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Analysis of genetic structure of a *Suillus grevillei* population in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR)

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SUMMARY

Clones of ectomycorrhizal fungi can colonize new areas through production of vegetative mycelium or spore dispersal, but the relative importance of these processes in nature is not known. In this study, sporocarps of an ectomycorrhizal fungus, *Suillus grevillei*, were mapped and sampled from a *Larix kaempferi* stand at the foot of Mt Fuji. DNA was extracted directly from each sporocarp, and DNA polymorphism was analysed by polymerase chain reaction (PCR) amplification of inter-simple sequence repeat (ISSR) regions primed by $(GTG)_5$, $(GCC)_5$ and $(GACA)_4$. Different sensitivities to detect polymorphism were found among the three primers, with $(GACA)_4$ showing the highest sensitivity. Forty seven sporocarps were analysed by the three ISSR primers and divided into 34 genets based on combination of PCR fingerprints. In the population 28 genets were represented by individual sporocarps. In most cases, sporocarps grown in aggregation (within a circle of 50 cm diameter) showed some different ISSR band patterns. These results suggest that genets of *S. grevillei* at the test site are relatively small. The genetic similarities between the 34 genets were also calculated and similarity groups were determined by the criterion that all similarity F values of genets within a group were not $<80\%$. In general, the genets within a similarity group located close to each other. The results of multiple different but highly related genets in a small area suggest that the population of *S. grevillei* in this stand is not spread and maintained by clonal mycelium extension but is reproduced by spore dispersal.

Key words: ectomycorrhiza, genet, inter-simple sequence repeat (ISSR), *Larix kaempferi*, mycelium extension, population, spore dispersal, *Suillus grevillei*.

INTRODUCTION

In forest ecosystems, trees interact with many soil microorganisms. Tree species in boreal and temperate forests usually form ectomycorrhizas by symbiosis with fungi. Many studies have highlighted the importance of ectomycorrhizal (ECM) fungi in material dynamics (Smith & Read, 1997), and the contribution of ECM fungi to material dynamics depends on many factors including fungal reproduction in the soil. Thus, a better understanding of clonal diversity of ECM fungi would cast light on a novel aspect of forest dynamics.

Most ECM fungi are basidiomycetes and form sporocarps. The fungi are considered to disperse by mycelium extension or by spores, but the relative importance of these processes in nature is not known. If single clones of ECM fungi extend their colonial area mainly by hyphal development, they would form large genets with genetically identical sporocarps. If they extend mainly by spore dispersal, they would form a number of small genets and sporocarps within small area would be genetically distinct. Therefore, the manner of proliferation of an ECM fungus could be inferred by examining genetic differences between sporocarps and thereby determining genets from which sporocarps have emerged.

Several investigations on ECM fungal genet sizes have already been reported. For example, Dahlberg & Stenlid (1994) determined the distribution of *Suillus bovinus* genets within different aged *Pinus sylvestris* stands by isolating clonal cultivations from sporocarps sampled in the stands and testing their somatic incompatibility, and showed that *S. bovinus* genets enlarge as trees become older. This means that *S. bovinus* genets may expand by mycelial growth. The same situations have also been found in *S. granulatus* populations (Jacobson *et al.*, 1993) and

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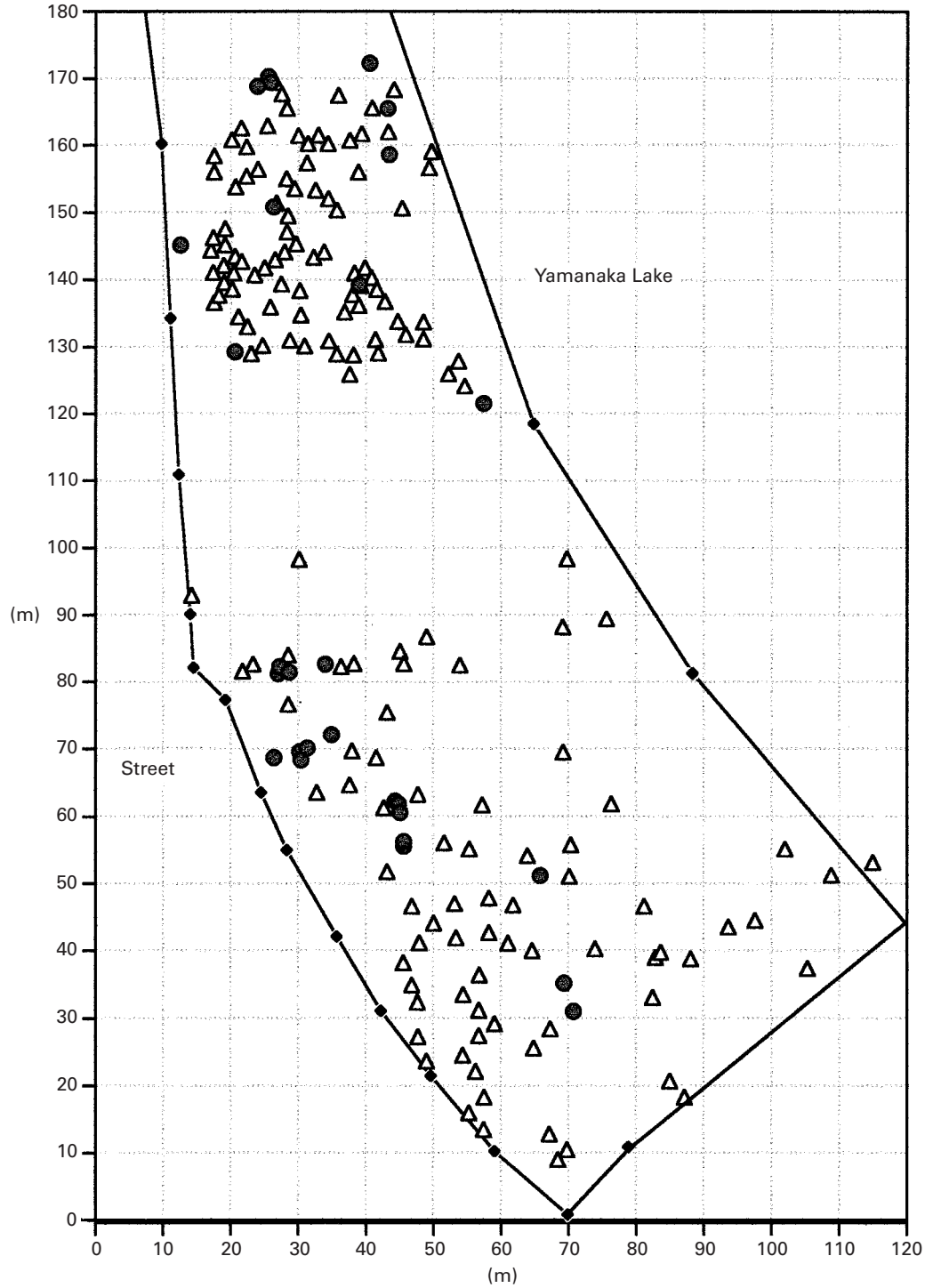

Fig. 1. The distribution of *Larix kaempferi* trees and *Suillus grevillei* sporocarps developed at the test site. *Larix* trees (open triangles), *S. grevillei* sporocarps (closed circles), the boundary line (closed diamonds) of the test site.

S. pungens (Bonello *et al.*, 1998). Similarly, genets of *Laccaria bicolor* have been observed to remain associated with same host for several years and extended their distribution by mycelial growth (de la Bastide *et al.*, 1994; Selosse *et al.*, 1998). By contrast, Anderson *et al.* (1998) reported that many *Pisolithus tinctorius* genets at their field site identified by analyses of random amplified polymorphic DNA (RAPD) and ISSR were relatively small in size, suggesting that this species expands its population by sexual reproduction, i.e. spore dispersal. The features of ECM fungal reproduction would be influenced by fungal species, age of the hosts and environmental factors. Further case studies on genets should be collated to reveal the general reproductive features of ECM fungi in nature.

Table 1. *Screening of 13 inter-simple sequence repeat (ISSR) primers, with the annealing condition and results*

ISSR primer	Annealing temperature (°C)	Band visibility	Remarks
$(CA)_8$	48	Smear	–
$(CT)_8$	48	Smear	–
$(GGAT)_4$	48	Faint	Strong background
$(CA)_8G$	45	Good	Strong background
$(GTG)_5$	**55**	**Good**	**Polymorphic**
$(CATA)_4$	42	None	–
$(GCC)_5$	**65**	**Good**	**Polymorphic**
$(GAT)_6$	48	Faint	–
$(ACA)_6$	45	None	–
$(CA)_8CT$	48	Good	Strong background
$(CA)_8GT$	48	Good	Strong background
$(GACA)_4$	**48**	**Good**	**Polymorphic**
$(GAA)_6$	45	None	–

Primers printed in bold were used in this study

In many studies on the genetic structure of ECM fungal populations (Fries, 1987; Dahlberg & Stenlid, 1990, 1994; Guillaumin *et al*., 1996; Dahlberg, 1997), genets have been identified by somatic incompatibility tests, as well as by mating systems of haploid hyphae (Fries & Mueller, 1984; de la Bastide *et al*., 1994) and by electrophoretic patterns of isoenzymes (Sen, 1990). However, these tests have only been successfully applied to a limited number of ECM species (Dahlberg & Stenlid, 1995). Recently, thanks to the rapid development of techniques for DNA analysis, especially polymerase chain reaction (PCR)-based ones, more direct and sensitive analyses of genetic difference have become possible. Martin *et al*. (1997) and Longato & Bonfante (1997) used methods involving amplification of the inter-simple sequence repeats (ISSRs) to study genetic variation within species as well as between species of ECM fungi and suggested that microsatellite-primed PCRs were reliable, sensitive and technically simple methods for assaying genetic variability.

In this paper, we analyse the genetic structure of a population of an ECM fungus, *S. grevillei*, at a *Larix kaempferi* stand in Japan by ISSR polymorphism.

MATERIALS AND METHODS

Test site location and situation

The present test site of *c*. 10000 m² was located by the Lake Yamanaka at the foot of Mt Fuji, Japan (35° 24′ N, 138° 52′ E). *Larix kaempferi* (Lamb.) Carrière trees over 85 yr old were sparsely and unevenly distributed, and mixed with Japanese red pines and broad-leaf trees. The site is constantly disturbed as it faces a street and is separated by a low fence, allowing people and animals free access.

Sporocarp sampling

All of the sporocarps of *S. grevillei* (Klotz.) Sing emerging at the test site were collected once a week during the period of sporocarp development. The position of each sporocarp was measured by a survey laser instrument (Criterion 300, Laser Technology, Englewood, CO, USA). Positions of sporocarps aggregated together within a circle of 50 cm in diameter were represented by that of a sporocarp within the aggregate. In total, 47 sporocarps were sampled. Pieces of basal tissue of each sporocarp cap were frozen by liquid nitrogen and stored at −80°C until use.

DNA extraction from the sporocarps

About 100 mg of frozen basal tissue of sporocarp caps were crushed into powder with a pestle in a mortar pre-cooled by liquid nitrogen. The powdered samples were thawed and homogenized in a washing buffer (0.1 M Tris–HCl (pH 8.0), 2% 2-mercaptoethanol, 1% polyvinylpyrrolidone and 0.05 M ascorbic acid). After centrifugation at 19100 ***g*** for 2 min, the pellet was washed five times by homogenization in the washing buffer and centrifuged at 19100 ***g*** for 2 min. DNA was then extracted from the washed pellet by a modified CTAB (cetyltrimethylammonium bromide) method (Gardes & Bruns, 1993; Martin *et al*., 1997). The pellet was suspended and incubated in CTAB buffer (2% CTAB, 0.1 M Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 0.5% 2-mercaptoethanol) at 65°C for 1 h, and extracted once by an equal volume of a phenol-chloroform-isoamyl alcohol mixture (25:24 : 1, v/v) and twice by an equal volume of a chloroform-isoamyl alcohol mixture (24:1, v/v). DNAs were precipitated by adding an equal volume of isopropanol. After collection by centrifugation at 5400 ***g*** for 5 min, DNAs were resolubilized in 100 µl of sterilized distilled water, then treated with 1 µl of 10 mg ml^{-1} ribonuclease solution (RNase, Nippon Gene Co., Tokyo, Japan) for 30 min at room temperature and precipitated again by adding 60 µl of a PEG solution (20% polyethylene glycol (6,000) and 2.5 M NaCl). After centrifugation at 19100 ***g*** for 10 min and washing in 70% ethanol, the pellet was resuspended in 50 µl of TE buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA) and stored at −30°C until use.

PCR amplification and electrophoresis

PCR amplification was performed in 20 µl reaction mixture which contained *c*. 10 ng of template DNA, 0.2 mM each of dNTP, 1.5 mM $MgCl_2$, 0.5 µM inter-simple sequence repeat motif primer (Table 1),

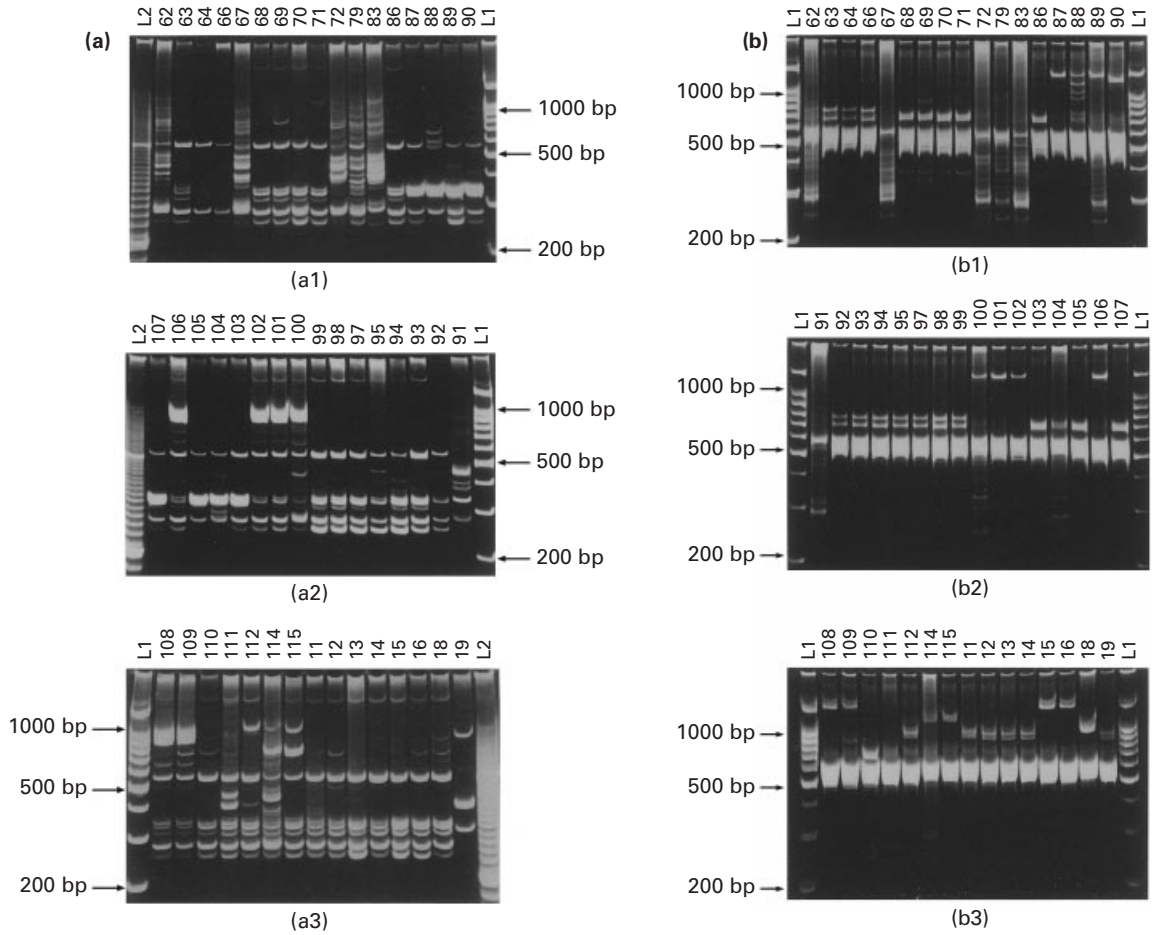

Fig. 2. PCR-assisted inter-simple sequence repeat (ISSR) fingerprints of 47 *Suillus grevillei* sporocarps from a middle-aged *Larix kaempferi* stand using the primers (a) $(GTG)_5$, (b) $(GCC)_5$ and (c) $(GACA)_4$. Lane numbers refer to individual sporocarps sampled from the test site (No. 19 was a sporocarp of *Suillus lutues* found at the same test site). L1 is the 100 base pair (bp) ladder and L2 is the 20 bp ladder. PHY is the pHY DNA MW standard marker (Takara Shuzo Co., Tokyo, Japan).

2.0 µl of 10 X buffer and 0.5–0.6 U of AmpliTaq Gold (AmpliTaq Gold kit, Perkin Elmer, Branchburg, NJ, USA) where 1 U of AmpliTaq Gold is defined as the amount that will incorporate 10 nmoles of dNTPs into acid insoluble material per 30 min in a 10 min incubation at 74°C under analysis conditions) by a PCR Thermal cycler (TP 3000, Takara Shuzo Co., Tokyo, Japan). The thermal cycling schedule was as follows: the first cycle consisted of 9 min at 94°C, 1 min at the annealing temperature (Tm) and 1 min at 72°C, followed by 38 cycles of 1 min at 94°C, 1 min at Tm and 1 min at 72°C, and by the final cycle of 1 min at 94°C, 1 min at Tm and 10 min at 72°C. Then the reaction mixture was cooled at 4°C for >5 min to terminate the PCR reaction and stored at -30°C until use.

PCR products, 5 µl aliquots, were mixed with 2 µl loading buffer (50% glycerine, 1 mM EDTA, and 0.25% xylene cyanol FF; Wako Co. Osaka, Japan) and subjected to electrophoresis on 8–12% polyacylamide gels. After staining with ethidium bromide, band patterns were visualized on a UV transilluminator.

Genetic grouping

The sporocarps with the same PCR profile patterns for each primer were regarded as a single group. Sporocarps which were commonly classified to the same group by the PCR fingerprints of any primers used in the following polymorphic analyses were regarded as belonging to the same genet.

Similarity between genets

Similarity between genets was evaluated by applying the equation $F = 2n\mathrm{AB}/(n\mathrm{A}+n\mathrm{B})$ (Nei, 1987), in which nA and nB are the numbers of PCR fragment bands present in genet A and genet B, respectively, and nAB is the number of bands common to genet A and B.

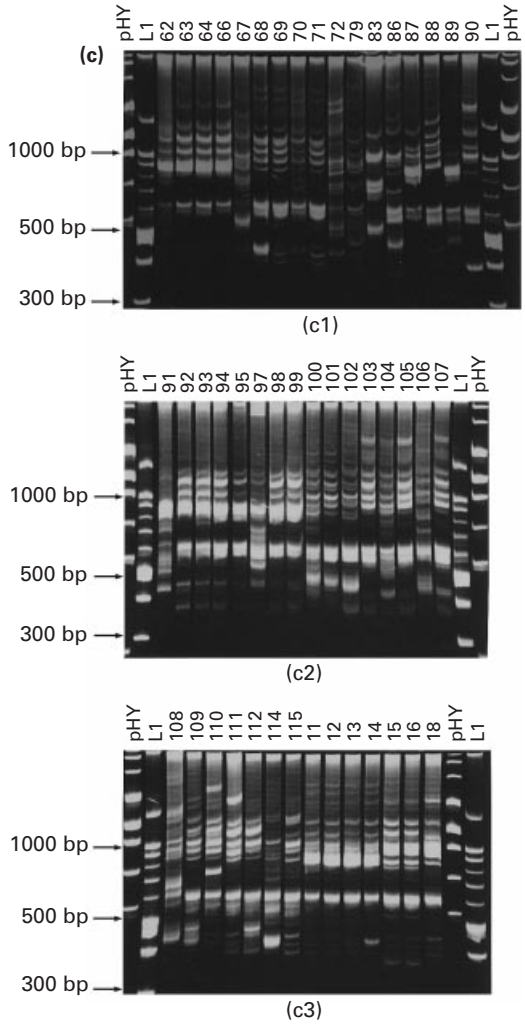

Fig. 2. For legend see facing page.

RESULTS

Emergence of S. grevillei *sporocarps at the test site*

The peak of sporocarp emergence lasted only *c*. 1 wk. Of 47 sampled sporocarps, two sporocarps were found on 2 October 1997, 44 on 9 October and only one on 16 October. No sporocarp of *S. grevillei* was found thereafter. Sporocarps in this test site sometimes emerged in aggregation and most occurred in two areas (Fig. 1). Most sporocarps were found near *L. kaempferi* trees.

ISSR fingerprints and ISSR groupings

Thirteen ISSR primers were selected by referring to the primers used by Martin *et al*. (1997) and Longato & Bonfante (1997) and to the probes used by Edwards *et al*. (1996). Screening of the 13 primers was performed with seven sporocarps which emerged at the test site separately from each other (Table 1). Of these 13 primers, only three, $(GTG)_5$, $(GCC)_5$ and $(GACA)_4$, provided polymorphic band patterns with little background staining (Table 1). Reproducibility of amplifications primed by the three primers was checked on three occasions. Amplifications by 0.5 U of DNA polymerase, AmpliTaq Gold, in a 20 µl of reaction solution with primers, $(GTG)_5$ and $(GCC)_5$, always gave identical fingerprints. In the case of $(GACA)_4$, the distinct and clear bands showed high reproducibility but the detection of certain faint bands was less reproducible. However, when the amount of the polymerase was increased from 0.5 to 0.6 U, band patterns of $(GACA)_4$ also showed high reproducibility. Thus, 0.6 U of AmpliTaq gold were used for further amplifications primed by $(GACA)_4$. As PCR profiles of $(CA)_8$ and $(CT)_8$ were smeared, whereas $(GGAT)_4$, $(CATA)_4$, $(GAT)_6$, $(ACA)_6$ and $(GAA)_6$ gave unclear or no bands, and $(CA)_8G$, $(CA)_8CT$ and $(CA)_8GT$ gave clear bands but with strong background staining (Table 1), the above indicated primers were not used in the following polymorphism analyses.

PCR fingerprint profiles generated using each of the three primers, $(GTG)_5$, $(GCC)_5$ and $(GACA)_4$, are presented in Fig. 2a,b,c, respectively. A total of

Table 2. *Inter-simple sequence repeat (ISSR) groups determined by identical band patterns of the three primers and genets of* S. grevillei *at the test site identified by combination of the three kinds of ISSR groups*

ISSR group			Genet	
GTG	GCC	GACA	Encompassed sporocarps	Genet number
62	62	62	62	G1
63	63	63	63	
64	64	64	64	G2
66	66	66	66	
67	67	67	67	G3
69	69	69	69	G4
71	71	71	71	G5
68	68	68	68	G6
70	70	70	70	
72	72	72	72	G7
79	79	79	79	G8
83	83	83	83	G9
86	86	86	86	G10
87	87	87	87	G11
88	88	88	88	G12
89	89	89	89	G13
90	90	90	90	G14
91	91	91	91	G15
95	95	95	95	G16
92	92	92	92	
93	93	93	93	
94	94	94	94	G17
98	98	98	98	
99	99	99	99	
97	97	97	97	G18
100	100	100	100	G19
101	101	101	101	G20
102	102	102	102	G21
106	106	106	106	G22
108	108	108	108	G23
109	109	109	109	G24
103	103	103	103	
105	105	105	105	G25
107	107	107	107	
104	104	104	104	G26
110	110	110	110	G27
111	111	111	111	G28
112	112	112	112	G29
114	114	114	114	G30
115	115	115	115	G31
11	11	11	11	
12	12	12	12	G32
13	13	13	13	
14	14	14	14	
18	18	18	18	G33
15	15	15	15	G34
16	16	16	16	

Numbers in columns refer to individuals of *S. grevillei* sporocarps sampled from the test site.
Numbers of sporocarps divided by solid lines constitute ISSR groups or genets. The numbers following G refer to genet number.

64 reproducible bands were detected; 26 bands by $(GACA)_4$, 17 bands by $(GCC)_5$ and 21 bands by $(GTG)_5$. Based on the polymorphic band patterns, $(GTG)_5$, $(GCC)_5$ and $(GACA)_4$ divided the 47 sporocarps into 27, 27 and 32 groups, respectively (Table 2). The three primers shared some similarities in grouping the sporocarps. For example, sporocarps nos. 63, 64 and 66, sporocarps Nos 103, 105 and 107 and sporocarps nos. 92, 93, 94, 98 and 99 were included in the same group by all three primers. Primer $(GACA)_4$ detected more groups than the other two primers. $(GTG)_5$ and $(GCC)_5$ grouped

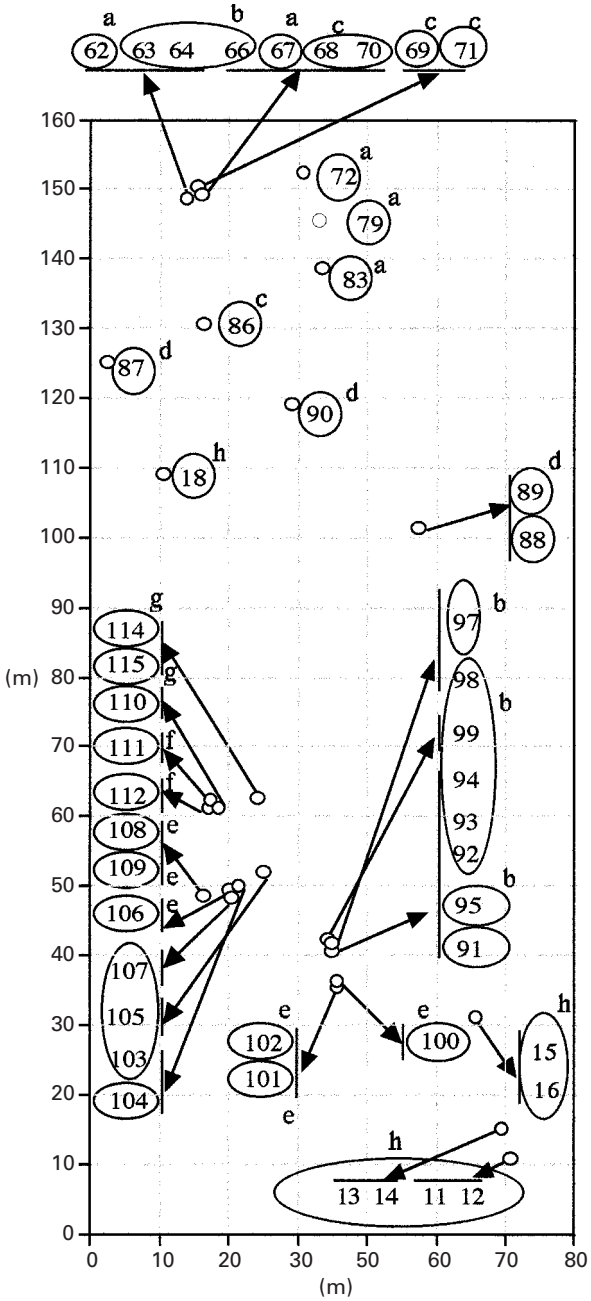

Fig. 3. Distribution of *Suillus grevillei* genets at the test site. Numbers refer to individual sporocarps sampled from the test site. The numbers of sporocarps belonging to the same genet were encompassed by a circle. The open circles indicate the positions of one sporocarp or an aggregation of sporocarps. Genets superscripted with the same letter were constituted in a same high similarity group (*F* values were $>80\%$).

sporocarps Nos 101, 102, 106, 108 and 109 into one group, whereas $(GACA)_4$ divided the five sporocarps into five groups. This result indicated that $(GACA)_4$, of the three primers, had the highest resolution to detect polymorphism in *S. grevillei*.

Genet distribution

Based on the combination of the three ISSR groups, 47 sporocarps were classified into 34 genets (Table 2), of which 28 genets were each represented by individual sporocarps. Most sporocarps which emerged in single aggregations belonged to different genets (Fig. 3). The longest axis representing the largest genet encompassing sporocarps nos. 103, 105 and 107 was *c.* 6 m.

Similarity value (F) *and similarity groups*

F values between the 37 genets were calculated, and similarity groups were determined based on the criterion that *F* values between any two genets were $\geqslant 80\%$, i.e. all *F* values between genets within a similarity group were not $<80\%$ (Fig. 3). In most cases, genets in the same similarity group tend to locate closely to each other. However, several genets, such as genet G32 (Table 2) and genet G33 in group h, and genet G2 and the other genets in group c, belonged to the same similarity group located far away from each other.

DISCUSSION

Martin *et al.* (1997) and Longato & Bonfante (1997) demonstrated that $(GTG)_5$-primed fingerprints of ECM fungi showed intraspecific polymorphism and proposed that ISSR analysis was a potentially useful tool for fungal population study. Our results of ISSR analyses confirmed their proposal. Fingerprints by the three ISSR primers used in the present analyses were identical on three occasions of amplification to check reproducibility and distinguished sporocarps located close together, into different genetic groups. These ISSR markers seem to be reliable and sensitive for DNA polymorphic analyses of *S. grevellei*.

The results in our study showed that there were 34 genets in the population, of which only six genets produced multiple sporocarps, the remainder being represented by individual sporocarps (Table 2). Genet sizes of this *S. grevillei* population seem to be relatively small, compared with other *Suillus* species. Large sizes of *S. bovinus* genets were found in mature stands and a relatively high number of sporocarps per genet were calculated (Dahlberg & Stenlid, 1990). In a study of *S. variegatus* population, only 38 genets were identified from 120 tested sporocarps and most of them produced multiple sporocarps (Dahlberg, 1997).

The length of the biggest genet in long axis in this test site was *c.* 6 m. In many cases, even sporocarps close to each other (<0.5 m apart) belonged to different genets (Fig. 3). Different situations have been found in the studies of other *Suillus* species populations. The first study dealing with an ECM fungal population revealed that *Suillus luteus* genets in a forest stand were at least 2 m width (Fries, 1987). Studies of *S. bovinus* populations showed that genets were 1–30 m across (Dahlberg & Stenlid, 1990, 1994a), whereas the average genet size of *S.*

variegatus was 20 m in old Scots pine stands and 10 m in young stands (Dahlberg, 1997). Similarly, genet sizes of *S. granulatus* (Jacobson *et al.*, 1993) and *S. pungens* (Bonello *et al.*, 1998) have been reported to be >20 m and 40 m, respectively. The above comparison also shows that *S. grevillei* genets seem to be rather smaller than those of other *Suillus* species. Since large and small genet sizes may reflect extension of their colonial area mainly by hyphal development and spore dispersal, respectively, the smaller genets of *S. grevillei* suggest that more genets in this population have only recently become established from spore dispersal.

Most genets grouped together by the 80% similarity criterion were located close to each other (Fig. 3). Since a single genet that originates from a dikaryotic mycelium inherits the genetic composition of two different spores, the genetic similarity of genets may mean that the genets originate from closely-related spores such as those from the same sporocarp. Thus, the close location of genetically similar genets suggested that the spores from the same sporocarps do not tend to be dispersed far.

It is possible that a population of newly established genets might be in its immature development stage or reproduces through sexual reproduction. Dahlberg & Stenlid (1994) demonstrated that sizes and density of *S. bovinus* genets in Scots pine forests varied with forest age, ranging from 15 to 160 yr old, and there are fewer, larger genets in the older than the younger stand. A similar situation was also found in *S. variegatus* populations (Dahlberg, 1997). It was suggested that a population of *Suillus* species was first established by spore dispersal, forming a number of small genets, and, as a population becomes mature, expanded by mycelial extension, resulting in a fewer number of larger genets (Dahlberg & Stenlid, 1995). At our test site, since the age of this *Larix* stand was >85 yr old, and sporocarps of *S. grevillei* had been observed there for many years (S. Chishima (manager of the forest), pers. comm.), the *S. grevillei* population seems to be well-established. However, the extensions of clonal mycelia there have been limited. This suggests that the present *S. grevillei* population seems to follow a different development course from other *Suillus* species and reproduce by sexual reproduction, that is, by spore dispersal even at its mature stages.

The differences between the population biology of *S. grevillei* and other *Suillus* species may be due to characteristics of the species or to environmental factors. For example, disturbance may limit mycelial extension and promote establishment of clones from dispersed spores, and the test site studied here may be greatly disturbed by people and animals, since it faces a street across a low fence. It would be of interest to examine *S. grevillei* genet sizes in less disturbed *Larix* stands and compare these with the present results.

Based on this discussion, we conclude that the present *S. grevillei* population is reproduced mainly by spore dispersal. However, it should be noted that there are a few problems to be solved in future. One such problem is derived from the fact that the distribution of sporocarps is not always consistent with that of underground mycelia. To what extent both distributions of *S. grevillei* are consistent with each other should be investigated in future. Also, the present study was performed only in a disturbed test site. To estimate precisely the effect of environmental conditions including disturbance on the reproduction of *S. grevillei*, its populations in many other sites should be investigated.

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