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Construction of a genetic linkage map and analysis of quantitative trait loci associated with the agronomically important traits of *Pleurotus eryngii*

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ABSTRACT

Breeding new strains with improved traits is a long-standing goal of mushroom breeders that can be expedited by marker-assisted selection (MAS). We constructed a genetic linkage map of *Pleurotus eryngii* based on segregation analysis of markers in postmeiotic monokaryons from KNR2312. In total, 256 loci comprising 226 simple sequence-repeat (SSR) markers, 2 mating-type factors, and 28 insertion/deletion (InDel) markers were mapped. The map consisted of 12 linkage groups (LGs) spanning 1047.8 cM, with an average interval length of 4.09 cM. Four independent populations (Pd3, Pd8, Pd14, and Pd15) derived from crossing between four monokaryons from KNR2532 as a tester strain and 98 monokaryons from KNR2312 were used to characterize quantitative trait loci (QTL) for nine traits such as yield, quality, cap color, and earliness. Using composite interval mapping (CIM), 71 QTLs explaining between 5.82% and 33.17% of the phenotypic variations were identified. Clusters of more than five QTLs for various traits were identified in three genomic regions, on LGs 1, 7 and 9. Regardless of the population, 6 of the 9 traits studied and 18 of the 71 QTLs found in this study were identified in the largest cluster, LG1, in the range from 65.4 to 110.4 cM. The candidate genes for yield encoding transcription factor, signal transduction, mycelial growth and hydrolase are suggested by using manual and computational analysis of genome sequence corresponding to QTL region with the highest likelihood odds (LOD) for yield. The genetic map and the QTLs established in this study will help breeders and geneticists to develop selection markers for agronomically important characteristics of mushrooms and to identify the corresponding genes.

1. Introduction

Pleurotus eryngii, an edible white-rot fungus, is widespread in Eurasia and northern Africa (Miles and Chang, 2004). It has become a major cultivated mushroom in Asia, with a current global production rate of approximately 3×10^5 metric tons/yr (Im et al., 2013). *P. eryngii* has a thick meaty white stem with good flavor and nutritional factors (Kim et al., 2014); it has potential medicinal uses due to its content of various biologically active compounds that exert antihypertensive, antioxidant, antihypercholesterolemic, antihyperglycemic, immunomodulating, and antitumor effects (Carbonero et al., 2006; Smith et al., 2002; Stajic et al., 2009). With the increase in demand for *P. eryngii*, customized production to improve yield, disease resistance, and quality has become major areas of focus. Using conventional breeding, the development of new cultivars with multiple superior traits is laborious and time-consuming because many of the economically important production traits, such as yield, quality, and disease resistance are controlled by polygenic inheritance

Abbreviations: AFLP, amplified fragment length polymorphism; CIM, composite interval mapping; InDel, insertion/deletion; LG, linkage group; LOD, logarithm of the odds; LR, Likelihood Ratio Test statistic; MAS, marker-assisted selection; Pm1, population of monokaryons 1; SNP, single nucleotide polymorphism; RAPD, random amplified polymorphic DNA; SSR, simple sequence-repeat; QTL, quantitative trait locus.

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(Chakravarty, 2011; Kim et al., 2011; Tester and Langridge, 2010). As an alternative method of locating the genomic regions associated with those quantitative traits, QTL mapping that couples genetic linkage maps and phenotypes has been attempted. MAS improves the accuracy and efficiency of the selection steps because the selection of progeny carrying the favorable traits of interest is performed using genotypic markers rather than phenotypes (Collard et al., 2005; Collard and Mackill, 2008). It is also possible to use these markers to screen multiple phenotypes in diverse environments (Bernardo, 2008). The application of genetic mapping and QTL analysis is a more recent development in mushroom species than in animal or plant species, and little is known about the potential of applying MAS in mushroom breeding (Ramírez et al., 2010; Sonnenberg et al., 2005). Genetic linkage maps have been constructed for several mushroom species, including *Agaricus bisporus*, *Pleurotus ostreatus*, and *Lentinus edodes*, using markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), single nucleotide polymorphisms (SNPs) and microsatellite markers (Foulongne-Oriol et al., 2010; Larraya et al., 2000; Okuda et al., 2009; Terashima et al., 2002). In the button mushroom *A. bisporus*, QTLs conferring yield, cap color and pathogen resistance have been reported (Foulongne-Oriol et al., 2012a, 2012b; Loftus et al., 2000; Moquet et al., 1999). Likewise, QTLs that control the growth rate of the mycelium, biomass production, and several production-related traits of *P. ostreatus* were described previously (Larraya et al., 2003, 2002). The first genetic linkage map of *P. eryngii* was constructed for the QTL analysis of sporulation-deficient mutants using AFLP markers (Okuda et al., 2012), but little is known about the genetic control of many other important agronomic traits such as yield, quality, cap color, earliness, and length. In this study, we constructed a new genetic linkage map for *P. eryngii* using mating-type factors, SSRs, and InDel markers. We used this map to identify markers that are closely associated with agronomically important traits. This work provides a foundation for the future development of molecular markers that may be useful for trait improvement in *P. eryngii* via MAS.

2. Materials and methods

2.1. Fungal strains and population development

All of the *P. eryngii* strains used in this study were obtained from the Gyeongnam Agricultural Research and Extension Services (GNARES) Korea. The KNR2312 and KNR2532 were used as the parental strains because of their differences in phenotypes and genetically distance in the *A* and *B* mating-type loci proved compatibility in all of the possible mating combinations between monokaryons from the both strains. A 98 haploid meiotic monokaryons (Pm1; population of monokaryons 1) from the KNR2312 was used for the construction of a genetic linkage map. Four second-generation hybrid populations (Pd3, Pd8, Pd14 and Pd15) were obtained by crossing the Pm1 with four monokaryotic testers (KNR2532-3, KNR2532-8, KNR2532-14 and KNR2532-15) from KNR2532 for fruiting body evaluation (Fig. S1). The crossing between two protoclones and testers was conducted for comparison of their genetic traits. Mycelial cultures of the Pm1 were grown on mushroom complete medium at 25 °C in the dark and crossed with the above monokaryotic testers as described in the previous report (Ryu et al., 2012b). For screening primers for polymorphism, two parental protoclones, P5 and P6, which were derived from KNR2312 using the protoplast homogenization method (Ryu et al., 2012b), were used.

2.2. SSR, InDel, and mating-type identification and marker development

A previously reported [http://112.220.192.2/per/, (Ryu et al., 2012a)] genomic sequence draft for P5 (previous version) was used as a source for the identification of SSRs, and the resequencing traces of P6 were used for finding InDels. Candidate SSR loci were identified using SSR Locator I (Maia et al., 2008) with the following conditions: the sequences at least contained monomers (×20), 2-mers (×10), 3-mers (×7), 4-mers (×5), 5-mers (×4), 6-mers (×4), or 7-mers (×4). Primers were designed using the Primer3 algorithms (Rozen and Skaletsky, 2000) with the following conditions: an optimal primer length of 20 nt (18–26 nt), an optimal melting temperature of 50 °C (45–55 °C), an optimal product size of 125 bp (100–350 bp), and an optimal G/C content of 50% (45–55%). To improve the resolution of the major QTL regions (LG6 and LG9) and the ends of the putatively fragmented LGs (LG1, LG8, LG13, and LG14), InDel loci were manually identified from the genomic sequences of P5 and P6 corresponding to the above LGs, and then 53 primer sets that flanked the InDels were designed. InDel loci with minimum 10 nt length of the insertions and deletions were selected to allow easy discrimination of polymorphisms using agarose gel (Table S1). The mating type of Pm1 individuals was identified in accordance with a previous report (Ryu et al., 2012b).

2.3. Genomic DNA isolation and PCR reaction

Mycelium was prepared, and genomic DNA was isolated as described previously (Ryu et al., 2012b). The polymorphic primers screened by preliminarily PCR using KNR2312, P5 and P6 were then used to genotype Pm1 individuals. PCR was performed in 96-well plates using a DNA Engine Dyad Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA) under the following conditions: denaturation at 95 °C for 2 min; followed by 35 cycles each of 95 °C for 30 s, 52 °C for 40 s, and 72 °C for 30 s; and a final extension at 72 °C for 5 min. The total volume of the PCR reaction mixture was 10 μl, which contained 15 ng of genomic DNA, 0.2 mM dNTPs, 0.25 U of *e-Taq* DNA polymerase (SolGent, Korea), 1× buffer containing 2.5 mM Tris-HCl (pH 8.2) and 1.5 mM $MgCl_2$, and 0.25 pM of each primer. Amplified PCR products were electrophoresed in a 3% agarose gel containing safeview classic (iNtRON Biotechnology, Korea). Polymorphism (dominant or codominant) of SSR and InDel was scored on a presence, absence, or size difference basis.

2.4. Linkage map construction

All of the informative SSR, InDel markers, and mating types were used to construct a genetic linkage map. Each marker was individually scored for the parental and mapping populations. The marker genotype data were compiled in a single Excel file. Linkage analysis of the markers, estimation of the recombination frequencies, and the determination of the linear order of the loci were performed using JoinMap 4.0 (Van Ooijen, 2006). Segregation patterns were assigned to each marker following "HAP" data entry notation (<a>, <b>). The markers showing deviated segregation ($P < 0.05$) from the expected Mendelian ratio of 1:1 for a haploid meiotic population were determined using χ^2 tests and are represented by asterisks on the map. Markers were placed into LGs using the "logarithm of the odds (LOD) groupings" and "create groups for mapping" commands with the Kosambi map function (Kosambi, 1943). The calculation parameters were set for a minimum LOD score of 4.0 (except LG9 [LOD = 3.0]) and a

recombination fraction of 0.45. The order of the markers in the groups was established using the "Calculate Map" command. The recombination frequencies were converted to genetic distances in centiMorgans (cM) using the Kosambi mapping function. MapChart for Windows (Voorrips, 2002) was used to draw the linkage maps.

2.5. Fruiting condition

Four dikaryotic populations (Pd3, Pd8, Pd14 and Pd15) were used to perform a fruiting test for phenotypic scoring. They were all fruited during the same period in the four different rooms of the mushroom culturing facility at the Gyeongsangnam-do Agricultural Research and Extension Services (Jinju, South Korea), as described previously (Ryu et al., 2012b) with one minor modification. The medium was prepared in an 850-ml PP (polypropylene) bottle by autoclaving 590 g of medium (with 70% *Populus* sp. sawdust, 15% wheat bran, and 15% rice bran in the formula, v/v) with 67% moisture at 121 °C for 100 min. The cooled substrate was inoculated with 3–4 pieces of 1 × 1 cm MCM-agar covered with mycelia and was then incubated for 35 days in a dark incubation room that was maintained at 20 °C with 65% relative moisture. The spawn-running period was estimated by determining the time that elapsed between the time of inoculation and the time that the all substrate was completely colonized during the incubation. An incomplete colonized bottle by 35th day was scored 36 days. At the 35th day of the spawn running period, the outer area of the substrate was removed by scraping to induce the formation of primordia, and the cultures were then placed in a room that was maintained at 15 °C with 95% relative humidity and cool-white fluorescence light (200 lx). When the fruiting bodies reached 1.5–2.0 cm in height, all of the fruiting bodies and primordia except the best one were removed (thinning) to evaluate the fruiting characteristics more clearly (which is a usual practice at mushroom farms). Thinning was performed twice, if necessary. Three repetitions (fruiting body) of 98 genotypes in each dikaryotic population were performed in phenotyping.

2.6. Phenotypic evaluation

Three growing units (bottles) were used to determine every trait. The period of pin-heading (the number of days from the removal of the old medium to the appearance of primordia) and earliness (the number of days from the removal of the old medium to harvesting) was measured. Other traits related to fruiting body were evaluated for three mushrooms from three growing units (one fruiting body was grown per bottle by thinning). The mean phenotypic values of genotypes were used for QTL mapping. Fruiting bodies were harvested before the pileus had opened completely (the ratio of pileus diameter and stipe thickness is about 1.3–1.7 depending on the shape of fruiting body) to determine yield, the diameter of the pileus, the thickness and length of the stipe using calipers (Mitutoyo, Tokyo, Japan) and a scale (HF400, Tokyo, Japan). The quality standards were comprehensively evaluated using a 9-point measurement scale (Ryu et al., 2007). Mostly the quality was correlated to weight of fruiting body. Thus, we employed the equation, "quality = 0.0895 × weight + 0.2523" with an adjustment point depending on the mushroom's shape and pileus color. The color of the surface of the pileus was determined at three points of a middle distance from center to the margin per pileus using a colorimeter (Minolta CR 310 Chroma Meter) based on the L (lightness), a (redness), and b (yellowness) system. The statistical analyses were performed by transforming the lightness values according to the logit scale, as recommended by Moquet et al. (1999), using the following equation: $\text{logit}L = \log[L/(100 - L)]$.

2.7. Statistical analyses

The data for each quantitative trait were subjected to a normality test (Kolmogorov-Smirnov) and drawing histograms for trait distribution. The data for each trait that were obtained in each population were analyzed according to a completely randomized design, as follows: $x_{ij} = \mu + \tau_i + \varepsilon_{ij}$, where μ is the mean value, τ is the treatment effect, and ε is the residual effect (Liu, 1997). The effects of the genotypes on the traits were determined by performing a one-way analysis of variance (ANOVA). The broad sense heritabilities (h^2) based on the mean genotypic values were estimated as follows: $h^2 = \sigma_G{}^2/\sigma_P{}^2$ where $\sigma_G{}^2$ is the genetic variance, $\sigma_P{}^2$ is the phenotypic variance. The correlation coefficients for pairs of traits were determined using Pearson's procedure. The data analyses were performed using the R open-source software (Team, 2013).

2.8. QTL analysis

QTL analysis was performed using the CIM program (Jansen and Stam, 1994; Zeng, 1994) of the QTL Cartographer software, version 2.5 (Wang et al., 2007). The five most significant cofactors identified with forward and backward regression were added as cofactors in the CIM step (model 6 using a 10-cM window size, 5 control markers, and a 1-cM step size). The LOD thresholds for discerning significance were calculated by a 1000-permutation test at the $P = 0.05$ and $P = 0.01$ levels using an experiment-wise error (α) of 0.05. The QTLs that were above the threshold level were considered significant, and the Likelihood Ratio Test statistic (LR) is expressed as a LOD score (LOD = 0.2171 LR). The confidence interval (CI) was calculated by the points on the genetic map that corresponds to a decrease of the LOD score of 1 unit from the highest peak. Approximately position a QTL was taken as a maximum LOD peak. The percent of phenotypic variance and their individual additive effects explained by a single QTL were calculated as an R^2 value at highest probability peaks. The R^2t is the total effect estimated by ANOVA based on the model: Y = μ + M1 + M2 + M3 + error where Y is trait value, μ is the mean of the trait, M1, M2, M3 the effect of the markers M1, M2, M3 closely linked to QTL1, QTL2 QTL3 respectively. The QTLs were named as follows: *(Y/L/Q/T/D/E/P/S/C)(3/8/14/15)x*, where *Y* is the yield, *L* is the length, *Q* is the quality, *T* is the stipe thickness, *D* is the pileus diameter, *E* is the earliness, *P* is the period of pin-heading, *S* is the period of spawn running, *C* is the cap color, *3/8/14/15* are the population numbers, and *x* is a consecutive QTL number.

2.9. Gene prediction and determination of gene function

A draft genomic sequence of P5 (Ryu et al., 2012a) was used to predict the candidate genes that are responsible for the yield. A sequence of approximately 1100 kb (from 83.4 to 94.5 cM on LG1) that corresponded to the sequence between SSR239 and SSR311 (the markers adjacent to the peak, Y3_I of LG1, with the highest LOD and R^2 score) was obtained using a *P. eryngii* genome browser (http://112.220.192.2/per/) and analyzed using the FGENESH (http://www.softberry.com/), UniProt (http://www.uniprot.org/), EMBL-EBI (http://www.ebi.ac.uk/) and PROSITE (http://www.expasy.ch/sprot/prosite.html) programs to predict the genes present and their functions. Among the predicted genes, expression regulator, mycelial growth rate, cell wall synthesis and hydrolysis genes were screened as a candidate yield-related gene.

3. Results

3.1. Marker development

Based on the genomic DNA sequence of P5, 523 SSR markers were identified, with trinucleotides (195 SSRs; 37%) and hexanucleotides (110 SSRs, 21%) as the most frequently observed SSRs (Table S2). Of the 484 primers that were designed, 441 (91%) primer sets amplified PCR fragments of the expected sizes. Finally 285 primer sets revealed polymorphism, with 174 (39%) being codominant and 111 (25%) being dominant markers in the preliminary test that was performed using the parental lines (P5 and P6). Of the 285 primer sets, 231 sets with distinct polymorphism were segregated in 98 progenies of the mapping population (Pm1). Additionally 53 InDel markers from major QTL region and putatively fragmented LGs were made and finally 28 polymorphic InDel markers were used for mapping. The order of the genetic distance and the physical location of some primers did not show consistency (Table S1).

3.2. Genetic map construction

A total of 256 markers were successfully assigned to the LGs. Five SSRs remained unlinked. Segregation analysis was performed using the χ^2 test, in which segregation distortion was determined for 23 (8.9%; $0.001 \leqslant P < 0.05$) markers, and significant distortion was determined for 6 (2.0%; $P < 0.001$) markers (Fig. 1). The markers were positioned on 14 LGs with LOD thresholds of 3.0 (only for LG9) to 4.0, with a maximum recombination fraction of 0.4. Because the number of LGs constructed during the initial mapping project was not consistent with the number of chromosomes (n = 11 or 12) of *P. eryngii* (Lee et al., 2009; Ryu et al., 2012a; Slézec, 1984) the putatively fragmented LGs, which could be assumed due to the changed integrity of the different LOD values, were joined by improving the resolution of their termini on the map using the InDel markers. Ultimately, LG1 and LG8 were joined with LG13 and LG14, respectively. Moreover, the resolution of the major QTLs on LG6 and LG9 was improved by the addition of InDel markers, particularly on the regions containing the major QTLs (Fig. 1). The map contained markers with distances ranging from 55.5 cM to 130 cM for LG12 and LG1, respectively (Table 1). The map spanned a total length of 1047.9 cM, with an average interval of 4.09 cM between two adjacent markers. The average ratio of the physical distance to the genetic distance was estimated 41.8 kb/cM based on the previously reported size of the genome (43.8 Mbp) of *P. eryngii* (Lee et al., 2009). The total number of markers in each LG varied from 7 to 38. The mean size of the LGs was 87.3 cM and contained 21.3 loci. Mating-type locus *A* was mapped to LG3, whereas mating-type locus *B* was mapped to LG9.

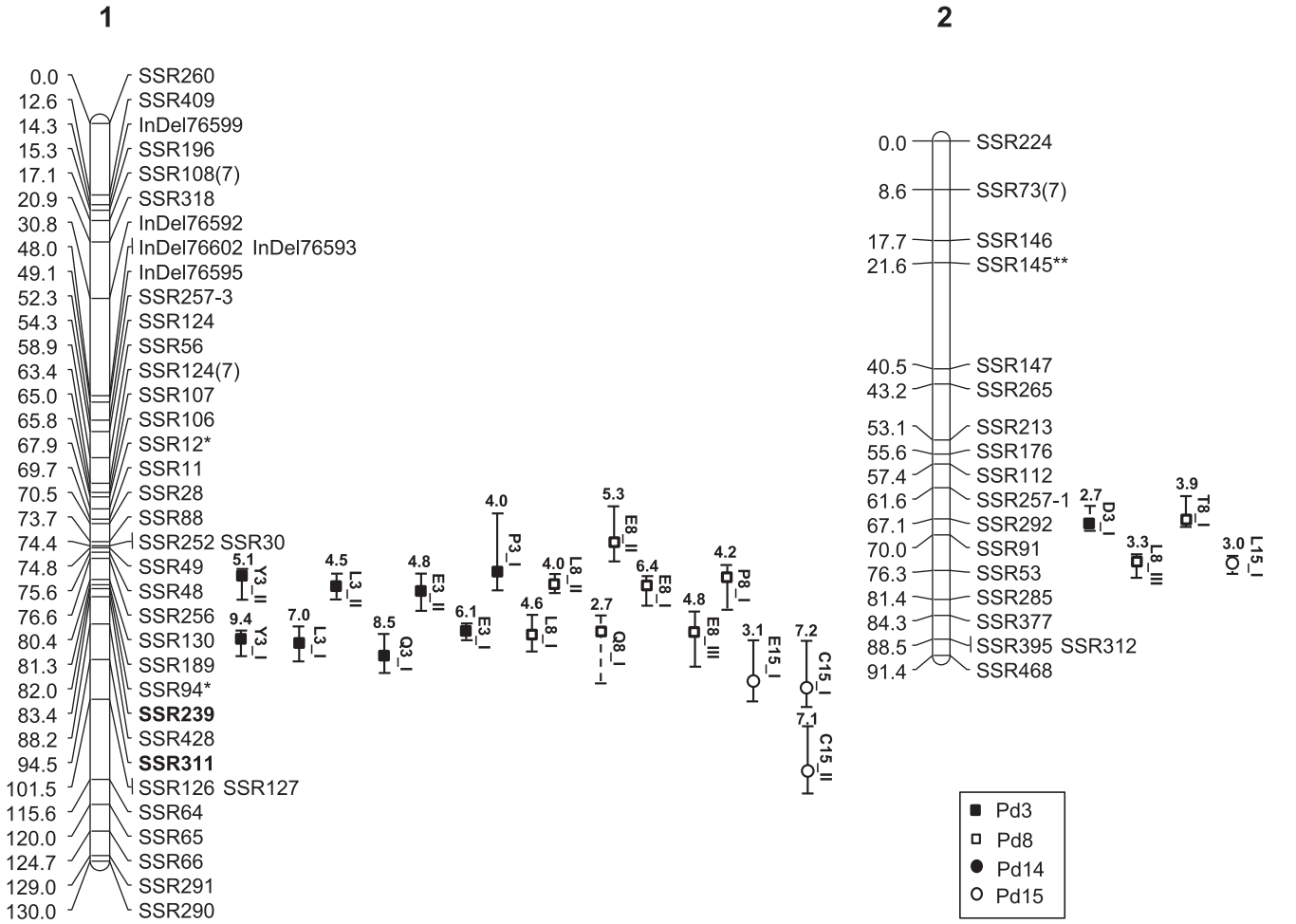

Fig. 1. Location of QTLs responsible for fruiting body traits on the *P. eryngii* linkage map. The genetic distance of markers in cM (Kosambi units) is indicated on the left side. The QTL nomenclature is described in Section 2. The markers that deviated from the expected segregation ratio (1:1) are denoted with various asterisks (∗, $P < 0.05$; ∗∗, $P < 0.01$; and ∗∗∗, $P < 0.001$) to the right of the name of the marker. The positions of the maximum LOD values are indicated in a black square (Pd3), a white square (Pd8), a black circle (Pd14) or a white circle (Pd15). 1-LOD decreases on either side of the LOD peak (>3.0) are represented by solid lines, and QTLs with $2.5 \leqslant$ LOD scores < 3.0 are represented by dashed lines. The genome region near Y3_I QTL on LG1 (from SSR239 to SSR311; shown in bold) was used to gene prediction.

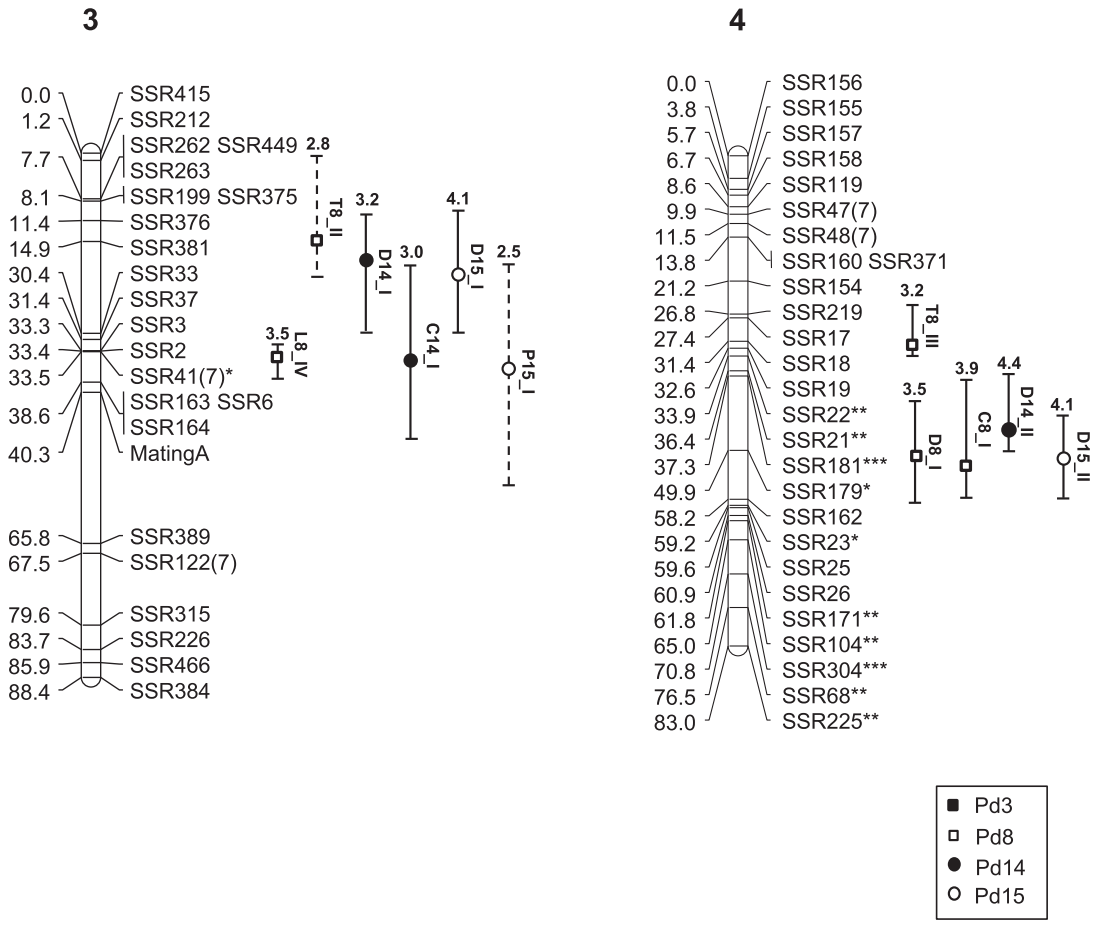

Fig. 1 (*continued*)

3.3. Trait analysis

The phenotypic performance of the parental strains (KNR2312 and KNR2532) such as their yield and their morphology-related traits, with their variation ranges and coefficient of variations (CV) is presented in Table S3. The parent strains showed significant differences in phenotypes. KNR2312 had better characteristics with respect to yield, quality, and the length and diameter of the stipe than did KNR2532. However, KNR2532 had a better earliness than KNR2312. The phenotypic data of dikaryons mated between two protoclones and testers are shown in Table S4. Fruiting bodies of KNR2532-3 × KNR2312P5 and -P6 were not matured after pin-heading. The hybrids shared P6 showed better traits in weight, quality, stipe thickness and pileus diameter, while P5 shared hybrids showed better in period of spawn running, harvest and pin-heading. The fruiting body from all populations showed around 1.4 of the ratio of pileus diameter and stipe thickness (Fig. S4). This is a similar value of fruiting body in market. Moreover, all variances of traits showed consistency, thus CV of three repetitions of traits and populations was reasonable for statistical analysis (Pd3 in Fig. S5). The effects of the testers on the populations were highly significant ($P < 0.001$) for most traits by ANOVA (Table S5). The data from each population were treated separately for the QTL analyses. Four populations showed continuous variation with a nearly normal distribution in all of the traits (Fig. 2); thus, no data transformation was required. The statistical analysis showed different CV for the different populations. The CV values for the traits of Pd3 were higher than those of the other populations (Table 2).

The broad-sense heritabilities of Pd14 and Pd15 were relatively lower than Pd3 and Pd8. In the case of Pd3, the broad-sense heritabilities of Y, E, L, Q, T, D and C were relatively high, ranging from 0.59 to 0.83. For Pd8, the values of Y8, D8 and Q8 were low, ranging from 0.33 to 0.38, whereas the values of L8 and E8 were high. Heritabilities of cap color were above 0.60 at Pd3, Pd8 and Pd14 (Table 3).

Table 4 shows the pairwise phenotypic correlation coefficients for all of the traits by populations. The quality, yield and length traits were positively correlated with each other, indicating that these parameters have a genetic relationship. These components showed a significantly ($P < 0.001$) high correlation coefficient (0.63–0.93) regardless of the population (Table 4 and Fig. S2). Negative correlations between yield, length and earliness were obtained, and these correlations varied depending on the population (−0.82 and −0.78 for Pd3 and −0.28 and −0.30 for Pd14), indicating that precocious individuals tended to produce high-quality mushrooms. No significant correlation was found between cap color and any of the other traits; therefore, this trait was assumed to be under independent control.

3.4. QTL analysis

The empirical thresholds that were determined using the 1000-permutation LR test were in the range of 1.73–5.17, depending on the traits (Table S6). The mean LR values of traits were in the range of 2.29–3.24; therefore, an average LOD threshold of 3.0 was used to declare a QTL significant. To compare the results of each

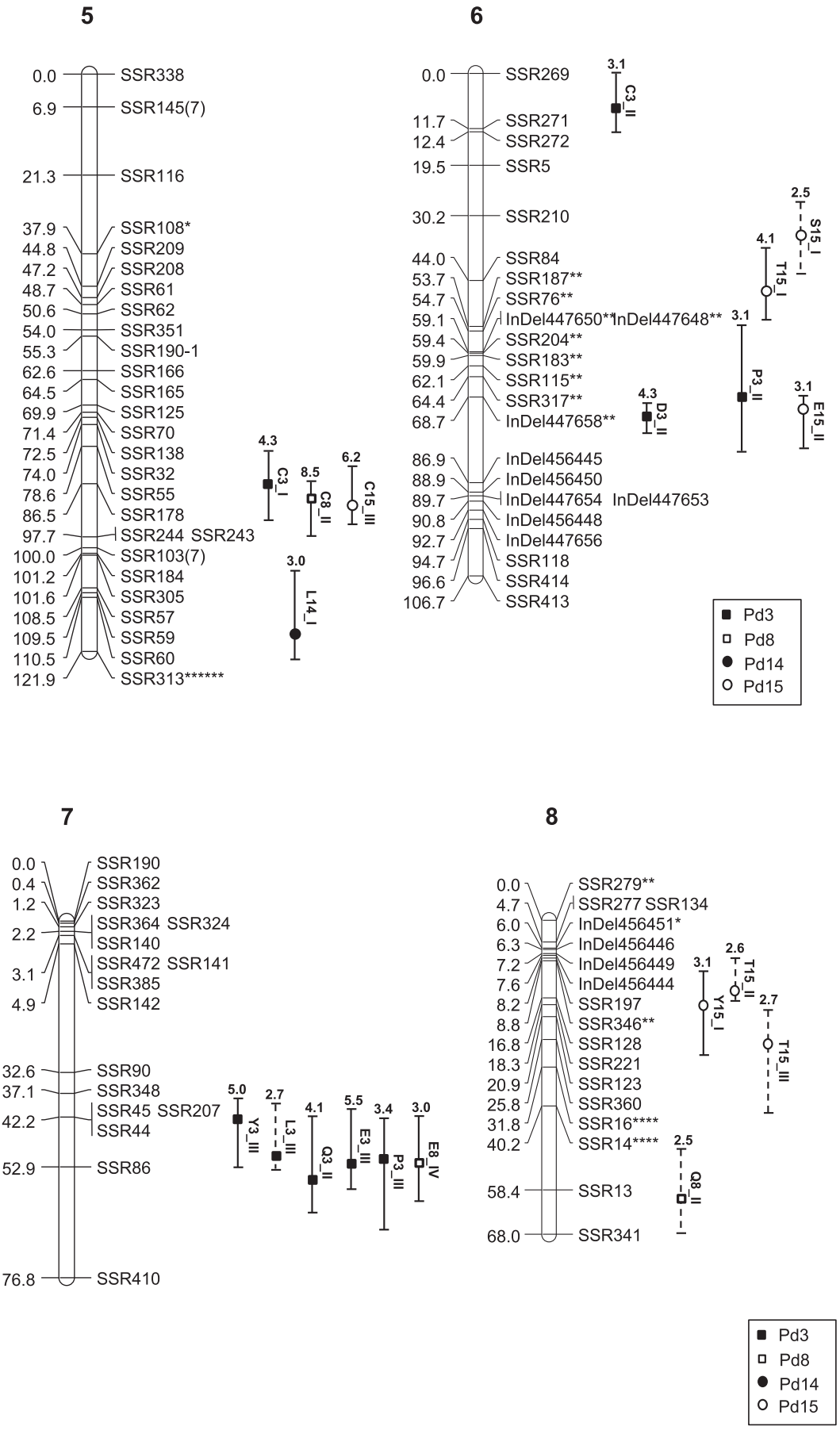

Fig. 1 (*continued*)

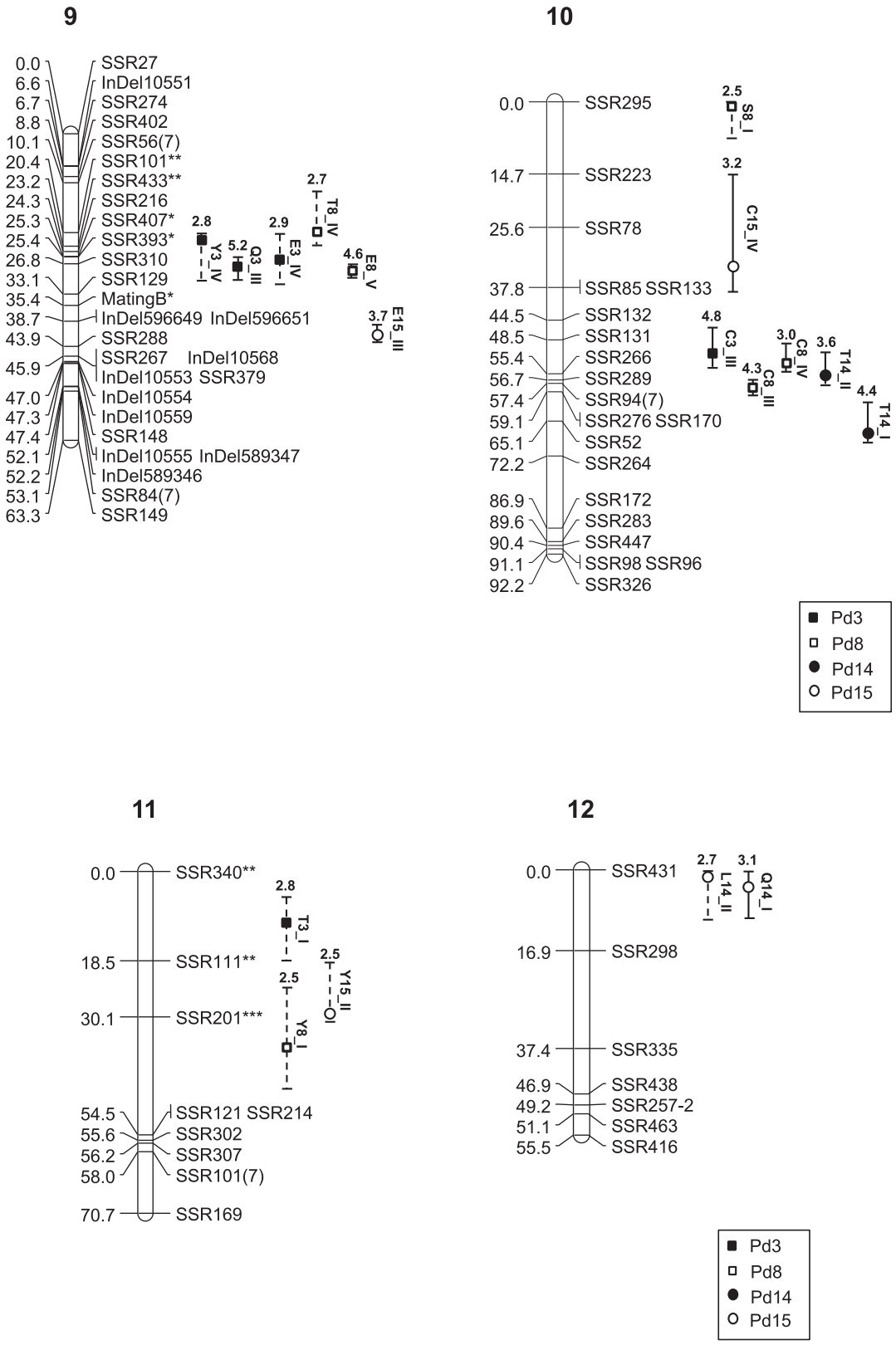

Fig. 1 (*continued*)

population, the QTLs possessing LOD values of less than the significant thresholds (2.5⩽, <3.0) are also denoted by dashed lines (Fig. 1).

The QTLs for each trait and each population that were obtained using the CIM procedure are shown in Table 5. The QTLs showed a very high level of significance ($P < 0.0001$ to 0.01), with LOD scores that ranged from 2.5 to 9.4. Depending on the traits, up to five QTLs exhibiting individual R^2 values in the range of 5.82% (Pd3, yield) to 32.57% (Pd15, length) were detected. The Rt^2 was as high as 52.33% for yield (Pd3).

Table 1
Distribution of the genetic markers among the *P. eryngii* linkage groups.

Linkage group	Size		No. of markers[b]	Average marker interval (cM)
	Genetic (cM)	Physical (Mbp)[a]		
LG 1	130.0	5.27	38	3.42
LG 2	91.4	2.77	18	5.08
LG 3	88.4	4.46	24	3.68
LG 4	83.0	4.27	27	3.07
LG 5	121.9	3.94	27	4.51
LG 6	106.9	3.02	24	4.45
LG 7	76.8	3.30	17	4.52
LG 8	68.0	2.91	17	4.00
LG 9	63.3	2.30	28	2.26
LG 10	92.2	1.85	20	4.61
LG 11	70.7	1.53	9	7.86
LG 12	55.5	1.27	7	7.93
Ave	87.3	3.07	21.3	4.09
Total	1048.1	36.89	256	

[a] The size is approximate and estimated based on the draft sequence of *P. eryngii* (http://112.220.192.2/per/).
[b] 226 SSR markers, 28 InDel markers and 2 mating types.

Considering all nine of the traits, a total of 23, 8 and 17 QTLs were detected in Pd3, Pd8, Pd14 and Pd15, respectively. Most of the QTLs were co-located at a narrow region across populations and traits. The placement of a few QTLs was specific to the population, such as C3_II on LG6 and L14_I on LG5, which were found only in Pd8 and Pd15, respectively. Hot spots for 18 and 6 QTLs that control yield and morphology, respectively, were found at 65.4–115.3 cM on LG1 and at 38–65 cM on LG7, respectively (Fig. 1).

3.5. QTLs for yield-related traits

3.5.1. Yield (Y) and length (L)

Seven QTLs for yield traits were identified at three locations in three populations (Pd3, Pd8 and Pd15). The QTLs Y3_I and Y3_II were located at positions ranging from 76.7 to 91.4 cM on LG1 and explained 22.89% and 13.12% of the phenotypic variation, respectively. The QTLs of Pd3 located on LG1 showed a plotting pattern similar to that of L3, Y3, Q3 and E3 (Fig. S3). SSR428 was significantly associated with yield traits (LOD = 9.4) in Pd3 (Fig. 1). These yield-related regions on LG1 are involved in length, quality, and earliness as well as with the period of pin-heading. Another yield QTL, Y3_III, found on LG7, had an LOD of 5.0 and an R^2 value of 10.92%. The collective phenotypic variation explained by these three QTLs was 52.33% in the case of Pd3. Moreover, we detected yield QTLs on LG8 and LG11 in Pd8 and Pd15. These QTLs had lower LOD values (2.5–3.1) than that of Pd3 and explained a much smaller portion of the phenotypic variance (8.95–16.96%) (Table 5). The additive effects were more positive than negative, indicating that the yield trait was most likely derived from KNR2312.

Ten QTLs for length were identified in three populations. The highest LOD score was 7.0 for L3_I, which explained 13.70% of the phenotypic variation. The QTL confidence intervals of LG1 (L3_I, L3_II, L8_I and L8_II), LG7 (L3_III) and LG12 (L14_II) overlapped with the QTLs for length, quality, and earliness. However, the QTLs for length on LG2 and LG5 were individually scattered.

3.5.2. Quality (Q)

The product obtained by multiplying stipe length by pileus diameter is significantly correlated with yield and quality (Ryu et al., 2006). In light of this observation, these quality traits were comprehensively evaluated by considering the yield and length values and their ratio. Six QTLs with LOD scores ranging from 2.5 to 8.5 and R^2 values ranging from 7.62% to 23.42% were identified. The collective phenotypic variation explained by these three QTLs was 48.5% in the case of Pd3.

3.5.3. Stipe thickness (T) and pileus diameter (D)

Ten QTLs for stipe thickness were identified; they had LODs of 2.6–4.4 and a maximum R^2 of 20.07% and were scattered across the LGs. The confidence intervals for stipe thickness overlapped with those for pileus diameter on LG2 and LG3. The additive effects of the QTLs were mostly negative, indicating that the alleles appeared to be from KNR2532. Seven QTLs for pileus diameter were identified, the highest LOD of which was 4.4 with an R^2 value of 16.59%. In all of the populations, the QTLs for pileus diameter were detected at consistent chromosomal locations on LG3 (D14_I and D15_I) and LG4 (D8_I, D14_II and D15_II).

3.5.4. Earliness (E)

A total of 12 QTLs for earliness were identified at four locations on LG1, LG6, LG7 and LG9 in three populations, which had R^2 values ranging from 6.85% to 21.22%. The highest LOD score was 6.4 on LG1 in Pd8, which had an R^2 value of 17.25% (E8_I). Total R^2 of earliness was 49.11% (Pd3) and 40.32% (Pd8). Three genomic regions for earliness, one on LG1 (65.4–88.0 cM), one on LG7 (42.2–61.2 cM) and one on LG9 (21.1–31.0 cM), were shared by the QTLs for the yield-related and morphology-related traits (Fig. 1). The earliness QTLs were positively affected by the KNR2532 allele.

3.5.5. Period of pin-heading (P) and period of spawn running (S)

The confidence intervals of the QTLs associated with P and S traits were relatively wider than those of other traits. The most significant QTLs for the period of pin-heading were found on LG1, which had a LOD of 4.2 and an R^2 value of 13.13% (P8_I). The period of pin-heading was associated with earliness on LG1, LG6 and LG7. However, the QTLs for the period of spawn running on LG2 and LG5 were independent of those of the other traits and had low LOD values.

3.5.6. Cap color (C)

The number of QTL for cap color was the largest among traits. Twelve QTLs were detected on LG1, LG4, LG5, LG6 and LG10, with the highest LOD score, 8.5, occurring on LG5 (C8_II). The cap color QTLs were clustered on LG5 (C3_I, C8_II and C15_III) and LG10 (C8_III, C8_IV and C15_IV) (Table 5, Fig. S3), indicating the importance of this genomic region for the trait. Total cap color variance of QTL in Pd15 explained 44.87%.

3.5.7. Candidate genes for a yield QTL on LG1

Approximately 1100 kb was obtained between nearest markers to yield QTL, SSR239 (83.4 cM) and SSR311 (94.5 cM). A total of 310 genes were predicted by manual curation based on FgeneSH and transcriptome data in the browser (Table S7). It has been reported that crop yield was related to growth rate of biomass (Larraya et al., 2003; Salmones et al., 1997), signal transduction (Coello et al., 2010) and transcription factor (Cabello et al., 2016; Ferreira et al., 2016). Thus, each sequence was analyzed by UniProt, EMBL-EBI and PROSITE to predict function, then finally, multiple candidate genes encoding expression regulator, signal transduction, mycelial growth and hydrolase that appeared likely to be associated with the yield trait were suggested (Table S7). Interestingly, one transcription factor and two enzyme genes were found to regulate transcription and degradation or synthesis biomass components, cellobiohydrolase and 1,3-beta-D-glucan synthase, respectively. Moreover, other genes related to biomass production, including the 60S ribosomal protein L27, carboxylic ester

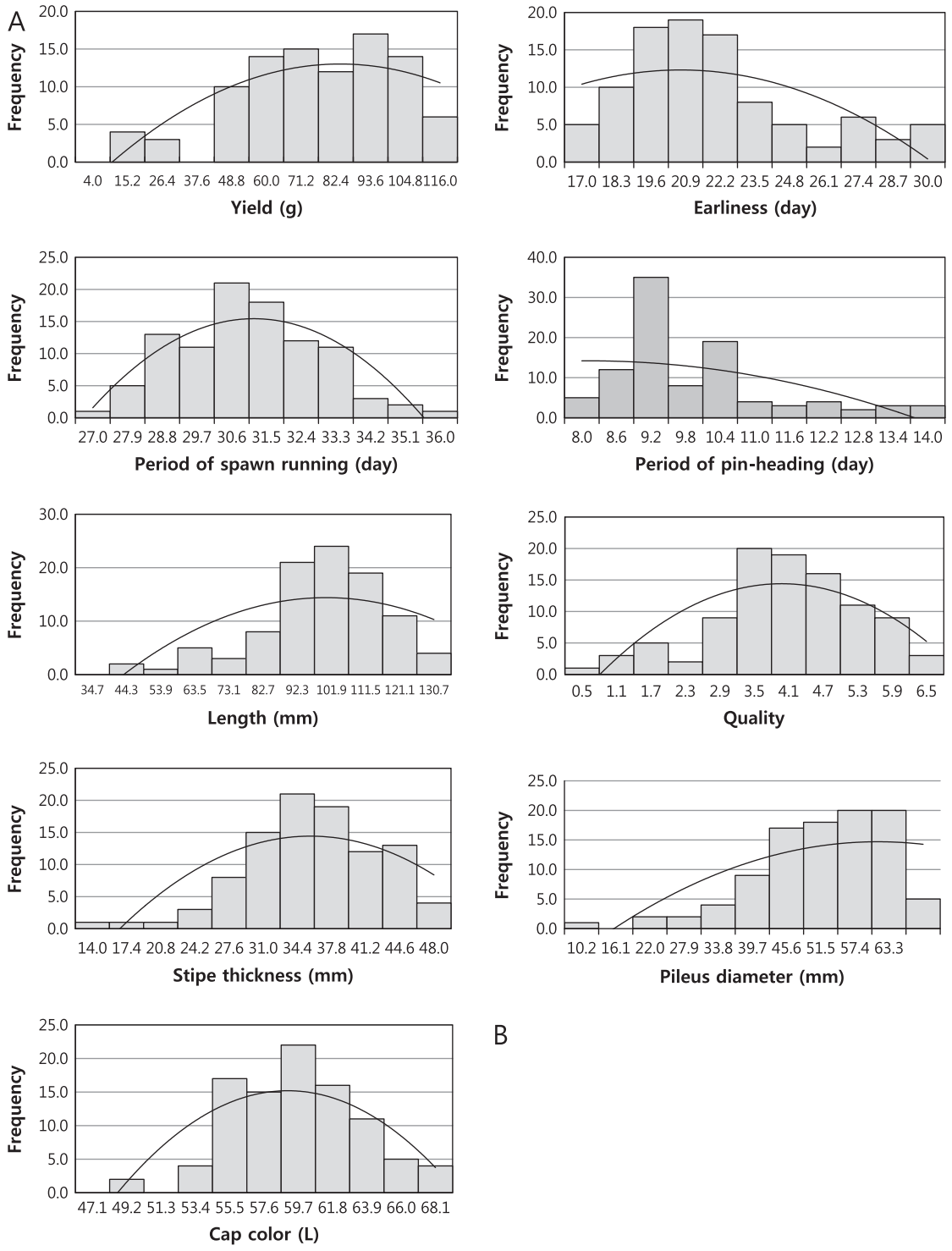

Fig. 2. Frequency distribution histograms for the yield-related traits (A) and morphology-related traits (B) of Pd3 population.

hydrolase, helicase, hexokinase and glycoside hydrolase were found in this region.

4. Discussion

4.1. Experimental design

Construction of genetic linkage map and QTL mapping was conducted to elucidate the genetic basis of agronomic traits in *P. eryngii*. Because main agronomic traits could be accessed only from fruiting body, four monokaryons harboring different mating loci were used as a compatible gamete. Although experimental repetition was limited due to the restricted resources, we built various dikaryotic populations from crossing with multiple testers. It might be better than larger repetition of a single population. This allowed us to detect the various and reliable QTLs from the combining of two distant varieties, KNR2312 and KNR2532. Dikaryons in each population shared the identical nucleus, phenotypic variances reflected the nucleus of segregant and its interaction with tester's nucleus. The segregants (Pm1) were from recombination

Table 2
Phenotypic characteristics of the test-crossed populations.

Trait	Pd3				Pd8				Pd14				Pd15			
	Code	Mean (SD)	[min-max]	CV (%)	Code	Mean (SD)	[min-max]	CV (%)	Code	Mean (SD)	[min-max]	CV (%)	Code	Mean (SD)	[min-max]	CV (%)
Yield-related traits																
Yield (g)	Y3	70.4 (25.45)	[4.5–116.0]	36.2	Y8	79.0 (14.0)	[41.3–120.0]	17.8	Y14	75.8 (15.4)	[35.7–101.3]	20.3	Y15	75.1 (15.2)	[26.5–104.0]	20.2
Earliness (day)	E3	21.5 (3.36)	[16.7–30.0]	15.7	E8	17.5 (2.1)	[14.3–24.0]	12.0	E14	17.0 (1.6)	[11.7–24.0]	9.5	E15	18.3 (2.1)	[15.3–26.3]	11.4
Period of spawn running (day)	S3	30.6 (1.83)	[27.0–36.0]	5.98	S8	31.7 (2.1)	[27.0–36.0]	6.6	S14	30.6 (2.0)	[26.7–36.0]	6.6	S15	31.6 (2.0)	[27.5–36.0]	6.4
Period of pin-heading (day)	P3	9.6 (1.70)	[8.0–14.0]	17.7	P8	9.1 (1.48)	[7.3–15.0]	16.26	P14	8.7 (0.96)	[5.7–13.3]	11.03	P15	9.4 (1.12)	[5.7–13.0]	11.91
Morphology-related traits																
Length (mm)	L3	94.6 (18.70)	[35.0–130.7]	19.8	L8	101.6 (12.7)	[76.7–134.3]	12.5	L14	85.1 (15.0)	[47.7–119.3]	17.6	L15	102.3 (13.8)	[65.5–134.0]	13.5
Quality	Q3	3.8 (1.28)	[0.5–6.5]	33.7	Q8	4.2 (0.8)	[2.3–6.0]	18.2	Q14	3.5 (0.9)	[1.2–5.5]	26.0	Q15	4.4 (1.0)	[1.5–6.5]	21.6
Stipe thickness (mm)	T3	34.3 (6.67)	[14.0–48.0]	19.4	T8	31.8 (5.8)	[22.0–50.7]	18.3	T14	39.6 (6.5)	[25.7–59.0]	16.4	T15	34.6 (5.7)	[22.0–54.0]	16.6
Pileus diameter (mm)	D3	43.1 (11.57)	[4.0–63.3]	26.8	D8	53.9 (6.9)	[31.7–69.7]	12.8	D14	47.7 (9.4)	[17.7–69.3]	19.8	D15	44.4 (9.9)	[14.3–63.3]	22.2
Cap color (LogitL)	C3	0.15 (0.07)	[−0.05–0.33]	46.5	C8	0.24 (0.07)	[0.01–0.39]	30.5	C14	0.15 (0.07)	[−0.88–0.36]	47.1	C15	0.20 (0.08)	[0.02–0.39]	38.4

Table 3
Broad-sense heritability of the yield-related and morphology-related traits.

Population	Source of variation	Yield (Y)		Earliness (E)		Period of spawn running (S)		Length (L)		Quality (Q)		Stipe thickness (T)		Pileus diameter (D)		Cap color (C)	
		df	MS[a]	*df*	MS	*df*	MS	*df*	MS	*df*	MS	*df*	MS	*df*	MS	*df*	MS
Pd3	Genotype[b]	96	1963.57	97	34.30	94	15.81	96	1059.53	96	4.97	96	133.50	96	401.81	94	56.49
	Error	194	265.57	196	2.21	190	6.48	194	131.78	194	0.59	194	25.15	194	58.42	190	9.13
	h^2	0.68		0.83		0.32		0.70		0.71		0.59		0.66		0.63	
Pd8	Genotype	97	593.61	97	13.32	94	13.72	97	493.56	97	1.81	97	102.51	97	142.28	97	44.63
	Error	196	238.36	196	2.47	190	3.17	196	83.56	196	0.67	196	25.91	196	50.23	196	6.76
	h^2	0.33		0.59		0.53		0.62		0.36		0.50		0.38		0.65	
Pd14	Genotype	97	714.20	97	7.95	97	12.54	97	681.34	97	2.51	97	126.83	97	267.30	97	49.83
	Error	196	259.19	196	2.30	196	4.90	196	92.83	196	0.53	196	42.00	196	60.31	196	9.20
	h^2	0.37		0.45		0.34		0.68		0.55		0.40		0.53		0.60	
Pd15	Genotype	97	697.34	97	13.18	83	12.32	97	577.48	97	2.78	97	98.95	97	291.90	97	61.62
	Error	196	207.78	196	2.45	168	3.48	196	119.31	196	0.67	196	18.02	196	53.28	196	22.57
	h^2	0.44		0.59		0.46		0.56		0.51		0.60		0.60		0.37	

[a] MS, mean square.
[b] 98 genotypes were evaluated however some traits were not able to be accessed.

of KNR2312P5 and -P6 during meiosis. The segregation efficiency might be enough because the phenotypic differences of two protoclones were distinct, 108–134% in yield, 114–139% in earliness and so on (Table S4).

4.2. Linkage map

A genetic linkage map of *P. eryngii* was constructed firstly using SSR, InDel and mating-type markers. Some primer's physical locations were obscure and the order of the genetic distance and the physical location showed inconsistency possibly due to the differences between previous and current version of genome sequence. Distorted segregation was observed in 12.7% of the markers, and was lower than that reported for *P. ostreatus* (14%) and *P. eryngii* (20.4%) (Larraya et al., 2000; Okuda et al., 2012). The germination of basidiospore and growth rate of monokaryon depend on genotypes including mating-type and other loci (Larraya et al., 1999, 2002, 2001), bias might be from the use of early-germinated monokaryons. The Pm1 was consisted of randomly collected monokaryons germinated from spore incubated for 30 days. It might be attributed to a short confidence interval compared with others (Foulongne-Oriol et al., 2012a). The present map consisted of 12 LGs covering 1048.1 cM, with an average interval length of 4.09 cM (Table 1 and Fig. 1). These values were similar to those reported for other *Pleurotus* species, in which 11–13 LGs covering approximately 1000 cM were found (Larraya et al., 2000; Okuda et al., 2009, 2012). The average ratio of the physical distance to the genetic distance (41.8 kb/cM) was slightly higher than previously reported ratios (Larraya et al., 2000; Okuda et al., 2012). The average interval length (4.09 cM) was less than the 5.3 cM value reported for *P. ostreatus* (Larraya et al., 2000) and the 6.17 cM value reported for *P. eryngii* (Okuda et al., 2012). These results suggested that our map has a uniform marker density and is sufficient for providing useful genetic data for basic and applied studies of this mushroom.

4.3. Traits

In general, heritability was associated with QTL number, LOD and R^2, depending on more population than trait. In case of Pd3

Table 4
Pearson's correlation coefficients between all the traits in four populations.

Pd8	Pearson's correlation coefficient (significance)								
	Pd3								
	Y	L	T	D	Q	E	S	P	C
Y	–	0.88 (<0.001)	0.51 (<0.001)	0.68 (<0.001)	0.93 (<0.001)	−0.82 (<0.001)	−0.06 (0.55)	−0.55 (<0.001)	−0.32 (<0.01)
L	0.63 (<0.001)	–	0.28 (<0.01)	0.64 (<0.001)	0.89 (<0.001)	−0.78 (<0.001)	−0.13 (0.23)	−0.61 (<0.001)	−0.15 (0.16)
T	0.43 (<0.001)	0.30 (<0.01)	–	0.23 (<0.05)	0.34 (<0.001)	−0.29 (<0.01)	0.05 (0.65)	−0.12 (0.25)	0.24 (<0.05)
D	0.49 (<0.001)	0.08 (0.45)	0.23 (0.02)	–	0.72 (<0.001)	−0.70 (<0.001)	0.04 (0.99)	−0.47 (<0.001)	0.39 (<0.001)
Q	0.67 (<0.001)	0.70 (<0.001)	−0.03 (0.80)	0.31 (<0.001)	–	−0.78 (<0.001)	−0.03 (0.75)	−0.56 (<0.001)	−0.14 (<0.05)
E	−0.26 (0.05)	−0.41 (<0.001)	0.26 (<0.01)	−0.21 (<0.05)	−0.34 (<0.001)	–	0.06 (0.59)	0.73 (<0.001)	0.27 (<0.05)
S	−0.30 (<0.01)	−0.38 (<0.001)	0.04 (0.69)	−0.12 (0.23)	−0.31 (<0.01)	0.23 (0.02)	–	0.08 (0.47)	−0.074 (0.48)
P	−0.04 (0.06)	−0.37 (<0.001)	0.27 (<0.005)	−0.16 (0.11)	−0.22 (0.03)	0.88 (<0.001)	0.19 (0.06)	–	0.0001 (0.99)
C	−0.05 (0.60)	−0.03 (0.70)	0.01 (0.95)	0.01 (0.91)	−0.07 (0.46)	0.10 (0.32)	−0.17 (0.02)	−0.06 (0.57)	–
P15	Pearson's correlation coefficient (significance)								
	P14								
	Y	L	T	D	Q	E	S	P	C
Y	–	0.64 (<0.001)	0.41 (<0.001)	0.35 (<0.001)	0.74 (<0.001)	−0.28 (<0.01))	0.08 (0.42)	−0.07 (0.51)	0.10 (0.33)
L	0.72 (<0.001)	–	−0.24 (<0.05)	0.26 (<0.01)	0.90 (<0.001)	−0.30 (<0.01)	0.12 (0.24)	−0.04 (0.90)	0.10 (0.34)
T	0.36 (<0.001)	−0.18 (0.08)	–	0.23 (<0.05)	−0.12 (0.23)	0.10 (0.34)	0.26 (<0.05)	0.03 (0.78)	0.35 (<0.001)
D	0.51 (<0.001)	0.29 (<0.01)	−0.10 (0.308)	–	0.39 (<0.001)	0.25 (<0.05)	0.03 (0.78)	−0.05 (0.63)	0.28 (<0.05)
Q	0.79 (<0.001)	0.76 (<0.001)	0.007 (0.94)	0.45 (<0.001)	–	0.36 (<0.001)	0.07 (0.52)	−0.04 (0.71)	−0.07 (0.49)
E	−0.47 (<0.001)	−0.55 (<0.001)	0.31 (<0.01)	0.38 (<0.001)	0.58 (<0.001)	–	0.17 (0.08)	0.54 (<0.001)	0.13 (0.21)
S	−0.08 (0.42)	−0.19 (0.07)	0.19 (0.08)	0.18 (0.32)	−0.13 (0.23)	0.16 (0.14)	–	0.35 (<0.001)	0.01 (0.91)
P	0.14 (0.17)	0.38 (<0.001)	0.33 (<0.001)	0.08 (0.44)	0.23 (0.02)	0.55 (<0.001)	0.30 (<0.05)	–	0.14 (0.17)
C	0.05 (0.60)	0.14 (0.17)	0.13 (0.21)	−0.02 (0.85)	0.003 (0.97)	0.22 (0.002)	−0.008 (0.93)	0.05 (0.62)	–

and Pd8, 0.50–0.64 of coefficient of determination for heritability and genetic factors (QTL number, LOD and R^2) was observed (data not shown), while no traits showed consistent significant value throughout the populations. This indicates that, the recessive or cooperative alleles of tester might be critical to express alleles from postmeiotic segregants. In that context, heritability seems not to be a sufficient factor but a necessary condition with a proper interaction of alleles. Inconsistency in QTL number and R^2 of some traits including cap color and extent of heritability was observed. Actually, there was no obvious relationship between two factors in *A. bisporus* (Foulongne-Oriol et al., 2012a). Thus, not all heritabilities were explained by QTL.

Low CV of traits reduced precision of detecting of QTL (Larraya et al., 2003), and it might be cause of low discrimination upon genotype. In this study, QTL number and R^2 were related to CV, however, low coefficient of determination, 0.16 and 0.27 was observed (data not shown). The exact correlation of value (CV and heritability) and QTL was not achieved possibly because QTL detection was dependent on the allelic substitution effects of the meiotic recombinant and interaction with the tester and small population which were suggested by previous report (Foulongne-Oriol et al., 2012a).

Although limited repetition was adapted for phenotyping, the CV of repetitions in every trait showed relatively low, it mainly because of the conditioned cultivation room and thinning. The mushroom cultivation facility has been built like a "multispan panel house" and each room shares a big hallway controlled under the same conditions as the growing rooms so that air from outdoors could be buffered well. The thinning treatment and an appropriate harvest time were determined by comparing the ratio of pileus diameter and stipe thickness, allowing fruiting bodies to be treated as regular traits for statistical analysis.

Analyzing the correlations between traits showed that the quality increased as the gain of yield and length increased in four *P. eryngii* populations (0.67–0.93) (Table 4). In the case of *A. bisporus*, the yield (kg/m^2) was positively correlated with the number of fruiting bodies (no./m^2) but was negatively correlated with the weight (g/mushroom), suggesting a triangular relationship among these variables (Foulongne-Oriol et al., 2012a). Given the positive relationships observed in *P. eryngii*, the development of genotypes with a high yield and/or long length might be beneficial for the production of high-quality mushrooms. A tendency toward an increased yield and length of the mushrooms was observed in the earliest individuals obtained. However, a negative relationship between weight (g/mushroom) and earliness (the period from casing until the harvest of the first fruiting bodies) was observed previously in *A. bisporus*, which is likely due to differences in the cultivation methods. The earliest genotypes tended to produce numerous but small mushrooms of *A. bisporus,* which are cultivated without thinning the fruiting bodies; however, *P. eryngii* was cultivated using the thinning method (cutting off small fruiting body in the early stage and maintaining the best one), and thus, the number of fruiting bodies was irrelevant.

4.4. QTL

CIM analysis revealed 71 QTLs with high LOD scores that ranged from 2.5 to 9.4 (Table 5). Most of the QTLs were co-located at hot spots across populations and traits. The placement of a few QTLs was specific to the population, such as C3_II on LG6 and L14_I on LG5, which were found only in Pd8 and Pd15, respectively. Hot spots for 18 and 6 QTLs that control yield and morphology, respectively, were found at 65.4–115.3 cM on LG1 and at 38–65 cM on LG7, respectively (Fig. 1). Likewise, not all populations conferred their QTL at the alleles possibly due to allelic substitution effects, interaction with the tester and small population (Foulongne-Oriol et al., 2012a). In particular, in this study, four testers harboring different genetic traits and Pm1 obtained through meiotic recombination of P5 and P6 whose traits were significant different (Table S4). Alleles of each progeny in Pm1 and its interaction with testers might be various and explain the inconsistency of QTL detection.

Clustering of the QTLs for diverse traits was detected in several chromosomal regions. The QTLs for yield, length, quality, earliness and the period of pin-heading were clustered in four major genomic regions on LG1, LG3, LG7 and LG9 (Fig. 1). Among the QTLs detected for all of the traits and populations, 18 QTLs were located in a specific genomic region on LG1. This result suggests that a pleiotropic control mechanism is involved in the expression of

Table 5
QTL for yield- and morphology-related traits found in fruiting bodies of the Pd3, Pd8, Pd14 and Pd15.

	Pd3									Pd8								
Trait	QTL	LG	CI (cM)[a]	LOD[b]	Nearest marker[c]	Position (cM)[d]	R^2(%)	Total of variance explained (%)	Additive effect	QTL	LG	CI (cM)	LOD	Nearest marker	Position (cM)	R^2 (%)	Total of variance explained (%)	Additive effect
Yield (g)	Y3_I	1	87.0–91.4	9.4	SSR428	88.4	22.89	52.33	12.9286	Y8_I	11	23.6–43.7	2.5	SSR201	34.2	16.96	16.96	6.0334
	Y3_II	1	76.7–81.7	5.1	SSR256	76.7	13.12		10.1500									
	Y3_III	7	38.1–52.2	5.0	SSR45	42.2	10.92		−8.8979									
	Y3_IV	9	21.1–30.4	2.8	SSR433	21.3	5.82		6.8132									
Length (mm)	L3_I	1	86.4–92.4	7.0	SSR428	88.4	13.70	38.00	8.1402	L8_I	1	85.8–91.8	4.6	SSR428	88.4	10.97	36.04	4.3632
	L3_II	1	76.8–80.9	4.5	SSR256	76.8	20.21		9.6914	L8_II	1	76.7–80.1	4.0	SSR256	76.9	12.32		4.6505
	L3_III	7	39.1–52.4	2.7	SSR86	49.6	11.59		−7.2423	L8_III	2	73.1–76.9	3.3	SSR53	73.9	29.09		−6.9447
										L8_IV	3	32.3–37.9	3.5	SSR3	33.5	9.02		−3.9262
Quality	Q3_I	1	87.3–94.0	8.5	SSR428	90.3	23.42	48.50	0.6613	Q8_1	1	85.5–97.1	2.7	SSR428	88.1	8.39	20.41	0.2295
	Q3_II	7	42.3–62.1	4.1	SSR86	54.6	11.25		−0.4550	Q8_II	8	48.7–66.0	2.5	SSR13	58.5	7.62		−0.2213
	Q3_III	9	25.2–29.4	5.2	SSR310	26.9	12.06		0.4732									
Stipe thickness (mm)	T3_I	11	0–12.5	2.8	SSR340	0	9.49	9.49	2.4594	T8_I	2	62.4–67.6	3.9	SSR292	67.2	13.32	27.68	2.1705
										T8_II	3	1.3–21.1	2.8	SSR381	14.8	8.88		1.7697
										T8_III	4	25.7–33.9	3.2	SSR18	31.3	9.99		−1.8967
										T8_IV	9	12.4–22.8	2.7	SSR101	20.5	8.51		−1.8001
Pileus diameter (mm)	D3_I	2	64.0–68.1	2.7	SSR292	67.2	8.94	18.01	−3.7545	D8_I	4	41.0–57.5	3.5	SSR179	49.8	11.75	11.75	−2.4537
	D3_II	6	68.6–74.9	4.3	Indel447658	68.7	13.81		6.8087									
Earliness (days)	E3_I	1	85.3–88.0	6.1	SSR428	87.2	16.47	49.11	−1.3967	E8_I	1	75.7–80.8	6.4	SSR256	76.5	17.25	40.32	−0.8936
	E3_II	1	76.8–83.1	4.8	SSR130	79.1	13.70		−1.2931	E8_II	1	65.4–74.9	5.3	SSR28	71.2	15.21		−0.8509
	E3_III	7	42.2–58.7	5.5	SSR86	53.0	13.77		1.2842	E8_III	1	83.4–87.8	4.8	SSR428	86.2	14.98		−0.8237
	E3_IV	9	21.1–31.0	2.9	SSR433	23.3	6.85		−0.9422	E8_IV	7	43.6–61.2	3.0	SSR86	52.5	6.97		0.5876
										E8_V	9	23.2–25.9	4.6	SSR216	24.3	11.89		−0.7578
Period of pin-heading (days)	P3_I	1	67.0–80.2	4.0	SSR256	76.8	12.05	33.39	−0.6128	P8_I	1	75.9–83.2	4.2	SSR256	76.8	13.13	13.13	−0.5465
	P3_II	6	53.6–79.6	3.1	Indel447658	67.6	9.81		−0.5521									
	P3_III	7	42.2–65.2	3.4	SSR86	50.1	12.17		0.5987									
Period of spawn running (days)										S8_I	10	0–7.0	2.5	SSR295	0	8.60	8.60	−0.5502
Cap color (Logit*L*)	C3_I	5	79.0–93.0	4.3	SSR178	84.9	14.86	32.91	0.0297	C8_I	4	36.9–56.0	3.9	SSR179	50.1	10.62	35.24	−0.0251
	C3_II	6	1–12.1	3.1	SSR271	6.8	14.17		0.0288	C8_II	5	82.1–93.2	8.5	SSR178	88.4	33.17		0.0427
	C3_III	10	46.1–53.8	4.8	SSR131	50.3	17.73		0.0322	C8_III	10	56.0–59.0	4.3	SSR289	57.5	11.91		0.0354
										C8_IV	10	49.3–54.8	3.0	SSR266	52.6	14.65		0.0375
	Pd14									Pd15								
	QTL	LG	CI (cM)	LOD	Nearest marker	Position (cM)	R^2 (%)	Total of variance explained (%)	Additive effect	QTL	LG	CI (cM)	LOD	Nearest marker	Position (cM)	R^2 (%)	Total of variance explained (%)	Additive effect
Yield (g)		–								Y15_I	8	11.7–29.0	3.1	SSR221	17.9	11.16	17.53	−5.1725
										Y15_II	11	18.4–30.0	2.5	SSR201	29.4	8.95		5.4126
Length (mm)	L14_I	5	103.6–121.9	3.0	SSR60	115.7	16.47	18.00	6.3756	L15_I	2	72.8–75.7	3.0	SSR53	73.9	32.57	32.57	−8.0157
	L14_II	12	0–9.7	2.7	SSR431	0.0	9.36		−4.6879									
Quality	Q14_I	12	0–9.1	3.1	SSR431	3.1	21.58	21.58	−0.4453									
Stipe thickness (mm)	T14_I	10	60.4–70.8	4.4	SSR52	67.1	20.07	4.15	−3.1906	T15_I	6	35.9–50.5	4.1	SSR84	43.9	13.82	9.25	−2.1928
	T14_II	10	50.3–56.5	3.6	SSR266	54.2	14.62		−2.6685	T15_II	8	8.8–17.8	2.6	SSR128	15.0	10.15		−1.8785
										T15_III	8	19.6–30.9	2.7	SSR360	25.7	8.94		−1.7508

(*continued on next page*)

Table 5 (*continued*)

	Pd14									Pd15							
	QTL	LG	CI (cM)	LOD	Nearest marker	Position (cM)	R^2 (%)	Total of variance explained (%)	Additive effect	QTL	LG	CI (cM)	LOD	Nearest marker	Position (cM)	R^2 (%)	Total of variance explained (%)	Additive effect
Pileus diameter (mm)	D14_I	3	10.8–29.9	3.2	SSR381	17.8	11.60	28.37	3.2562	D15_I	3	10.2–30.0	4.1	SSR381	20.2	16.71	27.98	3.2562
	D14_II	4	36.8–49.4	4.4	SSR179	45.1	16.59		−3.9357	D15_II	4	43.7–57.1	4.1	SSR179	50.1	12.14		−3.5193
Earliness (days)		–								E15_I	1	88.4–98.8	3.1	SSR48	94.7	9.61	17.47	−0.6610
										E15_II	6	66.0–76.6	3.1	Indel447658	68.8	9.60		−0.6770
										E15_III	9	38.9–42.4	3.7	Indel596649	40.8	21.22		−0.970
Period of pin-heading (days)		–								P15_I	3	19.5–55.5	2.5	SSR163	35.4	9.31	9.31	3.5690
Period of spawn running (days)		–								S15_I	6	26.0–43.3	2.5	SSR210	32.5	11.80	11.80	4.0544
Cap color (Logit*L*)	C14_I	3	17.8–46.1	3.0	SSR3	32.2	10.37	10.37	−0.0230	C15_I	1	88.8–100.1	7.2	SSR311	96.2	27.61	44.87	−0.0414
										C15_II	1	103.3–115.3	7.1	SSR64	110.4	22.63		−0.0376
										C15_III	5	82.2–94.1	6.2	SSR178	89.3	24.08		0.0393
										C15_IV	10	14.8–37.8	3.2	SSR85	32.9	9.89		0.0248

[a] 1-LOD support interval.
[b] LOD score value at the LOD score peak.
[c] Nearest marker to the LOD score peak.
[d] Position of the LOD score peak in cM.

multiple traits or that tight genetic linkage between independent loci for different traits exists within a narrow genomic region. Pearson's correlation analysis of phenotypes also showed that quality, length, thickness, diameter, and earliness were highly correlated (Table 4). The presence of yield and length QTLs in the same chromosomal region was consistent with their synergistic effect on quality (Table 5 and Fig. 1). However, in some cases, beneficial QTLs were associated with undesirable QTLs (the clustering region on LG1). For example, a QTL responsible for yield, length and quality was present in the same region as a QTL for earliness and the period of pin-heading. Therefore, careful selection would be required to avoid undesirable traits. Moreover, it is difficult to determine whether these clustered QTLs are the results of a pleiotropic effect or the effects of closely linked genes. Thus, fine mapping would be valuable in determining the nature of any effects.

Based on the additive effect, the alleles of KNR2312 conferred a higher level of yield and quality, as expected, whereas the favorable alleles for earliness and the period of pin-heading were most likely derived from the testers (KNR2532). One of the main goals of mushroom breeding is increasing the yield, which has been reported to be correlated with the mycelial growth rate (Kües and Liu, 2000; Larraya et al., 2002, 2003; Salmones et al., 1997). However, no significant correlations were detected between the period of spawn running and other yield-related traits in our study (Table 4). Only two weak QTLs for the period of spawn running were identified, and they showed no association with yield or the morphology-related traits. One possible reason for this result might be the discordance between the mechanisms of vegetative (mycelial) and reproductive (fruiting) growth and low variation in the spawn running period. Cap color is an important factor for determining the preference of consumers in markets and was reported to be associated with pathogen resistance in *A. bisporus* (Foulongne-Oriol et al., 2012a; Moquet et al., 1999); a bimodal distribution has been observed for each color parameter that has a high level of heritability (Moquet et al., 1999). The major QTLs controlling cap color were near the *PPC1* locus, and they explained 89.8% of the variance. Unexpectedly, the distribution of cap color in *P. eryngii* showed continuous variation with diverse levels of heritability according to the population (Fig. 2). This continuous pattern of cap color might be due to two parents having a slight difference in cap color (lightness) (Table S3). However, all of the QTLs related to cap color were located on LG5 and LG10 regardless of the population, and the total amount of variance they explained was relatively high (44.87% in Pd15), indicating that the genes that control chromaticity may be located in these regions.

4.5. Candidate genes

Ultimately, identifying the genes responsible for the quantitative traits of interest and elucidating the factors controlling their expression and regulation can be achieved. The yield trait is important to breeders and farmers due to its association with profitability. Computational prediction and manual curation resulted in 310 genes from the genomic region near the yield QTL (Y3_I). We scrutinized whether the function of the genes was related to yield. Wide regulatory gene such as transcription factor or signal transduction was expected to be found because crop yield has been reported to be a complex trait (Boyles et al., 2016; Shi et al., 2009). One transcription factor and several signal transduction-like genes, kinase, GTPase and ATPase were found (Table S7). Transcription-factor gene in *A. bisporus* has been reported to involve in the regulation of fruiting process (Morin et al., 2012). ATPase is regulatory proteins that are involved in several cellular functions, such as signal transduction, translation and intracellular transport. These proteins appear to be critical for growth and development (Leipe et al., 2002). It is compelling that genes

associated with biomass production were found to be related to the yield trait because the fruiting body is a biomass consisting of various glycosidic polymers. It is assumed that, initially, the cellulose in substrates is degraded to monomers by cellobiohydrolase, and then, these monomers are used by 1,3-beta-D-glucan synthase to synthesize 1,3-beta-D-glucans for the construction of cell walls. Cellobiohydrolase has been reported to play a major role in the degradation of crystalline cellulose to cellobioside (Baldrian and Valášková, 2008; Ryu et al., 2011; Sánchez, 2009). Generally, a fungus has multiple glucan synthases for constructing cell walls (Rop et al., 2009). The 60S ribosomal protein L27, a component of the 60S ribosomal subunit, is known to function in rRNA processing, ribosomal assembly and protein synthesis. There is no direct clue to narrow down the candidate gene from whole genes so far. Further investigation using the gene knockout method will be required to determine whether these genes affect the yield trait. Likewise, the other QTLs identified here will be good targets for gene identification. The findings presented here might be useful in understanding the genetic inheritance of agronomically significant characteristics of *P. eryngii* and useful for the breeders and geneticists to link phenotypic and genotypic traits. Thus, it could be helpful in gene pyramiding, backcrossing and recurrent selection using a MAS.

4.6. Conclusions

We constructed a 12 LGs genetic linkage map of *P. eryngii* based on segregation analysis of markers comprised of 256 loci in post-meiotic monokaryons. Total 71 QTLs explaining between 5.82% and 33.17% of the phenotypic variations of the nine traits were identified using CIM. The candidate genes encoding transcription factor, signal transduction, mycelial growth and hydrolase that appeared likely to be associated with the yield trait were suggested. However, limited population, low repetition and primer caused that the QTLs detected are readily useful in narrow range of *P. eryngii* cultivars. The QTLs detected in this study, together with candidate genes could be a strong probe to elucidate of corresponding genes. With improving of the genome sequence and fine mapping of QTL using larger population and primers, to fine the strong selection markers applicable to breeding programs deserves to go further.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2016.05.002.

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