Sporocarps were collected from the other six sites during fruiting periods in 2001, 2002 and 2003. During fruiting periods, sites were visited daily to ensure comprehensive sampling of sporocarps. The locations of all matsutake sporocarps were recorded before the sporocarps were collected. In 2001, 2002 and 2003, we sampled 51, 164 and 65 sporocarps, respectively. Stem pieces were excised from each sporocarp, desiccated with silica gel, and stored at room temperature until further analysis.

Sampling of ECM root tips

Below-ground ECM tips were collected in 2002 and 2005. In 2002, we selected two sporocarps (1D2 and 2D2) that occurred in close proximity (1.7 m) in a fairy ring at site 4. We established two parallel plots (90 × 210 cm), with one sporocarp at the center of each plot. To assess the fine-scale distribution of below-ground matsutake genets, each plot was subsequently divided into 21 square subplots (30 × 30 cm), as shown in Fig. 4. A soil sample (10 cm³) was collected from the center of each subplot 2 wk after the last sporocarp occurrence. Samples were kept at 4°C until further processing. We randomly subsampled one-sixteenth of the mixed root fragments based on the biomass contained in each soil sample. After careful washing, all ECM root tips were collected, classified according to morphology, and counted under a dissecting microscope. Surface color, texture, emanating hyphae and rhizomorphs were used for morphotyping. We randomly selected 10 ECM root tips from each morphotype group. Each ECM root tip was placed in a 2.0 ml centrifuge tube, desiccated with silica gel, and stored at room temperature until further analysis.

Since replicated fairy rings were unavailable in 2002, we could not unequivocally show differences in ECM communities among different spatial positions in fairy rings, (i.e. inside, beneath and outside the rings). Thus, five different fruiting fairy rings were newly selected for ECM sampling at the same site in October 2005. Within each fruiting fairy ring, two sporocarps were arbitrarily chosen. The positions of selected fruiting fairy rings and sporocarps are shown in Fig. 3. For each sporocarp, three soil samples (10 cm³) were collected beneath the fairy

rings (beneath the sporocarp), inside the fairy rings (50 cm from the sporocarp toward the inside) and outside the fairy rings (50 cm from the sporocarp toward the outside), respectively. The ECM root tips were collected from soil samples following the method of Nara *et al.* (2003). Briefly, all roots contained in each soil sample were carefully removed and washed. Roots of plants other than *P. densiflora* were excluded. Approximately 200 root tips were then randomly collected from each soil sample. If a soil sample contained < 200 root tips, all root tips were used. The ECM root tips were morphotyped as described above. One or two ECM root tips were selected from each morphotype except the one specific to matsutake. From the morphotype specific to matsutake, 10 root tips were selected. Each of the selected ECM root tips was placed in a 2.0 ml centrifuge tube, desiccated with silica gel, and stored at room temperature until further analysis.

Mapping and sampling of host *P. densiflora* at site 4

The diameter at breast height (d.b.h., 1.3 m above ground level) and positions of all *P. densiflora* trees growing within site 4 were measured in 2005. A bark fragment containing the cambial zone was excised from each *P. densiflora* tree. Fresh cambial tissue was peeled from each sample, placed in a 2.0-ml centrifuge tube, and used immediately for DNA extraction.

DNA extraction

Crude genomic DNA was extracted from dried sporocarps and fresh cambial tissue using a modified cetyl-trimethylammonium bromide (CTAB) method (Lian *et al.*, 2003b) and from individual ECM root tips using the method described by Nara *et al.* (2003). DNA extracted from sporocarps, cambial tissue and ECM root tips was resuspended in 100, 50 and 20 µl of sterilized water, respectively, and stored at –30°C.

Simple sequence repeat analysis of matsutake sporocarps and host *P. densiflora* trees

We used five microsatellites (*Trma01*, *Trma02*, *Trma07*, *Trma14* and *Trma16*) developed by Lian *et al.* (2003a) to identify genotypes of matsutake sporocarps. Microsatellites were amplified using a polymerase chain reaction (PCR) thermal cycler (TP3000; Takara Shuzo Co., Tokyo, Japan) in a 5-µl reaction mixture containing 0.5 µl of template DNA, 0.4 mM of each dNTP, 0.2 µM of each designed primer pair, of which one primer was labeled with Texas Red (Hitachi Instruments Service Co., Tokyo, Japan), 1× LA PCR buffer (Takara Shuzo Co.), 2.5 mM of Mg²⁺, and 0.25 U of LA *Taq* DNA polymerase (Takara Shuzo Co.). The PCR conditions were as follows: 1 min at 94°C for one cycle, followed by 29 cycles of 30 s at 94°C, 30 s at the annealing temperature of the primer pair and 30 s at 72°C, followed by one cycle of 30 s at 94°C, 30 s at the annealing temperature of each primer

pair (see Lian *et al.*, 2003a), and 5 min at 72°C. The reaction products were electrophoresed on a 6% Long Ranger sequencing gel (FMC BioProducts Co., Rockland, ME, USA) using an SQ-5500 sequencer (Hitachi Electronics Engineering Co., Tokyo, Japan). Electrophoretic patterns were analysed using FRAGLYS, version 3 software (Hitachi).

To identify the genotypes of host *P. densiflora* trees, four SSR markers were used: Pde3, Pde5, Pde14 (developed by Lian *et al.*, 2000) and pdms09 (developed by Watanabe *et al.*, 2006). The samples were amplified in a 5- μ l reaction mixture containing 1 μ l of template DNA, 2.5 μ l Hotstar *Taq* Mastermix (Qiagen, Hilden, Germany), 0.3 μ M of each designed primer pair and 1.35 μ l of sterilized water. The PCR amplification was performed as follows: 15 min at 95°C for one cycle, 40 cycles of 30 s at 95°C, 90 s at the annealing temperature of the primer pair (Lian *et al.*, 2000; Watanabe *et al.*, 2006) and 60 s at 72°C, followed by a final 5-min extension step at 72°C. The amplified PCR products were detected as described earlier.

Internal transcribed spacer–terminal restriction fragment length polymorphism analysis of ECM root tips

Polymorphisms of the ITS region were used for species-level identification of ECM root tips. Briefly, template DNA was amplified by PCR using the fungal-specific primer pair ITS1f and ITS4 (Gardes & Bruns, 1993), of which the primer ITS4 was labeled with Texas Red. The PCR amplification was conducted using a KOD-Plus-DNA Polymerase kit (Toyobo Co., Osaka, Japan) in 2002 and an Ampli *Taq* Gold kit (Applied Biosystems, Foster City, CA, USA) in 2005 following the manufacturers' instructions. For the samples in 2002, only ITS fragment lengths were analysed by gel electrophoresis using the sequencer. For the samples in 2005, each PCR product (1 μ l) was further digested in a 5- μ l reaction solution with a restriction enzyme, *Hinf*I (0.6 U) or *Alu*I (0.75 U), at 37°C for 4 h. Terminal restriction fragment length polymorphisms (T-RFLP) were then analysed using the sequencer.

One or two root tips from each ITS-T-RFLP group in 2005 and from each of the dominant ITS groups in 2002 were used for sequencing. The ITS fragments were amplified using the unlabeled primer pair ITS1f and ITS4. The PCR products were purified using a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) according to the manufacturer's instructions. The purified products were sequenced using a DTCS Quick Start Master Mix (Beckman Coulter Inc., Fullerton, CA, USA) and a capillary sequencer (CEQ8800; Beckman Coulter). For the unsuccessfully sequenced samples, the PCR products were subcloned using the pT7 Blue Perfectly Blunt Cloning kit (Novagen, Madison, WI, USA). The insert in each positive clone was amplified using the M13 reverse and U19 forward primers, and its size was determined using agarose gel electrophoresis to confirm that the clone contained

the full length of the PCR product. The PCR products of the target inserts were then sequenced as described above.

The sequences were compared with GenBank sequences by conducting BLAST searches. Similarity to the sequences of known species was used to assign fungal species identities. Furthermore, to identify accurately the below-ground ECM fungal species, we also collected sporocarps of ECM fungi found in the study area in 2005. Sequence data of these sporocarps were also used for the identification of fungal species on ECM tips.

SSR analysis of matsutake ECM root tips

The ECM root tips from the matsutake morphotype (Gill *et al.*, 2000) were also analysed by ITS polymorphisms to confirm matsutake colonization. After this confirmation, we conducted SSR analysis for both symbionts (i.e. matsutake and its host *P. densiflora*). Five SSR markers were used to identify below-ground genotypes of matsutake. These markers were confirmed not to amplify the host *P. densiflora* DNA. For the identification of host genotypes, we used four SSR markers that did not amplify matsutake DNA. Amplified products were electrophoresed and analyzed as described above.

Data analysis

Population genetic structure of matsutake sporocarps All sporocarps with an identical allele pattern in all loci were considered to be one genet. We used the method described by Kretzer *et al.* (2003) to calculate the probability that two samples having the same allelic combinations at all unlinked microsatellite loci belong to different genets. The size of each genet was measured as the greatest distance between sporocarps of the same genet. Sites 1 and 2, which were separated by 5 m, were treated as a single population for the analysis of genetic parameters between site pairs. Nei's (1987) unbiased genetic distances between site pairs were calculated using POPGENE, version 1.31 (Yeh *et al.*, 1997). To test for isolation by distance, genetic distances and geographical distances among sites were analysed with Mantel tests using GENALEX, version 5 (Peakall & Smouse, 2001). Weir & Cockerham (1984) estimators of F_{ST} and F_{IS} were estimated using GENEPOP, version 3.4 (Raymond & Rousset, 1995), available on the Internet (<http://wbiomed.curtin.edu.au/genepop/index.html>). The Hardy–Weinberg and linkage equilibrium of SSR loci and genetic differentiation between pairs of sites were also tested using GENEPOP, version 3.4. We estimated the relatedness of pairs of matsutake genets by the method of Queller and Goodnight (1989) using RELATEDNESS, version 5.0. Correlations of relatedness between pairs of genets and corresponding geographical distances were tested with Mantel tests using GENALEX, version 5.

Below-ground community structure Since the number of replications was not sufficient in the 2002 samples, we used 2005 data for the quantitative analysis of the below-ground

ECM community. The ECM root tips of the same fungal species in different morphotypes were pooled in each soil sample. The relative abundance of a given ECM species was expressed as the percentage of the total number of mycorrhizal root tips in each spatial position (i.e. inside, beneath, or outside fairy rings). The frequency of each fungal species was represented by the number of soil samples containing the fungus out of 10 soil samples collected at each spatial position. Bootstrap and second-order jackknife estimates of true species richness beneath, inside and outside matsutake fairy rings were estimated separately using ESTIMATE S, version 7.5 (Colwell, 2005). Shannon and Simpson's indices to evaluate the diversity of ECM fungi in different spatial positions of the fairy rings were also calculated using ESTIMATE S. Significant differences in observed species, estimated species richness, and Shannon and Simpson's indices among different positions of the fairy rings were evaluated with a χ^2 test. Analysis of variance (ANOVA) was used to test the difference in observed species per soil sample among the spatial positions.

Results

Genet distribution of matsutake sporocarps

Most matsutake sporocarps formed fairy rings that were distributed at the six sites examined (Fig. 2). We used five microsatellite markers to determine the genet structures within fairy rings. Of the five microsatellite markers, the locus *Trma02* was monomorphic for all sporocarps collected in 2001. Therefore, this locus was excluded from further analysis. The other four markers, *Trma01*, *Trma07*, *Trma14* and *Trma16*, were polymorphic and yielded 2, 2, 3 and 6 alleles, respectively. The expected probability that two sporocarps having the same multilocus genotype combined with four SSR loci belong to the different genets was calculated as values from 1.1×10^{-4} to 2.7×10^{-2} , with an average of 6.5×10^{-3} . Thus, this low probability indicates that the four SSR loci were sufficient to distinguish all genets in this study.

A total of 38 multilocus genotypes were detected in 280 sporocarps collected from the six sites using the four microsatellite markers. The distribution of sporocarps and genets among sites is shown in Fig. 2. Over 3 yr, 2, 5, 4, 8, 5 and 14 genotypes were identified at sites 1, 2, 3, 5, 6 and 7, respectively. Of the 38 genets, 10 were found in all 3 yr, six in 2001 and 2002, two in 2001 and 2003, four in 2002 and 2003, and one, nine and six only in 2001, 2002 and 2003, respectively.

In total, 13 fruiting fairy rings, shown in light blue in Fig. 2, were observed at the six sites. Of these, six contained one genet, five contained two genets, and the other two fairy rings contained three and four genets. For most genets, the sporocarps of each genet were aggregated. However, sporocarps of genet 3-3 at site 3 were found in two separate patches (Fig. 2c). The largest genet (genet 3-3) was 11.5 m (Fig. 2c). Other genets ranged from 0 to 5.0 m, with an average of 2.0 m.

All four SSR loci of matsutake were consistent with Hardy-Weinberg equilibrium at the study sites. Nei's (1987) unbiased genetic distances between site pairs ranged from 0.0497 to 0.1410, with an average of 0.0844. These values were not correlated with geographic distances ($r = 0.316$; $P = 0.706$; Mantel tests). The overall mean F_{ST} and F_{IS} among sites were 0.0132 and -0.1340 , respectively. No significant genetic differentiation between site pairs was found; neither was there significantly high relatedness between pairs of genets within sites ($r = 0.01$, $P = 0.645$; Mantel tests).

Below-ground community structure of ECM fungi

In 30 soil samples collected in 2005, 39 fungal species were detected based on base-sequence homology (Table 1). Only six species matched sporocarps based on T-RFLP patterns and sequences. Of the 39 fungal species detected, 21 were isolated from only one soil sample. The second-order jackknife and bootstrap estimates were calculated as the presence of 74 and 48 species, respectively (Table 2).

Beneath the fairy rings, four species of ECM fungi were identified. Matsutake was exclusively dominant in all samples. Of 10 soil samples, eight contained only matsutake root tips. In the other two samples, *Rhizopogon* sp., *Russula* sp., and *Tomentella* sp. were detected, but their relative abundances were low ($< 0.5\%$).

Twenty-five species were found inside the fairy rings and 25 species were found outside (Tables 1 and 2). Of these, 11 species occurred both inside and outside. Frequently observed species were common inside and outside the fairy rings (*Rhizopogon* sp. with five soil samples inside and three outside; *Russula* sp. with four inside and four outside; and *Craterellus* sp. with three inside and three outside) (Table 1).

The ECM fungal diversity was significantly lower beneath the fairy rings than inside and outside the rings in terms of observed species richness, estimated species richness, species richness per soil sample and Shannon and Simpson's indices (Table 2). No significant differences in these indices were detected between inside and outside the fairy rings (Table 2).

In the 2002 samples, we found 55 distinct fungal ITS_{1f-4}-types based on the combined analysis of morphotype and ITS_{1f-4} patterns. Matsutake dominated 70–86% of below-ground ECM tips beneath the fairy rings. Frequently observed species were also common between inside and outside the fairy rings. *Russula* sp. occurred in 23 of 42 soil samples and was the most frequently observed species. *Craterellus* sp. and an unknown fungus followed *Russula* sp. in frequency, occurring in 10 soil samples.

Below-ground genet distribution of matsutake

To reveal the correspondence between the spatial distribution of matsutake fruit bodies and the mycorrhizas that formed below ground by the corresponding genets, above- and below-ground

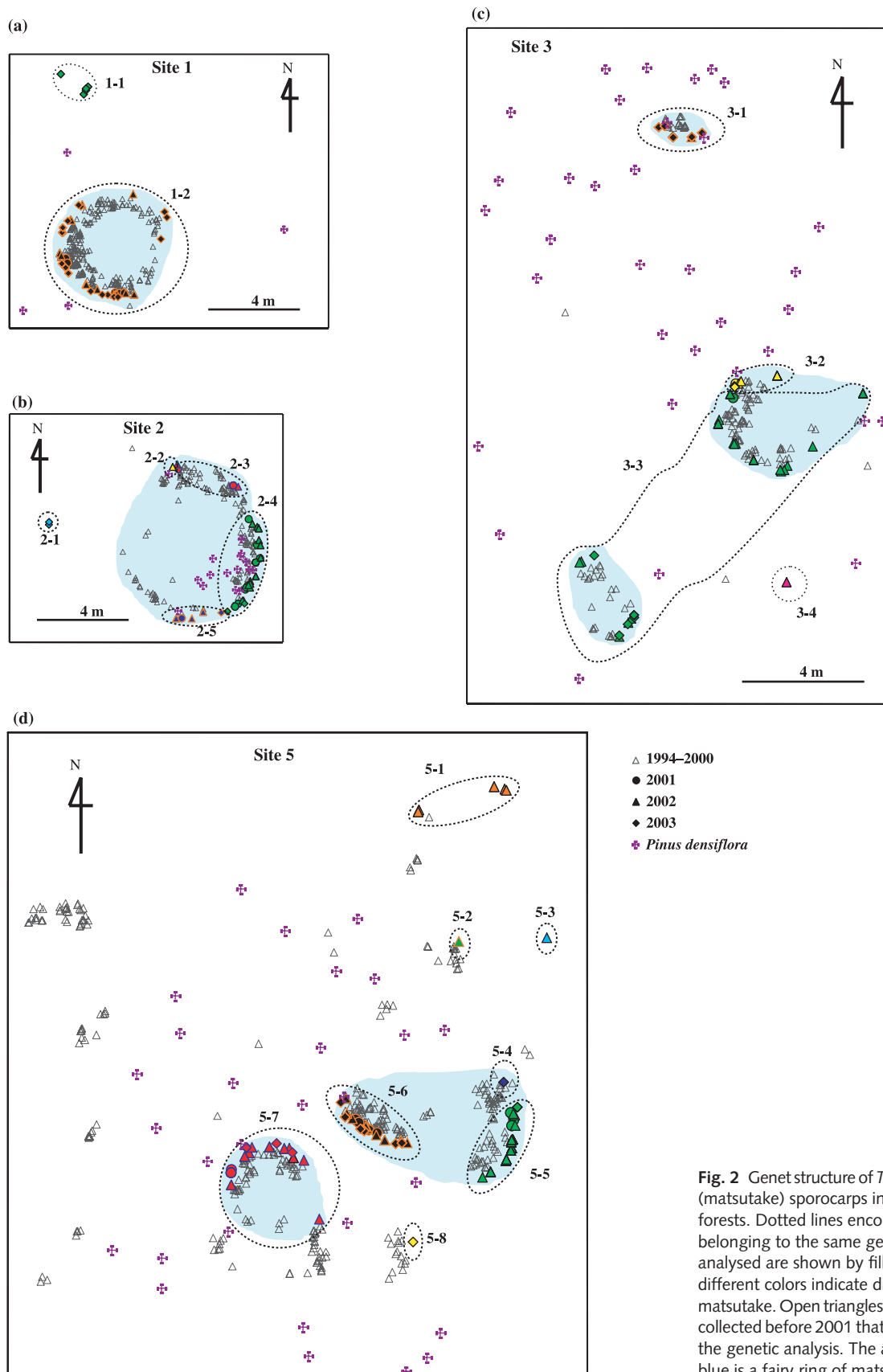


Fig. 2 Genet structure of *Tricholoma matsutake* (matsutake) sporocarps in *Pinus densiflora* forests. Dotted lines encompass sporocarps belonging to the same genet. Sporocarps analysed are shown by filled symbols, where different colors indicate different genets of matsutake. Open triangles represent sporocarps collected before 2001 that were not included in the genetic analysis. The area shown in pale blue is a fairy ring of matsutake.

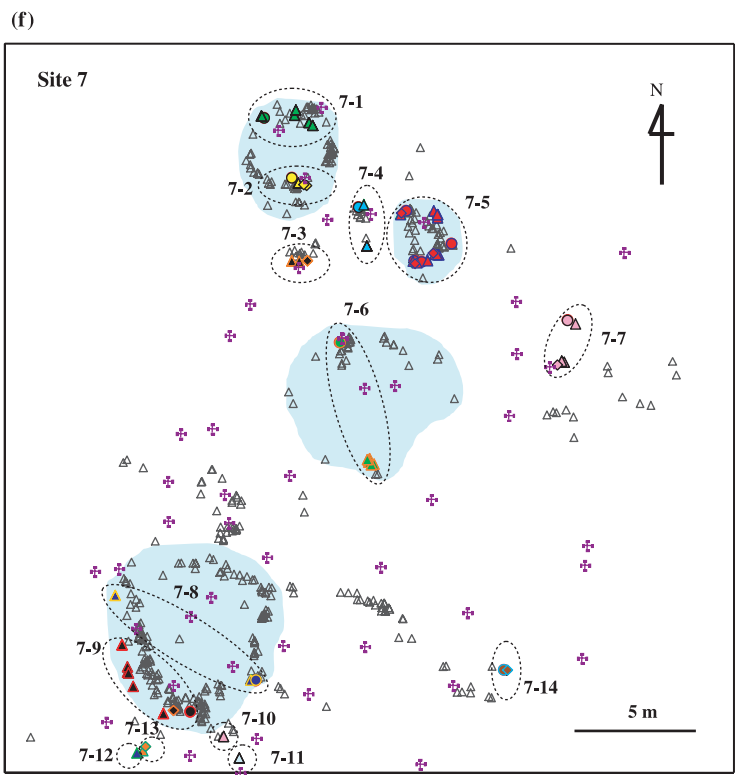
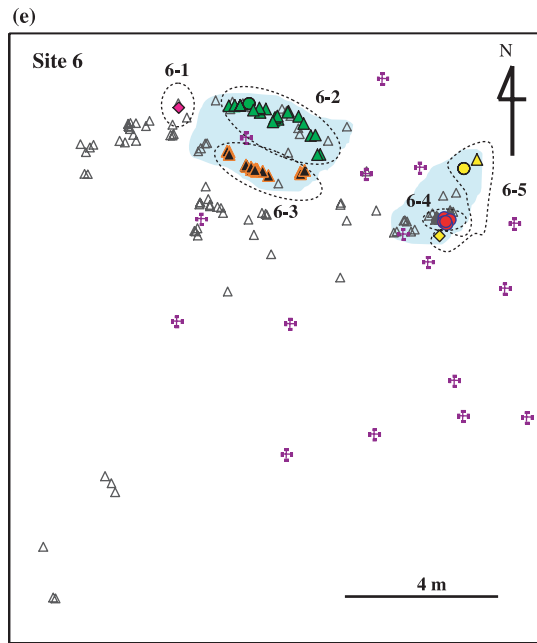


Fig. 2 continued

genets of matsutake were investigated in the fruiting fairy rings. The two sporocarps sampled in each of the five fruiting fairy rings in 2005 were identified as belonging to the same genet, except in ring 3 (Fig. 3). The matsutake genets of five rings were different from each other. We identified six genets. Matsutake ECM root tips were found in all 10 soil samples collected beneath the sporocarps. All matsutake root

tips in each soil sample belonged to the same genet observed as the sporocarp. The correspondence between below-ground and above-ground genets was also confirmed by fine-scale sampling in 2002, in which three genets were identified (Fig. 4). Furthermore, below-ground ECM tips of matsutake were distributed only within a close distance (usually < 40 cm) from the corresponding sporocarps in 2002 (Fig. 4).

Table 1 Relative abundance (RA) and frequency (Fr) of ectomycorrhizal (ECM) fungi detected beneath (Shiro), inside and outside *Tricholoma matsutake* (matsutake) fairy rings

ECM fungal species	Inside RA (%)	Fr (/10)	Shiro RA (%)	Fr (/10)	Outside RA (%)	Fr (/10)	Accession number	Closest BLAST match
<i>Amanita</i> sp. 1	2.1	2	0	0	2.1	1	AB251821	AB015696; 97%; 619 bp
<i>Amanita</i> sp. 2	0.0	0	0	0	0.5	1	AB251844	AF438560; 99%; 316 bp
Ascomycota	1.1	1	0	0	0.0	0	AB251839	AB089660; 100%; 293 bp
<i>Boletus</i> sp.	0.0	0	0	0	3.1	1	AB251823	AY680966; 98%; 452 bp
<i>Cenococcum geophilum</i>	0.0	0	0	0	3.1	1	AB251837	AB089816; 99%; 478 bp
<i>Coltricia</i> sp.	0.3	1	0	0	0.0	0	AB251822	DQ234561; 96%; 162 bp
<i>Cortinarius</i> sp. 1	0.8	1	0	0	2.1	1	AB251830	DQ097877; 96%; 551 bp
<i>Cortinarius</i> sp. 2	2.6	1	0	0	10.6	1	AB251842	AY174813; 94%; 408 bp
<i>Cortinarius</i> sp. 3	4.6	1	0	0	0.0	0	AB251825	AY083191; 94%; 479 bp
<i>Cortinarius</i> sp. 4	0.0	0	0	0	0.3	1	AB251840	AY174853; 93%; 481 bp
<i>Cortinarius</i> sp. 5	9.5	2	0	0	1.7	1	AB251817	AJ438981; 97%; 397 bp
<i>Cortinarius</i> sp. 6	0.0	0	0	0	7.8	2	AB251815	AF136740; 92%; 445 bp
<i>Cortinarius</i> sp. 7	11.2	3	0	0	8.2	3	AB251810	AY082606; 98%; 537 bp
Ectomycorrhizal sp. 1	0.0	0	0	0	0.6	1	AB251835	AF476977; 98%; 376 bp
Ectomycorrhizal sp. 2	0.4	1	0	0	0.0	0	AB251811	AB089818; 99%; 399 bp
<i>Hydnellum</i> sp.	0.8	1	0	0	3.2	1	AB251828	AY569021; 98%; 211 bp
<i>Hygrophorus</i> sp.	10.7	1	0	0	0.0	0	AB251834	DQ097884; 93%; 330 bp
<i>Lactarius</i> sp.	1.0	1	0	0	0.0	0	AB251813	AF096983; 97%; 503 bp
<i>Leucophleps</i> sp.	6.7	2	0	0	3.9	1	AB251819	AY621801; 98%; 173 bp
<i>Pseudotomentella</i> sp.	0.0	0	0	0	0.4	1	AB251831	AF274768; 93%; 502 bp
<i>Rhizopogon</i> sp.	11.5	5	0.3	1	9.6	3	AB251807	AF062936; 99%; 309 bp
<i>Russula</i> sp. 1	0.0	0	0.0	0	3.2	1	AB251827	AY061652; 94%; 523 bp
<i>Russula</i> sp. 2	0.0	0	0.0	0	0.3	1	AB251832	AY750164; 93%; 586 bp
<i>Russula</i> sp. 3	13.8	4	0.5	1	16.8	4	AB251814	AY061719; 94%; 378 bp
<i>Sistotrema</i> sp.	0.1	1	0.0	0	10.6	2	AB251812	AJ606041; 91%; 520 bp
<i>Thelephora</i> sp.	2.1	1	0.0	0	0.0	0	AB251809	AJ889980; 95%; 370 bp
Thelephoraceae sp.	0.8	2	0.0	0	0.5	1	AB251836	U83468; 91%; 487 bp
<i>Thelephoroid</i> sp.	0.0	0	0.0	0	7.9	1	AB251833	AF351870; 98%; 572 bp
<i>Tomentella</i> sp. 1	1.4	1	0.0	0	0.0	0	AB251843	U83482; 91%; 557 bp
<i>Tomentella</i> sp. 2	16.0	3	0.0	0	1.2	1	AB251820	AF272901; 98%; 515 bp
<i>Tomentellopsis</i> sp.	0.8	2	0.3	1	0.0	0	AB251829	AJ410760; 99%; 346 bp
<i>Tricholoma matsutake</i>	0.0	0	99.1	10	0.0	0	AB251845	DQ323063; 98%; 471 bp
<i>Tricholoma</i> sp. 1	0.0	0	0.0	0	0.5	1	AB251824	AB036895; 99%; 488 bp
<i>Tricholoma</i> sp. 2	0.4	2	0.0	0	0.0	0	AB251816	AF349695; 96%; 591 bp
<i>Tricholoma</i> sp. 3	0.0	0	0.0	0	1.0	2	AB251826	AB036894; 100%; 572 bp
<i>Tylospora</i> sp.	0.6	1	0.0	0	0.0	0	AB251838	AJ534920; 98%; 179 bp
<i>Xerocomus</i> sp.	0.5	1	0.0	0	0.0	0	AB251818	AF402140; 95%; 261 bp
Unknown 1	0.3	1	0.0	0	0.0	0	AB251808	
Unknown 2	0.0	0	0.0	0	0.7	1	AB251841	

The RA of a given ECM fungus is expressed as the percentage of the total number of ECM root tips examined from the 10 soil samples (/10). Fr is the number of soil samples that contained each fungus in the 10 soil samples. The closest BLAST match is shown along with the matching accession number, the percentage match and the length of the sequence read.

Host *P. densiflora* trees colonized by matsutake

We successfully identified individual host trees using matsutake ECM root tips found in 10 soil samples collected beneath the corresponding sporocarps from five fairy rings in 2005. We confirmed that 22 *P. densiflora* trees were associated with matsutake. Sixteen of these 22 trees were located within the study site (Fig. 3).

Single below-ground genets of matsutake were confirmed to colonize three to seven host trees (average, 4.5) after pooling the ECM samples from the same matsutake genet. Similarly,

in 2002, all three below-ground genets were hosted by multiple trees, up to a maximum of seven different *P. densiflora* trees per genet.

Of 22 *P. densiflora* trees hosting matsutake, 19 hosts were associated with single genets of matsutake (Fig. 3). Exceptions included trees A826, A132 and A136, which were associated with four, two and two genets of matsutake, respectively (Fig. 3). Of seven *P. densiflora* hosts identified in 2002, six were associated with at least two genets of matsutake.

The size (d.b.h.) of *P. densiflora* trees associated with matsutake ranged from 9.9 to 54.4 cm, with an average \pm SD of

Table 2 Species richness (S) of underground ectomycorrhizal (ECM) fungi detected beneath (shiro), inside and outside *Tricholoma matsutake* (matsutake) fairy rings

Positions	ECM root tips examined	Observed species ^a	Species per soil sample ^b	Estimated S by Jack 2 ^a	Estimated S by bootstrap ^a	Shannon's index ^a	Simpson's index ^a
Inside	1595	25	4.2 ± 0.4	46	31	2.53	9.76
Shiro	2000	4***	1.3 ± 0.2**	7***	5***	0.06***	1.02***
Outside	1904	25	3.4 ± 0.5	53	31	2.64	11.15
Total	5499	39	3.0 ± 1.7	75	48	2.50	6.20

^aSignificant differences were determined with χ^2 tests. ***, Significant differences at $P < 0.001$.

^bThe number of ECM species detected is shown as mean ± SE. **, Significant differences at $P = 0.01$ by ANOVA tests.

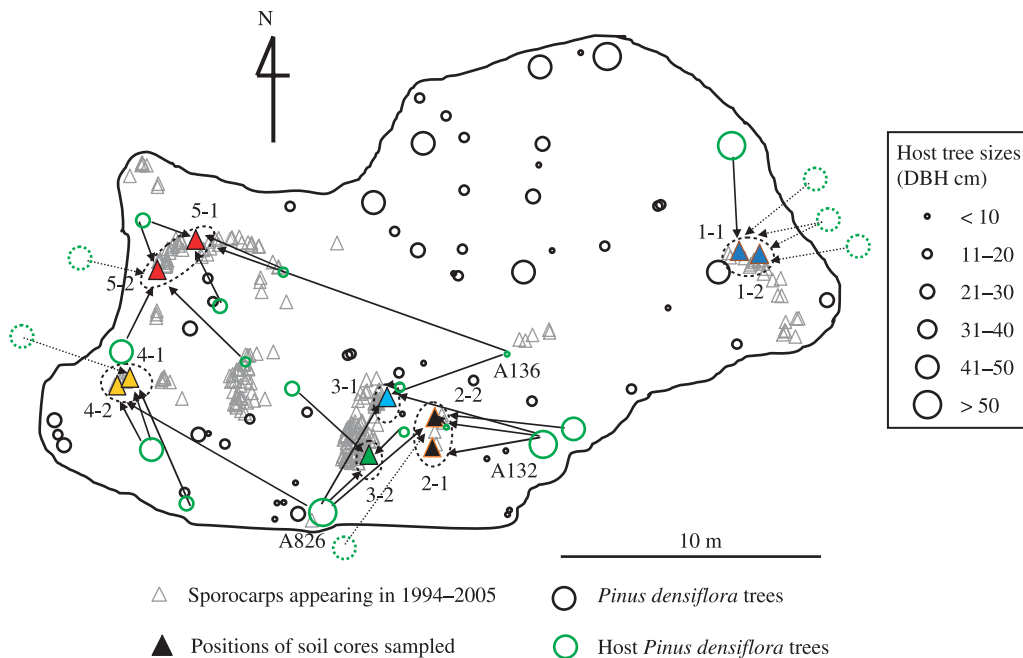


Fig. 3 Associations between below-ground genets of *Tricholoma matsutake* (matsutake) and its host *Pinus densiflora* trees. The data shown here were obtained from samples collected in 2005. Filled triangles indicate the positions of below-ground sampling, where different colors indicate different matsutake genets in the soil samples. Dotted lines encompass sporocarps to the same genet. *Pinus densiflora* trees are shown by open circles (green and black) of different sizes in relation to d.b.h. (diameter at breast height) sizes. Host trees (green circles) are connected to the associated matsutake genets by arrows. Dotted arrows indicate associations with unidentified hosts (green dotted circles) in surrounding forests, where these unidentified hosts do not represent the actual positions. Black circles represent trees on which matsutake ectomycorrhizas were not detected.

28.9 ± 15.2 cm. The average size of host trees was significantly larger than that (20.0 ± 12.5 cm) of all trees identified in the site ($P < 0.05$, ANOVA). The distance between matsutake root tips and the presumed host trees ranged from 0.5 to 12.5 m, with an average of 4.0 ± 2.6 m. No correlations were found between host sizes and distances of associated matsutake genets ($r = 0.268$, $P > 0.1$).

Discussion

The presence of different matsutake genets in each study site indicates that sexual reproduction is involved in the establishment of novel matsutake shiros in this forest. In our

study sites, several sporocarp aggregations were newly produced during the last 10 yr (Narimatsu, 2004) and all belonged to different genets. This indicates that the frequency of genet establishment by sexual reproduction at this site is several times per decade.

Most matsutake genet sizes (32/38) found in this study were < 3.0 m in the largest distance between sporocarps of the same genet. Similar genet sizes have been reported from *Russula brevipes*, a late-stage ECM fungus, in mature stands of lodgepole pine and Sitka spruce (Bergemann & Miller, 2002). Genet sizes of ECM fungi under natural conditions depend on mycelium extension rates and persistent duration, and vary greatly among species, ranging from < 1 m, such as *Laccaria*

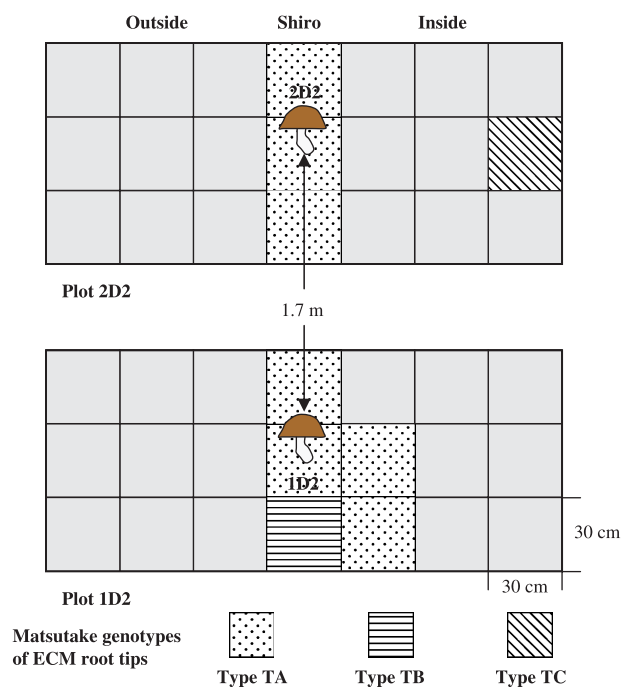


Fig. 4 Genet distribution of *Tricholoma matsutake* (matsutake) ectomycorrhizal (ECM) root tips in a fruiting fairy ring. All data were obtained from samples collected in 2002. A soil sample (10 cm³) was obtained from the center of each subplot (30 × 30 cm) in each of the two plots. The sporocarp in the center of each plot indicates the position where the analysed sporocarp developed. The genotypes of these two sporocarps (1D2 and 2D2) were the same and belonged to genotype TA. Two other genotypes (TB and TC) were also found in two subplots, both of which were apart from the sporocarps. The gray squares indicate subplots in which no ECM root tips of matsutake were detected.

amethystina (Gherbi *et al.*, 1999) and *Suillus grevillei* (Zhou *et al.*, 1999), to tens of meters, such as in *S. variegatus* (Dahlberg, 1997), *S. pungens* (Bonello *et al.*, 1998), and *S. pictus* (Hirose *et al.*, 2004). The average size of matsutake genets was 2.0 m, with the largest being 11.5 m (Fig. 2c). Narimatsu (2004) mapped the spatial distribution of matsutake sporocarps between 1994 and 2003 at the same sites. He found that most fairy rings were maintained during the 10-year investigation and expanded at a rate of 6.0–15.7 cm yr⁻¹, with an average of 10.3 cm (Narimatsu, 2004). This is significantly slower than the expansion rates of other ectomycorrhizal species such as *Hebeloma cylindrosporium* (45–60 cm yr⁻¹; Gryta *et al.*, 2000) and *Laccaria* spp. (20–100 cm yr⁻¹; Selosse *et al.*, 1999). Although the number of matsutake sporocarps that developed in each fairy ring differed significantly among years, the expansion rate was relatively constant (Narimatsu, 2004). If the expansion rate is constant for every stage of fairy ring development, the largest genet we examined would have persisted for > 50 yr.

We found sporocarps of different genets within an individual fairy ring, such as at site 2 (Fig. 2b), which contained four different genets. Based on field observations over several years,

Ogawa (1975) previously reported that several small shiros that had established close together expanded yearly, fused with each other and finally formed one large fairy ring. Since sporocarps of the same genet within each fairy ring in our study sites formed aggregates with a relatively clear genet boundary (Fig. 2), the fusion of shiros observed by Ogawa (1975) may also have occurred at these sites. Moreover, we also found that matsutake sporocarps of each genet were spatially aggregated, and neighboring genets did not overlap (Figs 2 and 4). This is similar to the cases of *S. bovinus* (Dahlberg & Stenlid, 1994) and *S. variegatus* (Dahlberg (1997)). Since the analysis of below-ground genets demonstrated that matsutake ectomycorrhizas were distributed in a relatively limited area (i.e. only beneath the fruiting fairy ring or shiro) and had the same genotype as the above-ground sporocarps (Figs 3 and 4), the restricted sporocarp genet distribution may reflect the restricted distribution of the below-ground genets in the shiro. Murata *et al.* (2005) using IRAP analysis also reported that there were different genets within an individual fairy ring of matsutake. They also pointed out heterogeneity of genets even in an individual sporocarp of matsutake, based on genetic analysis of cultured mycelia isolated from the same sporocarp. If such fine heterogeneity often occurs on the scale of individual sporocarps, spatial genet distribution within a fairy ring of matsutake may be intermingled but not aggregated. Since we found only clearly aggregated genets within a fairy ring, such genet heterogeneity within a sporocarp described by Murata *et al.* (2005) seems to be an exceptional event at our study site.

The establishment of new individual genets at the sites was mediated by basidiospore dispersal. The analysis of codominant SSR markers enables us to infer geographical gene flow of matsutake. Allele frequency distribution of the four SSR markers used here did not deviate significantly from Hardy–Weinberg equilibrium and these four markers were not linked with each other. Moreover, relatedness analysis indicated that related matsutake genets did not appear over short distances, suggesting that matsutake genets are established by long-distance dispersal of basidiospores. This conclusion is also supported by the absence of genetic differentiation among sites. Generally, wind dispersal results in a biased establishment of genetically close genets over short distances (Zhou *et al.*, 2001a; Bergemann & Miller, 2002), but no such pattern was observed in this study. Although wind dispersal seems to be predominant for matsutake, enthusiastic gatherers of matsutake sporocarps or other mammals may partly contribute to the spore dispersal.

High diversities of below-ground ECM fungi were observed inside and outside the fairy rings of matsutake, as has been reported in many other forests (Gardes & Bruns, 1993; Matsuda & Hijii, 1998; Zhou & Hogetsu, 2002). Beneath the fairy rings, however, we did not observe high ECM diversity; instead, matsutake dominated exclusively and only a few fungal species appeared as very minor components. This result indicates that matsutake mycelia completely alter the ECM fungal communities with the expansion of the fairy rings. Our

results also indicated that the ECM community could readily recover after the passage of the fairy rings because we found no significant difference in ECM communities between inside (after the passage) and outside (before the passage) of the fairy rings.

The SSR markers have repeatedly been used to study fungal (Zhou *et al.*, 2001a; Kretzer *et al.*, 2003, 2005; Wu *et al.*, 2005) or plant populations (Valdes *et al.*, 1993; Lian *et al.*, 2003b). However, no previous studies have used both fungal and plant SSR markers simultaneously to investigate interactions between host plants and ECM fungi. Here, we applied SSR markers to identify genotypes of both the host and the fungus of a matsutake root tip. As a result, we found that most matsutake genets were sustained by multiple pine trees, and larger trees were more likely to be hosts. This indicates that each genotype of the mycobiont (matsutake) is not strictly compatible with only a single genotype of the host, *P. densiflora*. Host roots may function simply as a relay substrate for the extension of matsutake extraradical mycelia. Although common mycelial networks that interconnect multiple trees have been assumed in forest ecosystems (Harley & Smith, 1983; Horton & Bruns, 1998), no direct evidence has unequivocally demonstrated the existence of such networks in nature. In this study, densely packed ectomycorrhizas and extraradical mycelia were located beneath each sporocarp, in which multiple host genotypes were found to be colonized by a single matsutake genotype. To our knowledge, this is the first direct evidence to clearly show the dominance of the mycelial connection among trees.

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