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BELOW-GROUND GENET STRUCTURE OF AN ECTOMYCORRHIZAL FUNGUS, *LACCARIA AMETHYSTINA* IN THE VOLCANIC DESERT ON MOUNT FUJI

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Abstract: Below-ground genet structure of an ectomycorrhizal (ECM) fungus *Laccaria amethystina* was studied 9 months after 2005 fruiting season and in a fruiting season of 2006 in the volcanic desert on Mount Fuji, Microsatellite markers were used for the genetic study of *L. amethystina*. In the sporocarp season, the genet identical to the centered sporocarp in each plot was always detected in soil and often dominated the below-ground genets in each plot. In contrast, nine months after the sporocarp formation, the genet identical to the previous year's centered sporocarp was not detected in soil in more than half plots, and instead, there were many other small genets. Majority of them shared at least one common allele with the centered sporocarp in every SSR locus. These results indicate that genets of *L. amethystina* tend to disappear within nine months after sporocarp formation and that 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 June than in the sporocarp season. This suggests that most of the genets newly generated until June disappear through competition with a sporocarp-producing genet that becomes dominant in the sporocarp season.
Key words: Ectomycorrhiza, below-ground, genet, *Laccaria amethystina*, Mount Fuji,

Introduction

ECM symbioses are not only morphological coexistence of two organisms but also mutually beneficial relationships between plants and fungi. Fungal hyphae can effectively scavenge soil nutrients, because they are thinner and longer than plant roots and root hairs. In addition, by producing enzymes and organic acids, hyphae can use many organic and inorganic compounds, which are difficult to be utilized by plants alone (Smith & Read 1997). Therefore, both partners in ECM symbiosis obligately depend on each other to grow and survive in natural environments. Because of the physiological importance of ECM symbioses and their dominance in forest ecosystems, ECM fungi play important roles in various ecological processes in developed forests. Indeed, ECM root tips and ECM mycelia account for more than 50% soil respiration (Hogberg & Hogberg 2002) and a substantial part in carbon and nutrient cycling in forest ecosystems (Forgel and Hunt 1979, Vogt *et al.* 1982). ECM symbioses are also observed in the volcanic desert on Mount Fuji which is under primary successional condition (Nara & Hogetsu 2004). In this desert, vegetation has been gradually recovering from the last devastating eruption in 1707, and is now patchily distributed forming isolated vegetation islands in a sea of scoria desert. In total, 23 ECM fungal species have been recorded (Nara *et al.* 2003a) in this desert and among them *L. amethystina* was most dominant. Considering dominance of this ECM fungus seems to be the key fungal species in this ecosystem, thus investigated in this study.

Usually sporocarps were used for genetic study of ECM fungi. The use of sporocarps in ECM fungal population studies has some major disadvantages. Because most *Laccaria* sporocarps in this desert appear only in a few weeks, it is impossible to study *Laccaria* populations in other seasons using sporocarps. Moreover, sporocarp differentiation is affected by many biotic and abiotic conditions (Grades & Bruns 1996; Dahlberg *et al.* 1997). Thus, there would be many belowground genets that do not produce any sporocarps during a study period. It is also possible that belowground ECM tips and mycelia of a genet spread far beyond its sporocarp distribution. Therefore, reproduction characteristics found in sporocarp populations of both *Laccaria* species, e.g. small genet sizes and rapid genet turnover, may not be conclusive without corresponding belowground genet data.

Microsatellite (or simple sequence repeat; SSR) markers have many advantages over other genetic markers and species specific. So, SSR markers can easily identify the target species from below-ground samples. Moreover, SSR markers are not available for any *Laccaria* species so far. This study investigates the belowground genet structures of *L. amethystina* in the volcanic desert on Mount Fuji, using species specific SSR markers.

Materials and Methods

Research site: The 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 160 vegetation patches in the research quadrat (Nara et al., 2003 a, b) and study samples were collected from the *Salix* habitat patches.

Soil sampling: Soil samples were collected 9 months after 2005 fruiting season (June 14, 2006) and in a fruiting season (September 9, 2006) from different vegetation patches. At 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.

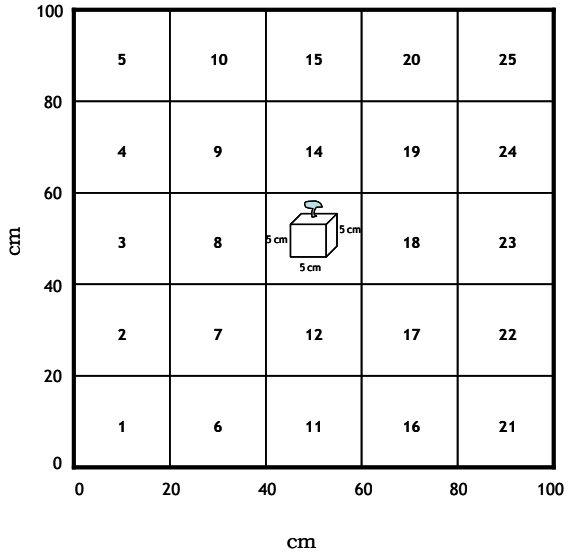

Fig. 1 A below-ground sampling plot (1 × 1 m), divided into equal 25 sub-plots (20 × 20 cm).

ECM root tip sampling: *L. amethystina* ECM root tips were collected from soil samples following the method of Nara *et al.* (2003a). *Laccaria* 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.1, 5, 7, 9, 13, 17, 19, 21 and 25; Fig. 1) from a plot, 10 randomly collected *L. amethystina* 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 amethystina* ectomycorrhizal root tips

DNA extraction: 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.

Microsatellite (SSR) analysis: To identify the *L. amethystina* fungal genotypes, nine microsatellites (*La03, La06, La07, La12, La 14, La17, La21, La23,* and *La32*) developed by Wadud *et al.* (2006) were used. Microsatellites were amplified using a polymerase chain reaction (PCR) and amplified products were electrophoresed and analyzed as described by Wadud *et al.* (2006).

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

Results

Below-ground *L. amethystina* population structure in a fruiting season

Of 250 soil samples collected from 10 plots in the fruiting season, 181 contained *L. amethystina* ECM tips which belonged to 29 genets and 69 samples were blank. (Table 1 and Fig. 3). Each genet of *L. amethystina* was unique to a single plot, and no genet was found in ≥2 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 0.53 ± 0.04 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.

Below-ground *L. amethystina* population structure nine months after fruiting season

Of 250 soil samples collected from *L. amethystina* plots in June 2006 i. e., nine months after the fruiting season, 188 contained *L. amethystina* ECM tips, which belonged to 118 genets and rest 62 samples contained no *L. amethystina root tips* (Table 1 and Fig 4). As in the 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. amethystina* 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. amethystina	Blank	
in a fruting season	250	181	69	29
nine months after fruiting season	250	188	62	118

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

Sampling time	Number of genets (mean ± SE)
in fruting season	2.9 ± 0.28***
nine months after fruiting season	11.8 ± 1.23

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

In contrast to the fruiting season, there were many small below-ground genets of *L. amethystina* in June 2006. A maximum of 19 genets were found in a plot (S-395) and average numbers of genets per plot was 11.8 ± 1.2 which was significantly higher than in fruiting season 2.9 ± 0.3 (mean ± SE, Table 2). Average areas per genet, which are the sum of sub-plot areas that include each genet, was 0.06 ± 0.01 m^2 in June. This was significantly smaller than in the fruiting season, 0.25 ± 0.04 m^2 (mean ± SE, Table 3).

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

Season	Area per genet (m^2)	
	Mean ± SE	Range
in fruting season	0.25 ± 0.039***	0.04 - 0.68
nine months after fruiting season	0.06 ± 0.007	0.04 - 0.60

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

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

In June 2006, the below-ground genets identical to the previous year's sporocarp were detected in four plots (S-684, S-455, S-818, S-643, Figs. 4). All of these persistent genets remaining in soil after fruiting season were comparatively larger in size compared with the other genets (Fig. 4).

Majority of below-ground genets (51 %) sampled nine months after fruiting season shared common alleles with the previous year's sporocarps (Fig. 4), indicating that these below-ground genets may be offsprings of the previous year's sporocarps.

Discussion

In the sporocarp season, the below-ground genets of *L. amethystina* identical to current year sporocarps were found in all plots. Such sporocarp-producing genets were detected in larger areas than other below-ground genets. Thus, it may be necessary to occupy a certain area (≥0.32 m^2) to obtain enough resources for sporocarp formation.

In the sporocarp study of *L. amethystina*, the majority of sporocarp genets found in 2005 were not detected in the next year (Wadud 2007). In this study, the genets identical to previous year's sporocarps were not detected in more than half plots nine months after fruiting season. Thus, the below-ground result confirms that the majority of *Laccaria* genets actually disappear after sporocarp formation. Frequent disappearance of genets after sporocarp formation has also been described in *Suillus grevillei* (Zhou *et al.* 2001b) 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). Although most of *Laccaria* ECM tips would be laid off within several months after sporocarp decay, some can survive over the long winter and start to colonize surrounding new roots. Even if the survival rate per root tip was constant among the genets, larger genets that colonize many ECM roots may have a greater chance to survive and enlarge.

With or without persistent genets, many small genets were found in below-ground in June. 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 in June 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). Because both types of mating require a spore-originated hypha, this result indicates importance of spore-mediated reproduction in the annual genet dynamics *L. amethystina* in either event.

Below-ground genets in June were significantly smaller and richer than in the sporocarp season when the sporocarp-forming genets become larger and dominate in below-ground. Thus, most of below-ground genets found in June are considered to disappear without formation of any sporocarps before sporocarp 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 seasonal dynamics of below-ground *L. amethystina* genets is much greater than ever thought.

Conclusion

In conclusion, the present study demonstrates that *L. amethystina* have spore-dependent reproduction strategies in the volcanic desert on Mount Fuji, showing rapid generation and turnover of genet. Such reproductive characteristics of this species would enable it to first colonize on newly established *S. reinii* in nonECM habitat and to remain dominant in the disturbance-rich environment.

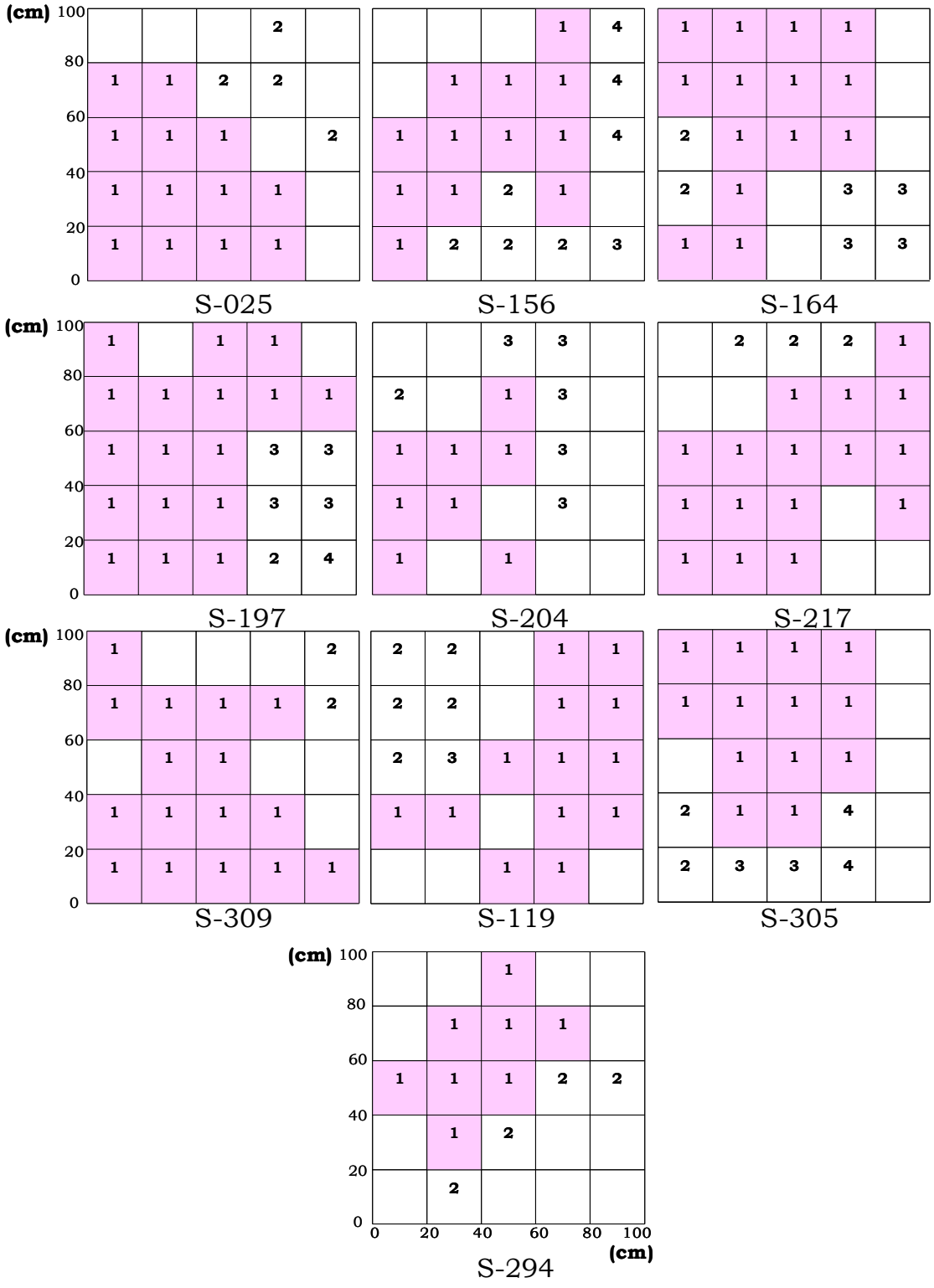

Fig. 3 Spatial distribution of below-ground *L. amethystina* genets in 10 plots (S-025, S-156, S-164, S-197, S-204, S-217, S-309, S-119, S-305 and S-294) in a fruiting season. Black Arabic numbers represent *L. amethystina* genets, blank positions indicate no *L. amethystina* ECM root tips were found. Highlighted genets are the genets that produced sporocarps in this season.

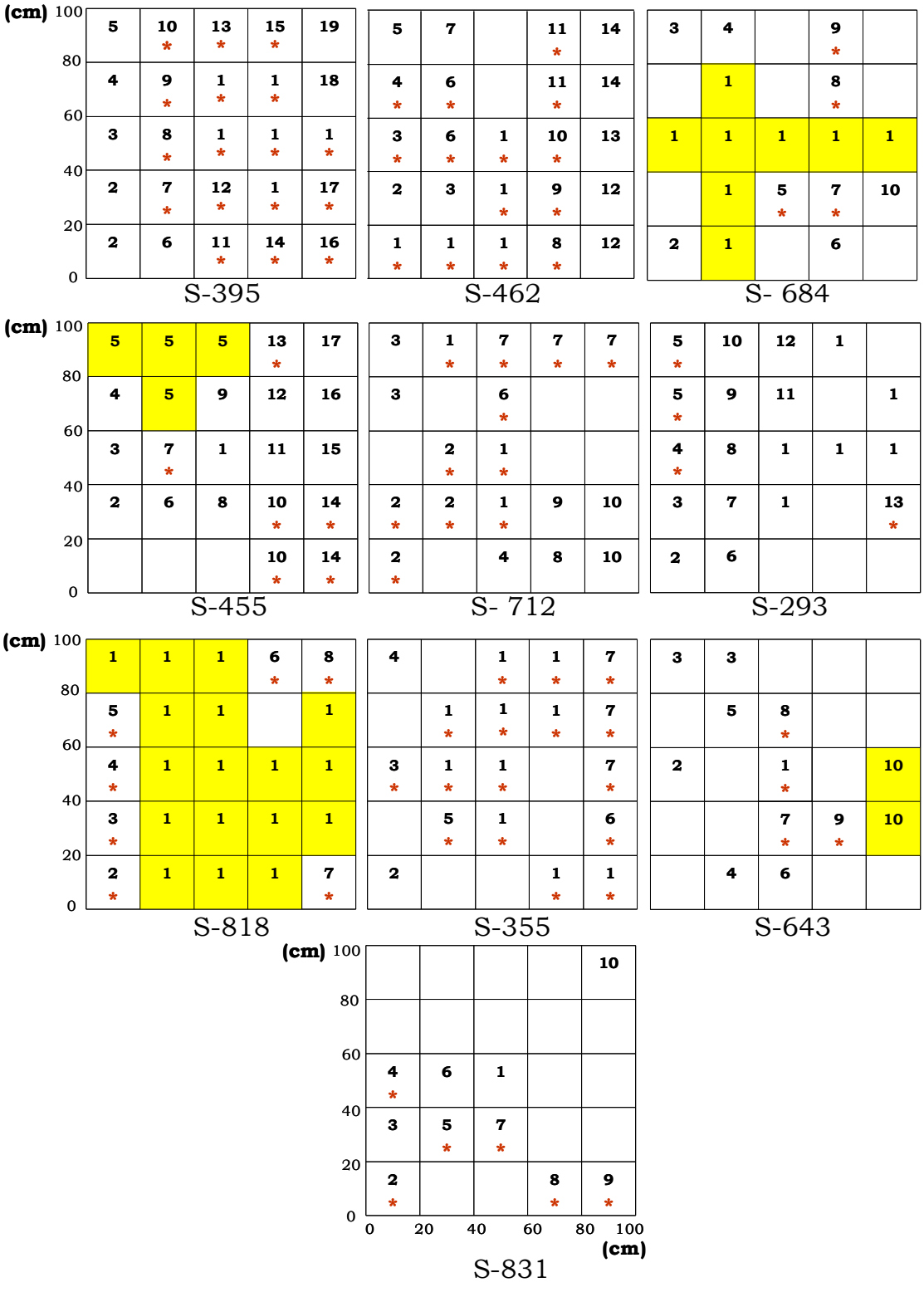

Fig. 4 Spatial distribution of below-ground *L. amethystina* genets in 10 plots (S-395, S-462, S-684, S-455, S-712, S-293, S-818, S-355, S-643 and S-831) nine months after fruiting season. Black Arabic numbers represent *L. amethystina* genets, blank positions indicate no *L. amethystina* ECM root tips 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.

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