Genetic map of a basidiomycete fungus, *Lentinula edodes* (shiitake mushroom), constructed by tetrad analysis

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We constructed a framework map of the edible mushroom *Lentinula edodes* (shiitake mushroom) using tetrad analysis. The map is based on the segregation of 264 randomly amplified polymorphic DNA (RAPD) markers, 14 structural genes, 1 expressed sequence tag (EST) marker, 2 mating factors (*matA* and *matB*), and 8 sequence-characterized amplified regions (SCARs) among 92 basidiosporic strains in 23 tetrads. We identified 11 linkage groups on a support interval of a minimal LOD score of 3.0 and a maximum distance of 25 centimorgans (cM). The length of the 11 linkage groups (LGs) ranged from 157.2 cM to 24.4 cM, and they covered a distance of 908.8 cM. One of the quantitative trait loci controlling the vegetative growth rate on potato dextrose agar (PDA) for *L. edodes* was detected between the markers D18CCA-360t and S08-1000c on LG2.

**Key Words:** *Lentinula edodes*, framework map, tetrad analysis, quantitative trait loci.

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**Introduction**

*Lentinula edodes* (Berk.) Pegler, which originated in the Asia-Australasia region, is a basidiomycete fungus and a kind of popular edible mushroom known as shiitake mushroom (Kobayashi and Shimizu 1951, Kobayashi 1966, Aoshima and Furukawa 1980). *L. edodes* contains medical compounds including elitadenin, lentinan, ergosterol and others (Aoyagi *et al.* 1980, 1982a, 1982b, Chang 1980, Chang and Miles 1984). Many varieties of *L. edodes* have been developed in Japan. Breeding of *L. edodes* has progressed as a result of mating between superior varieties and selection from many progenies. Genetic analysis of this mushroom may contribute to the continued development of varieties.

The application of DNA markers enables to achieve genotype identification and molecular tagging for gene isolation as well as to identify various agronomic traits. Randomly amplified polymorphic DNA (RAPD) is especially useful for analyzing a large number of marker loci and this method was used for constructing genetic maps of filamental fungi also (Williams *et al.* 1990, Xu and Leslie 1996, Kerrigan *et al.* 1993). Single strand conformation polymorphism (SSCP) is useful for the detection of point mutations in genomic regions and has been applied for mapping known sequence data of structural genes (Hunter *et al.* 1993, Orita *et al.* 1989). Two genetic maps have been reported in *L. edodes* using RAPD markers (Kwan and Xu 2002) and AFLP markers (Terashima *et al.* 2002). These maps were constructed based on the analysis of monokaryotic strains randomly isolated, and the number of offsprings used for constructing the genetic maps was 32 (Kwan and Xu 2002) and 95 (Terashima *et al.* 2002).

Kwan and Xu (2002) employed quantitative trait locus (QTL) analysis to examine the growth rate of monokaryotic strains. Agronomic traits have also been characterized by QTL analysis in other edible basidiomycetes, *Agaricus bisporus* and *Pleurotus ostreatus* (Moquet *et al.* 1999, Larraya *et al.* 2002, 2003).

Tetrad analysis can be applied to construct highly reliable genetic maps, because it is a procedure of genetic analysis using four daughter cells carrying different chromosomes as a result of meiosis from one mother cell (Mather and Beale 1942). Tetrad analysis is useful for detecting linkage,
Materials and Methods

Strains

Parental dikaryon, MCR14, was generated from the monokaryon D703PP-9 (mating-type: \(A_B\)) obtained from D703, a New Zealand wild strain, and from the monokaryon G408PP-4 (\(A_B\)) obtained from G408, a Japanese wild strain (Miyazaki and Neda 2004). The mitochondrial genome of the parental strain, MCR14, was derived from G408. Basidiospores were isolated by a micromanipulator and maintained on potato dextrose agar (PDA) slants. In the present study, we constructed a moderately dense map of \(L.\ edodes\) using tetrad analysis and mapped 289 loci, including 14 structural genes, 2 mating factors, 1 expressed sequence tag (EST) marker gene, 8 sequence-characterized amplified regions (SCARs), and 264 RAPDs to 11 linkage groups. The total genetic distance was 908.8 centimorgans (cM). We then analyzed QTL to control the growth rate of \(L.\ edodes\). To our knowledge, this is the first genetic map of basidiomycete fungi constructed based on tetrad analysis.

determination of the position of the centromere, and mating type analysis (Perkins 1953, Whitehouse 1958). Miyazaki et al. (2005) determined the distances between centromere and mating factors in \(L.\ edodes\) using tetrad analysis. Even strains with a low spore germination ability or growth rate after germination are not omitted when isolated as a set of tetrads. In populations obtained by tetrad isolation, each tetrad carries all the genetic information of the parental strain. In addition, when the segregation of heterozygous DNA markers is analyzed, all the heterozygous DNA markers can be detected in a segregation ratio of 2:2 in a tetrad. These characteristics of tetrad analysis are convenient for the construction of a genetic map and QTL analysis.

In the present study, we constructed a moderately dense map of \(L.\ edodes\) using tetrad analysis and mapped 289 loci, including 14 structural genes, 2 mating factors, 1 expressed sequence tag (EST) marker gene, 8 sequence-characterized amplified regions (SCARs), and 264 RAPDs to 11 linkage groups. The total genetic distance was 908.8 centimorgans (cM). We then analyzed QTL to control the growth rate of \(L.\ edodes\). To our knowledge, this is the first genetic map of basidiomycete fungi constructed based on tetrad analysis.

Determination of growth rate

To estimate the growth rate on PDA (Nissui Inc.) at 25°C, a disk (diameter, 4 mm) of mycelia previously cultured on PDA was placed in the center of a PDA plate (diameter, 90 mm). Six repetitions were performed for the samples grown on PDA. The diameter of each colony found to be perpendicular in two directions was measured every 1–3 days. The regression line slope (mm/day) calculated by the ANOVA test indicate of the mycelial growth rate of each strain.

DNA extraction

Crude genomic DNA was prepared by the modified cetyltrimethylammonium bromide (CTAB) procedure (Murray and Thompson 1980). After RNase treatment, this crude genomic DNA was purified using a Geneclean-III kit (BIO101) or ELU-QUIK-KIT (Schleicher and Shuell), as previously described (Tanaka et al. 2004). The concentration and purity of the DNA solution were assayed based on the spectrophotometric absorbance and ratio of OD260:OD280 (GeneQuant II, Pharmacia Biotech).

Polymerase chain reaction (PCR) amplification

PCR amplification was used to analyze the SCAR markers, detect polymorphisms by designing specific primers, and amplify fragments for SSCP. Each reaction mixture consisted of 20 mM Tris-HCl (pH 8.5), 50 mM KCl, 2.5 mM MgCl\(_2\), 0.16 mM of each dNTP, 0.08 \(\mu\)M of each primer, 5 ng of genomic DNA, and 0.25 unit of Platinum Taq DNA polymerase (Invitrogen). The total volume of each reaction mixture was 12.5 \(\mu\)l, and each was overlaid with mineral oil. The thermal cycling program (PE480, Perkin Elmer) was as follows: 15 cycles at 95°C for 90-s, 55°C for 90-s, 72°C for 120-s, and then 15 cycles at 95°C for 30-s, 55°C for 90-s, 72°C for 120-s. This was followed by a 10-m incubation at 72°C and subsequent cooling to 4°C. To detect the SCAR markers, we used the primers developed by Hisaeda et al. (unpublished). The sequences of the primers used for the detection of polymorphisms by PCR, SSCP, and SCARs are shown in Table 1.

RAPD analysis

For RAPD analysis, two methods were adopted. In one method, the analysis was performed in a microtube, and in the other method, in a glass capillary. In the case of the microtube, the amplification was performed on a 10-\(\mu\)l mixture that contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl\(_2\), 0.2 mM of each dNTP, 0.5 \(\mu\)M of primer, 10 ng of genomic DNA, and 0.5 unit of Platinum Taq DNA polymerase (Invitrogen). After pre-denaturation at 95°C for 1-m, the mixture was subjected to 45 cycles of 30-s of denaturation at 95°C, 30-s of annealing at 55°C, and a 90-s extension at 72°C, prior to a final extension of 7-m at 72°C and subsequent cooling to 4°C. Amplification was performed on T1 (Biometra). In the case of the glass capillary, the reaction was performed on a 15-\(\mu\)l mixture that contained 50 mM

For QTL analysis, dikaryotic strains derived from crosses between 92 monokaryotic strains isolated as tetrads and a tester monokaryotic strain, H600PP-39, were used to determine the growth rate. These dikaryotic strains were designated as MCR101-MCR192, inheriting mitochondria from H600PP-39. Similarly, the monokaryons D703PP-9 and G408PP-4 were crossed with H600PP-39 to obtain dikaryotic strains, and designated as MCR205 and MCR206, respectively.
Tris-HCl (pH 8.5), 5 mM MgCl₂, 500 μg/ml of bovine serum albumin, 0.5 mM of each dNTP, 2.0% (W/V) of Ficoll400, 4 mM of tartrazine, 0.01 mM of EDTA, 0.25 μM of primer, and 0.6 unit of Tth DNA polymerase (TOYOBO). After pre-denaturation at 94°C for 1-m, the mixture was subjected to 60 cycles of 10-s of denaturation at 94°C, 30-s of annealing at 36°C, and 60-s extension at 72°C, prior to a final extension of 5-m at 72°C and subsequent cooling to room temperature (at about 25°C). The amplified fragments were separated by electrophoresis in 1.2% or 1.0% agarose gels and observed by staining with ethidium bromide. The primers consisted of 10-mer primers (Operon Inc.) and 13-mer primers synthesized with 3-base in reference to the 10-mer primers. Each marker was designated by a combination of the primer name and the fragment length (in bp), and the markers amplified by the microtube were denoted by the addition of ‘t’ at the end, and ‘c’ for capillary PCR.

**SSCP analysis**

Biotin-labeled PCR products were diluted 50- to 100-fold in 1× TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM of EDTA, pH 8.0), 6% (w/v) of sucrose, and 0.33% of tartrazine. Double-stranded DNA in a diluted solution was denatured and maintained at 96°C for 5-m, and then the mixture was cooled on ice. The mixture was subsequently loaded on a 15 × 40 cm vertical 5% Hydro-Link Long Ranger acrylamide gel (AT Biochem) in 1× TBE buffer and subjected to electrophoresis at 14°C for 90-m at 30 watts. DNA strands were transferred to a nylon transfer membrane (MSI) and were visualized using a Phototope-Star Detection Kit (Biolabs).

**Linkage and QTL analysis**

Only the data of segregation in a 2:2 ratio in each tetrad were used for linkage analysis. The linkage of the markers was tested based on the LOD score (the threshold of the LOD score was 3.0, and the maximum distance was 25 cM). The MAPMAKER version 3.0 computer program was used for the linkage analysis. In this program, an efficient algorithm that allowed a simultaneous multipoint analysis of any number of loci (Lander et al. 1987) was used. The loci were grouped using the “GROUP” (two-point analysis) and “COMPARE” commands. The Kosambi mapping function was applied to determine the distance between two loci. QTL analysis was performed using Mapmaker/QTL 1.1 (Lincoln et al. 1992). A threshold score value of 2.0 was selected to indicate the presence of a QTL.

**Results**

**RAPD markers used for the construction of a genetic map of L. edodes (MCR14)**

A total of 692 primers, which included 379 10-mer oligonucleotide primers and 313 13-mer oligonucleotide primers, were used for the construction of a genetic map of L. edodes (MCR14). The primers were grouped using the “GROUP” command. The Kosambi mapping function was applied to determine the distance between two loci. QTL analysis was performed using Mapmaker/QTL 1.1 (Lincoln et al. 1992). A threshold score value of 2.0 was selected to indicate the presence of a QTL.
primers, were screened for the amplification of heterozygous RAPD markers in order to perform linkage analyses to select polymorphisms between the monokaryotic strains D703PP-9 and G408PP-4, which generated the parental strain MCR14. Among 667 primers, 507 primers (258 10-mer primers and 249 13-mer primers) could amplify the heterozygous RAPD(s). Thirty 10-mer primers and seventy-five 13-mer primers were used to construct the linkage map of _L. edodes_. These primers were selected based on the number of fragments per primer and the clarification of RAPD fragments amplified in agarose gel after electrophoresis. These conditions were important for efficient construction of the genetic map using fewer PCR reactions and selecting more reliable markers. The size of the RAPD fragments ranged from 150 bp to 3500 bp, with an average value of about 720 bp. Ultimately, 264 RAPDs were mapped on the linkage map of _L. edodes_.

**Mating factors and tightly linked SCAR markers**

Two SCAR markers tightly linked to the mating factors, namely S0PH90-590 linked to the A mating factor (matA) and S0PO19-560 linked to the B mating factor (matB), had been developed using 18 tetrads (72 meiotic strains) (Tanaka _et al_. 2004). In the present study, these markers were analyzed using 23 tetrads. In conclusion, both SCAR markers were confirmed to be linked to each mating factor perfectly.

**Analysis of structural genes and one EST marker (LEEST359)**

Fourteen structural genes, _priA_ (Kajiwara _et al_. 1992), _priB_ (Endo _et al_. 1994), _mfbA_ (Kondoh _et al_. 1995), _mfbB_ (Ishibashi _et al_. 1996), _mfbC_ (Miyazaki _et al_. 2004b), _uck1_ (Kaneko _et al_. 1998), _hyd1_ (Ng _et al_. 2000, Nishizawa _et al_. 2002), _hyd2_ (Ng and Kwan 2000), _cyp1_ (Akiyama _et al_. 2002), _cap_ (Zhou _et al_. 1998), _recQ_ (Katsukawa _et al_. 2004), _rnr2_ (Kaneko and Shishido 2001), _cdc5_ (Miyazaki _et al_. 2004a), and _rDNA_ (White _et al_. 1990), and one EST marker, LEEST359 (Kawamukai _et al_. unpublished), were analyzed for their segregation patterns and mapped on linkage groups. Polymorphisms were detected in four structural genes when the electrophoresis was performed using agarose gel (Fig. 1A and Table 1). The other genes were analyzed using the SSCP procedure. Figure 1B shows a typical result of SSCP analysis.

**SCAR markers**

Nineteen SCAR markers developed from specific RAPD markers for detecting differences in the varieties of _L. edodes_ were analyzed (Hisaeda _et al_. unpublished). The polymorphisms in a parental strain were detected on six SCAR markers, M09, M17, M18, M40, M47, and M53 out of the 19 SCAR markers analyzed. Figure 1C shows a typical result of SCAR analysis.

**Linkage analysis and detail of the genetic map of _L. edodes_ (MCR14)**

Segregation data were recorded for a total of 309 markers: 284 RAPD markers, 14 structural genes, 2 mating factors, 1 EST marker, and 8 SCAR markers. Linkage analysis using MAPMAKER enabled to identify 11 linkage groups with 289 loci (Fig. 2); while 20 markers remained unassigned to any linkage group. These groups covered a distance of 908.8 cM. The linkage group length ranged between 157.2 cM (LG1) and 24.4 cM (LG11). The number of loci for each linkage group ranged between 18 and 38. The average interval between two loci was 3.15 cM.

**Analysis of mycelium growth rates on PDA**

The analysis of the QTL(s) controlling the vegetative growth rate was performed on PDA. Frequency distribution in the dikaryotic population obtained by crossing with a monokaryotic tester, H600PP-39, is shown in Fig. 3. The growth rate (mm/day) ranged from 0.51 (MCR181) to 4.65 (MCR160). Growth rates of MCR205 (D703PP-9 × H600PP-39), MCR206 (G408PP-4 × H600PP-39) and MCR14 (D703PP-9 × G408PP-4) were 1.99, 1.28 and 3.2, respectively (Fig. 3). A QTL, _Legrpda1_, controlling the vegetative growth rate on PDA in _L. edodes_ was located between the D18CCA-360 and S08-1000c markers on LG2 by interval mapping (Fig. 2). The LOD value of this QTL was 3.26, and the percentage of variance explained was 15.1%.

**Discussion**

In the present study, a linkage map of _L. edodes_ was constructed with 289 loci including 14 structural genes. All the genes in the analysis were mapped based on PCR detection of SNPs or SSCP procedure (10 genes). In addition, one EST marker was mapped on LG3. It appears that the parental strain, MCR14, displayed a high heterozygosity level, because the outbred lines used in the study resulted from the crossing between a Japanese wild strain and a New Zealand wild strain (Miyazaki and Neda 2004). This strain exhibited convenient characteristics in attempt at mapping other structural genes or EST markers. The application of tetrad analysis was suitable for screening markers with a high confidence level (Miyazaki _et al_. 2000). Discarding data that did not reveal a segregation ratio of 2:2 in a tetrad enabled to avoid the problem of treating null data in RAPD markers. Therefore, a highly reliable linkage map could be constructed based on tetrad analysis, and to our knowledge, this is the first genetic map of basidiomycete fungi constructed by tetrad analysis. Since most of basidiomycete fungi produce four basidiospores on a basidium, tetrad analysis of basidiomycete fungi could be carried out more easily than in other organisms. However, it would be difficult to apply this method to basidiomycete fungi with a low germination rate of basidiospores. Nevertheless, we suggest that tetrad analysis should also be performed to analyze genetically other edible mushrooms.
The linkage map (threshold: LOD score 3.0 and maximum distance of 25 cM) in the present study consisted of 11 linkage groups, as in the map reported by Terashima et al. (2002). However, this number of linkage groups was different from the chromosome number of eight reported in this mushroom, based on light microscopic observation and pulsed field gel electrophoresis (Nakai, 1986, Arima and Morinaga, 1993). Terashima et al. (2002) indicated that the number of chromosomes of L. edodes was unclear. Our data revealed that the number of linkage groups in this fungus was similar to that reported by Terashima et al. (2002). However, matB belonged to the smallest linkage group, LG11 (24.4 cM) in our map, while in the report of Terashima et al. (2002), matB belonged to LG_V (199.4 cM), which was much larger than LG11. LG11 in our map could have combined with another linkage group on one chromosome, while the LG_V group described by Terashima et al. (2002) could have been dismantled, or the small linkage groups they reported could have combined to form one linkage group such as that in our report. The exact number of chromosomes in L. edodes is considered to range between 8 to 11. To detect this number in the fungus, it may be necessary to correlate the analysis by electrophoresis with the data of linkage analysis of L. edodes.

Since in our linkage map, the matA and priA genes were included on LG1 (Fig. 2), as also reported by Kwan and Xu (2002), it is likely that these two genes were located on the same chromosome. In addition, matA was found to belong to a relatively large linkage group in our report and was also described in two previous linkage maps of L. edodes. On LG4, uck1, priB and cdc5 were located in a narrow region. Kaneko et al. (1998) stated that these 3 genes were located in tandem on a chromosome. In the present study, these genes showed a perfectly cosegregation, suggesting that they were closely located in any strain of L. edodes.

LEEST359 displayed a high homology to the C-4 methyl sterol oxidase gene of Candida albicans (Kawamukai et al. unpublished). C-4 methyl sterol oxidase is a key enzyme in the ergosterol biosynthetic pathway. In fungi, ergosterol participates in cell organization and is known as provitamin D₂. It is considered that a gene with the sequence of LEEST359 could be involved in the production of ergosterol. The role of a gene including this EST marker could be determined by the analysis of the QTL controlling the amount

Fig. 1. Examples of the polymorphisms detected by the molecular markers. Lane M: 100-bp ladder marker (Amersham); lanes 1 through 2—monokaryotic strains, D703PP-9 and G408PP-4, composing a parental strain; lanes 3 through 6—tetrad strains, MCR14B-5-1, -2, -3 and -4; lanes 7 through 10—tetrad strains, MCR14B-7-1, -2, -3 and -4; lanes 11 through 14—tetrad strains, MCR14B-10-1, -2, -3 and -4; lanes 15 through 18—tetrad strains, MCR14B-16-1, -2, -3 and -4 in all the figures. (A) RAPD amplification using primer OPB15. The arrowheads indicate heterozygous RAPD markers, B15-1050c (upper) and B15-800c (lower). (B) PCR detection. The arrowhead indicates the amplification fragment of uck1. (C) SSCP analysis of hyd1. (D) Analysis of a SCAR marker. The arrowhead indicates the amplification fragment of M40.
Fig. 2. Genetic map of *L. edodes* constructed by using the outbred line crossed between a New Zealand strain (D703) and a Japanese strain (G408). The linkage group numbers, length of each linkage group and number of loci mapped on the linkage group are indicated on top. Distances (cM) between markers are shown on the left side. The names of the markers are shown on the right side. The confidence interval of QTL (LOD score > 2.0) is denoted by vertical blocks, and the peak position is indicated by a horizontal line.
of ergosterol in the fruiting body of *L. edodes*.

A QTL, *Legrpda1*, that controlled the vegetative growth rate of *L. edodes* on PDA at 25°C using a dikaryon population obtained by crossing with a tester strain, H600PP-39, was detected between the D18CCA-360t and S08-1000c markers on LG2. It is thus expected that this QTL mainly controls the growth rate of the mushroom at least on PDA. A strain with a high growth rate is tolerant to pathogenic fungi, such as *Trichoderma*, and forms rapidly colonies in the substrate in culture. We suggest that the use of a dikaryotic population obtained by crossing between isolated tetrads and common monokaryotic strain may provide on effective approach for QTL analysis. However, it is possible that other loci than this QTL control the growth rate of *L. edodes*. Larraya et al. (2002) identified QTLs controlling the dikaryotic growth rate in *P. ostreatus* using 5 populations derived from 5 monokaryotic tester strains on two kinds of media. To identify other QTLs that control the vegetative growth rate of *L. edodes*, it may be necessary to use populations obtained from monokaryotic strains except H600PP-39, and to vary the medium conditions. If a QTL could be mapped also between the D18CCA-360t and S08-1000c markers on LG2, based on the analysis of different populations and/or conditions, it could be concluded that the *Legrpda1* QTL detected in the present study controls the growth rate of the fungus. Information about the role of *Legrpda1* and other QTLs in the control of the growth rate of this fungus could be useful for agronomical applications.

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**Literature Cited**


