FULL PAPER

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Genetic variability among *Pholiota aurivella* isolates from a small natural population

Abstract Genetic differences between 36 *Pholiota aurivella* wild isolates collected from 13 decayed logs of Salicaceae trees distributed along about 1200 m of a streambed in a forest were characterized by somatic incompatibility and mating tests, and by restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA). There was a perfect correlation between somatic incompatibility and mating type groups, and isolates could be divided into 15 genets (genetically identical clones). Because the mtDNAs of the 36 wild isolates have 14 different *Eco*RI RFLP patterns, they likely originated from at least 14 distinct wild strains, indicating that multiple wild strains with distinct genetic compositions coexist in the forest investigated in this study. mtDNA variation of *P. aurivella* is apparently very high despite the close proximity of sample collection sites within the forest. The territories of single *P. aurivella* genets within a host log are apparently larger than other nonpathogenic wood-decaying basidiomycetes reported previously, such as *Flammulina velutipes* and *Lentinula edodes*.

Key words Genetic variability · Mating type · Mitochondrial DNA genotype · *Pholiota aurivella* · Somatic incompatibility

Introduction

Wild strains of wood-decaying basidiomycetes are valuable genetic resources for mushroom breeding. These fungi are highly valued as food, but are also used as a source for pharmaceuticals, and in industrial applications for lignin degradation and bioremediation (Kawagishi 2005). To use these wild strains most effectively, the genetic variability of each individual species must be understood. There have been some studies on the genetic diversity of geographically isolated populations of several cultivated wood-decaying basidiomycetes such as *Lentinula edodes* (Berk.) Pegler (Fukuda and Tokimoto 1991; Fukuda et al. 1994; Hibbett et al. 1995), *Pleurotus ostreatus* (Jacq: Fr.) Kummer (Matsumoto and Fukumasa-Nakai 1995; Matsumoto et al. 1995), *Pholiota nameko* (T. Ito) S. Ito and Imai (Obatake et al. 2002), and *Flammulina velutipes* (Curt.:Fr.) Sing. (Nishizawa et al. 2003), and genetic differences between wild strains growing on the same substrates have been studied within the context of the size of their inhabited territory in some wood-decaying basidiomycetes such as *P. ostreatus* (Kay and Vilgalys 1992), *L. edodes* (Chiu et al. 1999; Fukuda and Mori 2003), and *F. velutipes* (Fukuda et al. 2000). However, offspring derived from extensively cultivated strains may have increased in natural populations, which may be reflected in the genetic variability of the natural populations. Therefore, more information and additional case studies are necessary to further clarify the genetic variability among wild strains in natural populations for various wood-decaying basidiomycetes.

We accidentally found 13 decayed trees on which one or more fruiting bodies of *Pholiota aurivella* (Batsch: Fr.) Kummer were forming along about 1200 m of a streambed (mainly within 650 m) in a forested area of Nagano Prefecture, Japan. Because little is known about the artificial cultivation of *P. aurivella* in Japan, there is little possibility that cultivated strains have increased within the natural population. The purpose of the present study is to identify and quantify the genetic differences among the *P. aurivella* fruiting bodies within this small natural population. Somatic incompatibility and mating tests were combined with restriction fragment length polymorphism (RFLP) analyses of mitochondrial DNA (mtDNA) to provide an efficient and accurate assessment of genetic variation, strain distribution, and possibly population dynamics.

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Materials and methods

Wild isolates of *P. aurivella*

A total of 36 *P. aurivella* fruiting bodies were collected from 13 decayed logs of trees in the family Salicaceae located at Iriyamabe in Matsumoto City, Nagano Prefecture in autumn of 1998 and 1999 (Table 1). The collection sites are at an elevation between 1200 and 1250 m. Fruiting bodies were collected after their fruiting positions on the decayed logs, and locations of the decayed logs were recorded (Fig. 1). All specimens used in this study were identified as *P. aurivella* based on morphological characters, particularly basidiospore dimensions, rather than the very similar species *Pholiota adiposa* (Fr.) Kummer, following Imazeki and Hongo (1987). To make a pure culture from each fruiting body, small tissue blocks were aseptically excised and transferred to MYG agar (10 g malt extract, 4 g yeast extract, 10 g glucose, and 20 g agar per liter of distilled water). Samples were incubated at 25°C, and newly grown hyphae were aseptically transferred to fresh MYG agar.

Somatic incompatibility tests

Somatic incompatibility interactions, i.e., the interactions between two dikaryotic strains when paired together in culture, were tested among the wild isolates by placing 5-mm-diameter mycelial plugs from the isolates about 30 mm apart onto M agar (20 g malt extract and 20 g agar per liter of distilled water) in a 90-mm Petri plate. All isolates were paired in every possible combination, including self-pairings. Interactions were observed after 30 days incubation at 25°C. Each of the parings was repeated at least twice.

Mating tests

Basidiospores were collected from wild fruiting bodies and allowed to germinate on MYG agar plates, or if no basidiospores could be collected from the wild fruiting body, fruiting bodies were produced by inoculating 100 g sawdust medium (*Fagus crenata* Blume sawdust:rice bran; 4:1 v:v, with 65% moisture content) in 200-ml glass bottles with a mycelial plug (about 5 mm in diameter) of the wild isolate. After 4 weeks incubation at 25°C in the dark, the culture was transferred to lighted conditions at 16°C to induce fruiting. Up to 30 germinating basidiospores per fruiting body were randomly isolated, transferred individually to new M agar slants, and incubated at 25°C.

Single-spore isolates were mated by placing mycelial plugs (about 2 mm in diameter) on M agar plates at a distance of 5 mm. Dikaryosis of the paired monokaryons was confirmed microscopically by the appearance of clamp connections.

mtDNA isolation and restriction analyses

To prepare mycelium for mtDNA isolation, 100 ml MYG liquid medium in a 500-ml Erlenmeyer flask was inoculated with an M agar disk (about 5 mm in diameter) from the mycelial culture and incubated without shaking in the dark at 25°C for 7 days. Mycelium was aseptically fragmented in a Waring blender, and 20 ml was used to inoculate 100 ml of fresh MYG liquid medium. Flask cultures were incubated without shaking in the dark at 25°C for 5–7 days, harvested, washed with water, and lyophilized. Isolation of mtDNA from the lyophilized mycelia was by the procedure of Fukumasa-Nakai et al. (1992).

mtDNA isolated from each of the 36 wild isolates was digested with *Eco*RI (Nippon Gene, Tokyo, Japan), following the supplier's specifications, and separated by electrophoresis on 0.8% agarose (Nippon Gene, Type S) gels at 5 V/cm for 4 h, followed by staining with ethidium bromide (0.5 µl/ml). Restriction patterns were recorded by photographing the gels on a UV transilluminator. Lambda

Table 1. *Pholiota aurivella* wild isolates used in this study

Decayed log no.	Number of fruiting bodies from the log	Fruiting area on log[a] (m)	Isolate no.	Date isolated
Log #1	1	–	Pa1	10/10/1999
Log #2	1	–	Pa2	10/10/1999
Log #3	2	0.90	Pa3.1, 3.2	10/10/1999
Log #4	1	–	Pa4	9/29/1999
Log #5	1	–	Pa5	10/4/1998
Log #6	8	2.73	Pa6.1–6.8	9/11/1999 for Pa6.1–6.5
				10/10/1999 for Pa6.6–6.8
Log #7	5	1.02	Pa7.1–7.5	9/29/1999
Log #8	3	0.85	Pa8.1–8.3	9/29/1999
Log #9	3	2.28	Pa9.1–9.3	10/4/1998
Log #10	7	1.20	Pa10.1–10.7	10/10/1999
Log #11	1	–	Pa11	10/4/1998
Log #12	1	–	Pa12	9/11/1999
Log #13	2	1.52	Pa13.1, 13.2	10/4/1998

[a] The longest distance between fruiting bodies on decayed log from which multiple fruiting bodies were collected

Fig. 1. Spatial distribution of decayed logs (#1–#13) on which one or more fruiting bodies of *Pholiota aurivella* were forming in a forest in Nagano Prefecture, central Japan. The number of fruiting bodies collected from each decayed log is shown in *parentheses*. Positions of three genetically different fruiting bodies on log #8 are also shown
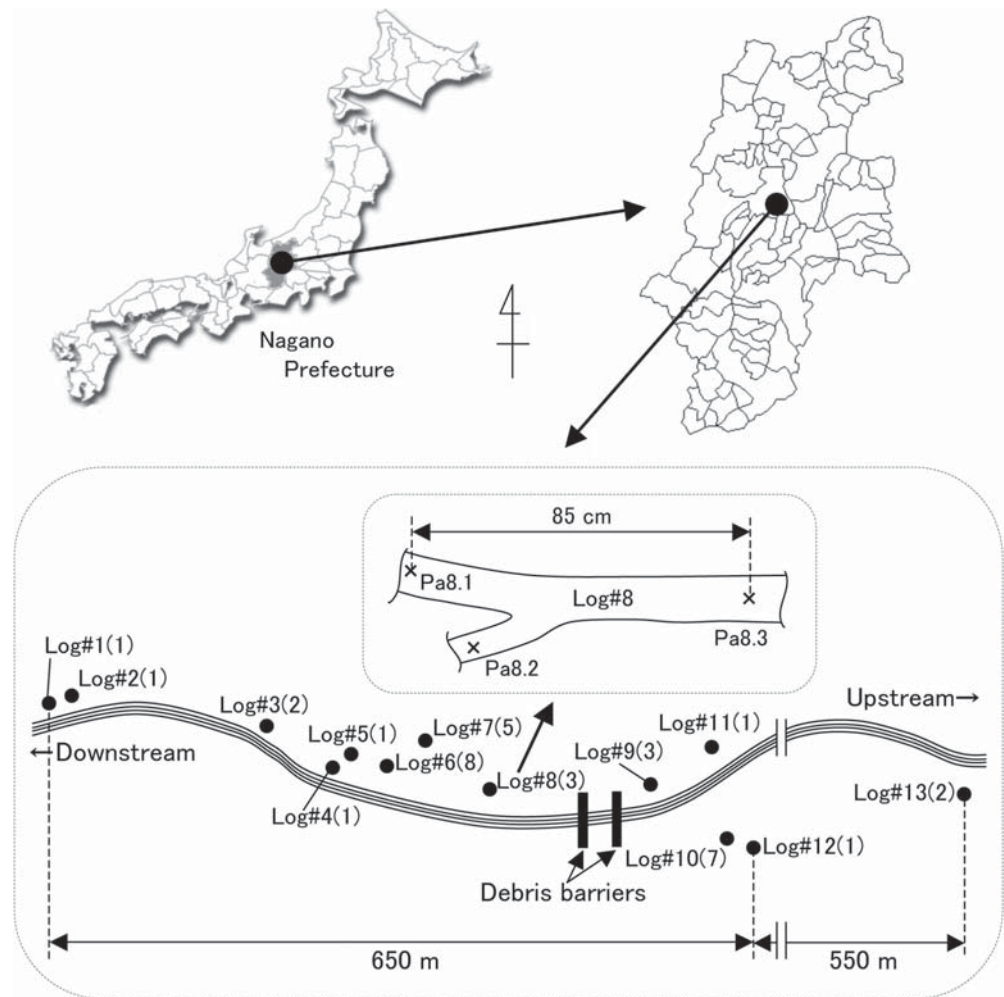
phage DNA digested with *Hin*dIII served as a molecular standard.

RFLP numerical analysis

The presence or absence of individual *Eco*RI restriction fragments was scored, and a distance value (D) was calculated between pairs of different mtDNA types as $1 - 2(Nxy)/(Nx + Ny)$, in which Nxy is the number of restriction fragments shared between pairs of mtDNA types, and Nx and Ny are the total numbers of restriction fragments in all digests in mtDNA types X and Y, respectively.

Results

Somatic incompatibility tests

Self-pairing always resulted in complete intermingling without any evidence of antagonism (Fig. 2). The interisolate interactions most similar to self-pairing were observed among isolates collected from the same log, except for three isolates from log #8, indicating that the paired isolates are somatically compatible. Incompatible interactions resulted in the formation of white lines at junction zones of the paired isolates (Fig. 2). Of the 630 interisolate parings, 556 were somatically incompatible, and resulted in segregation of the 36 wild isolates into 15 somatic incompatibility groups, each of which consisted of one to eight members (Table 2).

Mating tests

Although a bipolar mating system has previously been demonstrated in the related species *P. adiposa* (Arita and Mimura 1969), the mating system of *P. aurivella* has not been described. To determine the mating system of this fungus, at least 12 single-spore isolates derived from three representative wild isolates (Pa2, Pa4, and Pa6.1) were selected randomly and paired in all possible intraisolate combinations. The results of these crosses indicate that *P. aurivella* has a bipolar mating system (data not shown). To obtain monokaryons of two distinct mating types (mating-
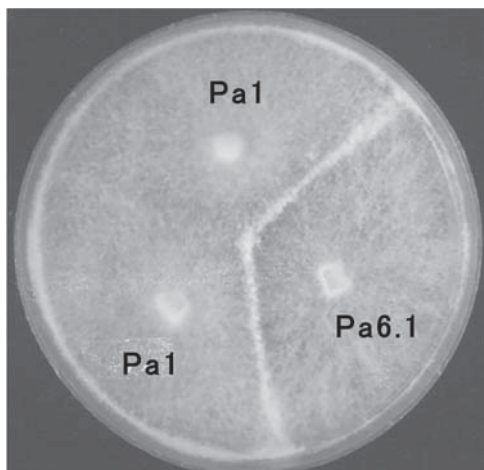
Fig. 2. Somatic incompatibility interactions between paired *Pholiota aurivella* wild isolates. The junction between two Pa1 clones is nearly indistinguishable and represents a compatible somatic interaction. The junction between isolates Pa1 and Pa6.1 shows incompatibility (*white line*)

Table 2. Somatic incompatibility groups (SIGs), mating types, and mtDNA genotypes among *Pholiota aurivella* wild isolates used in this study

Isolate no.	Number of isolates	SIG	Mating type	mtDNA genotype
Pa1	1	SIG-1	*A1, A2*	A
Pa2	1	SIG-2	*A3, A4*	B
Pa3.1, 3.2	2	SIG-3	*A5, A6*	C
Pa4	1	SIG-4	*A7, A8*	D
Pa5	1	SIG-5	*A9, A10*	E
Pa6.1–6.8	8	SIG-6	*A1, A11*	F
Pa7.1–7.5	5	SIG-7	*A12, A13*	G
Pa8.1	1	SIG-8	*A14, A15*	H
Pa8.2	1	SIG-9	*A16, A17*	I
Pa8.3	1	SIG-10	*A18, A19*	J
Pa9.1–9.3	3	SIG-11	*A20, A21*	K
Pa10.1–10.7	7	SIG-12	*A7, A22*	L
Pa11	1	SIG-13	*A22, A23*	L
Pa12	1	SIG-14	*A24, A25*	M
Pa13.1, 13.2	2	SIG-15	*A26, A27*	N

type testers), at least 8 single-spore isolates derived from each wild isolate were selected randomly and paired in all possible combinations. As mating-type testers could be obtained from each wild isolate, they were paired in inter-isolate combinations to determine whether the incompatibility factors of the wild isolates were identical.

Mating tests were initially performed within the isolate groups derived from the same log. Isolate groups were found to possess identical incompatibility factors within each group, except for the three wild isolates from log #8. Mating tests were then performed in all interisolate combinations among the mating-type testers from 15 representative wild isolates (Pa1, -2, -3.1, -4, -5, -6.1, -7.1, -8.1–3, -9.1, -10.1, -11, -12, and -13.1). Of the 105 interisolate combina-

tions, only combinations between tester no. 1 of Pa1 and no. 1 of Pa6.1, no. 1 of Pa4 and no. 1 of Pa10.1, and no. 2 of Pa10.1 and no. 1 of Pa11 were incompatible. On the basis of these mating reactions, mating types of two mating-type testers from each representative wild isolate were designated as shown in Table 2. Twenty-seven different *A* factors were present in the 36 wild isolates, and there was a perfect correlation between somatic incompatibility group and mating type (Table 2).

mtDNA RFLP analyses

*Eco*RI digests of mtDNA from the 36 wild isolates produced 14 distinct RFLP patterns (Fig. 3). mtDNA genotypes generally correlated with the somatic incompatibility groups and the mating types, although some isolates belonging to different somatic incompatibility and mating-type groups (Pa10.1 through 10.7, and Pa11) had the identical mtDNA genotype "L" (Table 2). Distance values calculated between all pairs of the 14 mtDNA genotypes ranged from 0.034 (between genotypes "A" and "G") to 0.926 (between "E" and "I", "J" or "M") (Table 3).

Discussion

Somatic incompatibility and mating tests, and mtDNA RFLP analysis were effective for examining genetic differences among the 36 *P. aurivella* wild isolates used in this study, and resulted a high group correlate for all three tests, although some isolates belonging to different somatic incompatibility and mating-type groups (Pa10.1 through 10.7, and Pa11) had the mtDNA of genotype "L" (Table 2). The group consisting of Pa10.1 through 10.7 and Pa11 may be derived from a common wild strain, as their mtDNA genotypes are identical and one of the *A* factors (*A22*) was common between them (Table 2). In addition, two isolate groups, one from log #1 and log #6 and the other from log #4 and log #10, had incompatibility factors *A1* and *A7* in common, respectively (Table 2). There is a possibility that the wild isolates carrying the same *A* factors are derived from common wild strains, although mtDNA genotypes differ among isolates from different logs. In addition, because the distribution of incompatibility factor *A* is apparently random in the forest, there may be no prepotent strain, and sexual matings may often occur randomly among the various strain combinations.

The 36 wild isolates could be divided into 15 distinct groups based on somatic incompatibility and mating type (see Table 2). This result implies that multiple wild *P. aurivella* genotypes coexist in a remarkably small area of the forest investigated in this study. In addition, judging from the number of mtDNA genotypes detected, the 36 wild isolates examined in this study could be derived from at least 14 distinct wild strains, assuming that mtDNA genomes remain unchanged throughout the life cycle of *P. aurivella*, as has been described in *L. edodes* (Fukuda et al. 1995; Fukuda and Fukumasa-Nakai 1996).

Fig. 3. Fourteen representative mtDNA restriction fragment patterns produced from 36 *Pholiota aurivella* wild isolates with *Eco*RI digestion. The strains associated with respective restriction fragment length polymorphism (RFLP) patterns of A to N are presented in Table 2. *Lane m* shows *Hin*dIII-digested lambda phage DNA
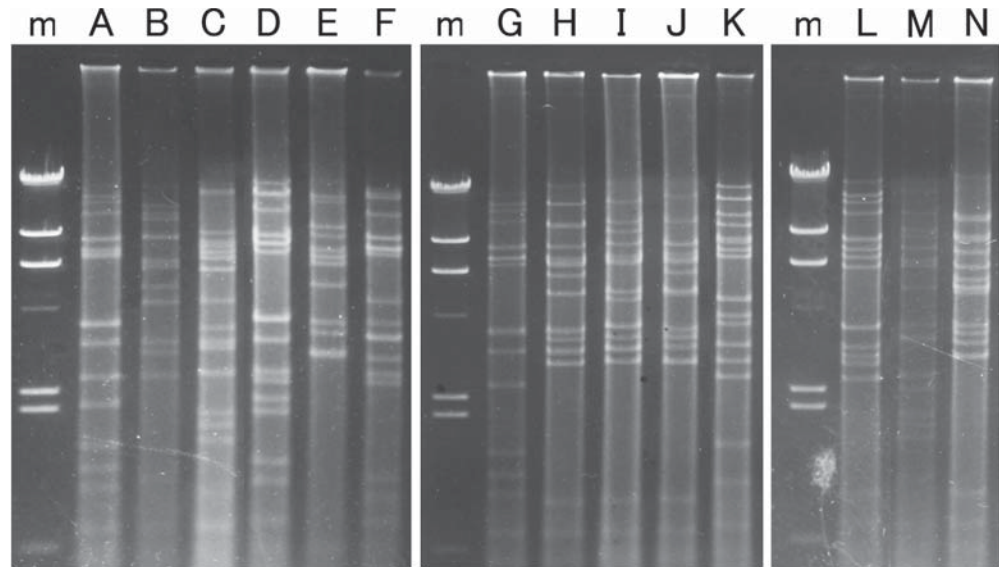

Table 3. Distance matrix based on mtDNA genotypes of *Pholiota aurivella* wild isolates

mtDNA genotype	A	B	C	D	E	F	G	H	I	J	K	L	M	N
A	–													
B	0.586	–												
C	0.576	0.750	–											
D	0.419	0.600	0.647	–										
E	0.704	0.692	0.867	0.786	–									
F	0.548	0.733	0.706	0.688	0.643	–								
G	0.034	0.571	0.563	0.400	0.692	0.533	–							
H	0.517	0.714	0.625	0.667	0.692	0.600	0.500	–						
I	0.867	0.862	0.879	0.871	0.926	0.806	0.862	0.793	–					
J	0.733	0.793	0.818	0.806	0.926	0.871	0.724	0.655	0.400	–				
K	0.355	0.667	0.647	0.438	0.857	0.750	0.333	0.600	0.806	0.742	–			
L	0.267	0.517	0.576	0.548	0.630	0.548	0.241	0.517	0.800	0.667	0.484	–		
M	0.800	0.793	0.818	0.871	0.926	0.806	0.793	0.724	0.733	0.667	0.806	0.733	–	
N	0.742	0.800	0.824	0.875	0.786	0.813	0.733	0.467	0.548	0.548	0.813	0.677	0.806	–

Distance values among the 14 mtDNA genotypes ranged from 0.034 to 0.926, a larger range than those for 38 Japanese *L. edodes* (0.026–0.300), 18 Japanese *P. ostreatus* (0.171–0.543), and 36 Japanese *P. nameko* (0.011–0.610) wild strains (Fukuda et al. 1994; Matsumoto and Fukumasa-Nakai 1995; Obatake et al. 2002). In addition, the maximum distance values among samples of *L. edodes* and *P. ostreatus* collected worldwide are 1.000 and 0.909, respectively (Fukuda et al. 1994; Matsumoto and Fukumasa-Nakai 1995). Thus, the mtDNA variation of *P. aurivella* wild isolates seems to be very high despite the small sampling area. Judging from high mtDNA variability and number of *A* factors found in the 36 wild isolates, the genetic variability in the natural population of *P. aurivella* may be quite high compared with *L. edodes*, *P. ostreatus*, and *P. nameko*.

It is possible that extensive cultivation using a specific cultivar has accidentally caused genetic erosion in the natural populations of some mushroom species, thus reducing mtDNA variation. For example, Tokimoto et al. (1973) reported random geographical distribution of two incompatibility factors of *L. edodes*, and they suggested that offspring derived from a few major commercial strains had increased within the wild populations of this fungus. Such reproduction of a specific cultivar is possibly reflected in the mtDNA genotypes of wild strains and decreases mtDNA variation. As little is known about the cultivation of *P. aurivella*, it is likely that high mtDNA variability, as demonstrated by the results in limited sampling in the Nagano forest, may be maintained in the natural population. In addition, mtDNA RFLP patterns have been used as markers to monitor cytoplasmic inheritance in mating between sexually compatible strains in *Agaricus bitorquis* (Quél.) Sacc. (Hintz et al. 1988), *L. edodes* (Fukuda et al. 1995), and *P. ostreatus* (Matsumoto and Fukumasa-Nakai 1996). The distinct mtDNA RFLP patterns of *P. aurivella* detected in this study allowed us to examine the mode of mitochondrial

inheritance in this fungus. Recombination of mtDNA in sexual crosses has been reported for a few mushrooms including *Coprinus cinereus* (Schaeff: Fr.) S.F. Gray (Economou et al. 1987) and *P. ostreatus* (Matsumoto and Fukumasa-Nakai 1996). mtDNA recombination during sexual crosses may increase mtDNA variation in *P. aurivella*, although further studies would be required to clarify the relationship between inheritance and variation of *P. aurivella* mtDNA.

Large territories of individual genets (genetically identical clones) have been reported for some root-infecting basidiomycetes (e.g., Kile 1993; Bae et al. 1994; Worrall 1994), as they can spread by vegetative growth from tree to tree. For example, the largest known territory of a single fungal genet is the 15 hectares colonized by *Armillaria bulbosa* (Barla) Romagn. (Smith et al. 1992). Basidiospore dispersal is probably the main means of spread of nonpathogenic wood-decaying basidiomycetes, and small single genet territories within a single fallen tree have been reported for *P. ostreatus* (Kay and Vilgalys 1992), *L. edodes* (Chiu et al. 1999; Fukuda and Mori 2003), and *F. velutipes* (Fukuda et al. 2000). From the results of these studies, we can estimate that the individual genets of these wood-decaying basidiomycetes do not extend much more than about 1 m in diameter. In the present study, distribution of three genetically distinct isolates from log #8 support this estimate (Fig. 1, Table 2). However, only single genets were found on each of the remaining six logs (#3, #6, #7, #9, #10, and #13), from which multiple fruiting bodies were collected. Eight isolates from log #6 were collected within about 2.73 m, and three isolates from log #9 within about 2.28 m (see Table 1). As already described, because multiple wild strains carrying different genetic compositions probably coexist within the forest, multiple genotypes would be expected to colonize a single log if basidiospores from the strains fell onto the same host log, germinated to produce monokaryons, and newly established dikaryons grew vegetatively after mating between compatible monokaryons. Although the reasons that *P. aurivella* genets colonize much larger areas than the other wood-decaying basidiomycetes are not clear, a low basidiospore germination rate may be a contributing factor, as it is in the related species *P. adiposa* (Arita and Mimura 1969). The spore germination percentage of *P. aurivella* is very low (below about 0.5%). If spore germination is also rare in nature, only a very few monokaryotic strains would grow in the host wood. In addition, vegetative growth of *P. aurivella* mono- and dikaryotic mycelia was relatively faster than other wood-decaying basidiomycetes such as *P. ostreatus*, *L. edodes*, and *F. velutipes* on MYG agar plates (data not shown). If rapid vegetative growth also occurs in nature, a single genet can be expected to expand its territory in a relatively short period. However, additional case studies are necessary to determine how single genets of *P. aurivella* establish colonial territory.

According to Imazeki and Hongo (1987), *P. aurivella* is distributed in Japan, China, Far Eastern Russia, Europe, North America, and Morocco. To fully understand the extent of genetic diversity in natural populations of *P. aurivella*, samples from the geographically distant regions must be analyzed. Such a survey could make it possible to understand how the genetic variability of *P. aurivella* is maintained in natural populations.

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