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Construction of a Genetic Linkage Map of Shiitake Mushroom *Lentinula Edodes* Strain L-54

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From fruiting bodies of *L. edodes* strain L-54, single-spore isolates (SSIs) were collected. Two parental types of L-54 were regenerated via monokaryotization. By means of random-amplified polymorphic DNA (RAPD), DNA samples from L-54, its two parental types, and 32 SSIs were amplified with arbitrary primers. Dedikaryotization was demonstrated, and 91 RAPD-based molecular markers were generated. RAPD markers that were segregated at a 1:1 ratio were used to construct a linkage map of *L. edodes*. This RAPD-linkage map greatly enhanced the mapping of other inheritable and stable markers [such as those that are linked to a phenotype (the mating type), a known gene (*priA*) and a sequenced DNA fragment (MAT)] with the aid of mating tests, bulked-segregant analysis, and PCR-single-strand conformational polymorphism. These markers comprised a genetic map of *L. edodes* with 14 linkage groups and a total length of 622.4 cM.

Keywords: Genetic map, Genetic mapping, *Lentinula edodes*, Random-amplified polymorphic DNA (RAPD)

Introduction

Shiitake mushroom, *Lentinula edodes*, is worldwide the second most popular edible mushroom (Chang, 1999; Chiu *et al*., 1999). In order to improve the quality and increase the production of *L. edodes*, many studies have been carried out. However, the genetics of *L. edodes* have not been extensively studied. The lack of genetic information has handicapped the breeding of this mushroom.

Only scanty genetic information and a few genetic markers have been available. The traditional methods for generating genetic markers, such as auxotrophic and drug-resistant markers, are time-consuming and require characterization of the mutations before they can be used. There are also not enough earlier molecular markers [e.g., isozyme and DNA restriction fragment length polymorphism (RFLP) based-markers of *L. edodes*] to construct a useful genetic map (Royse *et al*., 1983; Castle *et al*., 1987). A more contemporary method uses PCR, such as random-amplified polymorphic DNA (RAPD), to generate molecular markers (Williams *et al*., 1990). In *L. edodes*, the parental monokaryons and single-spore progeny can be regarded as F1 and F2 generations, respectively (Kwan, 1992). With this "well-planned cross", RAPD polymorphisms among a reasonable number of single-spore isolates (SSIs) can be produced, analyzed, and linked in order to construct a genetic map.

The objectives of this study are as follows: (1) Construct a genetic map of *L. edodes*, mainly by the RAPD method with standard mapping. (2) Locate phenotypic markers with the aid of bulked-segregant analysis, a known gene and sequenced DNA fragment by means of PCR-single-strand conformational polymorphism (PCR-SSCP).

Materials and Methods

Strain and growth conditions Professor Chang Shu-Ting (Department of Biology, the Chinese University of Hong Kong) kindly provided the *L. edodes* dikaryotic strain L-54. Working stocks were grown on PDA plates (24.0 g/l of potato dextrose broth and 15.0 g/l of Difco Bacto agar) at 26°C for 7 (of dikaryon), or 14~16 (of monokaryon) days. For DNA extraction, mycelia were inoculated in a PDB medium (24.0 g/l of potato dextrose broth) at 26°C for 10 days (of dikaryon), or 15~20 (of monokaryon) days with shaking at 250 rpm.

Single-spore isolation Basidiospores were isolated from fresh-fruiting bodies by holding the cap, gills downward, in a sterile petri dish for 8 h at room temperature. Spore deposits were suspended in distilled water and spread on PDA plates. After incubation for 3-4 days at 26°C in the dark, individually-germinated basidiospores were transferred onto fresh PDA plates. SSIs were distinguishable

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from dikaryons by their slow growth and the absence of clamp connections.

Recovery of parental types Two parental types were recovered from L-54 dikaryon by protoplast isolation and regeneration (Zhao and Chang 1993). Two parental monokaryons (P1 and P2) were identified by their positive-mating reaction when they mated with each other.

DNA extraction DNA samples were extracted from the mycelia of strains L-54, P1 and P2, and SSIs by the method of Lee and Taylor (1990), which was followed by RNase treatment and cesium chloride precipitation (Yoon *et al.* 1991). The concentration and purity of the DNA preparations were assayed by spectrophotometric measurements (Milton Roy Spectronic 3000, GeneQuant RNA/DNA Calculator) and 0.7% agarose gel electrophoresis in a 1× TBE buffer (prepared from a 10× TBE stock solution containing 0.9 M Tris base, 0.9 M boric acid, and 2 mM EDTA).

Generation of RAPD markers RAPD markers were generated by RAPD amplification of DNAs of strains P1 and P2, and then 32 SSIs. RAPD protocol was an optimization of that described by Sobral and Honeycutt (1993) with the *AmpliTaq* Stoffel fragment (Perkin-Elmer Cetus) as the DNA polymerase. All of the tests were in a 50 ml reaction that contained 1× Stoffel buffer (10 mM Tris-HCl, 10 mM KCl, pH 8.3), 6.0 mM $MgCl_2$, 0.2 mM dNTP, 50 ng template DNA, 0.4 mM primer, and 5 U Stoffel fragment. The reaction mixture was subjected to 40 cycles in a PTC-100 Programmable Thermal Controller (MJ Research) through a thermal profile of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min, followed by a 20-min elongation. RAPD products were detected by electrophoresis on 3% Nuseive:Seakem (3:1) agarose gel and stained with 0.5 mg/ml ethidium bromide for 15 min, then destained with distilled water for 10 min. The gel was transferred to a UV transilluminator and photographed with Polaroid 667 film (ASA3000). The primers (Operon 10-mer Kit A-D, OPA-OPD, Operon Technologies) were 10-base oligonucleotides with arbitrary sequences without self-complementary ends. Their G + C content was 60-70%.

Linkage analysis A series of pairwise chi-square (χ^2) tests of independent assortment were conducted to screen for RAPD markers with the expected 1 : 1 segregation pattern among the progeny (Weir 1990). The RAPD markers that showed significant deviation ($P<0.05$) from the null hypothesis of the independent assortment were not used for the subsequent analysis.

The linkage of the markers was tested by LOD Score (the threshold of Lod score was 3.00). The MAPMAKER/MAP version 3.0b computer program (Whitehead Institute for Biomedical Research) performed the linkage analysis. This program uses an efficient algorithm that allows a simultaneous multipoint analysis of any number of loci (Lander *et al*., 1987). MAPMAKER does not recognize linkages for markers in repulsion, so the repulsion-phase linkages can be detected by analyzing re-coded data (i.e., presence re-coded to absence, and vice versa) together with the original data set using the F2 backcross data type (http://www-genome.wi.mit.edu/genome_software/other/mapmaker.html). Grouping of the marker loci was conducted using the "GROUP" (two-point analysis) and "COMPARE" commands. The "ORDER" command was used to determine a linear order using a multi-point analysis.

Mating tests Mating types of the two parental monokaryons, P1 and P2, were designated as A1B1 and A2B2, respectively. The mating type of an SSI was determined by mating reactions between this SSI and two parents. It was confirmed by mating reactions between all of the SSIs. To test for a mycelial interaction, two 5-mm^2 plugs of agar and mycelia from two monokaryons were placed 1.5-2.0 cm apart on a 9-cm petri dish that contained a PDA medium, and cultivated at 26°C for 7-15 days. Mycelial interactions were observed continuously and recorded as positive, common A (Flat), common B (Barrage), and negative (Miles & Chang 1986). Morphological characteristics of 'common A' and 'common B' were compared by reference mating.

Bulked-segregant analysis Bulked-segregant analysis or pooled sampling is a method for rapid identification of markers that are linked to any specific gene or genomic region (Michelmore *et al*., 1991). The success of the bulked-segregant analysis depends on the genetic divergence between the two parents in the target region. Within each pool, individuals are identical for the trait, but are arbitrary for all others. In this study, the bulked-segregant analysis was carried out to isolate RAPD markers that were closely linked to the mating type factors A and B of *L. edodes*. Sixteen SSIs were grouped into 4 pools. Each pool had 4 SSIs with the same mating type. DNAs from 4 pooled samples were extracted and amplified by RAPD. The polymorphisms were confirmed to link to the mating type loci by RAPD amplification of the 32 SSIs segregation with the same primer pairs. Although there were only 4 SSIs per pool to a specific mating type factor (either A or B), 8 individuals were used for analysis because the mating system of *L. edodes* is bifactorial. Theoretically, for a dominant marker that segregates in the F2 population, the probability of a pool of 8 individuals that have a band, and a second pool of equal size does not have this band, is $2 \times (1/4)^8 \times [1 - (1/4)^8] = 3.0 \times 10^{-5}$, when the locus is unlinked to the target gene.

PCR-single-strand conformational polymorphism (PCR-SSCP) PCR-SSCP is a genetic-screening method to rapidly detect single nucleotide substitutions in genomic DNA or cDNA that are amplified by PCR (Orita *et al*., 1989). The double-stranded DNA target sequence is denatured to 2 single-stranded DNA molecules, which are separated by polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. Fragments of equal length that differ in sequence migrate to different positions, according to their conformational changes. The separated bands of the single-stranded DNA indicate the presence of mutations.

In order to map a known gene (*priA*) (Kajiwara *et al*., 1992) and a sequenced DNA fragment (MAT) (Leung *et al*., 1995), the fragments of interest that were inherited in 32 SSIs were amplified by PCR with designed primers (Table 1), then subjected to a PCR-SSCP analysis. One ml that contained approximately 100 ng of the PCR product was mixed with 1 ml of the denaturing Solution (98% formamide containing 0.05% bromophenol blue and 0.05% xylene cyanol). It was incubated at 95°C for 3 min, then quickly placed in an ice/water bath to cool for a few minutes. Before starting the

Table 1. Lengths of the target sequences and primer pairs used by PCR-SSCP.

Target DNA	Target Length (bp)	Primer	Sequence
*pri*A	302	Upper	CTACCCCCAACAAAGGAAATG
		Lower	ACACTACGAAACAAGAATCAG
MAT	200	Upper	ATGCTCACCAACAATCAACC
		Lower	CTGGTGTTGCGGGAGTGAGG

electrophoresis, PhastGel media [including PhastGel Homogeneous 12.5 (12.5% polyacrylamide) and PhastGel Native Buffer Strips (0.88 M L-alanine, 0.25 M Tris-HCl, pH 8.8, 3% agarose)] were placed in the PhastSystem (Pharmacia Biotech), and the stand-by temperature (4°C) was set. A 100 Vh of pre-run was performed to generate a continuous-buffer system. Soon after denaturation, 0.8 µl of the sample was pipetted onto an upturned 8/1 µl sample applicator and placed in the PhastSystem. The separation began to run immediately. According to the PCR fragment size, the separation was run from 150 Vh (for 200 bp) to 250 Vh (for 300 bp). After the separation-running, the gel was fixed and stained.

Results and Discussion

Generation of RAPD markers Primer pairs, rather than single primers, were used in this study. With 86 primer pairs, 7-10 amplified fragments were generated per primer pair. The first step of RAPD marker screening was the primer pair selection. A total of 48 primer pairs were tested; 27 of them were selected, based on their successful generation of polymorphic RAPD bands from two parents. This step was also the dedikaryotization demonstration: with the same primer pairs, the RAPD profiles of P1 and P2 could be distinguished as containing a subset of the bands that were seen in L-54.

With the 27 primer pairs, RAPD markers were identified by detection of the polymorphic fragments that were amplified among 32 SSIs. Each primer pair generated 3-4 polymorphic bands on average. The markers ranged in size from 70 to 740 bp. Figure 1 shows a typical RAPD amplification among 32 SSIs with the primer pair of OPC-07 and 08. There were two polymorphic bands of 182 bp and 340 bp in size. Each marker was given a name that was designated by a combination of the primer names and the size (in bp) of the polymorphic band. For clarity, the serial alphabet "a-t" were used in place of the primer numbers "01-20" in the Operon 10-mer Kits. For example, a 182 bp band that was produced with the primer pair of OPC-07 and 08 was designated as CgCh182.

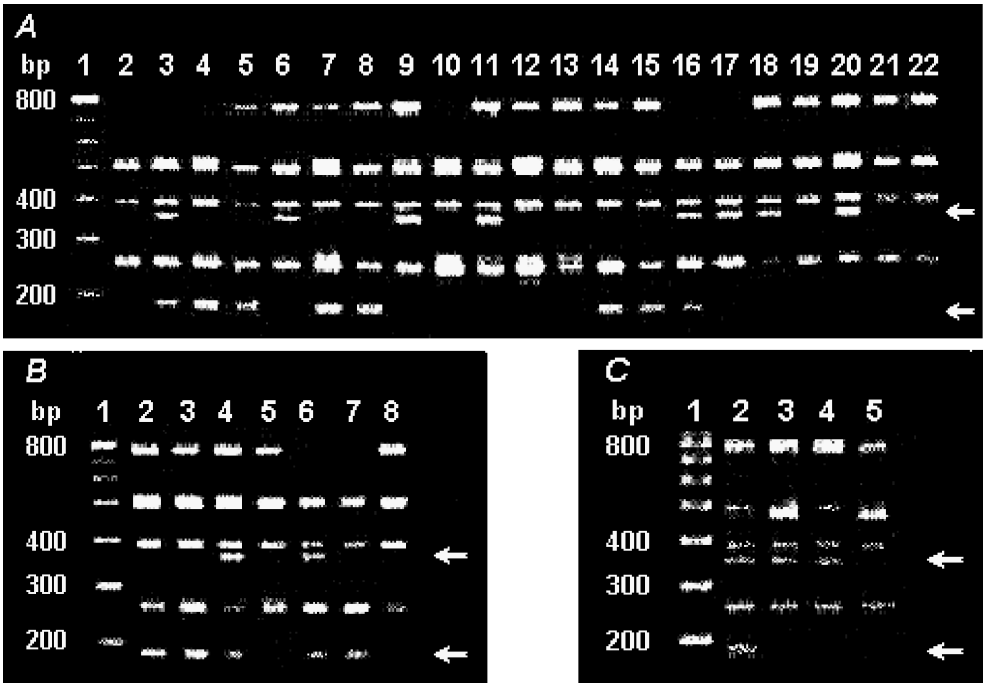

Fig. 1. RAPD bands of 32 SSIs with Operon 10-mer primer of OPC-07 and OPC-08 as the primer pair. Each lane 1 is the size marker (100-bp ladder); lane 2~22 in *A*, lane 2~8 in *B*, and lane 2~5 in *C* are SSIs. *Arrows* indicate the RAPD markers, CgCh182 and CgCh340.

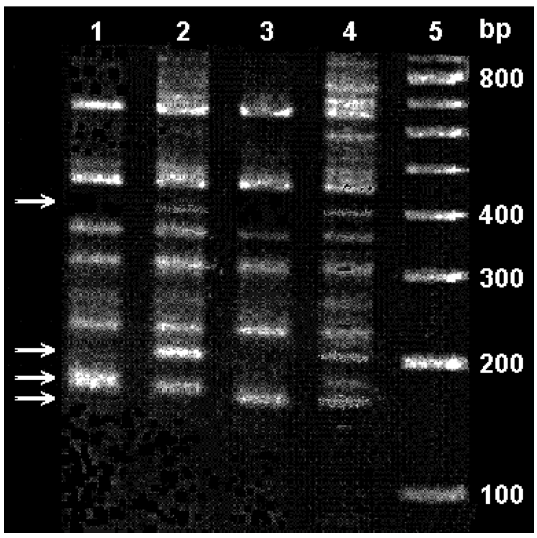

Fig. 2. RAPD bands of pooled samples of SSIs with the primer pair of OPB-05 and OPC-05. Land 1~4 are pools with mating types of A1B1, A1B2, A2B1 and A2B2, respectively; lane 5 is the size marker (100-bp ladder). *Arrows* indicate the bulked segregant analysis-based RAPD markers, BeCe160, BeCe170, BeCe210, and BeCe400.

Linkage analysis and linkage map construction RAPD with primers of short oligonucleotides enhanced the development of the genetic linkage map. A total of 91 polymorphic bands were identified; 78 had the 1 : 1 segregation ratio at the 0.05 level of significance. Of the 78 markers that were analyzed, 62 were mapped to 14 linkage groups by MAPMAKER/MAP. Two markers were considered linked when the Lod score between them was larger than 3.00. Relative positions of these markers were estimated by maximum likelihood. The remaining 16 RAPD markers were difficult to score with confidence, and were not included on the map. In most cases, the separate RAPD markers that were amplified with the same primer pair assorted independently. Sometimes, however, these fragments demonstrated linkage or even tight linkage.

Once a genetic map is constructed, some traits can be extensively characterized and inheritance patterns can be determined. In this study, the following strategies were employed to map the mating type factors, *priA* gene, MAT fragment, and the growth rate loci of *L. edodes*. The RAPD linkage map facilitated the mapping of these genetic loci.

Mapping of mating type loci Mating type genes are essential to the cultivation of *L. edodes*, because the breeding of this mushroom is always limited by the incompatibility of its mating system (Chang & Miles, 1991). *L. edodes* has a bifactorial-incompatibility mating system that is mediated by two phenotypic, loci A and B. This situation is similar to that of *Schizophyllum commune* in that it has a multiallelic-tetrapolar mating system, where the allelic forms of the incompatibility locus A are passed on to sexual progenies independently from those of the incompatibility locus B (Chang & Miles, 1991).

The tetrapolar incompatibility of *L. edodes* was confirmed in mating tests, because only the mating system with two incompatibility factors could give the four different mating patterns -A1B1, A2B2, A1B2 and A2B1- that are found within SSIs of L-54. From the mating types of 32 SSIs that were used in the RAPD amplification, the mating types loci A and B were analyzed by the MAPMAKER/MAP program with the 62 RAPD markers on the linkage map, and localized on linkage groups III and II, respectively (see Fig. 4).

Mapping of markers linked to mating-type genes RAPD amplification of the 4 pooled DNA samples generated 11 bulked-segregant analysis-based RAPD markers. Of the 11 markers that were analyzed by RAPD amplification of 32 SSIs, 3 were rejected by the segregation data analysis. Eight markers were confirmed to link to mating-type loci by a MAPMAKER/MAP linkage analysis; 3 of them were new markers that were not obtained by the RAPD analysis of P1 and P2. Four SSIs per pool was a practical and efficient pool size. Figure 2 is an example of the RAPD amplification of the pooled DNAs with the primer pairs OPB-05 and OPC-05, where 4 polymorphic bands were produced. BeCe160 and BeCe400 were previously observed in the first round of the RAPD amplification. BeCe170 was a new marker that was confirmed to link to the mating-type locus A, but BeCe210 was rejected.

A bulked-segregant analysis consolidated the linkage groups II and III by identifying the markers at the positions around the mating type loci. About 90% of the 11 linked-bulked-segregant analysis-based markers had a distance of less than 10.0 cM away from the mating types locus A or B, and 40% were closely linked (map distance <5.0 cM).

Mapping of the priA gene and MAT fragment Since the mapping of known genes or sequenced DNA fragments facilitates the identification of candidate genes or DNA fragments for mutations, a strategy for mapping genes or DNA fragments was developed using PCR-SSCP. With DNAs from strains L-54, P1 and P2, and 32 SSIs as the templates, and designed upper and lower primers (from published sequences) of *priA* and MAT as the primer pairs, the target sequences were amplified by PCR, and analyzed by SSCP. Figure 3 shows the MAT polymorphism by a SSCP analysis with SSIs of L-54 as the inherited progeny.

The first cloned gene of *L. edodes*, *priA*, may play a role at the beginning of fruiting (Kajiwara *et al.*, 1992). Primordia contain higher levels of the *priA* transcript than preprimordial mycelia and mature-fruiting bodies. A 302-bp fragment of the *priA* gene was amplified by PCR and analyzed by SSCP with 32 SSIs as the inherited progeny. The segregation data from SSCP were tested by chi-square calculation ($\chi^2 = 0.5$). Through the likelihood estimation by MAPMAKER/MAP,

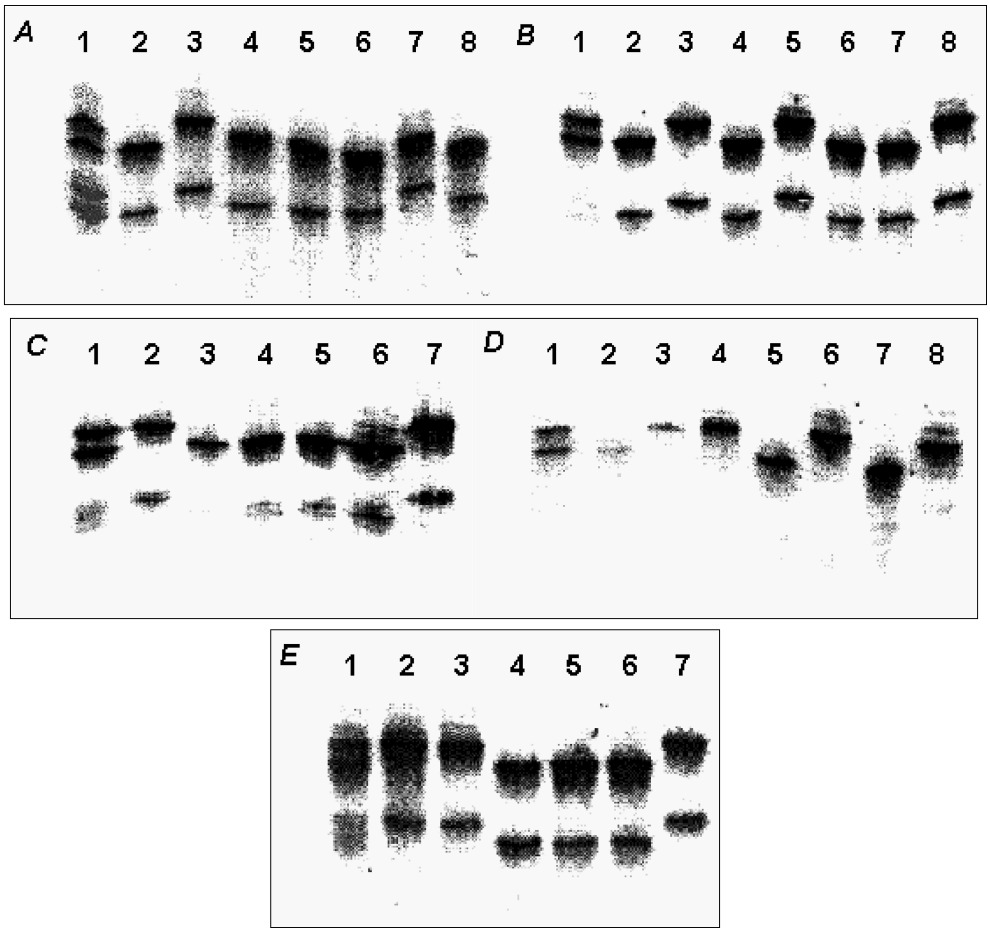

Fig. 3. Detection of MAT polymorphism by PCR-SSCP analysis in SSIs of L-54. Each lane 1 is L-54; lane 2 & 3 in *A* are P1 & P2, respectively; the rest are SSIs.

priA locus was localized on the linkage group III of the linkage map of *L. edodes* at the position of 23.1 cM from AiCi500 (see Fig. 4).

The MAT fragment is a differentially-expressed cDNA that is amplified by RNA arbitrarily-primed PCR (RAP) from the RNAs of vegetative mycelia, primordia, as well as young and mature fruiting bodies of the strain L-54 (Leung *et al*., 2000). A 200-bp of the MAT fragment was picked for a PCR-SSCP analysis. MAT locus was mapped on the linkage group IX at the position of 12.2 cM from AtBt210 (see Fig. 4).

Genetic map of *L. edodes* strain L-54 The genetic map of the *L. edodes* strain L-54 with 14 linkage groups is summarized in Fig. 4. The map included 62 RAPD markers, 3 bulked-segregant analysis-based RAPD markers, 2 phenotypic loci (mating type factors A and B), 1 known gene locus (*priA*), and 1 sequenced DNA fragment locus (MAT). Linkage groups ranged in length from 0.1 to 122.6 cM with a total length for the map of 622.4 cM. The distance between the adjacent markers ranged from 0.0 to 30.3 cM with an average of 9.0 cM.

It was reported that with a contour-clamped homogeneous electric field (CHEF) gel electrophoresis, the chromosome-sized DNA was separated into 8 bands in *L. edodes* (Arima & Morinaga, 1993). The genetic map of *L. edodes* in this study contained 14 linkage groups, which was 6 more than the number of haploid chromosomes. The deviation was probably due to some linkage groups that could be linked together when more SSIs were analyzed and more genetic markers were generated.

Based on the consideration of the unfilled gaps, the total length of the *L. edodes* genome (in cM) can be estimated (Postlethwait *et al.* 1994). The unfilled gap distance from the two end markers on each chromosome to their adjacent telomeres is estimated to be **2 telomeres per chromosome** $\times$ $d/2 \times C$, where C is the number of chromosomes, d is the average interval, and $d/2$ indicates an average distance from the end marker to the telomere. The distance for other unfilled gaps is estimated to be $(L - C + N) \times d_m$, where N is the number of unlinked markers, L is the number of linkage groups, and d_m is the maximum interval that is accepted in the map. Therefore, the estimate of the complete map size of *L. edodes* is about $622.4 + (8 \times 2 \times 9.0/2) + (14 - 8 + 16) \times 30.3$, or 1,360 cM.

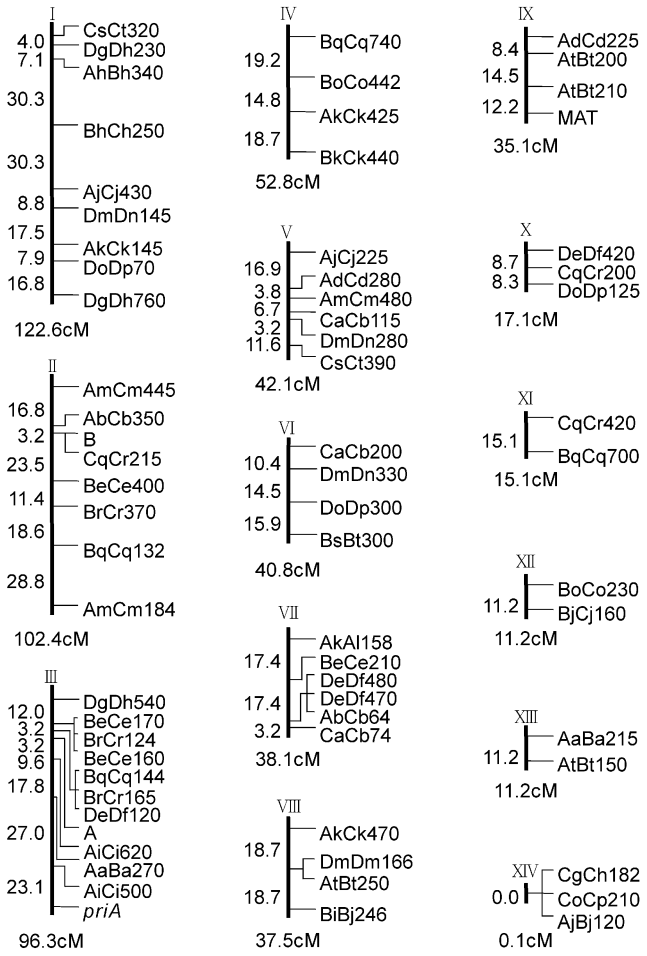

Fig. 4. Genetic map of *Lentinula edodes* strain L-54 with 14 linkage groups, including 62 RAPD markers, 3 bulked segregant analysis-based RAPD markers, 2 phenotypic loci (mating type factors A and B), 1 known gene locus (*priA*) and 1 sequenced DNA fragment locus (MAT). Roman numerals at the top are numbers of linkage groups. Names indicated on the right side are markers of each locus. Distances indicated on the left side are estimated percent recombination between the markers (in cM), which are depicted as the maximum-likelihood solutions provided by MAPMAKER/MAP (version 3.0b).

Another approach to estimate the size of a complete map is based on a partial-linkage map and consideration of the marker density (Hulbert *et al*., 1988). Supposing the complete map length is G (in cM), let $P(T, G)$ denote the probability that the Lod score for the random pair of markers (Z) will not be less than the Lod score threshold ($\geq T$), and let N denote the number of markers that are genotyped. Then the expected number of marker pairs that have $Z \geq T$ is $C_N^2 P(T, G) = [(1/2)N(N\text{-}1)]P(T, G)$. If the actual number of such pairs is K, $P(T, G) = K/[(1/2)N(N\text{-}1)]$, then $P(T, G)$ can be given by the chance that a randomly-chosen pair of loci lies within X cM, $P(T, G) = 2X/G$, where $X = X(T)$ is the distance between the two markers for which the expected Lod score is T. Accordingly, the estimate of the genome size is given by $G = N(N\text{-}1)X/K$. In this study, if X is ≤ 18 cM (T = 3.00), then the estimate G is 1,200 cM. If X is ≤ 24 cM (T = 2.00), then G is estimated to be 1,000 cM. These estimates are consistent with that based on the consideration of the unfilled gaps. The length of the complete map on average is given by 1,200 cM, which suggests that the current genetic map covers more than 50% of the whole genome of *L. edodes*.

The utility of a genetic map is related to its degree of completeness. Assuming that the N markers are randomly distributed in a genome the size of G cM, if there is at least one marker that lies within d cM of the genome, then each marker will sweep an area of $2d$ cM; therefore, the N markers provide $2Nd/G$-fold coverage of the genome, ignoring end effects (Jacob *et al*., 1991). So, the proportion of the genome within d cM of at least one marker is approximately $1 - \exp(-2Nd/G)$. As the genetic length of *L. edodes* is estimated to be 1,280 cM, it is expected that about 86% of the genome should be located within 20 cM of a marker, about 77% should be located within 15 cM of a marker, and 62% should be within 10 cM of a marker. The average distance between the adjacent markers is 9.0 cM, the fact that 29 of the 91 RAPD markers remain unlinked to any other marker supports this expectation.

The average map distance of one cM in kb of DNA is usually estimated by comparing the size of a physical map (in kb) to the length of its genetic map (in cM). Because the karyotype analysis showed that the total genome size of *L. edodes* was about 33 Mb (Arima & Morinaga, 1993), then this estimate of the genetic map length gives an average of 27 kb per cM. As references, in fungi, one cM represents 25 kb in *Bremia lactucae* (Hulbert *et al*., 1988), 23 kb in *Cochliobolus heterostrophus* (Tzeng *et al*., 1992), and 40 kb in *Magnaporthe grisea* (Skinner *et al*., 1993).

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