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Research Paper

# Construction of a genetic linkage map of *Lentinula edodes* based on SSR, SRAP and TRAP markers

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Genetic mapping is a basic tool for eukaryotic genomic research. It allows the localization of genes or quantitative trait loci (QTLs) and map-based cloning. In this study, we constructed a linkage map based on DNA samples from a commercial strain L808, including two parental monokaryons and 93 single spore isolates considered with segregating to 1:1:1:1 at four mating types ($A_1B_1$, $A_1B_2$, $A_2B_1$ and $A_2B_2$). Using Simple Sequence Repeats (SSR), Sequence Related Amplified Polymorphism (SRAP), Target Region Amplified Polymorphism (TRAP) molecular markers, 182 molecular markers and two mating factors were located on 11 linkage groups (LGs). The total length of the map was 948.083 centimorgan (cM), with an average marker interval distance of 4.817 cM. Only two gaps spanning more than 20 cM was observed. The probability of 20 cM, 10 cM, 5 cM genetic distance cover one marker was 99.68%, 94.36%, 76.43% in our genetic linkage map, respectively. This is the first linkage map of *Lentinula edodes* using SSR markers, which provides essential information for quantitative trait analyses and improvement of genome assembly.

**Key Words:** *Lentinula edodes*, genetic linkage map, SSR.

## Introduction

*Lentinula edodes* (Berk.) Pegler, Shiitake, is the second highest yield edible mushroom in the world, especially in China and Japan (Bruhn *et al.* 2009, Chang 1999). In China, the output has reached 90% of the total output in the world. Apart from its importance as an edible fungus, it also contains many medicinal compounds, such as the lentinan, the cortinellin and eritadenine which has hypocholesterolemic activity (Aoyagi *et al.* 1980, 1982a, 1982b, Shimada *et al.* 2003, Wasser 2002).

Many varieties of *L. edodes* have been developed in the world, breeding of *L. edodes* has progressed as a result of mating between superior varieties and selection from many varieties. The life style of *L. edodes* can be considered typical of many other mushroom species. The haploid sexual spores germinate to form monokaryotic mycelia. And two monokaryons which have compatible mating types combined and leads to the formation and growth of a sexually competent dikaryon. When, in response to environmental stimuli, the dikaryon develops into the characteristic fruit body, nuclear fusion finally occurs, but it is immediately followed by meiosis and sporulation (Brown and Casselton 2001). *L. edodes* is a typical tetrapolar heterothallic basidiomycete. Its mating system is controlled by two mating type factors (*Mat A* and *Mat B*). *Mat A* controls the pairing of nucleus and the formation of clamp connection. *Mat B* controls the migration of nucleus and the fuse of clamp connection. Only two monokaryons with different mating alleles at both the A and B mating-type loci can fuse and generate a dikaryon (Foulongne-Oriol 2012, Murakami and Takemaru 1975, Takemaru 1961, Tokimoto *et al.* 1973). In the subsequent meiosis, the fruiting body generates sexual spores of four kinds of mating types: AxBx, AxBy, AyBx and AyBy. However, it was reported that not all kinds of mating types are existing in the basidiospores of some strains and distorted segregation may also appear (Raper *et al.* 1958).

A genetic linkage map is a representation of the genome that can show the relative positions and distances between markers or genes along the chromosomes as determined

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by their recombination frequency (Doganlar *et al.* 2002, Muraguchi *et al.* 2003). The closer the two genes are on a chromosome, the lower the chances are that crossing over will occur between them. Thus, how often those two markers are inherited together in a population after sexual reproduction reflects their linkage. The genetic linkage analysis allows clarifying the meiotic behavior and genome structures in various species of fungi and provides genetic markers that are useful for quantitative trait loci (QTLs), cross-breeding and map-based cloning of genes of interest (Farman 2001, Lukowitz *et al.* 2000).

The application of DNA markers enables to achieve genotype identification and molecular tagging for gene isolation as well as to identify various agronomic traits (Kerrigan *et al.* 1993, Williams *et al.* 1990). The usefulness of a genetic linkage map depends on the choice and number of polymorphic markers used. For *L. edodes*, several molecular genetic maps have been reported previously and different types of molecular markers are utilized in their experiment (**Table 1**). The first molecular genetic map constructed by Kwan and Xu (2002) was based on random amplified polymorphic DNA (RAPD) and spanned 622.4 cM. Amplified fragment length polymorphism (AFLP) was also used to construct a linkage map by Terashimia *et al.* (2002). Furthermore, Miyazaki *et al.* (2008) generated a linkage map by tetrad analysis in 2008. In 2014, Gong *et al.* (2014) constructed another linkage map by using SRAP, TRAP and Insertion-deletion (INDEL) markers.

Previously we constructed a moderately dense map based on SSR, SRAP and TRAP markers. SSR markers have the advantages as codominant, abundant throughout genomes and highly polymorphic, which are the most popular and versatile markers for a wide range of application in ecology, biology, and genetics presently (Foulongne-Oriol *et al.* 2010, Goodwin *et al.* 2007, Kaye *et al.* 2003, Manzo-Sánchez *et al.* 2008). SSR markers can also be easily detected by polymerase chain reaction (PCR) and gel electrophoresis (Varshney *et al.* 2005), so they have been widely applied in many fungi for verification of cultivar identity, diversity studies (Ren *et al.* 2014, Smith *et al.* 2000, Tang *et al.* 2007), linkage map construction (Shirasawa *et al.* 2012), and QTL analysis (Christians *et al.* 2011, Shen *et al.* 2005). SSR markers are classified into genomic SSR markers and expressed sequence tag-SSR (EST-SSR) markers depending on the origin of the sequences used for the initial identification of these markers. As EST-SSR markers result in multiple sets of markers at the same locus, genomic SSR markers are more useful to construct the genetic map (Wang *et al.* 2010). However, construction of high-density genetic maps requires genotyping a large population at a large number of loci (Akhunov *et al.* 2009). Dominant markers, such as SRAP and TRAP are not as efficient as SSR for comparative mapping, but they are able to enhance the density of the maps. SRAP generates PCR-based markers targeting open reading frames (ORFs), which combines simplicity, reliability, moderate throughput ratio and facilitate sequencing of selected bands. Further, it targets coding sequences in the genome and results in a moderate number of co-dominant markers (Li and Quiros 2001). TRAP markers are improved from SRAP, using known partial sequence of a candidate gene as a fix primer (Hu and Vick 2003). The application of these three kinds of markers has efficiently and cost-effectively generated a massive amount of credible genetics sequence data.

In the present study, we employed a monokaryotic population including two parental monokaryons and 93 single spore isolates (SSIs) of L808 to construct a moderately dense map of *L. edodes* and mapped 184 loci including two mating type factors, 96 SSR markers, 12 SRAP markers, and 74 TRAP markers to 11 linkage groups. To our knowledge, this is the first genetic map of *L. edodes* constructed based on SSR markers.

## Materials and Methods

### *Mapping population*

Strain L808 (Accession No.: 2008009) stored at Shanghai Academy of Agricultural Science was selected as the

**Table 1.** Present status of *L. edodes* linkage maps

| No. | Map population | Main markers | No. of markers | Total length (cM) | No. of linkage groups | Marker spacing (cM) | Reference |
|---|---|---|---|---|---|---|---|
| 1 | Two protoplasted monokapyons and 32 sporulated monokaryons of L54 | RAPD | 62 | 622.4 | 14 | 9.0 | Kwan *et al.* 2002 |
| 2 | | Markers based on low-coverage resequence | 200 | 637.1 | 13 | 3.4 | Au *et al.* 2013 |
| 3 | 95 basidiospore progeny generated by a single cross between the A567-S8 strain from Japan and the NZ 1569-S3 strain from New Zealand | AFLP | 200 | 1956.7 | 11 | 9.5 | Terashima *et al.* 2002 |
| | | AFLP-H, AFLP-S | 166 | 1398.4 | 11 | 8.4 | Terashima *et al.* 2006 |
| 4 | 23 tetrads (92 basidiospores) generated by a cross between monokaryon strains D703PP-9 and G408PP-4 | RAPD, EST, SCAR | 289 | 908.8 | 11 | 3.1 | Miyazaki *et al.* 2008 |
| 5 | 171 dikaryons derived from randomly mating individuals of the full-sib monokaryons of L205-6 and W1-26 | SRAP, TRAP, Indel | 459 | 989.7 | 15 | 2.2 | Gong *et al.* 2014 |

parental strain. Experimental material was two parental monokaryons 808A and 808B (mating type: $A_1B_1$ and $A_2B_2$) of L808 that had compatible mating types and 93 SSIs was generated by using the method of monokaryon isolation and regeneration. Mating-types of the SSIs were determined as previously reported (Darmono and Burdsall 1992, Li *et al.* 2007). The tested materials were cultivated in potato dextrose broth (PDB) medium at 25°C for 15–20 days with shaking at 200 rpm. The collected mycelia were freeze-dried and stored at –20°C.

### *DNA extraction*

DNA samples were extracted from the mycelia of the two parental monokaryons and 93 SSIs according to the modified cetyltrimethylammonium bromide (CTAB) procedure (Kuhad *et al.* 2004, Murray and Thompson 1980). The concentration and purity of the DNA solution were assayed after RNase treatment based on the spectrophotometric absorbance and the ratio of OD260: OD280. DNA was then diluted to 100 ng/μL.

### *SSR marker development and PCR amplification*

595 pairs of SSR markers were designed based on the whole genome sequencing of *L. edodes* (unpublished data of our own lab). Markers were screened by SSR amplification of DNAs of two parental monokaryons. We selected sequence-specific primers for direct PCR amplification of polymorphisms in the population (strains) selected for mapping. Each reaction mixture consisted of 1 μL 10 × PCR buffer, 1 μL $MgCl_2$ (25 mmol/L), 0.2 μL dNTP mix (2.5 mmol/L for each), 0.1 μL *Taq* polymerase (5 U/μL) (Promega, USA), 1 μL genomic DNA (100 ng/μL), 1 μL forward primers (10 μmol/L) and 1 μL reverse primers (10 μmol/L). The total volume of each reaction mixture was 10 μL. The PCR amplifications were carried out in a Mycyler Thermal Cycler (Bio-Rad, USA) under the following conditions: 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, then was subjected to a elongation at 72°C for 7 min and finally cooling to 4°C.

### *SRAP and TRAP marker development and PCR amplification*

Referring to the experimental results of Wen-bing Gong, we synthesized 56 SRAP markers and 111 TRAP markers (Gong *et al.* 2014). Markers with considerable polymorphism were selected for use on follow-up subsequent amplification test. In the SRAP and TRAP analysis, the PCR reaction mixture and the amplification program used were similar to that for the SSR analysis except for some details. The specific reaction system is listed in **Table 2**. Conditions of the amplification system were as follows: 94°C for 5 min, 5 cycles (at 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min), then 35 cycles (at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min), finally was the elongation at 72°C for 7 min and subsequent cooling to 4°C.

**Table 2.** Reaction system of SRAP and TRAP

| Reactants | SRAP | TRAP |
|---|---|---|
| 10 × PCR buffer (without $Mg^{2+}$) | 2 μL | 2 μL |
| 25 mmol/L $MgCl_2$ | 1.6 μL | 1.6 μL |
| 2.5 mmol/L dNTP | 0.6 μL | 0.6 μL |
| 5 U/μL Taq polymerase | 0.35 μL | 0.35 μL |
| 10 μmol/L forward primer | 0.8 μL | 1.25 μL |
| 10 μmol/L Reverse primer | 0.8 μL | 0.3 μL |
| 100 ng/μL DNA | 1 μL | 1 μL |
| $ddH_2O$ | 12.85 μL | 13.2 μL |
| Total volume | 20 μL | 20 μL |

### *Detection of SSR, SRAP and TRAP markers*

The PCR products mixed with 6 μL Loading buffer were denatured at 95°C for 10 min, and then cooled on ice. The denatured products were detected by electrophoresis on 6% polyacrylamide gel and stained with silver nitrate solution for 10 min, then developing color using sodium hydroxide solution.

### *Linkage constructions and analysis*

At each locus, a genotype identical to 808A was marked as "a", the same to 808B was marked as "b". And the uncertain ones are marked as "–". All markers were evaluated by the chic-square test and those showed significant deviation were not used for the subsequent analysis. Linkage analysis was performed using JOINMAP version 4.0 with a likelihood of odds (LOD) score of 2.0–10.0 and data of the mapping population were coded by the double haploid (DH) model (Van Ooijen and Voorrips 2001).

We used two methods to calculate the length of the linkage map. For the first method, twice of the whole map's average interval add to the length of each LG equal to the total length of the linkage map (Fishman *et al.* 2001, Labbé *et al.* 2008). For the second method, the length of each LG multiplied by (m + 1)/(m – 1) is the total length, m is the number of markers on each LG (Chakravarti *et al.* 1991, Martin *et al.* 2008). We took the average as the terminal length of the linkage map. The theoretical map coverage was estimated using the equation $c = 1 - e^{-2dn/G}$ (c is the proportion of the marker coverage, n is the number of markers, G is the estimated length of the linkage map (Ma *et al.* 2009, Remington *et al.* 1999).

## Results

### *Development of SSR, SRAP and TRAP markers*

We assayed 595 pairs of SSR primers for selective amplification by comparing SSR profiles generated from two parents of the mapping population used in this study, and those showing clear polymorphic bands between the two were selected. 103 pairs were selected and used for the entire population (**Supplemental Table 1**). For SRAP analysis, we performed with 56 (7 × 8) primer combinations and 7 out of 56 primers were chosen for polymorphism detection, each primer pair generated 2–15 polymorphic bands on

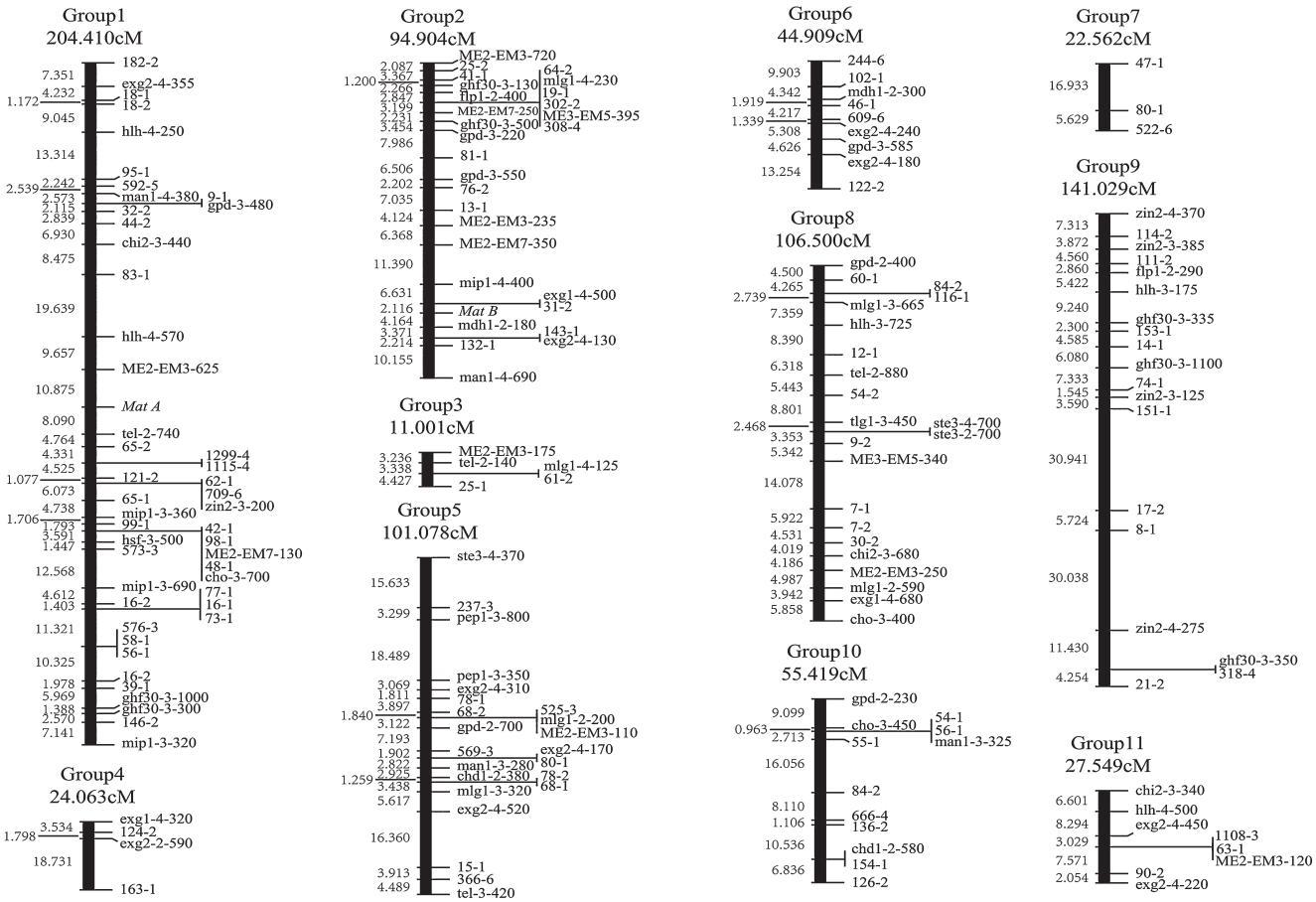


**Fig. 1.** Genetic linkage map of *L. edodes* L808 based on 182 molecular markers and two mating type factors. The linkage group numbers are indicated on top. Distances (cM) between markers are shown on the left side and the names of the markers are shown on the right side. SSR markers are named by the primer number, followed by the batch number of the primer synthesis (e.g. 182-2); SRAP and TRAP markers are named by the forward and reverse primer, followed by the band size (e.g. ME2-EM3-625, hlh-4-450).

average (**Supplemental Table 2**). 33 out of 111 (37 × 3) TRAP markers were confirmed to be polymorphic in the mapping population and the number of polymorphic TRAP markers amplified per primer pair varied from one to eight in this study (**Supplemental Table 3**).

### *Linkage map constructions and analysis*

After the segregation analysis of the polymorphic markers generated in the mapping population, we selected 203 markers including two mating factors (*Mat A* and *Mat B*), 103 SSR markers, 12 SRAP markers and 86 TRAP markers to construct the linkage map (**Fig. 1**). Among the remaining 203 markers, 182 showed segregation patterns fitting the expected segregation ratio at $P < 0.05$, the 182 markers together with the two mating factors were assigned to 11 LGs, the LG length varied between 11.001 cM (LG3) and 204.410 cM (LG1), with an average LG length of 75.766 cM. The number of markers used to construct the linkage groups ranged from 3 (LG7) to 49 (LG1). *Mat A* and *Mat B* were mapped on LG1 and LG2 respectively.

The average marker interval distance was s = 833.424 cM/(184 – 11) = 4.817 cM according to the approach of Fishman. For the calculate of the total length we used two methods and took the average between $L_1$ and $L_2$ ($L_1$ = 833.424 + 2 × 11 × 4.817 = 939.398 cM, $L_2$ = 956.767 cM), the estimated total length was found to be 948.083 cM. The average marker interval ranged from 2.200 cM (LG3) to 7.423 cM (LG9), with the minimum and maximum marker interval being 0.0 cM and 30.941 cM, respectively (**Table 3**). Based on our current linkage map and the genetic length of

**Table 3.** Characteristics of the linkage groups

| Linkage group | Length (cM) | No. of markers | Average marker spacing (cM) | Largest interval (cM) |
|---|---|---|---|---|
| LG1 | 204.410 | 49 | 4.172 | 19.639 |
| LG2 | 94.904 | 30 | 3.163 | 11.390 |
| LG3 | 11.001 | 5 | 2.200 | 4.427 |
| LG4 | 24.063 | 4 | 6.016 | 18.731 |
| LG5 | 101.078 | 23 | 4.395 | 18.489 |
| LG6 | 44.909 | 9 | 4.990 | 13.254 |
| LG7 | 22.562 | 3 | 7.521 | 16.933 |
| LG8 | 106.500 | 22 | 4.841 | 14.078 |
| LG9 | 141.029 | 19 | 7.423 | 30.941 |
| LG10 | 55.419 | 12 | 4.618 | 16.056 |
| LG11 | 27.549 | 8 | 3.444 | 8.294 |

*L. edodes* estimated by Kwan and Xu (2002), it was expected that about 99.68% of the genome should be located within 20 cM of a marker, about 94.36% should be located within 10 cM of a marker, and 76.43% should be within 5 cM of a marker.

## Discussion

The first requirement to perform linkage mapping is to have a segregating population. It is tightly linked to the primary goal of the mapping project (Young *et al.* 2001). Two parental monokaryons of L808 are distantly related and exhibit contrasted behavior for the traits of interest according to the previous laboratory result. It can thus be expected that their progeny will have a large number of polymorphic markers to be detected by mapping. Besides, population size has an appreciable impact on the accuracy of the linkage map, there is a balance between precision and population size. Although the genetic map will be more accurate using more single spore isolates as the mapping population, the population size rarely exceeds a hundred for practical reasons (Ferreira *et al.* 2006). Therefore, we chose two parental monokaryons and 93 basidiospore progeny as the mapping population which can be considered to be an ideal material for further mapping construction.

The second step in the construction of the linkage map is to identify polymorphic markers that will be used for genotyping the entire mapping population. In this study, we used SSR, SRAP and TRAP together with mating type factors to construct a medium-density linkage map. These molecular markers are all popular and efficient as valuable tools for genotyping of mapping population, which makes a good foundation for quantitative trait loci (QTLs) mapping, the genetic map-based cloning and molecular marker-assisted breeding. In the present report, we used the most SSR markers based on the whole-genome sequencing for genetic linkage mapping in *L. edodes*. Compared to anonymous markers, these simple, stable and gene-targeted SSR markers provide ample opportunities to a high-precision linkage map and can make a horizontal comparison with other macro fungi. We designed 595 pairs of SSR primers which are widely and evenly distributed based on the whole genome sequences and 96 pairs are identified. The polymorphism ratio (16.13%) is lower than the expected. Apart from SSR markers, SRAP and TRAP were also used in this study, which are highly efficient in generating a large number of markers, 2 to 15 markers were produced per primer pair. The polymorphism ratio of SRAP was 12.5% and TRAP was 29.7%. The application of SRAP and TRAP markers greatly improved both the marker density and the total coverage of the genetic map in *L. edodes*. The above results indicate that the polymorphism ratio is relatively low, this may be related to the genetic distance of the two nuclei, the screening conditions of the bands and the electrophoresis conditions.

The number of DNA markers on *L. edodes* linkage maps ranges from tens to several hundreds. The expected density of a linkage map depends obviously not only on the number of the molecular markers but also on the main goal of the mapping project. A comprehensive genetic map covering all chromosomes evenly is essential for effective QTL mapping, genome characterization, or marker-assisted breeding. For example, it has been demonstrated that the power of QTL detection and genetic effect estimates are not affected by an increase of marker density beyond 10 cM (Piepho 2000). The average marker interval distance of this study was 4.817 cM and showed a high precision, making it possible for the use of the present results in the follow-up studies.

It is known that lumps containing highly homologous sequences are scattered on the genome of *L. edodes*. It is considered that such scattered, highly homologous sequences bias the ratio. In other words, it is highly likelihood that markers which exist in different places are handled as the same locus, which may lead to lower accuracy of the linkage map. To minimum false linkage, we removed the markers showing a ratio different from the predicted ratio for linkage mapping.

Of the 203 markers that were analyzed, 184 were mapped to 11 linkage groups and spanned 948.083 cM, with an average marker distance of 4.817 cM, shorter than those reported genetic maps of Kwan and Xu (2002) and Terashima *et al.* (2002). The largest interval between two adjacent markers were found to be 30.038 cM and 30.941 cM on LG9, and no other intervals larger than 20 cM were found. The markers are evenly-distributed on the map, this could be due to the use of SSR markers which covers the whole genome. In terms of total length, the map in this study (948.083 cM) was closer to that constructed by Miyazaki *et al.* (2008) (908.8 cM) and Gong *et al.* (2014) (989.7 cM), longer than that of the RAPD-based map by Kwan and Xu (2002) (622.4 cM), but shorter than those of other two maps by Terashima *et al.* (2002).

The linkage map constructed in this study consists of 11 linkage groups, similar to the consequences as reported by Terashimia *et al.* (2002) and Miyakazi *et al.* (2008). In the previous reports of Kwan and Xu (2002), 14 linkage groups were revealed. According to the light microscopic observations and electrophoretic karyotype analysis, *L. edodes* was suggested that have eight chromosomes (Arima 1993), the deviation was probably may due to the analysis parameters set and the joining of some linkage groups. It is also possible to underestimate the number of chromosomes if some chromosomes are small. Therefore, whether *L. edodes* has eight or more chromosomes remains unclear and more research is needed.

Mating type factors are essential to the cultivation of *L. edodes*, as the breeding of this mushroom is always limited by the incompatibility of its mating system. The *Mat A* and *Mat B* loci were found to be located on relatively large linkage groups LG1 and LG2, respectively, in our analysis as also reported by Terashima *et al.* (2002), Kwan and Xu (2002). LG1 and LG2 in our map corresponded respectively

to LG2 and LG5 in the map constructed by Terashima *et al.* (2002).

Genetic linkage maps provide foundation for studying genome structure and organization and are highly valuable tools to identify the location of loci controlling important traits of interest. In our present study, a high-quality genetic map of *L. edodes* was constructed and as a whole it was roughly the same as the other genetic maps published about *L. edodes*. Specific features of our linkage map are as follows: (1) The markers are evenly distributed on the map and only two intervals larger than 20 cM was found. (2) The SSR markers on this map will provide bridges for comparative mapping as well as be a direct link to the whole-genome sequence of *L. edodes*. Previously, the most detailed linkage maps of L. *edodes* have been constructed by Gong *et al.* (2014) with SRAP, TRAP and INDEL markers, such markers are useful for genetic linkage map construction. However, they have the disadvantage of not being transferable to other populations, which greatly limits their application. The reason to construct this map was to create a more saturated and anchored linkage map for *L. edodes*, using the newly developed SSR markers. The high information content and transferability of SSRs are decisive for comparative alignment. Additionally, because SSRs are sequence-tagged markers, integration with linkage maps constructed by other laboratories or physical maps in feasible. In summary, this study contributes to our understanding of *L. edodes* genome organization and lays a foundation for establishing efficient genetic and breeding programs in *L. edodes*.

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