FULL PAPER

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Construction of a linkage map of *Lentinula edodes* (shiitake) with the HEGS (high-efficiency genome scanning) system: use of versatile AFLP and PCR-based gene markers

Abstract An improved linkage map of *Lentinula edodes* (shiitake) was constructed with an HEGS (high-efficiency genome scanning) system. Two hundred twenty-one HEGS-derived amplified fragment length polymorphism (AFLP-H) markers and 21 gene markers were developed and combined with 203 previously developed sequencer-derived AFLP markers (AFLP-S markers) and 3 mating factor loci (*A*, *B*α, and *B*β) to construct a comprehensive linkage analysis. As a result, a novel linkage map with 166 markers including 2 mating factors (*A* and *B*), 10 HEGS-derived gene markers, 72 AFLP-H markers, and 82 AFLP-S markers was obtained. Of the total 448 markers, 273 could not be located on a linear map and thus were assigned to linkage groups as accessory markers. The map covers a total length of 1398.4 centimorgans (cM) with an average marker interval distance of 8.4 cM. The map consists of 11 linkage groups (LGs) in agreement with our previous map, and 7 LGs among them were found to contain branched linkages, which may be the result of reciprocal translocations representing dynamic reorganization of the shiitake genome. The previously reported linkage map was improved in terms of number of markers, marker density, linear order of markers, and total map length.

Introduction

Lentinula edodes (Berk.) Pegler, shiitake, is one of the most popular edible mushrooms in east Asia and recently also in Western countries. Genetic improvement of cultivated strains for this mushroom has, therefore, been pursued for more than 50 years (Hashioka et al. 1961; Hasebe 1991; Tokimoto and Komatsu 1995). In addition to its importance as an edible mushroom, *L. edodes* has been found to contain medicinal compounds (Shimada et al. 2002; Wasser 2002), and trials on breeding for specific medicinal or dietary qualities of this fungus have recently begun.

It has been known that the fungal genome organization is unique in the existence of the chromosome length polymorphisms (CLPs) among isolates within species, which could be examined by electrophoresis of intact chromosomes, and their genomes are dynamically reorganized during meiosis (Muraguchi et al. 2003; Tzeng et al. 1992; for a review, see Zolan 1995). Although these cytogenetic studies have accumulated much information about the fungal genome organization, even the number of chromosomes of *L. edodes* has not yet been determined. It is inferred to be eight by examining basidia with light microscopy (Tanaka and Koga 1972; Nakai 1986). It is possible, however, by light microscopy to underestimate the number of chromosomes if some chromosomes are small. The electrophoretic karyotype of *L. edodes* was reported by Arima and Morinaga (1993), but it could not be clearly resolved because an adequate number of protoplasts (more than 10^8 protoplasts ml^{-1}), which is required for karyotype detection, could not be generated.

Genetic linkage analysis allows clarifying the meiotic behavior and genome structures in various species of fungi and plants (Tzeng et al. 1992; Harushima et al. 1998; Doganlar et al. 2002; Muraguchi et al. 2003) and provides

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genetic markers that are useful for efficient cross-breeding and for map-based cloning of genes of interest (Lukowitz et al. 2000; Farman 2001; Podlich et al. 2004). Four linkage maps with a low to medium number of genetic markers have been constructed for *L. edodes* using auxotropic markers (Hasebe 1991), isozyme markers (Bowden and Royse 1991), random amplified polymorphic DNA (RAPD) markers (Kwan and Xu 2002), or amplified fragment length polymorphism (AFLP) markers (Terashima et al. 2002), respectively. Hereafter, a precise linkage map of *L. edodes* should be constructed using genetic markers well suited for molecular genetics and molecular breeding in this fungus.

AFLP analysis was developed by Vos et al. (1995), and this technique allows reliable detection of numerous DNA polymorphisms. We have previously constructed a linkage map of *L. edodes* based on 203 AFLP markers and 3 mating factor loci (*A*, *B*a, and *B*β) (Terashima et al. 2002). These AFLP markers on the map were detected by using a DNA sequencer (ABI PRIZM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). However, these sequencer-derived AFLP markers could not be isolated for use in sequence and hybridization analyses. This drawback prevents researchers from using AFLP markers for variable application such as sequence-characterized amplified region (SCAR) markers and DNA probes for hybridization analysis. Kawasaki and Murakami (2000; reviewed in Kawasaki et al. 2003) have devised a simple and efficient electrophoresis system, high-efficiency genome scanning (HEGS), consisting of small- to medium-sized slab gels and a discontinuous Tris-glycine buffer, which can be used with silver or fluorescent staining. The use of this system permits direct isolation of DNA fragments from its gels and enables the fragments to be used for sequence and hybridization analyses. In addition, it was demonstrated that the HEGS system with AFLP analysis allows a single person to construct a linkage map in a short time at reasonable costs.

In *L. edodes*, several genes involved in development of the fruit body (Endo et al. 1994; Kajiwara et al. 1992; Leung et al. 2000), degradation of wood (Lee et al. 2001; Zhao and Kwan 1999), and signal transduction (Hori et al. 1991; Zhou et al. 1998, 2000) have been cloned and characterized. Mapping of gene markers is of special importance for comparison between the various maps of *L. edodes* or comparison with other fungal maps. In addition, the use of simple gene markers could allow rapid identification of genes responsible for quantitative trait loci (QTL) involved in agronomic and pharmacological traits in *L. edodes*.

In this study, we generated gene and AFLP markers by using the HEGS system (Kawasaki and Murakami 2000; Kawasaki et al. 2003). These genetic markers were very useful and efficient to construct a linkage map of *L. edodes*, along with the AFLP markers previously derived by sequencer. The previously reported linkage map was improved in terms of number of markers, marker density, linear order of markers, and total map length, and became more versatile by addition of the HEGS-derived gene and AFLP markers.

Materials and methods

Mapping population and culture conditions

A mapping population for constructing our previous linkage map of *L. edodes* (Terashima et al. 2002) was analyzed in this study. The A567-S8 strain from Japan and the NZ1569-S3 strain from New Zealand were used as parental strains to generate the mapping population. The mapping monokaryotic population was derived from a single cross between the parental strains.

AFLP markers

In this study, AFLP markers were detected by using the electrophoresis and detection system devised by Kawasaki and Murakami (2000) and described in Kawasaki et al. (2003) and Hori et al. (2003), whereas those in our previous map (Terashima et al. 2002) were detected with a DNA sequencer (ABI PRIZM 310 Genetic Analyzer; Applied Biosystems). The HEGS-derived and sequencer-derived AFLP markers were designated as AFLP-H and AFLP-S markers, respectively.

Polymerase chain reaction (PCR) amplification for AFLP analysis was performed using a procedure described by Vos et al. (1995) with a small modification (Terashima et al. 2002). Preselective amplification was performed with the E+0 (5′-GAC TGC GTA CCA ATT C-3′) and the M+0 (5′-GAT GAG TCC TGA GTA A-3′) primers. The reaction mixtures of preselective amplification were diluted 50 fold with TE [10mM Tris-HCl, ethylen-diamine-tetraacetic acid (EDTA) pH8.0] before selective amplification. Selective amplification was carried out using the E+2 and the M+3 primers. The E+2 and the M+3 primers had two and three arbitrary nucleotide extensions of the E+0 and the M+0 primers, respectively.

HEGS electrophoresis was performed with a modified procedure of Kawasaki and Murakami (2000) and Kawasaki et al. (2003). Electrophoresis was employed using an electrophoresis apparatus (model NA-1214A; Nippon Eido, Tokyo, Japan). The nondenatured gel consisted of a stacking gel containing 5.6% acrylamide solution and 150mM Tris-HCl (pH 6.8) and a separating gel containing 14% acrylamide solution and 410mM Tris-HCl (pH 8.8). The run buffer contained 240mM Tris and 2.5M glycine. PCR products were electrophoresed at 65V for 18h at room temperature. Detection of AFLP fragments was performed by using the Sil-Best Stain for Protein/PAGE kit (Nacalai Tesque, Kyoto, Japan).

HEGS-derived gene markers

Candidate genes for gene markers and PCR primers for amplification of the genes are listed in Table 1. Of these genes, 14 have been submitted to the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/Welcome-e.html) as genomic DNA sequences and 6 as cDNA sequences.

Table 1. Candidate genes and primers for high-efficiency genome scanning (HEGS) gene markers

Candidate gene	Protein encoded by candidate gene	Accession number	Primer 1 (sense)	Primer 2 (antisense)
Genome DNA sequence				
Ras	RAS	D00742	ATGAGAGACAGGTCGGGATG	CAGTCATCATTCGGCCAGTA
Tyr	Tyrosinase	AB033993	TGGAGAAATGATTCGGTACTCA	ATGTCTTTACCCTCGCTTGC
Gpd	Glyceraldehyde-3-phosphate dehydrogenase	AB013136	AAGCTCAAATCCTGTTGTACTTACC	GCGCTCGAAATTCAAAGAGA
priA	PRI A (putative zinc-binding motif)	X60956	GTCTTTAAAACGCGTCGTTGC	TTTCCTGAAAGGGGGAGTAGG
Cap	CAP (adenylyl cyclase-associated protein)	AB007849	GTGTTGTCCCCAGTGGTTCT	TGAGCTCAACTCCATCAACAA
14.3.3	Protein interacting with CAP	AB029308	TGCCTGAAACTCGTGAAGACT	CCCTTGGATTCCTCCTTTTG
uckl	UMP-CMP kinase	AB005742	TCTTTGTCCTTGGTGGTCCT	CGGTAACTTCCATTGGCACTA
lcc1	Laccase 1	AB055157	TCCATACGCTGACCTATACGA	ATCGGCTGGACCGTTGAC
lcc2	Laccase 2	AB055158	AGCACAGTCGCCTCAAGATT	AAACCAAGGGCAGGATTAT
hyd1	Hydrophobin 1	AF217807	TTGCTAGCCGTCTGGTAAT	ATACCCAAAAGCCCACAAAG
ncl1	Nuclease 1	AB035255	CGGAGTACTCAGAATCTCTAGGTC	ATTTGCTCAACAGAGCACGA
ncl3	Nuclease 3	AB075026	CCATTCTTCTTCTTGGACTTGC	CACAATCGCGCTTCTGATT
Ppa	Ser/Thr protein phosphatase 2A regulatory subunit A	AB027711	ATAAGAGTTGGCGGGTTCGT	ATGCGAGCCAAAATGACTTC
Shp	Small heme-binding protein 1	AB015310	TCGAAACCCAAATGTATATCAAGA	TTCCATCTTTGGCTTTACCC
IGS	Intergenic spacer (ribosome RNA)		AGTCCTATGGCCGTGGAT	CTGAAACGCCCTCTAAGTCAGAA
cDNA sequence				
priB	PRI B (DNA-binding motif)	D14489	TGGAAGTCCATGCTGAACAA	GTTCGGCGGTAGCTCGATTGG
Mapk	Mitogen-activated kinase	AF173376	TTCGTACCAACCACTGGACA	TTATGCCCACCGCATAACTT
mfbC	Unknown	AB067692	CGCCTTTCTCCAAAGTTGAC	CTAGCGGTGCTTGCTTCAA
gla1	Glucoamylase	AF220541	TCTGCTGTATTCCTCGCCTTA	CCTCCAAGTGTCATTCGTAGC
Xyl	Xylanase	AF411252	TACTTTTCCTCGCTTTGATCG	AGACACTGAGAATAGTACGCGTTG
cel6B	Cellulase CEL6B	AF411251	TCCACTGGCTACTTGCTCTC	TAGAGGAGGGTTGGCCTTTT

For all the genomic DNA sequences except for the *gpd* and *tyr* genes, PCR primers were established on coding regions such that each PCR product is 270–420 bp in length and encompasses one or more intron sequences. For each of the *gpd* and *tyr* genes, one primer was established on the 3′-end of its coding region and another primer was designed approximately 300 bp downstream of its coding region. For cDNA sequences, PCR primers were designed on the 5′- and 3′-ends of coding regions. PCR primers were designed using the Internet program, Primer 3 on the World Wide Web (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). For the ribosomal RNA gene, an intergenic spacer (IGS) was amplified using the O-1 and the LR12R primer (Anderson and Stasovski 1992).

Genomic DNAs extracted in our previous study (Terashima et al. 2002) were used in this study. PCRs were carried out containing 0.25 U Takara Ex Taq (Takara Biomedicals, Shiga, Japan), Ex Taq buffer supplied by Takara Biomedicals, 0.2 mM of all four deoxynucleotide triphosphates (dNTPs), and 0.5 μM of a forward and a reverse primer, using a iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Conditions of the thermal cycler involved initial denaturation at 95°C for 5 min, followed by 35 cycles at 55°C for 30 s, 72°C for 1 min, and 95°C for 30 s. Finally, the samples were subjected to a final cycle of annealing at 55°C for 30 s and elongation at 72°C for 10 min. PCR products amplified by primers designed based on cDNA sequences were digested with a endonuclease (*Hae*III or *Hha*I): PCR products for *priB*, *mfbC*, *gla1*, *xyl*, and *cel6B* were digested with *Hae*III whereas that for *mapk* was treated with *Hha*I.

Electrophoresis for PCR products was carried out in the HEGS system as already described. Gels were stained with ethidium bromide for 30 min to visualize fragment patterns on an UV transilluminator.

Linkage analysis

Segregation data of 203 AFLP-S markers and 3 mating factor loci, which were previously reported, and those of 221 AFLP-H markers and 21 gene markers, which were obtained in this study, were combined and subjected to linkage analysis. Linkage analysis was performed using MAPMAKER version 3.0 software (Lander et al. 1987) with a back-cross-type data set, which was recoded from the original data set of the parent strain NZ1586-S3 and the mapping population. Two-point analysis with likelihood of odds (LOD) scores of 3.0–5.0 and map distance of 25–35 cM generated 11 linkage groups (LGs) with 439 genetic markers, corresponding to 11 LGs reported previously (Terashima et al. 2002) and an additional LG with only 2 markers. The additional small LG was excluded from a final linkage map because the LG had only 2 markers and was supported only within narrow ranges of recombination values. To obtain reliable marker order, markers in each of the 11 LGs were subjected to two-point analysis at LOD 5.0 and map distance 5–10 cM, and marker ordering was carried out in each of the resulting subgroups. The subgroups were

then ordered based on the distances between markers on their termini. We examined whether markers were ordered linearly in each LG by using recombination rate tables generated by a command "lod table," and markers disturbing linear order were excluded from the map. The linkage map constructed in this procedure was designated as the main-map. Markers excluded from the main-map were assigned to closest markers on the main-map, and the markers in the resulting groups were ordered. When marker order on the groups was of cross or branched relationship with main-map order, they were added to the main-map. Thus, a final linkage map with branched linkages was constructed. The length of the final linkage map was calculated by MAPMAKER with Kosambi map function.

Results

HEGS-derived AFLP markers

We assayed 128 primer combinations for selective amplification by comparing AFLP profiles generated from two parents of the mapping population used in this study, and those showing clear polymorphic bands between the two were selected. Twenty-six primer combinations that generate 221 AFLP-H markers in all were selected and used for selective amplification for the entire population. The number of polymorphic AFLP-H markers amplified per primer pair in this study varied from 3 (for the primer pair E+AA/M+CGC) to 14 (E+AG/M+CTA, E+GA/M+CGA, and E+GT/M+CAT), with an average of 8.5 (Table 2). Of 221

Table 2. Distribution of amplified fragment length polymorphism (AFLP) markers on linkage groups

Primer pair[a]	No. of markers on linkage groups												Total no. of markers
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	u[b]	
AFLP-S													
E+AC/M+CA	6	3	6	1	4	2	5	1	–	2	1	–	31
E+AC/M+CC	5	5	–	1	3	3	1	2	–	1	–	–	21
E+AC/M+CG	5	6	2	5	–	1	2	1	–	1	1	–	24
E+AC/M+CT	3	5	2	5	–	1	4	2	–	–	1	–	23
E+AT/M+CA	6	3	3	3	3	3	3	1	–	1	–	1	27
E+AT/M+CC	3	3	–	3	7	4	1	6	1	1	–	–	29
E+AT/M+CG	6	4	3	1	–	2	–	–	4	1	–	–	21
E+AT/M+CT	3	4	5	5	3	2	2	2	–	–	1	–	27
													203[c]
AFLP-H													
E+AA/M+CAC	2	1	–	–	2	1	–	–	–	1	–	–	7
E+AA/M+CGC	2	–	–	–	1	–	–	–	–	–	–	–	3
E+AA/M+CGG	2	2	–	–	1	1	–	–	–	–	–	1	7
E+AA/M+CGT	–	2	–	2	1	–	1	–	1	–	–	–	7
E+AC/M+CCA	3	2	–	–	7	–	1	–	–	–	–	–	13
E+AC/M+CGT	4	1	1	1	–	–	–	–	1	–	–	–	8
E+AC/M+CTC	–	3	–	3	–	–	–	–	1	–	1	–	8
E+AC/M+CTG	2	1	2	1	1	–	–	–	1	1	–	–	9
E+AG/M+CCT	–	2	–	1	1	1	–	1	–	–	–	1	7
E+AG/M+CTA	1	1	–	3	1	–	–	4	–	4	–	–	14
E+AG/M+CTT	1	1	–	1	–	–	–	1	–	1	–	1	6
E+GA/M+CCA	4	1	–	–	–	–	–	1	–	–	–	–	6
E+GA/M+CCG	–	1	–	1	–	1	–	1	–	1	–	–	5
E+GA/M+CGA	3	2	2	3	–	4	–	–	–	–	–	–	14
E+GA/M+CGG	–	3	–	1	1	1	4	1	1	–	–	1	13
E+GA/M+CTA	1	3	–	–	1	1	1	2	–	–	–	–	9
E+GA/M+CTC	3	2	–	1	1	1	3	–	1	–	–	–	12
E+GA/M+CTT	3	1	–	–	–	1	1	–	–	–	–	2	8
E+GC/M+CAT	–	–	2	1	2	1	–	–	–	–	–	–	6
E+GG/M+CAA	–	2	–	–	1	1	1	–	1	1	–	–	7
E+GG/M+CCC	1	–	1	1	2	–	1	–	1	–	–	–	7
E+GG/M+CGG	1	–	1	2	2	1	1	–	–	–	–	–	8
E+GT/M+CAT	1	2	2	1	1	–	1	2	1	1	1	1	14
E+GT/M+CGA	–	1	–	1	1	–	1	2	–	–	–	–	6
E+GT/M+CTC	2	2	1	–	2	–	–	1	–	–	–	1	9
E+GT/M+CTG	3	–	–	1	–	1	2	–	–	1	–	–	8
													221[d]
Total	76	69	33	49	49	34	36	31	14	18	6	9	424

[a] The DNA fragments generated by the E+2 and M+2 primers and those by the E+2 and M+3 were detected using an ABI 310 genetic analyzer (Terashima et al. 2002) and a modified procedure devised by Kawasaki and Murakami (2000) and Kawasaki et al. (2003), respectively
[b] Unlinked to any linkage groups
[c] Total number of sequencer-derived AFLP markers (Terashima et al. 2002)
[d] Total number of HEGS-derived AFLP markers in this study

AFLP-H markers, 26 (11.8%) showed significant segregation distortion ($0.01 \leq P < 0.05$), and 40 (18.1%) expressed highly distorted segregation ratios ($P < 0.01$). Correspondence of AFLP markers with similar molecular weight between the two detection systems, the ABI Genetic Analyzer 310 and the HEGS system, was not detected (discussed next).

HEGS-derived gene markers

For all the genes reported as genomic DNA sequences, the PCR products showed polymorphism between the parental strains, A567-S8 and NZ1569-S3, in HEGS. Except for the *gpd* gene, these polymorphisms could not be detected on small agarose gels. For six genes reported as cDNA sequences, PCR products showed polymorphisms in electrophoresis after treatment with an endonuclease (*Hae*III or *Hha*I). Also, PCR products for IGS were also polymorphic. Consequently, 21 PCR-based gene markers were developed using the parental strains in this study. Some of them could be run in the same lane on the gel as they are different enough in size to be distinguished from one another.

Linkage analysis

Of a total of 448 makers, 439 were assigned to 11 LGs, corresponding to the 11 LGs reported previously (Terashima et al. 2002), with LOD scores of 3.0–5.0 and

map distance of 25–35 cM. A final linkage map was constructed with 166 markers including 2 mating factors, 10 HEGS gene markers, 72 AFLP-H markers, and 82 AFLP-S markers (Fig. 1). The map in this study covers a total length of 1398.4 cM with an average marker interval distance of 8.4 cM. Linkage group length varied between 22.0 cM (LG XI) and 195.5 cM (LG I), and the number of markers used to construct the linkage groups on the final map ranged from 4 (LG XI) to 24 (LG I) (Table 3). Of markers that could not be located on the final linkage map, designated accessory makers, those exhibiting distances of less than 5.0 cM to any markers on the map, as well as all gene markers excluded from the map, are listed in Table 4. The average marker interval ranged from 7.3 cM (LG XI) to 14.4 cM (LG IX), with the maximum marker interval being 24.0 cM between *EatMcg159* and *EatMcg308* in LG IX. Of the 11 LGs, 7 contained branched linkages, of which LG V had the maximum number of branched linkage (4 linkages) and LG VI contained the largest branched linkage (58.9 cM). LG II had the largest number of accessory markers (57 markers), and *shp* in LG II was linked to the greatest number of accessory markers (14 markers).

All the 21 HEGS gene markers were assigned to the 11 LGs in this study. Of these markers, 10 (*gla1*, *hyd1*, *ncl1*, *shp*, *cap*, *priB*, *cel6B*, *lcc2*, *ras*, and *gpd*) were located on the final linkage map (see Fig. 1), and 11 (*lcc1*, *mapk*, *ncl3*, *ppa*, *priA*, *mfbC*, *xyl*, *tyr*, *uck1*, *IGS*, and *14.3.3*) were listed as accessory markers (see Table 4). *ncl1* and *ncl3* were tightly linked with 0.0 cM in LG II, suggesting a cluster of genes

Fig. 1. A genetic linkage map of *Lentinula edodes* based on 166 markers including 2 mating factors, 10 gene markers, 72 high-efficiency genome scanning (HEGS)-derived amplified fragment length polymorphism (AFLP-H) markers, and 82 sequencer-derived AFLP-F markers. AFLP markers were named according to the primer designation, by the size of the amplified fragment in base pairs. Markers with significant ($0.01 \leq P < 0.05$) and high ($P < 0.01$) segregation distortion are indicated by *single asterisks* (*) and *double asterisks* (**), respectively. The number of accessory AFLP markers exhibiting distances of less than 5 cM from the final linkage map, and gene markers that could not be on the final linkage map, are indicated in parentheses on the *right* of markers on the final linkage map
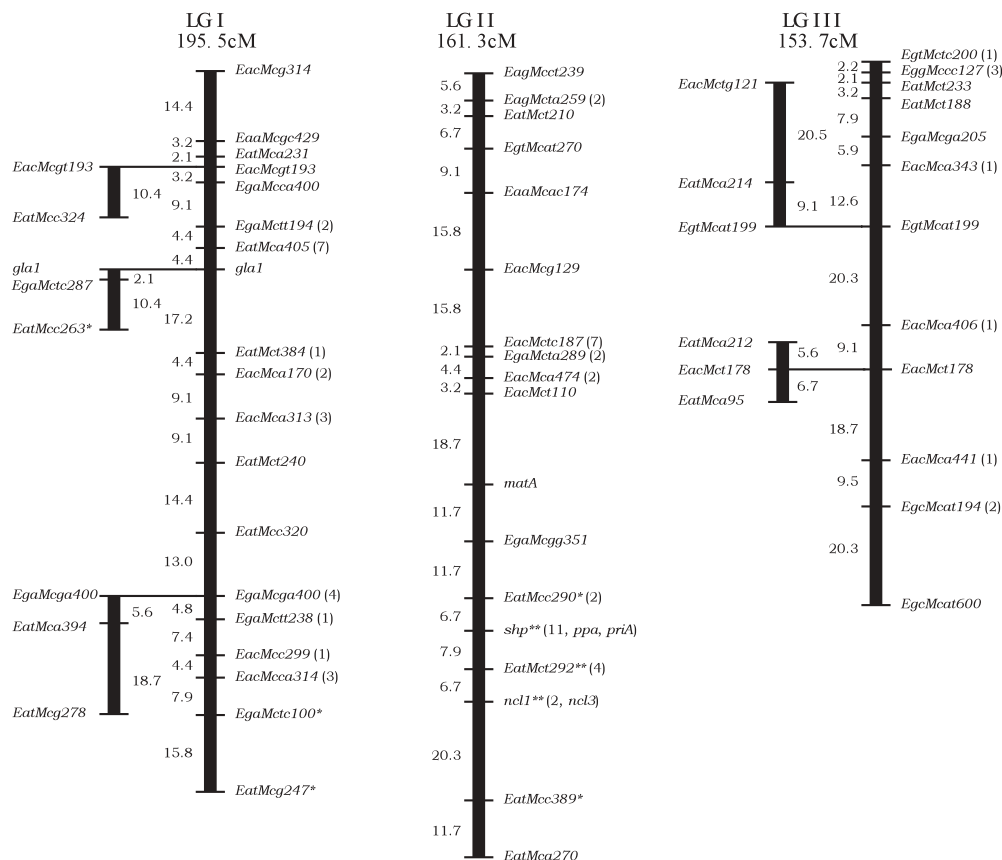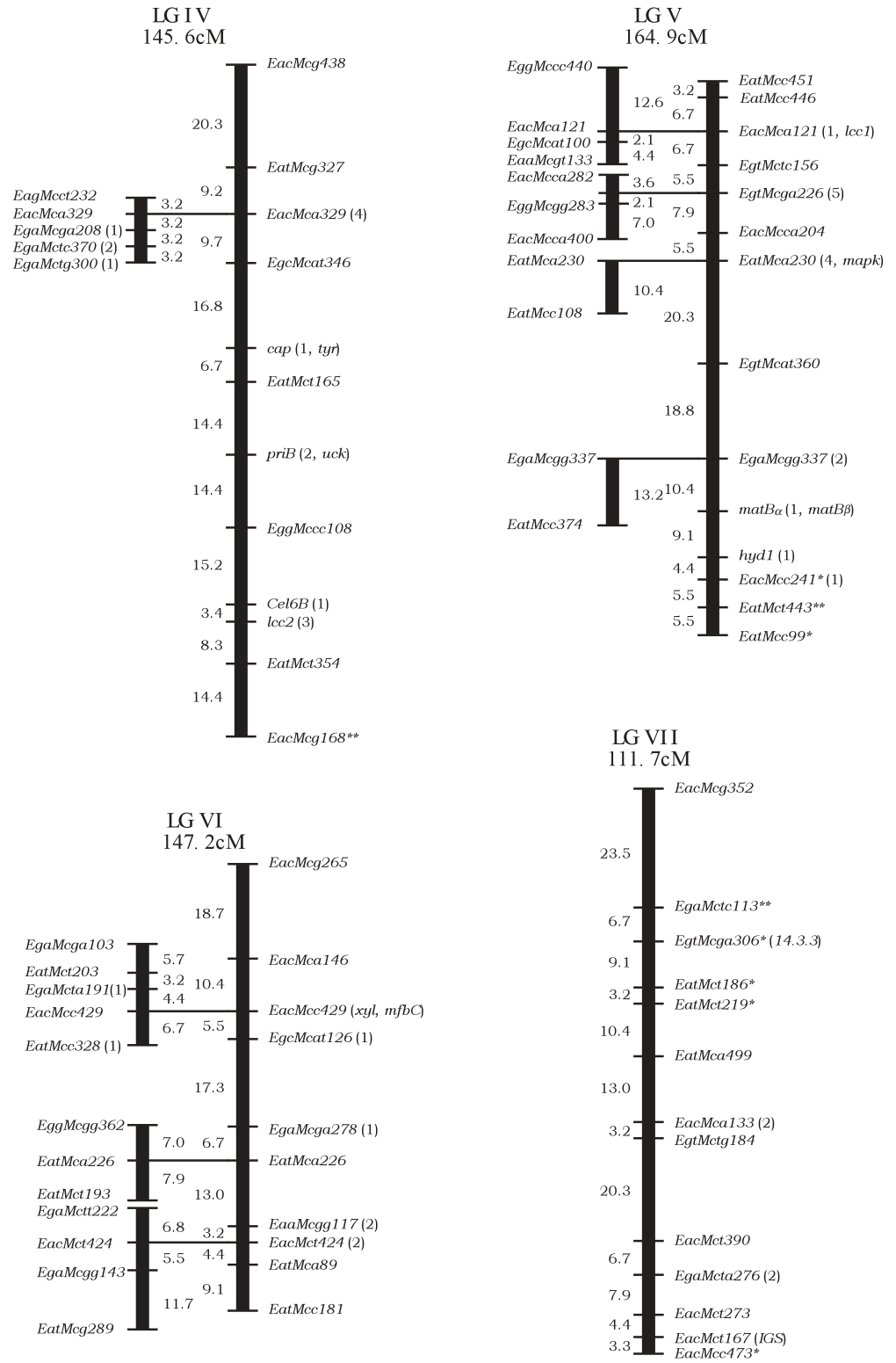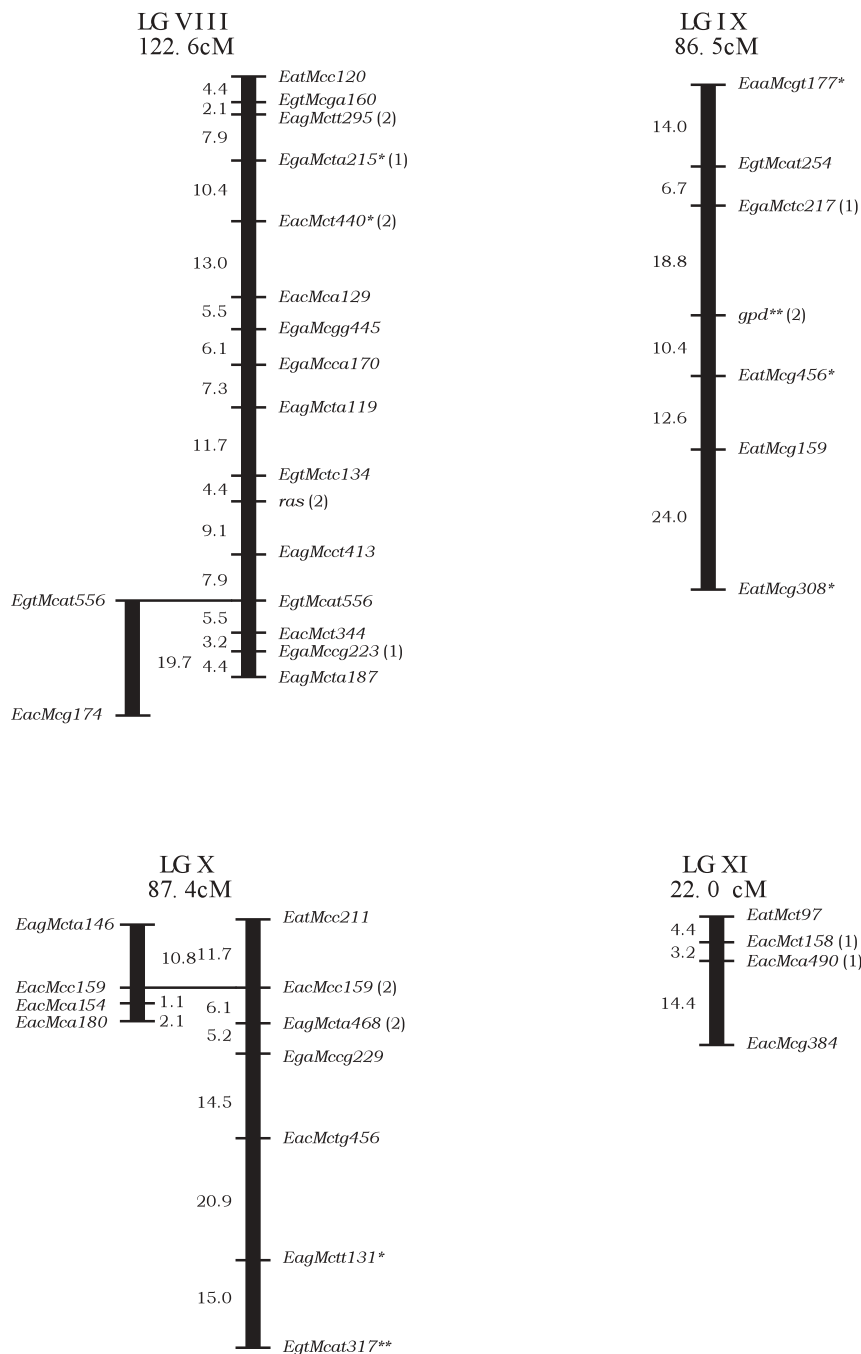
Fig. 1. *Continued*

LG I V
145. 6cM

EacMcg438

20.3

EatMcg327

9.2

EagMcct232
EacMca329
EgaMcga208 (1)
EgaMctc370 (2)
EgaMctg300 (1)
3.2
3.2
3.2
3.2

EacMca329 (4)

9.7

EgcMcat346

16.8

cap (1, tyr)

6.7

EatMct165

14.4

priB (2, uck)

14.4

EggMccc108

15.2

Cel6B (1)
3.4
lcc2 (3)
8.3

EatMct354

14.4

EacMcg168**

LG V
164. 9cM

EggMccc440

12.6
3.2
EatMcc451
EatMcc446
6.7

EacMca121
EgcMcat100
EaaMcgt133
2.1
4.4
EacMca121 (1, lcc1)
6.7

EacMcca282
3.6
EgtMctc156
5.5

EggMcgg283
2.1
EgtMcga226 (5)
7.9

EacMcca400
7.0
EacMcca204

EatMca230
5.5
EatMca230 (4, mapk)

EatMcc108
10.4
20.3

EgtMcat360

18.8

EgaMcgg337
EgaMcgg337 (2)
13.2
10.4
EatMcc374
matBα (1, matBβ)
9.1
hyd1 (1)
4.4
EacMcc241* (1)
5.5
EatMct443**
5.5
EatMcc99*

LG VI
147. 2cM

EacMcg265

18.7

EgaMcga103
5.7
EacMca146
3.2
EatMct203
10.4
EgaMcta191(1)
4.4
EacMcc429
EacMcc429 (xyl, mfbC)
6.7
5.5
EatMcc328 (1)
EgcMcat126 (1)

17.3

EggMcgg362
7.0
6.7
EgaMcga278 (1)
EatMca226
EatMca226
7.9
EatMct193
13.0
EgaMctt222
6.8
3.2
EaaMcgg117 (2)
EacMct424
EacMct424 (2)
5.5
4.4
EatMca89
EgaMcgg143
9.1
11.7
EatMcg289
EatMcc181

LG VII
111. 7cM

EacMcg352

23.5

EgaMctc113**

6.7

EgtMcga306* (14.3.3)

9.1

EatMct186*
3.2
EatMct219*

10.4

EatMca499

13.0

EacMca133 (2)
3.2
EgtMctg184

20.3

EacMct390

6.7

EgaMcta276 (2)

7.9

EacMct273
4.4
EacMct167 (IGS)
3.3
EacMcc473*

342

Fig. 1. *Continued*

LG VIII
122. 6cM

	EatMcc120
4.4	EgtMcga160
2.1	EagMctt295 (2)
7.9	EgaMcta215* (1)
10.4	EacMct440* (2)
13.0	EacMca129
5.5	EgaMcgg445
6.1	EgaMcca170
7.3	EagMcta119
11.7	EgtMctc134
4.4	ras (2)
9.1	EagMcct413
7.9	EgtMcat556
5.5	EacMct344
3.2	EgaMcg223 (1)
4.4	EagMcta187

EgtMcat556
19.7
EacMcg174

LG I X
86. 5cM

	EaaMcgt177*
14.0	EgtMcat254
6.7	EgaMctc217 (1)
18.8	gpd** (2)
10.4	EatMcg456*
12.6	EatMcg159
24.0	EatMcg308*

LG X
87. 4cM

EagMcta146
EacMcc159
EacMca154
EacMca180
10.8 11.7
1.1 6.1
2.1 5.2

	EatMcc211
	EacMcc159 (2)
	EagMcta468 (2)
	EgaMccg229
14.5	EacMctg456
20.9	EagMctt131*
15.0	EgtMcat317**

LG XI
22. 0 cM

	EatMct97
4.4	EacMct158 (1)
3.2	EacMca490 (1)
14.4	EacMcg384

with similar function, whereas *lcc1* and *lcc2* were located in LG V and LG IV, respectively (see Fig. 1).

Discussion

The AFLP-H markers generated in this study covered all linkage groups previously constructed based on AFLP-S markers (see Table 2). Of 26 primer pairs used in AFLP-H markers, 6 (E+AC/M+CCA, E+AG/M+CTA, E+GA/M+CGA, E+GA/M+CGG, E+GA/M+CTC, and E+GT/M+CAT) provided more than 10 AFLP-H markers, respec-

tively, which were almost evenly distributed on the linkage groups. Therefore, it is possible that use of the 6 AFLP primer pairs with HEGS could rapidly identify a genome region located by a gene for the phenotype of interest. The gene can be precisely mapped by additional markers including accessory markers listed in Table 4.

The total length of the linkage map in this study (1398.4 cM) was shorter than that of our previous linkage map (1956.7 cM). By the manual ordering of makers in this study, it was found that, in our previous map, only the automatic marker ordering with the MAPMAKER (Lander et al. 1987) and MAPL (Ukai et al. 1995) software has led to adopting several nonlinear marker orders,

Table 3. Characteristics of genetic linkage groups with gene-specific markers, AFLP markers, and mating factors in *Lentinula edodes*

Linkage groups[a]	Main map Length (cM)	No. of markers	Branched linkage Length (cM)	No. of branched linkage	No. of markers	Final map Length (cM)	No. of marker	Average marker interval (cM)	Accessory markers[b] Gene	<5 cM	≥5 cM
I	148.3	19	47.2	3	5	195.5	24	8.5	0	24	29
II	161.3	18	0	0	0	161.3	18	9.5	3	31	23
III	111.8	12	41.9	2	4	153.7	16	10.2	0	9	8
IV	132.8	12	12.8	1	4	145.6	16	9.7	2	16	21
V	109.5	14	55.4	4	8	164.9	22	7.9	3	14	14
VI	88.3	10	58.9	3	9	147.2	19	8.2	2	8	7
VII	111.7	13	0	0	0	111.7	13	9.3	2	4	19
VIII	102.9	16	19.7	1	1	122.6	17	7.7	0	8	7
IX	86.5	7	0	0	0	86.5	7	14.4	0	3	5
X	73.4	7	14	1	3	87.4	10	9.7	0	4	4
XI	22.0	4	0	0	0	22.0	4	7.3	0	2	0
–	–	–	–		–	–	–		–	–	9
	1126.5	132	249.9		34	1398.4	166		12	121	146

[a] A code of the linkage group corresponding to those in a previous map (Terashima et al. 2002)
[b] Markers that could not be located on the final linkage map

thereby overestimating the total length of the map. In addition, 21 branched linkages could not be recognized in our previous study. In this study, we took into consideration the branched linkages and manually confirmed linear marker orders by examining recombination rate tables. The linkage map of *L. edodes* was thereby improved in terms of the total length of the linkage map and the linear order of the markers.

All LGs except LG II, IX, and X included markers located on both terminal regions of the corresponding LGs in our previous map, suggesting that the linkage map in this study and our previous map cover almost the same area of the genome in *L. edodes*. Only for LG X in this study was the map length expanded from 47.6 to 87.4 cM. Comparison of LG X with the corresponding LG of our previous map revealed that the area of 72.5 cM in LG X and that of 25.9 cM in the LG of the previous map were not shared, suggesting that LG X in this study covers a larger genome area than that in the previous map.

Arima and Morinaga (1993) have documented that the rRNA gene is located on the largest chromosome, chromosome I, which is 7.0 Mb in physical length, using hybridization analysis after contour-clamped homogeneous electric field (CHEF) gel electrophoresis. Linkage analysis in this study revealed that *IGS*, which is an intergenic spacer of the rRNA gene, was included in LG VII. These results suggested that LG VII corresponds to a part of the chromosome I reported by Arima and Morinaga (1993). Both *matA* and *priA* were included in LG II in this study, whereas these markers have been reported to be located on LG III in a linkage map by Kwan and Xu (2002), suggesting that LG II in this study corresponds to LG III by indicated by Kwan and Xu (2002).

For linkage analysis of genes, restriction fragment length polymorphism (RFLP) analysis with probes of the cDNA has usually been carried out (Doganlar et al. 2002; Harushima et al. 1998; Muraguchi et al. 2003). However, this technique was tedious and time consuming. In this study, 21 gene markers exhibited polymorphism with the

HEGS system between the parental isolates, and all the genes were assigned to linkage groups. The HEGS gene markers can be established by trials with a few primer combinations. When additional genes are sequenced hereafter, these can be rapidly located on a linkage map of *L. edodes*.

It is expected that AFLP fragments that are derived from the two different electrophoresis systems, the ABI Genetic Analyzer 310 and HEGS, present the same length and will cosegregate: for example, an AFLP-S marker from the E+AC/M+CC primer combination is expected to cosegregate with an AFLP-H marker from E+AC/M+CCA. However, in this study, such corresponding markers could not be obtained. AFLP-H fragments were electrophoresed in nondenaturing condition and were detected as double-strand DNAs in this study, whereas AFLP-S fragments were electrophoresed in denaturing condition, and, of the double strands, only a single strand containing the end with the *Eco*RI site was detected (Vos et al. 1995). These differences between the electrophoresis systems might be the reason that the corresponding AFLP markers could not be obtained.

In this study, linkage analysis revealed that, of 11 LGs, 7 contained branched linkages. Because the parental strains used in this study belong to distinct phylogenetic lineages (Hibbett et al. 1995, 1998), these strains are likely to differ by reciprocal translocations, which might be responsible for the several branched linkages. In general, a branched linkage is attributed to a reciprocal translocation (Tamarin 1999). In fungi, chromosome length polymorphisms (CLPs), which are revealed by electrophoresis with intact chromosomes, have been reported within species: the cross between two parents differing in chromosome lengths produces a number of viable progeny with novel-sized chromosomes (Tzeng et al. 1992; Zolan et al. 1993; Zolan 1995). The CLP has been shown to be mainly generated during meiosis; one mechanism for generating CLPs has been reported to be an interchromosome recombination including the reciprocal translocation (Tzeng et al. 1992; Zolan et al. 1993). In the ascomycete *Cochliobolus heterostrophus*

Table 4. List of accessory markers on the linkage map of *Lentinula edodes*

LG	Markers on final linkage map[a]	Accessory markers[b]
LG I	*EgaMctt194*	*EatMca192* (0.0)[c], *EacMca192* (1.1)
	EatMca405	*EgaMctt311* (0.0), *EgaMcga300* (1.1), *EgtMctg222* (1.1), *EacMca89* (3.2), *EacMct480* (3.2), *EgtMctc96* (4.4), *EgtMctg131* (4.4)
	EatMct384	*EgaMctc431* (4.4)
	EacMca170	*EacMct144* (1.1), *EacMctg146* (2.1)
	EacMca313	*EggMcgg289* (2.2), *EgtMctg148* (3.3), *EacMcca289* (4.5)
	EgaMcga400	*EagMcta203* (1.1), *EgaMcga429* (2.2), *EacMca167* (3.3), *EacMct470* (3.3)
	EgaMctt238	*EacMcgt266* (2.2)
	EacMcc299	*EacMcgt267* (4.4)
	EacMcca314	*EggMccc161* (3.3), *EacMcca314* (3.3), *EacMcca272* (4.5)
LG II	*EagMcta259*	*EgaMctc189* (4.4), *EacMca93* (4.4)
	EacMctc187	*EacMctc164* (0.0), *EgaMctc194*(1.1), *EacMct149* (1.1), *EacMct160* (2.1), *EacMcgt181*(2.1), *EagMctt275* (3.2), *EacMcc188* (4.4)
	EgaMcta289	*EacMcca121*(2.2), *EacMctg105* (4.4)
	EacMca474	*EacMctc234* (2.1), *EaaMcgt247* (3.3)
	EatMcc290	*EatMca182* (4.4)
	shp	*EaaMcgt223* (0.0), *EatMct140* (0.0), *EgaMcta223* (1.1), *EgaMcgg245* (1.1), *EacMcc456* (1.1), *ppa* (2.1), *EacMct176* (2.1), *EgaMccg219* (2.2), *EggMcaa341* (3.2), *EgtMctc259* (3.2), *EgtMctc265* (3.2), *EgaMcgg205* (3.2), *priA* (21.9)
	EatMct292	*EagMcct151* (2.2), *EacMcc441* (3.2), *EgaMcta183* (4.4), *EatMcc285* (4.4)
	ncl1	*EacMcca300* (0.0), *ncl3* (0.0), *EacMcc305* (1.1)
LG III	*EgtMctc200*	*EacMct223* (2.2)
	EggMccc127	*EacMctg227* (0.0), *EggMcgg117* (1.1), *EacMct223* (2.2)
	EacMca343	*EacMcgt135* (4.7)
	EacMca406	*EgtMcat238* (4.4)
	EacMca441	*EacMca481* (4.4)
	EgcMcat194	*EacMca384* (1.1), *EacMca165* (2.2)
LG IV	*EacMca329*	*EacMctg247* (1.1), *EatMca287* (1.1), *EatMct198* (3.3), *EatMca251* (4.4)
	EgaMcga208	*EagMctt220* (3.3)
	EgaMctc370	*EgaMcga355* (3.3), *EagMcta381* (4.4)
	EgtMctg300	*EagMcta381* (4.4)
	cap	*tyr* (1.1), *EatMct161* (1.1)
	priB	*EggMcgg145* (0.0), *EaaMcgt163* (1.1), *uck* (1.1)
	EggMccc108	*EggMcgg344* (2.2)
	cel6B	*EatMcc203* (4.4)
	lcc2	*EagMcta410* (1.1), *EatMcc251* (4.4), *EatMct135* (4.5)
LG V	*EacMca121*	*lcc1* (2.1), *EacMcca167* (4.5)
	EgtMcga226	*EgaMcta295* (1.1), *EacMca375* (2.1), *EacMctg172* (2.2), *EatMca326* (2.2), *EacMca284* (3.2)
	EatMca230	*mapk* (0.0), *EatMct189* (2.2), *EgaMctc142* (3.2), *EggMccc267*(3.3), *EacMcca243* (3.3)
	EgaMcgg337	*EgtMctc124* (4.6)
	matBa	*matBβ*(1.1), *EaaMcgc278* (4.5)
	hyd1	*EggMcgg261* (3.3)
	EacMcc241	*EatMca106* (3.3)
LG VI	*EacMcc429*	*xyl* (3.2), *mfbC* (4.4)
	EgaMcta191	*EgaMctc300* (4.7)
	EatMcc328	*EatMcc369* (3.2)
	EgcMcat126	*EatMca228* (3.3)
	EgaMcga278	*EaaMcac198* (4.4)
	EaaMcgg117	*EgtMctg163* (2.2), *EacMca294* (2.2)
	EacMct424	*EgaMcga94* (2.2), *EggMcaa204* (3.3)
LG VII	*EgtMcga306*	*14.3.3* (1.1)
	EacMca133	*EgtMctg176* (2.1), *EacMca450* (2.2)
	EgaMcta276	*EgtMcat346* (1.1), *EgaMcgg486* (2.1)
	EacMct167	*IGS* (8.2)
LG VIII	*EagMctt295*	*EatMct417* (0.0), *EgaMcta234* (2.2)
	EgaMcta215	*EatMct213* (2.2)
	EacMct440	*EagMcta394* (2.2), *EatMcc366* (4.4)
	ras	*EatMcc112* (3.3), *EatMcc125* (4.4)
	EgaMccg223	*EagMcta180* (2.1)
LG IX	*EgaMctc217*	*EacMctg233* (2.1)
	gpd	*EacMcgt215* (0.0), *EggMcaa280* (4.5)
LG X	*EacMcc159*	*EgtMctg140* (1.1), *EagMcta270* (1.1)
	EagMcta468	*EaaMcac127* (3.3), *EagMcta474* (3.3)
LG XI	*EacMct158*	*EgtMcat207* (1.1)
	EacMca490	*EacMctc153* (1.1)

[a] Markers in the final linkage map of *L. edodes* that were most closely linked to an accessory marker

[b] Markers that could not be located on the final linkage map, which exhibited distances of less than 5.0 cM to any of the final linkage map, and gene markers excluded from the final linkage map are listed

[c] The number in parentheses shows map distances (cM) to a marker on the final linkage map of *L. edodes*

(Drech.) Drech., a combination of electrophoretic karyotyping and linkage analyses with RFLP markers revealed that a branch linkage on the map was attributed to a reciprocal translocation in parental strains (Tzeng et al. 1992), and in the basidiomycete *Coprinus cinereus* such a translocation has been implied by hybridization analysis of electrophoretic karyotypes with chromosome-specific probes (Zolan et al. 1993). At present, it remains unclear whether the reciprocal translocation is responsible for the branched linkage in this study. Improvement of electrophoretic karyotyping in *L. edodes* is necessary to examine the relationship. In addition, further linkage analysis using the spore-derived isolates expected to have the translocation in this study would provide more reliable information on the branched linkage.

In conclusion, AFLP-H markers and gene markers were generated with HEGS in addition to AFLP-S markers in our previous study, and the linkage map of *L. edodes* was improved in terms of number of markers, marker density, linear order of markers, and total map length. The AFLP-H markers reported here could be used for general purposes in molecular genetic studies. The HEGS gene markers make possible comparison between linkage maps and/or electrophoretic karyotypes and have the potential of microsynteny analysis between various fungal strains and probably rapid identification of genes involving QTL for agronomic and pharmacological traits of *L. edodes*. These genetic markers and the improved linkage map of *L. edodes* will contribute to our understanding of its genome organization and will be powerful tools for establishing efficient breeding programs for *L. edodes*.

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