Genet Variation of Ectomycorrhizal *Suillus granulatus* Fruiting Bodies in *Pinus strobus* Stands

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**Abstract** The genets of *Suillus granulatus* in a *Pinus strobus* stand (13 m × 60 m) were identified using random amplified polymorphic DNA molecular markers and the DNA of mushrooms that fruited for two years, and variations in genet size and distribution were analyzed. From a total of 116 mushrooms, 73 genets were identified and were grouped into three locations. The genets of mushrooms in close proximity differed from each other. The genet sizes varied at any of the three locations. The lengths of the identified genets in the pine stand ranged from 0.09 to 2.90 m. The average number of mushrooms per genet was 1.2 to 2.3, and the percentage of genets that were represented by a single mushroom was 44% to 94%. This variation in the genets of mushrooms in close proximity suggests that the ectomycorrhizal mycelial bodies of *S. granulatus* propagated sexually by fusing haploid spores derived from the mushrooms gills with below-ground mycelia. Therefore, it is necessary further to investigate the formation of new genets through spores in ectomycorrhizal fungal colonies.

**Keywords** Ectomycorrhizal colony, Fruiting body, Genet, *Pinus strobus*, *Suillus granulatus*

The collection and artificial cultivation of ectomycorrhizal mushrooms of high economic value, such as *Tricholoma matsutake*, *Sarcodon aspratus*, and Périgord black truffle, have been of great interest. These ectomycorrhizal mushrooms are known to initially spread by spores, form mycorrhizal symbioses with their host plant roots, and then produce mushrooms after forming mycelial colonies in the soil. Ectomycorrhizal symbioses in forest soils are generally formed in two processes: with newly germinated from spores or with continuously growing vegetative mycelium in the soil [1]. *Suillus granulatus* form ectomycorrhizas with white pines (*Pinus strobus*) and produce mushrooms that form dense and profuse colonies. However, the genets of these mushrooms are hardly known, and inoculations with either spores or mycelium have been unsuccessful in forming mycorrhizas in natural forests.

A genet is a colony of plants, fungi, or bacteria that come from a single genetic source. Whether the genets of mushrooms that occur closely are genetically the same or not requires genetic analysis. Recently, molecular markers, such as inter-simple sequence repeat, amplified fragment length polymorphism, and inter-retrotransposon amplified polymorphism, have become an effective way to investigate the structure of genets and genetic diversity in mushroom colonies [2, 3]. Various molecular markers have been used to identify the genets of ectomycorrhizal mushrooms: restriction fragment length polymorphism for *Rhizopogon vesiculosus* and *Rhizopogon vinicolor* [4], microsatellites for *Laccaria amethystina*, *Laccaria laccata* [5], and *Russula brevipes* [6, 7], random amplified polymorphic DNA (RAPD) for *Suillus pungens* [8] and *Marasmius oreades* [9], and inter-simple sequence repeat for *Suillus grevillei* [10]. Genets of early successional species, such as *L. laccata*, would appear or disappear every year [11]. On the other hand, the genet variations of the competition-tolerant ectomycorrhizal species, which can colonize from early to late successional stages, still need to be understood further.

In this study, the genet structure of the *S. granulatus* population in a 22-yr-old *Pinus strobus* stand was determined in terms of the size and location of the genets with fruiting bodies.

**MATERIALS AND METHODS**

**Occurrence of *Suillus granulatus* mushrooms.** *S. granulatus*, which belongs to the family of Boletaceae, forms symbiotic ectomycorrhizas with pine tree roots, and has a
Lee and Koo

brown sticky cap and lactating pored gills (Fig. 1). The genets of *S. granulatus* in a *P. strobus* stand at the Chungbuk National University in Korea were investigated to divide into a, b, and c groups. The host trees were planted in a line at 4.8 m intervals 22 years ago, and their present mean height and diameter at breast height are 18 m and 28 cm, respectively. The stand area was 60 m × 13 m, and its slope was 0° to 3°. The underlying vegetation was comprised mainly of herbaceous plants, lawn grasses, clovers, dandelions, and foxtails. *S. granulatus* mushrooms fruited at approximately 0.6 to 4.5 m away from the host trees. The number of *S. granulatus* fruiting bodies was 56 in 2013 and 60 in 2014, occurring mainly in late August (Fig. 2). Positions and distances of the fruiting bodies from the trees were measured, and these data were illustrated using AutoCAD to identify the patterns of the fungal colonies.

Collection and genet analysis of *S. granulatus* fruiting bodies. The fruiting bodies were collected, labeled, and stored at −20°C until analysis. The DNA of *S. granulatus* fruiting bodies was extracted using the HiGene Genomic DNA Prep Kit (BIOFACT, Daejeon, Korea). For the genet analysis, primers OPA07 (5’-GAA ACG GGT G-3’), OPA11 (5’-CAA TCG CCG T-3’), OPA13 (5’-CAG CAC CCA C-3’), OPA16 (5’-AGC CAG CTG GGA C-3’), OPB10 (5’-CTG CAG CGA A-3’), and OPB18 (5’-CCA CAG CAG T-3’) were used for the random amplification of fruit bodies genomic DNA fragment. DNA was amplified according to the method described by Williams et al. [12] using a gene amplifier (Swift MaxPro; Esco Micro Pte. Ltd., Singapore). The bands corresponding to the amplified PCR products were subjected to cluster analysis using the unweighted pair group method with arithmetic mean. Distribution of these mushrooms genets was assumed from match-delineated colony shapes, and was divided into genets based of RAPD analysis.

**RESULTS**

The RAPD primers OPA07, OPA11, OPA13, OPA16, OPB10, and OPB18 yielded 9, 9, 11, 14, 12, and 8 separate bands. The colonies of *S. granulatus* in the *P. strobus* stand were grouped into three locations A, B, and C, for genet analysis (Fig. 3).

*S. granulatus* mushrooms formed large, linear colonies at the stand, and the number of fruiting bodies was 56 from 25 genets in 2013 and 60 from 48 genets in 2014, i.e., a total of 116 fruiting bodies from 73 genets (Fig. 4). Among the 73 genets, 13 in 2013 and 41 in 2014 formed single

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**Fig. 1.** Fruiting body of *Suillus granulatus*. A, Brown sticky cap surface; B, Lactating pored gills.

**Fig. 2.** Occurrence of *Suillus granulatus* fruiting bodies in a *Pinus strobus* stand. The colonies were partly circular and partly linear.

**Fig. 3.** Map of the locations A, B, and C of the *Suillus granulatus* fruiting bodies for years 2013 (△) and 2014 (☆) in a 22-yr-old *Pinus strobus* (○) stand.
fruiting bodies. The average fruiting body numbers per genet were 2.2 in 2013, 1.25 in 2014, and 1.57 over 2 yr.

At location A, the fruitings in 2013 and 2014 were approximately 3.5 m apart. Eight mushrooms that fruited in 2013 were delineated into four genets, one of which was represented by a single mushroom (Table 1). In 2014, another eight mushrooms fruited and were delineated into three genets, two of which were represented by a single mushroom (Table 1). Thus, the percentage of genets represented by a single mushroom was 43% at this location.

The genet sizes varied from 0.20 m to 1.82 m (Table 1, Fig. 5).

At location B, 39 S. granulatus mushrooms fruited in 2013 and 37 in 2014, and the colony shapes were partly circular and partly linear (Fig. 6). The 39 mushrooms that fruited in 2013 were delineated into 18 genets, 8 of which were represented by a single mushroom (Table 2). Thus,

Table 1. Number of genets and fruiting bodies of *Suillus granulatus* and genet length at location A in 2013 and 2014

| Year | No. of fruiting bodies per genet | No. of genets | No. of fruiting bodies | Genet length (m) |
|------|--------------------------------|--------------|-----------------------|------------------|
| 2013 | One                            | 1            | 1                     | -                |
|      | Two                            | 2            | 4                     | 0.20, 0.72       |
|      | Three                          | 1            | 3                     | 0.88             |
| 2014 | One                            | 2            | 2                     | -                |
|      | Six                            | 1            | 6                     | 1.82             |
| Total|                                | 7            | 16                    |                  |

Fig. 4. Dendrograms of random amplified polymorphic DNA analysis of the collected all *Suillus granulatus* fruiting bodies for divide genets collected at the locations A, B, and C.
approximately 44% of the genets form a single mushroom. In 2014, the 37 mushrooms that fruited were delineated into 35 genets (Table 2). Thirty-three genets were represented by a single mushroom, which equates to about 94% of the genets. The percentage of genets represented by a single mushroom varied significantly from 44% to 94% over 2 yr. The average number of fruiting bodies per genet was 1.4, and the largest genet length was 2.9 m.

At location C, *S. granulatus* mushrooms fruited in fragments, in a complicated manner, or and in a line (Fig. 7).

### Table 2. Number of genets and fruiting bodies of *Suillus granulatus* and genet length at location B in 2013 and 2014

| Year | No. of fruiting bodies per genet | No. of genets | No. of fruiting bodies | Genet length (m) |
|------|---------------------------------|---------------|------------------------|------------------|
| 2013 | One                             | 8             | 8                      | -                |
|      | Two                             | 2             | 4                      | 0.09, 0.21, 0.30, 2.90 |
|      | Three                           | 2             | 6                      | 0.41, 1.40       |
|      | Four                            | 3             | 12                     | 0.28, 0.87, 1.01 |
|      | Five                            | 1             | 5                      | 0.51             |
|      | Total                           | 33            | 33                     |                  |
| 2014 | One                             | 33            | 33                     |                  |
|      | Two                             | 2             | 4                      | 0.15, 0.93       |
| Total|                                 | 51            | 62                     |                  |
Eight mushrooms that fruited in 2013 were delineated into four genets (Table 3). Three of the genets were represented by a single mushroom. In 2014, the 16 mushrooms that fruited were delineated into 11 genets, 7 of which were represented by a single mushroom (Table 3). In other words, 10 of the 15 (67%) genets that fruited at this location were represented by a single mushroom. The average number of mushrooms per genet was 1.60, and the longest genet was 2.79 m.

**DISCUSSION**

*Ectomycorrhizal fungal colonies, collective forms of fungal hyphae and roots.* *S. granulatus* mushroom colonies were partly irregular and partly circular. Fungal colonies of mushrooms are varied in shape under natural conditions. Ectomycorrhizal fungal colonies are grouped into three basic types: the fairy ring, irregular mat, and dispersed colony types [13]. The fairy ring type grows as a distinctly dense, circular mycelial colony below the ground, and mushrooms forming this type of colonies belong to the genera *Cantharellus*, *Tricholoma*, and *Agaricus*. Mushrooms forming irregular mat type colonies include those from the genera *Cortinarius*, *Sarcodon*, and *Suillus*. Mushrooms forming dispersed colonies include those from the genera *Amanita*, *Boletus*, *Russula*, and *Marasmius*, as well as litter-decomposing mushrooms.

Although the width of below-ground mycelial colonies of *T. matsutake* are about 1 m, the front edge of the live hyphal portion of the colonies are only approximately 20 cm, and only the front continues to grow in length [14]. The fruiting body of the mushroom appeared from a 1-yr-old mycelial colony [15]. Organic matter decomposing fungi living in plant residues on the soil surface also grow out in all directions from the center [16]. These mycelial colonies are from somatic cells with two nuclei, and thus, the mushrooms genets from the colonies should be the same. However, our molecular marker analysis showed that genets from the fruiting bodies in the colonies were different.

**Spores cause genet mutation.** In nature there are two types of processes that allow the formation of ectomycorrhizal fungal colonies: one is mating between two compatible monokaryotic (haploid) mycelia from spores, and the other is dikaryotic (haploid) mycelial growth [17]. Our study showed that the genets of fruiting bodies in the *P. strobus* stand that occurred very closely to each other in 2013 and 2014 were genetically different. This suggests that the genets of *S. granulatus* have been frequently changing due to the combination of the monokaryotic haploid hypha from introduced sexual basidiospores (Fig. 8) and the existing dikaryotic haploid ectomycorrhizal mycelia below the ground (Fig. 9).

The percentage of genets represented by a single...
mushroom ranged from 44% to 94% of the total genets depending on the year of occurrence and locations, and the average numbers of mushrooms per genet were 1.2 to 2.3. Similarly, in the case of *S. spraguei* in a natural *P. strobos* stand, 42 out of 46 genets from 50 sporocarps sampled were represented by a single sporocarp, while the remaining 4 genets were represented by 2 sporocarps, resulting in an average number of sporocarps per genet of only 1.09 [17].

In the late successional ectomycorrhizal species, *R. brevipes*, 33% to 50% of its genets were represented by a single fruiting body [6]. In *Russula vinosa*, the fruiting bodies that occurred in a limited space showed high genetic diversity, but the size of each genet did not exceed 1.0 m [18].

In the case of a pioneer and early successional species, *L. laccata*, the genets tend to disappear within a few months after sporocarp formation, and the new genets that appeared from the spores were significantly smaller in size and larger in quantity and dispersed during the fruiting season [19]. The high densities and annual renewal of *Laccaria* genets indicated frequent turnover by sexual reproduction via spores [11]. During its fruiting season, most genets of the *L. laccata* mushrooms were identical to the mycelial genets below the ground. However, nine months after the fruiting season, new genets were observed, and the genets from the previous year's fruiting bodies were not detected in 60% of the plots [19]. This means that, like in the case of the genets of *S. granulatus* in this study, the genets of *L. laccata* may have changed due to the combination of the dikaryotic ectomycorrhizal mycelia in the soil and the monokaryotic hyphae from new spores to change their genets and produced the fruiting bodies.

For mushrooms to reproduce and propagate, the spores disperse from mushroom gills and germinate when the humidity, temperature and nutrition are appropriate. Hyphae grown from germinated spores combine with hyphae from other spores to form a mycelial mat and develop primordial to mature mushrooms [20]. Meanwhile, in a stable ecosystem such as a fully grown forest, the new roots are surrounded by existing mycorrhizal roots, and therefore, the new spores have a rare chance to infect new roots. Thus, soil disturbances that destroy the existing fungal colonies can provide a place for the settlement of new spores [21, 22].

In nature, early successional ectomycorrhizal fungi form mycorrhizal symbiosis with their host plant roots by spores, which are dispersed into the soil [23-25], and the mycorrhizas can be replaced by late successional fungal species as the hosts grow [26]. On the other hand, if the vegetation is stable and early successional mycorrhizal species are persistent, then the transition of the spores becomes delayed [23]. Mushrooms that have a strong predisposition to form fairy-ring type colonies such as *T. matsutake* [27] maintain their colony through existing mycelium in the soil rather than newly dispersed spores [28]. However, our results suggest that the genets of ectomycorrhizal colonies may have been constantly diversifying as a result of fusion with hyphae from the basidiospores produced during fruiting season. This genetic diversification may contribute to the survival of the colonies under changing environmental conditions, such as the conditions of the host and soil and the weather.

**Diversity in genet sizes.** Many studies have shown that the genet sizes of ectomycorrhizal fungi were very diverse, depending on the host species, host ages and habitats [29]. The genet sizes were 300 m in *S. pungens* [8], 1 m or less in *L. amethystina* [30] and *Russula cremoricolor* [31], 12.5 m in *Laccaria bicolor* [32], 2.27 to 7.65 m in *S. spraguei* [17], and 3.5 m in diameter in *Hebeloma cylindrosporum* [33]. The size of *Collybia fusipes* genets in decomposing fallen leaves varied in size from very small to a few millimeter [34]. On the other hand, genets of *Armillaria bulbosa*, a root-disease pathogen in forest trees, can cover as much as 1 km [35]. The genet densities of ectomycorrhizal mushroom populations were also considerably varied between 30 and 5,000 per ha [29]. In this study, 44 to 94% of *S. granulatus* genets were represented by a single fruiting body, and the longest genet was approximately 2.9 m and composed of five fruiting bodies.

In conclusion, the genets of *S. granulates* mushrooms were varied even though they occurred very closely and became more varied every year. This means that the below-ground mycelial mat of *S. granulatus*, composed of vegetative hyphal cells, can be renewed sexually by fusing with haploid hyphae from spores produced in the mushroom gills. The small stand area and the short 2-yr survey period limits the interpretation of the formation of new genets of *S. granulatus*. Further studies will be needed to clarify the role of fusion between the hyphae from basiospores and the below-ground ectomycorrhizal mycelia in genet variation of *S. granulatus*.

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