J. Agrofor. Environ. 2(1): 1-6, 2008 ISSN 1995-6983

Below ground genet differences of an ectomycorrhizal fungus *Laccaria laccata* infecting *Salix* stands in primary successional stage

M. A. Wadud, C. L. Lian[1], K. Nara[1], M. S. Reza[2] and T. Hogetsu[2]
Department of Agroforestry, Bangladesh Agricultural University, Mymensingh; [1]Asian Natural Environmental Science Center, the University of Tokyo, Japan, [2] Graduate School of Agricultural and Life Sciences, the University of Tokyo, Japan

Abstract: Genet structure of an ectomycorrhizal (ECM) fungus *Laccaria laccata* was studied during a fruiting season and 9 months after the fruiting season in the volcanic desert on Mount Fuji. Below-ground samples of *L. laccata* were analyzed using SSR (Microsatellite) markers. During fruiting season, most of the below-ground genets were identical to sporocarp genets of each sampling plot. In contrast, nine months after the sporocarp formation, many small genets were observed in each plot but previous year's sporocarp genet was not detected in 60% plots. Majority of new genets shared at least one common allele with the previous year's sporocarp in every SSR locus. This indicates, genets of *L. laccata* tend to disappear after sporocarp formation (within few months) and new offspring genets are generated from the spores dispersed from the sporocarp to the vicinity. The size and number of the genet were significantly smaller and more in 'after fruiting season'. This result suggests, most of the newly generated genets disappear through competition with a sporocarp-producing genet that becomes dominant in the 'fruiting season'.

Key words: *Laccaria laccata*, Ectomycorrhiza, SSR, genet, Mount Fuji

Introduction

Ectomycorrhizal (ECM) symbiosis is a common association between plant roots and fungi; in nature the majority of terrestrial plant roots are colonized by symbiotic fungi forming ectomycorrhizas (Smith and Read, 1997). In this symbiosis, the fungus captures nutrients effectively from the soil and translocates part of them to the host plant. In return, the host plant supports ECM fungi by delivering photosynthesized carbohydrates. The fungi not only supply the host plant with nutrients but may also defend the host against drought, pathogens and heavy metals (Smith and Read, 1997). In general, ECM symbiosis refers to a mutualistic association that benefits both partners. In natural forests, ECM associations have a pivotal role in nutrient and water supply of trees as well as in nutrient cycling. This reinforces the link between above-ground and below-ground communities in the context of sustainable productivity of forest ecosystems. ECM symbioses are also observed in the volcanic desert on Mount Fuji which is under primary successional condition (Nara and Hogetsu, 2004). In this desert, vegetation under primary successional condition was destroyed by a volcanic eruption in 1707. Nara *et al.* (2003a) recorded 23 ECM fungal species in this desert of which *L. laccata* was most dominant. It is well recognized that the ECM community differs from the above-ground and below-ground points of view (Horton and Bruns, 2001). The use of above-ground samples i.e. sporocarps in ECM fungal population studies has some major disadvantages. Specially, most *Laccaria* sporocarps appear only in a few weeks and sporocarp differentiation is affected by many biotic and abiotic conditions (Grades and Bruns, 1996; Dahlberg *et al*., 1997). Therefore, sporocarp sampling is considered an inadequate method for ECM genet study (Dahlberg 2001). So, genet characteristics of above ground ECM fungi may not be conclusive without belowground genet study. SSR (Simple Sequence Repeat), popularly known as microsatellite markers have many advantages over other genetic markers and it is particularly species specific. So, SSR markers can easily identify the ECM fungal species from below-ground samples. So far, SSR markers are not available for any *Laccaria* species. Therefore, the present investigation have been undertaken to characterize the belowground genet structures of *L. laccata* using species specific SSR markers.

Materials and Methods

Study site: Research quadrat (100 × 550 m) was same as described in Nara *et al.* (2003a, b), located at altitudes of 1500-1600 m above sea level ($35^0$20'N, $138^0$48'E) on the south-east slope of Mount Fuji, Japan. During 1707 the vegetation on this slope was completely destroyed by Hoei eruption and now recovering patchily on scoria substrate. *Salix reinii* is the pioneer ECM woody tree species in this slope (Lian *et al*., 2003). *S. reinii* is distributed on 43 vegetation patches out of 160 vegetation patches in the research quadrat (Nara et al., 2003 a, b) and *L. laccata* samples were collected from the *Salix* habitat patches.

Soil sampling with ECM root tip: Soil samples were collected in a fruiting season (September 9, 2006) and 9 months after 2005 fruiting season (June 14, 2006) from different vegetation patches. In each sampling time, 10 sampling plots (1 × 1 m) were established and each plot contained a sporocarp at the center of the plot (Fig. 1). The previous year's sporocarp positions were used in the June sampling. Each plot was divided into 25 sub-plots (20 × 20 cm). From the center of each sub-plot, one 5 cm soil cube was sampled (Fig. 1). In total, 250 soil samples were collected at each sampling time. Soil samples were placed separately in plastic bags and kept at 4°C for further analyses.

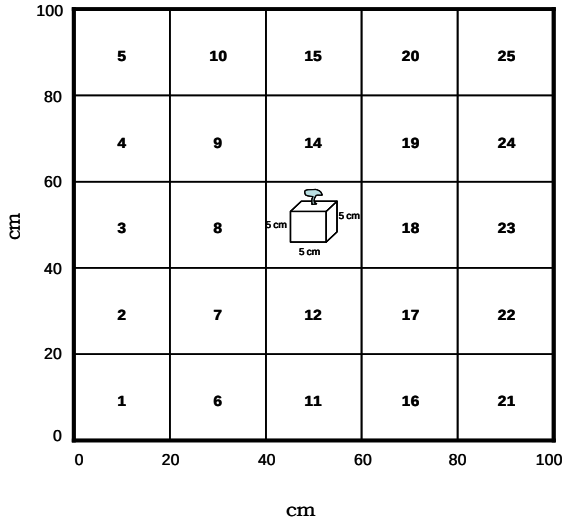

Fig. 1 A below-ground sampling plot (1 × 1 m), divided into equal 25 sub-plots (20 × 20 cm).

ECM root tip sampling from soil sample: ECM root tips were collected from soil samples following the method of Nara *et al.* (2003a). *L. laccata* root tips were characterized based on their specific color, texture and emanating hyphae by morphological examination under a dissecting microscope (Fig. 2). To determine the number of ECM root tips that are required for below-ground genet detection for each soil sample, SSR analyses were pilotted using 3 plots established in June 2006 for each *Laccaria* species. In each of nine soil samples (No.2, 4, 6, 10, 13, 16, 20, 22 and 24; Fig. 1) from a plot, 10 randomly collected *L. laccata* root tips were analysed by SSR markers. All replicate root tips in a sample, without exception, belonged to the same genet. Therefore, one root tip/soil sample was used in the following study.

Fig. 2. Typical *Laccaria laccata* ectomycorrhizal root tips

DNA extraction ECM root tips: DNA from individual root tips was extracted using a modified cetyltrimethyl ammonium bromide (CTAB) method described by Nara *et al.* (2003a). DNA pellets were dissolved into 20 µl sterilized water and kept at -30˚C until use.

SSR analysis: To identify the *L. laccata* fungal genotypes, five SSRs (*LL15, LL21, LL25, LL32* and *LL35*)) developed by Wadud *et al.* (2006b) were used. SSRs were amplified using a polymerase chain reaction (PCR) and amplified products were electrophoresed and analyzed as described by Wadud *et al.* (2006a).

Data analysis: ECM root tips that showed the same allelic patterns in all microsatellite loci were regarded as the same genotype. Significant differences in mean number of genets per plot and genet area between two sampling seasons were evaluated with *t*-tests.

Results

L. laccata population structure during a fruiting season

Total 250 soil samples collected from 10 plots in the fruiting season of which 156 contained *L. laccata* ECM tips which belonged to 31 genets and 94 samples were blank (Table 1 and Fig. 3). Every *L. laccata* genet was unique to a single plot, and no genet was found common in two plots. The genets found beneath the sporocarps were always identical to the corresponding sporocarps, often dominating the below-ground populations in the plots. The mean area occupied by the genets identical to the sporocarps was 0.34 ± 0.03 m^2 (mean ± SE). These sporocarp-producing below-ground genets were larger than other below-ground genets (P<0.0001). Samples belonging to the same genets always had continuous distributions and were not fragmented spatially.

L. laccata population structure nine months after fruiting season

Total 250 soil samples collected from 10 plots of *L. laccata* after the fruiting season of which 157 contained *L. laccata* ECM tips, which belonged to 81 genets and rest 93 samples contained no *L. laccata root tips* (Table 1 and Fig 4). Like fruiting season, individual genets were only observed in a single plot and never found in ≥2 plots. Samples belonging to the same genets also had continuous distributions and were not fragmented spatially.

Table 1 Genets of *L. laccata* ectomycorrhizal tips found below the sporocarps in a fruiting season and nine months after fruiting season

Sampling time	No. of soil samples	No. of samples identified as		No. of genets
		L. laccata	Blank	
in a fruting season	250	156	94	31
nine months after fruiting season	250	157	93	81

Table 2 Average number of genets per plot of *Laccaria laccata* during the fruiting period and nine months after sporocarp formation

Sampling time	Number of genets (mean ± SE)
in fruting season	3.1 ± 0.39***
nine months after fruiting season	8.1 ± 0.68

***, Significantly lower at $P < 0.0001$ by t-test.

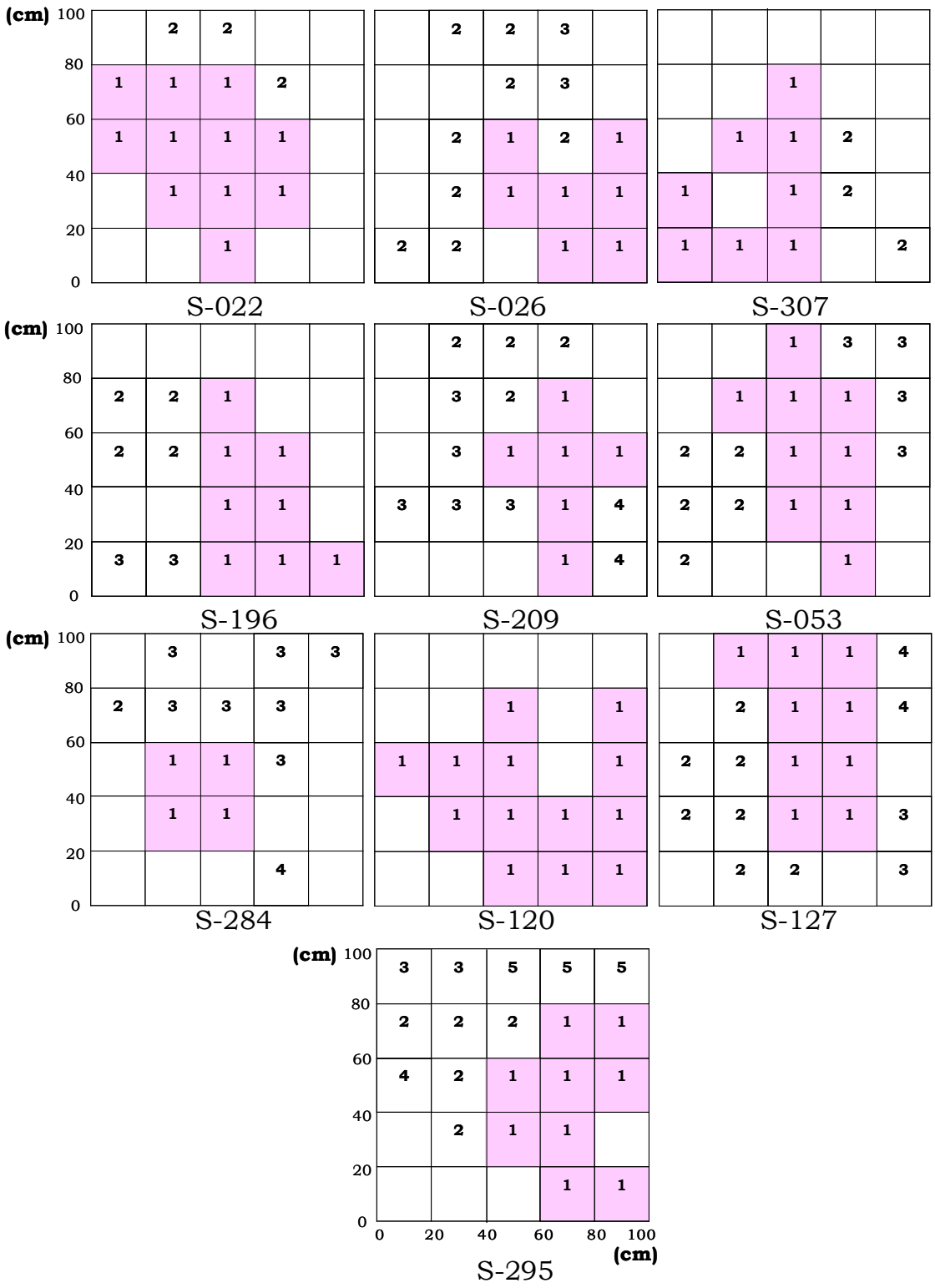

Fig. 3 Spatial distribution of below-ground *L. laccata* genets in 10 plots (S-022, S-026, S-307, S-196, S-209, S-053, S-284, S-120, S-127 and S-295) in a fruiting season. Black Arabic numbers represent *L. laccata* genets, blank positions indicate no *L. laccata* ECM root tips were found. Highlighted genets are the genets that produced sporocarps in this season.

In contrast to the fruiting season, there were many small genets of *L. laccata* in after the fruiting season. A maximum of 10 genets of *L. laccata* were detected in four plots (S-671, S-285, S-928 and S-369) and average numbers of genets per plot was 8.1 ± 0.68 which was significantly higher than in fruiting season 3.1 ± 0.39 (mean ± SE, Table 2). Average areas per genet, which are the sum of sub-plot areas that include each genet, was 0.08 ± 0.01 m^2 in June. This was significantly smaller than in the fruiting season, 0.20 ± 0.02 m^2 (mean ± SE, Table 3).

Table 3 Below-ground genet area of *Laccaria laccata* during and after fruiting season

Season	Area per genet (m^2)	
	Mean ± SE	Range
in fruting season	0.20 ± 0.02***	0.04 - 0.52
nine months after fruiting season	0.08 ± 0.01	0.04 - 0.32

***, Significantly higher at $P < 0.0001$ by t-test

Temporal persistence and parent-offspring relationship of below-ground genets

After the fruiting season, the below-ground genets identical to the previous year's sporocarp were detected in five plots (S-393, S-434, S-483, S-285, S-578, Fig. 4). The persistent genets were comparatively larger in size compared with the other genets (Fig. 4).

Most of the below-ground genets (53 %) sampled after fruiting season shared common alleles with the previous year's sporocarps genet (Fig. 4), indicating that these below-ground genets may be offsprings of the previous year's sporocarp genet.

Discussion

During fruiting season, the genets of *L. laccata* identical to current year sporocarps were found in all plots. Such sporocarp-producing genets were detected in bigger areas than other genets. Thus, it may be necessary to occupy a certain area (≥0.16 m^2) to obtain enough resources for sporocarp formation.

In this study, the genets identical to previous year's sporocarps were not detected in more than half plots after fruiting season. Frequent disappearance of genets after sporocarp formation has also been described in *Suillus grevillei* (Zhou *et al.* 2001) and *Hebeloma cylindrosporum* (Guidot *et al.* 2001, 2004).

Although the majority of genets disappeared after sporocarp formation, some genets persisted in the below-ground until next spring. These persistent genets occupied a relatively large area. This result is in accordance with the fact that sporocarp genets persisting for ≥2 consecutive years are relatively large (Wadud 2007) and large genets (>10 m) was observed in sporocarp populations of other ECM fungal species, due to continuous mycelial expansion for long years (Dahlberg and Stenlid 1994, Kretzer *et al.* 2003).

With or without persistent genets, many small genets were found after the fruiting season. Parentage analyses between these below-ground genets and the previous year's centered sporocarps revealed that more than 50 % of the genets shared at least one common allele with the sporocarp at every locus. Thus, the majority of below-ground genets after the fruiting season may be generated by sexual mating between monokaryotic hyphae originated from a spore of the centered sporocarp and another spore, and/or by di-mon mating between an extradadical hypha of the sporocarp and a monokaryotic hypha originated from a spore (Gardes *et al.* 1991).

Below-ground genets 'after the fruiting season' were significantly smaller and richer than in the 'fruiting season' when the sporocarp-forming genets become larger and dominate in below-ground. Thus, most of below-ground genets found 'after the fruiting season' are considered to disappear without formation of any sporocarps before 'fruiting season'.

Since we have to destructively sample below-ground mycorrhizae, we could not deal with a same sampling area in two seasons. However, the results of this study may allow us to infer that a seasonal difference of below-ground *L. laccata* genets is much greater than ever thought.

References

Dahlberg, A. 2001. Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. *New Phytologist* 150: 555−562.

Dahlberg, A., Jonsson, L., Nylund, J.E. 1997. Species diversity and distribution of biomass above and below ground among ectomycorrhizal fungi in an old-growth Norway spruce forest in South Sweden. *Canadian Journal of Botany* 75: 1323-1335.

Dahlberg, A., Stenlid, J. 1994. Size, distribution and biomass of genets in populations of *Suillus bovinus* (L. Fr.) Roussel revealed by somatic incompatibility. *New Phytologist* 128: 225-234.

Grades, M., Bruns, T.D. 1996. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: Above- and below-ground views. *Canadian Journal of Botany* 74: 1572-1583.

Grades, M., Mueller, G.M., Fortin, J.A., Kropp, B.R. 1991. Mitochondrial DNA polymorphisms in four *Laccaria species: L. bicolor, L. laccata, L. proxima and L. amethystina. Mycological Research* 95: 206-216.

Guidot, A., Debaud, J.C., Effosse, A., Marmeisse, R. 2004. Below-ground distribution and persistence of an ectomycorrhizal fungus. *New Phytologist* 161: 539-547.

Guidot, A., Debaud, J.C., Marmeisse, R. 2001. Correspondence between genet diversity and spatial distribution of above- and below-ground populations of the ectomycorrhizal fungus *Hebeloma cylindrosporum. Molecular Ecology* 10: 1121-1131.

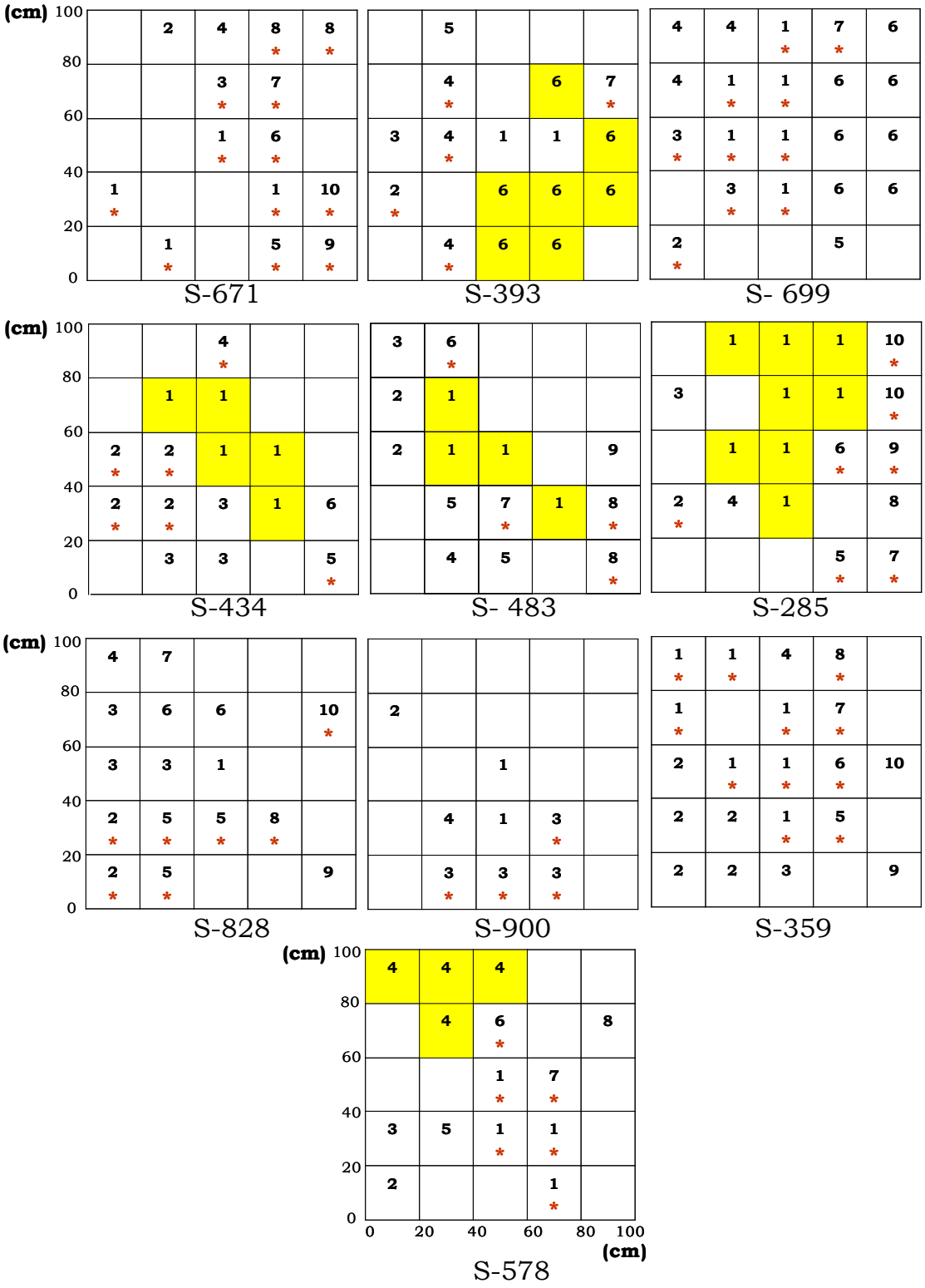

Fig. 4 Spatial distribution of below-ground *L. laccata* genets in 10 plots (S-671, S-393, S-699, S-434, S-483, S-285, S-828, S-900, S-359 and S-578) nine months after fruiting season. Black Arabic numbers represent *L. laccata* genets, blank positions indicate no *L. laccata* ECM root tips were found. Highlighted genets are the genets that produced sporocarps in the previous year. * shows the genet that share common alleles in all of nine loci with the genet that produced sporocarps in the previous year.

Horton, T.R. and Bruns, T.D. 2001. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Molecular Ecology* 10: 1855−1871.

Kretzer, A.M., Dunham. S., Molina, R., Spatafora, J.W. 2003. Microsatellite markers reveal the below ground distribution of genets in two species of *Rhizopogon* forming tuberculate ectomycorrhizas on Douglas fir. *New Phytologist* 161: 313-320.

Lian, C., Oishi, R., Miyashita, N., Nara, K., Nakaya, H., Zhou, Z., Wu, B., Hogetsu, T. 2003. Genetic structure and reproduction dynamics of *Salix reinii* during primary succession on Mt. Fuji, as revealed by nuclear and chloroplast microsatellite analysis. *Molecular Ecology* 12: 609-618.

Nara, K., Hogetsu, T. 2004. Ectomycorrhizal fungi on established shrubs facilitate subsequent seedling establishment of successful plant species. *Ecology* 85: 1700-1707.

Nara, K., Nakaya, H., Hogetsu, T. 2003a. Ectomycorrhizal sporocarp succession and production during early primary succession on Mount Fuji. *New Phytologist* 158: 193-206.

Nara, K., Nakaya, H., Wu, B., Zhou, Z., Hogetsu, T. 2003b. Underground primary succession of ectomycorrhizal fungi in a volcanic desert on Mount Fuji. *New Phytologist* 159: 743-756.

Smith, S.E., Read, D.J. 1997. *Mycorrhizal symbiosis*, 2nd edn. London, UK: Academic Press.

Wadud MA. 2007. Reproduction Ecology of pioneer ectomycorrhizal fungi, *Laccaria amethystina* and *L. laccata*, in the volcanic desert on Mount Fuji. Unpublished Ph D. dissertation. The University of Tokyo, Japan.

Wadud, M.A., Lian, C.L., Nara, K., Ishida, T.A., Hogetsu, T. 2006a. Development of microsatellite markers from an ectomycorrhizal fungus, *Laccaria amethystina*, by a dual-suppression-PCR technique. *Molecular Ecology Notes* 6: 130-132.

Wadud, M.A., Lian, C.L., Nara, K., Hogetsu, T. 2006b. Isolation and characterization of five microsatellite loci in an ectomycorrhizal fungus *Laccaria laccata. Molecular Ecology Notes* 6: 700-702.

Zhou, Z., Miwa, M., Matsuda, Y., Hogetsu, T. 2001. Spatial distribution of the subterranean mycelia and ectomycorrhizae of *Suillus grevillei* genets. *Journal of Plant Research* 114: 179-185.