Construction of a Genetic Linkage Map Based on Amplified Fragment Length Polymorphism Markers and Development of Sequence-Tagged Site Markers for Marker-Assisted Selection of the Sporeless Trait in the Oyster Mushroom (*Pleurotus eryngii*)

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A large number of spores from fruiting bodies can lead to allergic reactions and other problems during the cultivation of edible mushrooms, including *Pleurotus eryngii* (DC.) Quél. A cultivar harboring a sporulation-deficient (sporeless) mutation would be useful for preventing these problems, but traditional breeding requires extensive time and labor. In this study, using a sporeless *P. eryngii* strain, we constructed a genetic linkage map to introduce a molecular breeding program like marker-assisted selection. Based on the segregation of 294 amplified fragment length polymorphism markers, two mating type factors, and the sporeless trait, the linkage map consisted of 11 linkage groups with a total length of 837.2 centimorgans (cM). The gene region responsible for the sporeless trait was located in linkage group IX with 32 amplified fragment length polymorphism markers and the *B* mating type factor. We also identified eight markers closely linked (within 1.2 cM) to the sporeless locus using bulked-segregant analysis-based amplified fragment length polymorphism. One such amplified fragment length polymorphism marker was converted into two sequence-tagged site markers, SD488-I and SD488-II. Using 14 wild isolates, sequence-tagged site analysis indicated the potential usefulness of the combination of two sequence-tagged site markers in cross-breeding of the sporeless strain. It also suggested that a map constructed for *P. eryngii* has adequate accuracy for marker-assisted selection.

Pleurotus eryngii (DC.) Quél, known as the king oyster mushroom, is in high demand in Asia, Europe, and North America (41). Commercial production of this species began in Italy in 1970. In Japan and China, its production yield significantly increased from the 1990s to the 2000s (41). Species in the genus *Pleurotus* are known for their flavor and relatively low-cost methods of cultivation (42). Also, *P. eryngii* is medically valuable for its immunostimulatory, antifungal, and antiproliferative actions against cells (32, 33). The fruiting bodies of this genus, including *P. eryngii*, begin to release considerable numbers of spores at a very early stage in development and continue to do so throughout maturation (43). The release and spread of a large number of spores from the developing fruiting body can cause several serious problems during cultivation; the most serious of these is the inhalation of spores by workers, which can cause respiratory allergic reactions (mushroom worker's lung). These cases are reported and recognized medically as farmer's lung, related to occupational health in the cultivation of mushrooms, including the main commercial species *Lentinula edodes* (Berk.) Pegler (25, 51), *Agaricus bisporus* (J. E. Lange) Imbach (12), *Pleurotus ostreatus* (Jacq.) P. Kumm (28, 52), *P. eryngii* (43), *Hypsizygus marmoreus* (Peck) H. E. Bigelow (46), and *Pholiota nameko* (T. Ito) S. Ito & S. Imai (14). The large number of spores released by these cultivars also gives rise to various other problems, such as damage to cultivation facilities, reduced commercial value (due to spores deposited on the mushrooms), and depletion of genetic diversity in the natural population of the mushroom species cultivated (11, 34). Accordingly, eliminating overproduction of spores by *P. eryngii* is expected to yield many benefits, financial and otherwise, for mushroom cultivation.

Sporulation-deficient (sporeless) mutants are useful for preventing these problems in mushroom cultivation. Obatake et al.

(34) obtained a dominant sporeless mutant of *P. eryngii* by UV irradiation and then bred and registered a cultivar using the sporeless mutant in Japan (<http://www.hinsyu.maff.go.jp>) (35). However, this strain requires further breeding for crop improvement. To date, spontaneous sporeless mutants have been found in *Coprinopsis cinerea* (Schaeff.) Redhead, Vilgalys & Moncalvo (6, 9), *Schizophyllum commune* Fr (4), *Lentinus edodes* (10), *Pleurotus ostreatus* (7), and *Pleurotus pulmonarius* (Fr.) Quél (36). Artificial mutagenesis using UV radiation and chemical compounds has also been carried out to generate sporeless mutants of some mushroom species, including *C. cinerea* (15, 45), *Agrocybe cylindracea* (DC.) Maire (17, 31), *P. ostreatus* (13, 39), *Pleurotus sajor-caju* (Berk and Br.) (39), *P. pulmonarius* (13), and *P. eryngii* (34). To our knowledge, sporeless strains are currently cultivated commercially for only two species of mushrooms other than *P. eryngii* (35): *A. cylindracea* (30) and *P. ostreatus* (2, 3). Although sporeless strains are very valuable as breeding materials, traditional breeding for integration of the trait requires extensive time and labor. In the case of *P. eryngii*, it takes about 3 years to breed a cultivar from the sporeless mutant (34, 35). Thus, it would be extremely beneficial to establish a molecular breeding program based on a genetic linkage map and molecular markers for the sporeless strain.

Linkage maps are powerful tools in fundamental and applied genetic research. With a saturated linkage map, it is possible to

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efficiently carry out molecular analyses such as marker-assisted selection (MAS) and quantitative trait locus mapping. Linkage maps have been constructed for various basidiomycetes, including *C. cinerea* (29), *Laccaria bicolor* (Maire) P. D. Orton (19), *L. edodes* (18, 49), *A. bisporus* (5, 8, 16), *P. ostreatus* (23), *P. pulmonarius* (37), *Amylostereum areolatum* (Chaillat ex Fr.) Boidin (50), and *Heterobasidion annosum* (Fr.) Bref (24).

MAS is often adopted to improve selection efficiency in plant and animal breeding schemes. Its practical application for mushroom breeding lags behind that for plants and animals. With mushrooms, although molecular markers have been one of the most important tools used to differentiate cultivars for the protection of breeder's rights (44, 54, 55, 58), reports on the utilization of agronomic traits for breeding are few (38, 47). The reasons include the lack of necessary materials, such as a genetic linkage map for the establishment of molecular breeding.

In this study, we aimed to establish a molecular breeding scheme for cultivar improvement using the sporeless *P. eryngii* strain described by Obatake et al. (34). We present here an amplified fragment length polymorphism (AFLP)-based genetic linkage map of *P. eryngii* and sequence-tagged site (STS) markers for the sporeless trait. They were evaluated their usefulness in sporeless breeding.

MATERIALS AND METHODS

Fungal strains and mapping population. Obatake et al. (34) reported the generation of a sporeless dominant mutant, U2553, of *P. eryngii* by UV irradiation. They bred the sporeless cultivar PE2 from U2553 and registered it in Japan (35). PE2 consists of a PE2-1 and a PE2-5 monokaryotic isolate; PE2-1 is wild type, and PE2-5 is a sporeless mutant. The sporulation phenotype and mating type were determined for 140 progeny isolated from the sporeless cultivar PE2; 83 of the progeny were used as the mapping population to determine the segregation of AFLP markers and linkage relationships.

To map the two mating type loci (arbitrarily assigned names A and B) on a linkage map, the mating type for each progeny was determined using four tester strains selected from the mapping population. Mycelial plugs from a tester progeny and unknown progeny were inoculated 2 mm apart on 2% malt extract agar medium. Four to 5 days after incubation at 25°C, mycelia from the edge of the mating colony were examined for clamp connections under a light microscope. When the pairing was compatible, clamp connections were observed.

Fruiting trial and segregation of the sporulation phenotype. Test crosses for assessing the sporulation phenotype of each member of the mapping population were performed using the basidiospore progeny 90212-3 from *P. eryngii* wild strain ATCC 90212 (Table 1). Dikaryotic isolates generated by the above-described test crosses were cultured on a basal sawdust substrate, specifically, sawdust [*Cryptomeria japonica* (L.f.) D. Don]-corn cob-wheat bran-rice bran medium (3:1:1:1 [dry weight]); the moisture content was approximately 65%, for 30 to 40 days at 23 to 25°C, with 75 to 85% relative humidity. The cultures were then treated to stimulate fructification according to the method of Obatake et al. (34) and placed in a fruiting room kept at 15 to 17°C with 90% relative humidity and continuously illuminated (200 lx) by fluorescent lamps. Sporeless fruiting bodies were readily distinguishable from those of the wild type by the absence of white spore prints on black paper set under the fruiting bodies. The absence of a confirmed spore print was defined as the phenotype of the sporeless mutation. The sporulation phenotype data for the progeny were used for linkage analysis.

AFLP techniques. Cultures for DNA extraction were grown at 25°C for 2 weeks on liquid MYG broth (2% malt extract, 2% glucose, and 0.2% yeast extract), and then the mycelia were harvested and freeze-dried. Genomic DNA was extracted from mycelia of each progeny using a

TABLE 1 *Pleurotus eryngii* wild-type isolates used in this study

Isolate	Origin
NPE010	Cultivar (Japan)
ATCC 36047	Wild strain (Czechoslovakia)
KX-EG-9	Cultivar (Japan)
Germany	Wild strain (Germany)
Nara no.1	Cultivar (Japan)
ATCC 90212	Wild strain (Greece)
ATCC 90787	Wild strain (Spain)
ATCC 90887	Wild strain (France)
ATCC 90888	Wild strain (France)
UNICORN	Cultivar (USA)
WC827	Wild strain (Russia)
514	Wild strain from Pennsylvania State University
Tottoki-1	Cultivar (Japan)
Tottoki-2	Cultivar (Japan)

DNeasy plant minikit (Qiagen, Hilden, Germany). DNA from approximately 10 mg of dried mycelia was eluted with 100 μ l of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

AFLP analysis was carried out using a modification of the procedure described by Vos et al. (53) and the instruction manual for the AFLP core reagent kit (Invitrogen Corp., Carlsbad, CA). Genomic DNA was digested with restriction enzymes (EcoRI and MseI) and ligated to adaptors provided in the AFLP core reagent kit. The reaction mixtures were diluted 10-fold with TE buffer. The digested and ligated DNA was then used for preselective amplification with the E+0 (5'-GACTGCGTACCAATTC-3') and M+0 (5'-GATGAGTCCTGAGTAA-3') primers, which correspond to EcoRI and MseI ends, respectively. PCR for preselective amplification was performed as described by Vos et al. (53). Preselective amplification mixtures were diluted 20-fold with TE buffer and then used in selective amplification. Selective amplification was performed using the E+2 and M+2 selective primers, which contained two selective bases added to the core sequences of the E+0 and M+0 primers, respectively. The 19 primer combinations used for the construction of the genetic linkage map were as follows: E+AA/M+AA, E+AT/M+GG, E+AG/M+AA, E+AC/M+CA, E+AC/M+CC, E+TT/M+AC, E+TT/M+TT, E+TG/M+GT, E+TG/M+GG, E+GA/M+GC, E+GT/M+GT, E+GT/M+GG, E+GG/M+AT, E+GG/M+TA, E+GC/M+CG, E+CA/M+CA, E+CA/M+CC, E+CC/M+AC, and E+CC/M+TT. The EcoRI primers were labeled with the fluorescent dye 6'-carboxyfluorescein to enable detection with an ABI Prism 3130 genetic analyzer (Applied Biosystems, LLC, Foster City, CA). Reaction conditions and the thermocycler program used for selective amplification were as described by Terashima et al. (48, 49). AFLP fragments were detected using the ABI Prism 3130 genetic analyzer, and their sizes were analyzed using Gene Mapper v. 4.0 software (Applied Biosystems). TaKaRa Ex Taq (TaKaRa BIO Inc., Shiga, Japan) DNA polymerase and a MyCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) were used for all PCR procedures in this study. The AFLP patterns of each isolate were compared with those of the two parental isolates (PE2-1 and PE2-5) based on the fragment sizes, and all polymorphic markers present in one parent and absent in the other were scored. AFLP markers (Fig. 1) were designated based on the selective bases in each primer combination and their size in the electropherogram by the genetic analyzer relative to the 500 ROX size standard (Applied Biosystems).

BSA-AFLP. Bulk-segregant analysis (BSA) (27) was carried out for the identification of markers closely linked to the sporeless trait. Bulks comprised 14 isolates from each of the two categories, the sporeless type and the spore-forming type (wild type), based on sporulation phenotype data from 83 isolated progeny. Equal amounts of DNA from every isolate in the bulk were mixed for the following AFLP analysis. Out of 256 combinations, 19 combinations were already used for the construction of the

(A)

(B)

FIG 1 Genetic linkage maps of *Pleurotus eryngii* based on 294 AFLP markers, two mating type factors, and the sporeless trait. Underlines represent skewed markers ($0.001 \leq P < 0.05$), and daggers represent loci with more than four markers.

genetic linkage map, so the remaining 237 primer combinations were used in BSA-AFLP analysis.

Linkage analysis. Chi-square (χ^2) tests were performed to determine if the segregation ratio of the trait was significantly different from the expected 1:1 ratio among the progeny. Linkage relationships were determined for the assigned markers using MAPMAKER v. 3.0 software (21). Putative linkage groups (LGs) were initially determined using the “group” (two-point analysis) command with a minimum likelihood of odds (LOD) linkage score of 3.0 to 4.0 and a maximum recombination fraction of 0.5. The linear order of markers in each LG was ascertained using “three-point analysis” and the “try” command repeatedly as necessary. The final marker order was checked using a recombination rate table with the “LOD table” command.

Cloning techniques and sequencing analysis. Amplified fragments that showed both specificity and polymorphism between bulks in AFLPs were extracted from polyacrylamide gels and cloned using a DynaExpress TA PCR cloning kit (BioDynamics Laboratory Inc., Tokyo, Japan) according to the manufacturer’s instructions. Plasmid DNA was prepared using a QIAprep spin miniprep kit (Qiagen). The clones were sequenced using universal primers (T7 [5′-CGCGTAATACGACTCACTATAG-3′] and SP6 [5′-CATACGATTAGGTGACACTATAG-3′]) and a BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems) on an ABI Prism 3130 genetic analyzer according to the manufacturer’s recommendations.

Design of STS primers and STS analysis. The sequencing primers were designed with Primer3 software in the GENETYX v. 9.1.1 computer package (Genetex Co., Tokyo, Japan). The standard PCR procedure was started with 94°C denaturation for 2 min, followed by 30 cycles of 94°C for 10 s, 58 to 66°C for 10 s, 72°C for 1 min, and an additional extension for 10 min at 72°C. The PCR products from the genomic DNA of the two bulks and 83 isolates of the mapping population were electrophoresed on a 2.0% agarose gel to confirm that the cloned AFLP fragments had been converted into STS markers. STS analysis for candidate markers was performed in the same manner as described above for the sporeless mutant PE2 and 14 wild-type isolates (Table 1).

RESULTS

Characterization of mapping population. Sporeless *P. eryngii* strain PE2 produces a minuscule number of spores, but we could collect the required number of spores as a mapping population by a full examination. The spores had a high germination rate (more than 90%). The mating type factors of 140 progeny isolates were confirmed via crosses with appropriate mating type testers. Both mating type factors showed the same phenotypes as those of the parents (Tables 2 and 3), which indicates that no recombination occurred between the two linked mating subloci. Each component of A-factor and B-factor (A1, A2 or B1, B2) also clearly fit a 1:1 segregation (Tables 2 and 3). This result means that the spores produced went through meiosis and are good experimental material for a mapping population for linkage analysis.

We assessed sporulation phenotype by test crosses to map the sporeless-mutation-related gene region from PE2. As a result, the sporulation phenotype fit a 1:1 segregation (Tables 2 and 3), indicating that the sporeless mutation is controlled by a single dominant gene.

TABLE 2 Segregation of sporeless isolates (PE2-5) and wild-type isolates (PE2-1) by mating type

Mating type	No. of:		
	Sporeless mutants	Wild-type isolates	Total
A1B1	0	30	30
A1B2	38	2	40
A2B1	1	34	35
A2B2	32	3	35
Total	71	69	140

TABLE 3 F₁ analysis for a cross between a sporeless isolate (PE2-5) and a wild-type isolate (PE2-1)

Analysis	Type	No. of strains	Type	No. of strains	χ^2 (1:1)	P
Segregation of isolates of type:	Sporeless	71	Wild	69	0.029	0.90–0.80
A-factor	A1	70	A2	70	<0.001	>0.99
B-factor	B1	65	B2	75	0.714	0.40–0.30
Recombination between sporeless and B-factor strains		134 (parental)		6 (recombination)	117.03	<0.01

Marker development. A total of 339 AFLP markers were detected as showing genetic polymorphism among 83 progeny isolates by AFLP analysis with 19 selective primer pairs. The number of AFLP markers for each primer pair varied between 25 (E+AC/M+CA and E+AC/M+CC combinations) and 9 (E+GG/M+TA combination), with an average of 17.8 markers per pair (Table 4). Out of the 339 markers, 152 were inherited from the wild-type parent (PE2-1) and 187 from the sporeless parent (PE2-5) (Table 4). In the segregation analysis using χ^2 tests for the 339 AFLP markers, distortion was evident for 42 (12.4%; $0.001 \leq P < 0.05$) and significant distortion was evident for 27 (8.0%; $P < 0.001$) of the markers. Of the 42 distorted markers ($0.001 \leq P < 0.05$), 17 were from the wild-type parent and 25 from the sporeless parent, and of the 27 significantly distorted markers ($P < 0.001$), 12 were from the wild-type parent and 15 from the sporeless type parent. The 27 AFLP markers that showed significant distortion ($P < 0.001$) were not used for further analysis.

Linkage analysis and mapping. A linkage map of *P. eryngii* was constructed using MAPMAKER software v. 3.0 based on the sporeless trait, two mating type factors, and 312 AFLP markers. Of the 312 AFLP markers, the location of 18 could not be determined on the genetic linkage map (including six skewed markers [$0.001 \leq P < 0.05$]), so these were discarded. The remaining 294 AFLP markers, the sporeless trait, and two mating type factors were assigned to 11 LGs (LG I to LG XI) (Fig. 1; Table 5). The

genetic linkage map consisted of a total length of 837.2 centimorgans (cM), with an average marker interval of 6.17 cM. As shown in Table 5, the LG length varied from a minimum of 25.2 cM (LG XI) to a maximum of 116.8 cM (LG I), with an average of 76.1 cM per group. For each group, 19 (LG VII) to 41 (LG III) markers were mapped, with an average of 27 markers per group. The average marker interval ranged from 2.8 cM (LG XI) to 8.98 cM (LG I). The two mating type factors (A and B) were located on LG I and LG IX, respectively (Fig. 1). Some skewed markers were concentrated on specific regions of the LGs (6 markers on LG V, 9 markers on LG VI, 13 markers on LG VIII) (Fig. 1).

Localization of the gene region for the sporeless mutation and identification of linked AFLP markers by BSA-AFLP analysis. The gene region containing the sporeless mutation from PE2 was mapped based on the segregation of the sporeless phenotype in the mapping population. The sporeless region was found on LG IX, which was 56.1 cM in size, with 32 AFLP markers and B-factor (Fig. 1; Table 5). The map distance between the sporeless mutation locus and the B-factor locus was 1.2 cM.

BSA-AFLP analysis was carried out using 237 selective primer combinations and two bulked DNA sequences as PCR templates, composed of each of 14 isolates with a sporeless mutation trait or a wild-type trait. To obtain bands associated with the sporeless trait, we focused on those unique to either the sporeless-type pools or wild-type pools. Polymorphisms of screened fragments were

TABLE 4 Distribution of AFLP markers in parental sporeless mutant and wild-type *Pleurotus eryngii* isolates

Primer pair		No. of markers		Total no. of markers	No. of skewed markers ($0.001 \leq P < 0.05$)	No. of significantly skewed markers ($P < 0.001$)
EcoRI	MseI	Sporeless specific	Wild-type specific			
AG	AA	9	13	22	3	5
AC	CA	14	11	25	4	1
AC	CC	13	12	25	7	0
TT	AC	15	7	22	0	2
TT	TT	5	9	14	1	0
TG	GT	9	8	17	0	1
TG	GG	12	11	23	2	1
GA	GC	12	8	20	3	0
GT	GT	5	5	10	0	4
GT	GG	8	8	16	1	1
GG	AT	6	8	14	2	1
GG	TA	7	2	9	1	1
GC	CG	9	7	16	6	1
CA	CA	9	8	17	0	5
CA	CC	8	9	17	1	1
CC	AC	14	7	21	4	0
CC	TT	8	6	14	1	1
AA	AA	12	9	21	5	2
AT	GG	12	4	16	1	0
Total		187	152	339	42	27

TABLE 5 Characteristics of genetic linkage groups with AFLP markers, mating type factors, and the sporeless trait in *Pleurotus eryngii*

Linkage group	Length (cM)	No. of markers	Avg marker interval (cM)	No. of skewed markers (0.001 ≤ <i>P</i> < 0.05)
LG I	116.8	28	8.98	0
LG II	107.7	28	6.34	0
LG III	93.5	41	4.92	2
LG IV	90.1	28	5.30	4
LG V	80.7	21	8.07	6
LG VI	79.1	21	8.79	9
LG VII	70.6	19	5.88	0
LG VIII	63.3	21	6.33	13
LG IX	56.1	34	5.10	0
LG X	54.1	22	5.41	0
LG XI	25.2	34	2.80	2
Avg ^a	76.1	27	6.17	
Total	837.2	297		36

^a The average marker interval did not contain the same location markers.

identified among the 83 progeny isolates using AFLP analysis with each selective primer pair. We performed BSA-AFLP analysis in an attempt to obtain linked markers closer to the sporeless region than *B*-factor; however, we were unable to find closer markers, including cosegregating markers. Linkage analysis for the screening showed a total of eight BSA-AFLP markers linked to the sporeless region at a distance of 1.2 cM, which is the same locus as that of *B*-factor on LG IX (Fig. 2). Of them, three (ATCA280, TGAA326, and CGCG488) BSA-AFLP markers were from the sporeless mutation type and five (ATCG170, TACT419, CAAG386, CTAC175, and CGCG491) were from the wild type.

Development and evaluation of STS markers. Based on the cloned sequences of eight BSA-AFLP markers, corresponding STS primer pairs for each clone were designed (data not shown). Can-

didate STS primer pairs for detection of the sporeless trait were screened by PCR using template DNA from 83 progeny isolates and their parents, PE2-1 and PE2-5. Of them, seven primer pairs based on the sequences of seven markers (ATCA280, ATCG170, TACT419, CAAG386, CTAC175, CGCG488, and CGCG491) successfully amplified PCR fragments of the expected size and showed amplification corresponding to the sporulation phenotype of each isolate in the mapping population (data not shown). The seven candidate STS markers were evaluated for potential value in practical cross-breeding using various strains. STS analysis was performed using genomic DNA from 14 wild dikaryotic isolates (Table 1). All candidate markers were shared by many wild-type strains, suggesting that they are unsuitable for detection of the sporeless trait. Therefore, we designed two primer pairs, SD488-I and SD488-II, as STS markers using the sequence of CGCG488. The sequences of SD488-I and SD488-II were as follows: for SD488-I, 5'-CAAGATGAAGAGGACGGATTG-3' (forward) and 5'-ACGGACTTCAGCGACGAGCTCAATT-3' (reverse); for SD488-II, 5'-CGGCCATACACTTGCTCGTA-3' (forward) and 5'-ACTTCAGCGACGAGCTCAAT-3' (reverse). Using SD488-I and SD488-II, the efficiency of detection of the sporulation phenotype was 98.8% in the mapping population. STS analysis for these two primer pairs showed some negative amplification against some wild-type isolates; however, the combination of these could distinguish the sporulation phenotype of each isolate (Fig. 3). Furthermore, multiplex PCR using SD488-I and SD488-II showed results similar to those from their single use (Fig. 3).

DISCUSSION

We constructed an AFLP-based genetic linkage map of *P. eryngii* based on the sporeless trait, two mating type factors, and 294 AFLP markers. These values are similar to those of other *Pleurotus* species, *P. ostreatus* (23) and *P. pulmonarius* (37). Larraya et al.

FIG 2 Locus of the sporeless mutation and closely linked markers. (Left) map constructed by AFLP analysis; (right) map constructed by BSA-AFLP analysis. Daggers represent loci with more than four markers. Bold indicates markers selected as candidate STS markers. The description of each AFLP marker is given in Materials and Methods.

FIG 3 (A) Validation of STS markers SD488-I and SD488-II; (B) validation of both STS markers by multiplex PCR. The numbers from 1 through 17 represent, respectively, PE2-1, PE2-5, PE2, NPE010, ATCC 36047, KX-EG-9, Germany, Nara no. 1, ATCC 90212, ATCC 90787, ATCC 90887, ATCC 90888, UNICORN, WC827, 514, Tottoki-1, and Tottoki-2. These strains are listed in Table 1. M, 100-bp molecular ladder.

(23) reported 11 LGs covering a total length of 1,000.7 cM, with an average marker resolution of 5.3 cM for *P. ostreatus*; Okuda et al. (37) reported 12 LGs covering 971.1 cM, with an average resolution of 5.2 cM for *P. pulmonarius*. We therefore assess this map as having a medium marker density able to provide useful genetic data for basic and applied studies like gene isolation, mapping, and MAS for valuable breeding traits. Based on pulsed-field gel electrophoresis, *P. ostreatus* has 11 chromosomes (22). Interestingly, we identified the same number of LGs in *P. eryngii*. To investigate the phylogenetic relationship between *Pleurotus* species using genetic linkage maps and genome sequences, it will be necessary to accurately determine the number of chromosomes in *P. eryngii* by an analysis such as pulsed-field gel electrophoresis.

In this study, 339 AFLP markers were generated by AFLP analysis with 19 selective primer pairs, yielding 17.8 AFLP markers generated per selective primer combination. This efficiency is similar to the value obtained by AFLP analysis in the congeneric species *P. pulmonarius* (18.0 per pair) (37). Many of the generated AFLP markers showed the expected 1:1 segregation pattern ($P \geq 0.05$) in segregation analysis. However, 20.4% (69/339) of the AFLP markers had skewed segregation ratios ($P < 0.05$). Compared with percentages for other mushroom species, namely, *C. cinerea* (6.4%) (29), *L. edodes* (20.7%) (49), *A. bisporus* (32.8%) (16), *P. ostreatus* (14.0%) (23), and *P. pulmonarius* (11.1%) (37), this percentage is at a medium level. This result suggests that selection of the mapping population was random. Of the 69 skewed markers, 29 originated from the wild-type parent (PE2-1) and 40 from the sporeless parent (PE2-5). This result fits a 1:1 segregation, suggesting that the mutation does not affect segregation and that the sporeless locus is not linked to unfavorable traits related to survival.

We observed the clustered AFLP markers within several linkage groups. AFLP marker clustering has been referred to in the reports of various species (e.g., *Arabidopsis* [1] and oyster [56]), including a basidiomycete (*Heterobasidion annosum* [24]). Also, clustering has been found to be around the centromere regions (1, 56). Although the relationship between the location of marker clustering and the centromere in *P. eryngii* is still unclear, the implementation of genetic and cytogenetic studies of *P. eryngii* might reveal this phenomenon.

The sporulation phenotype of the sporeless and wild-type

traits segregated at the expected 1:1 ratio in a test cross. This suggests that the sporeless mutation is controlled by a single dominant gene, consistent with a previous report (34). Based on linkage analysis, the gene region for the sporeless mutation is located on LG IX with 32 AFLP markers and *B*-factor. A set of eight AFLP markers at 1.2 cM of the sporeless locus was identified by BSA-AFLP analysis. BSA is an efficient and effective method for identifying markers linked to a specific gene or genomic region (27). Although BSA is widely applied in plants, especially for disease resistance (20, 27, 57), its application to mushrooms is less well known; the identification of markers linked with mushroom weight in *A. bisporus* (40), mating type factors in *L. edodes* (18), and the sporeless trait in *P. pulmonarius* (38) has been reported. This method will be a useful tool for finding markers closely linked to important agronomic traits in mushroom breeding.

Fast selection for the sporeless trait has been limited due to the necessity of the presence of mature fruiting bodies, because the cultivation of *P. eryngii* usually requires at least 1 month to harvest the mature fruiting bodies. The ability for fruiting body formation differs between strains generated by cross-breeding, resulting in difficulty in cultivation for a definite period. Thus, selection of monokaryotic isolates from basidiospores based on their molecular profile is desired in the breeding of sporeless strains of *P. eryngii*. Based on the constructed genetic linkage map, we developed two STS markers, SD488-I and SD488-II, for the identification of the sporeless phenotype. These STS markers were able to distinguish sporeless strains from the wild type by a simple PCR method. Use of SD488-I and SD488-II led to 98.8% detection efficiency for the sporulation phenotype of isolates in the mapping population. This result suggests that the genetic linkage map of *P. eryngii* has high accuracy for molecular breeding schemes. To evaluate its potential use for practical cross-breeding of sporeless strains, STS analyses were carried out using 14 wild-type strains. Although each STS marker had some negative results, the combination of these markers could successfully distinguish the sporulation phenotype. Multiplex PCR using this primer combination showed amplification corresponding to sporulation phenotype. These results show that the two STS markers can be used for efficient MAS, enabling a reduction in labor and time-consuming processes used in cultivation, such as test crosses in large-scale screening for sporeless *P. eryngii* breeding.

MAS using strain-specific markers has frequently been studied in edible mushrooms (44, 54, 55, 58), but there have been few reports of MAS for agronomic traits in mushroom breeding (38, 47). Here we established MAS based on a genetic linkage map for the dominant sporeless trait in *P. eryngii*. The STS markers developed facilitate breeding of sporeless strains, and these marker sequences can be used as probes to enable the unprecedented isolation of the sporeless-mutation-related gene region in edible mushrooms by map-based cloning. The establishment of a genetic foundation, such as this genetic linkage map and a molecular breeding method, will support expanding its application to the commercially valuable mushroom *P. eryngii*.

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