Fungal Biology 123 (2019) 843–853

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

Map-based cloning of genes encoding key enzymes for pigment synthesis in *Auricularia cornea*

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ARTICLE INFO

Article history:
Received 13 February 2019
Received in revised form
10 June 2019
Accepted 3 September 2019
Available online 17 September 2019

Corresponding Editor: Simon Avery

Keywords:
Candidate genes
Color
Genetic linkage map
Genetic populations
SSR

ABSTRACT

Color is an important quality attribute of fungi, and a useful marker for classification, genetic, and molecular research. However, there is much debate over which enzymes play key regulatory roles in pigment synthesis pathways among different fungi and even within the same species. *Auricularia cornea* is the most widely cultivated mushroom in the genus *Auricularia*; 1.834 million tons of this mushroom were produced in 2016 in China. Thus, systematic studies on its color inheritance and the genes encoding key enzymes for pigment synthesis have high scientific and economic value. In this study, the white strain ACW001 and the purple strain ACP004 of *A. cornea* were used as dikaryotic parents. Selfing populations of ACW001 and ACP004 were constructed with their monokaryotic strains. The fruiting body color of the two populations was consistent with that of their parents, confirming that the two parents were color homozygotes. All strains in the hybrid population of the two parents produced purple fruiting bodies. A robust hybrid strain (ACW001-33×ACP004-33) was selected from the hybrid population, and 87 monokaryotic strains of ACW001-33×ACP004-33 were obtained as a mapping population. Finally, a testcross population was constructed by crossing the mapping population with the test strain ACW001-9. The color genotype of each monokaryotic strain in the mapping population was identified by a fruiting test. The genomes of the two monokaryotic strains ACW001-33 and ACP004-33 were sequenced, and then simple sequence repeat (SSR) and sequence-related amplified polymorphism (SRAP) molecular marker primers were developed. Then, 88 pairs of primers that could distinguish the genotypes of the mapping population were used to construct a genetic linkage map. The genetic linkage map consisted of 12 linkage groups (LGs) spanning 1315.2 cM. The color control locus was preliminarily located at 24.5 cM of the 11th LG. Fine-mapping primers were designed based on sequence differences between ACW001-33 and ACP004-33 in the primary location region. Four color control candidate genes were located in an 8.2-kb region of ACW001-33_contig733 and a 9.2-kb region of ACP004-33_contig802. Homologous alignment and prediction of conserved domain analyses indicated that two of the color control candidate genes encoded proteins with unknown function, and the other two, ACP004_g11815 and ACP004_g11816, encoded glutamyl aminotransferases. These two genes were consecutively arranged on ACP004-33_contig802, and were likely to encode key enzymes in the γ-glutamine-4-hydroxy-benzoate (GHB) pigment synthesis pathway. Primers were designed from the flanking sequences of the two genes and used to analyze the testcross population. Products were amplified only from the 30 testcross strains with purple fruiting bodies, confirming the accuracy of the localization results. We discuss the deficiencies and advantages of map-based cloning in fungi vs. plants, and summarize the steps and

Abbreviations: GHB, γ-glutamine-4-hydroxy-benzoate; LG, linkage group; LOD, logarithm of the odds; QTL, quantitative trait locus; SCAR, sequence characterized amplified region; SRAP, sequence-related amplified polymorphism; SSR, simple sequence repeat.

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https://doi.org/10.1016/j.funbio.2019.09.002

requirements of the map-based cloning method for fungi. This study has provided novel ideas and methods for locating functional genes in fungi.

1. Introduction

There are many kinds of edible fungi with rich and attractive colors. As an important commodity character, fruiting body color has attracted much attention. *Flammulina velutipes* and *Agaricus bisporus* are the main edible fungi and have been used in many studies on fruiting body color inheritance and on genes controlling color (Callac et al., 1998; Xie et al., 2004; Liu, 2004; Liao et al., 2007; Cai et al., 2014). However, the results of these studies were controversial. Some studies concluded that color was controlled by one pair of genes, but others concluded that it was controlled by many pairs of genes. Some partial color control genes or DNA fragments linked to them were obtained by converting selected molecular markers linked to color-control loci into sequence characterized amplified region (SCAR) markers. However, the gene sequences obtained using this method were incomplete, and could not be used in more in-depth analyses of the color control mechanism.

Previous studies have focused on the cloning of fungal functional genes related to lignocellulose degradation (Floudas et al., 2012; Nakazawa et al., 2017), mating type (Au et al., 2014; Wu et al., 2013), and fruiting body formation (Voigt and Poggeler, 2013; Wang et al., 2012). Most of the cloning methods relied on the design of degenerate primers based on sequences of relevant genes in model species. Alternatively, partial DNA fragments were obtained using molecular markers followed by chromosome walking. The lack of gene localization and cloning research on fungi means that it is difficult to identify unique functional genes in fungal species. In higher plants, techniques to locate and clone specific functional genes by map-based cloning are very advanced (Peters et al., 2003; Ronen et al., 2000; Wang et al., 2015b). It would be useful to develop a map-based cloning method to locate the color control locus in a fungus, identify the gene(s) controlling fruiting body color, analyze their function, and further explore the molecular mechanism of pigment synthesis.

A. cornea belongs to the Basidiomycota, Agaricomycetes, Auriculariales, Auriculariaceae, *Auricularia* Bull. ex Juss.. It is found widely across Asia, Europe, North and South America, and Africa. Its fruiting body is tender, has a crisp taste, and is rich in polysaccharides and other nutrients (Qing et al., 2009; Zhao et al., 2013). It is the most widely distributed and cultivated mushroom in the *Auricularia* genus worldwide (Wu, 2016). The dorsal surface (non-sporulation layer) of the fruiting body of *A. cornea* is usually brown or tan and the ventral surface (sporulation layer) is purple. However, pure white fruiting body strains have been reported from the wild (Wang et al., 1998) and from artificial cultivation (Wang et al., 2015a). Fortunately, we obtained a strain ACW001 with pure white dorsal and ventral surfaces. This provided a perfect experimental material to study color inheritance, locate the color control locus, and analyze the pigment synthesis pathway in *A. cornea*.

This is the first systematic study on the color control locus and genes encoding key enzymes in pigment synthesis in *A. cornea*. Using a combination of classical genetic methods and molecular methods, we uncovered the genetic basis of color control in *A. cornea*. These results lay the foundation for further breeding of new varieties with different colors to meet the diverse needs of consumers. We also describe a method for cloning target genes from *A. cornea* that can be used to locate, clone, and analyze functional genes in other fungi.

2. Materials and methods

2.1. Test strains

The white *A. cornea* strain ACW001 and the purple *A. cornea* strain ACP004 were provided by the Horticulture College of Jilin Agricultural University (Fig. 1).

2.2. Monokaryotic strain preparation

The ventral surface of a mature fruiting body was placed face-down in a sterilized empty Petri dish (9 cm in diameter). A white spore print was obtained after 6–12 h without shaking at 25 °C and the fruiting body was removed. A spore suspension mother liquor was made in a clean bench. The spore concentration was determined using a hemocytometer, and then the spore suspension was diluted to a concentration of 100–200 spores/mL with sterilized water. Then, 100 μL spore suspension was evenly spread onto PDA medium using a bent glass rod and the culture was incubated at 25 °C. The spores germinated after 4–7 d. A single colony was picked out and inoculated into a test tube containing PDA medium

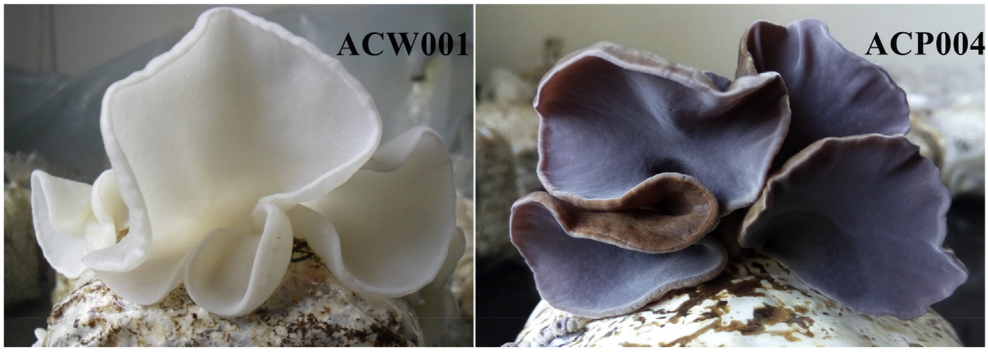

Fig. 1. Fruiting bodies of test strains.

and then labeled. Double-fluorescence staining technology (Kang et al., 1993; Yao et al., 2019) was used to identify monokaryotic strains. Fifty monokaryotic strains of the white parent ACW001 and 50 monokaryotic strains of the purple parent ACP004 were obtained to prepare the selfing populations and the hybrid population. In total, 87 monokaryotic strains were obtained from the hybrid ACW001-33×ACP004-33 as the mapping population, which was used to produce the testcross population.

2.3. Crossing between monokaryons

In a clean bench, 0.3–0.5 cm inoculation blocks of the two monokaryotic strains were inoculated in the middle of the same PDA plate medium at a distance of 3.0–4.0 cm, and then cultured at 25 °C. The mycelia were cultured for a further 5–7 d after the two colonies came into contact with each other, and then inoculation blocks were cut from the junction of the two colonies and transferred to fresh PDA medium. Dikaryotic mycelia were identified by observing clamp connections under an optical microscope (observation lens 40×, eyepiece 10×) and preserved.

2.4. Preparation of populations for genetic analyses

Selfing population: Twenty-one monokaryotic strains were randomly selected from the 50 monokaryotic strains of the white parent ACW001 for the monokaryons cross. The ACW001 selfing population consisted of 108 dikaryotic strains obtained from 210 pairs of selfing combinations. Twenty-one monokaryotic strains were randomly selected from the 50 monokaryotic strains of the purple parent ACP004 for the monokaryons cross. The ACP004 selfing population consisted of 108 dikaryotic strains obtained from 210 pairs of selfing combinations. The selfing populations were used to confirm the homozygosity of the parents' color and determine the mating type of parental monokaryotic strains.

Hybrid population: 10 monokaryotic strains of the white parent ACW001 (five of the monokaryons with the A_{w1} mating type and the other five with the A_{w2} mating type) were hybridized with 10 monokaryotic strains of the purple parent ACP004 (five of the monokaryons with the A_{p1} mating type and the other five with the A_{p2} mating type). One hundred successful hybrid strains were obtained as the hybrid population. This hybrid population was used to determine the dominance of the color character. A hybrid showing robust growth was selected for the collection of spores to prepare the mapping population.

Mapping population: Spores were collected from the hybrid strain ACW001-33 ×ACP004-33 showing strong mycelial growth, a short growth period, and normal fruiting body production. Then, 87 monokaryotic strains were obtained as the mapping population. These 87 strains resulted from meiosis and natural recombination of the prepared monokaryotic strains. The mapping population was used to generate the testcross population and to construct the genetic linkage maps.

Testcross population: The 87 dikaryotic strains of the testcross population were prepared by crossing the mapping population with the monokaryotic strain ACW001-9, whose mating type was A_{w2} different from those of ACW001-33 (A_{w1}) and ACP004-33 (A_{p1}). The testcross population was used to determine the color genotype of all monokaryotic strains of the mapping population and to identify the key genes in pigment synthesis.

2.5. Fruiting test and phenotyping based on fruiting body color

The strains of all genetic populations were inoculated onto PDA medium in Petri dishes, and then transferred to sterilized sawdust medium when the plate was fully covered. The sawdust medium comprised (w/w): 78 % hardwood sawdust, 20 % wheat bran, 1 % gypsum, and 1 % lime. The water content of the sawdust medium was 60 %. Each bag (15 cm × 30 cm) contained 300 g sawdust medium. The inoculated bags were placed in a dark cultivation room and kept at 25 °C constant temperature. A small cut was made in the middle of each bag using a sterile surgical blade when the bags were full of mycelia. Then, the bags were transferred to a well-ventilated room to induce fruiting under following conditions: light 500–600 lux, temperature 25–30 °C, humidity 90 %–95 %. When the fruiting body was mature, the ventral surface color was observed by the naked eye and scored as pure white (W) or non-white (P). The fruiting body phenotype data (P and W) were subjected to statistical analyses.

2.6. Genomic DNA extraction

Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The quality of purified genomic DNA was examined using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA) and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA, USA). The DNAs from the monokaryotic strains ACW001-33 and ACP004-33 were used for whole-genome sequencing and genotyping primer screening. The DNAs from 87 monokaryotic strains of the mapping population were used for genetic linkage map construction and for fine mapping of genes encoding key enzymes in pigment synthesis. The DNAs from 87 dikaryotic strains of the testcross population were used for functional verification of the genes encoding key enzymes in pigment synthesis.

2.7. Whole genome sequencing and development of molecular marker primers

Two genome sequencing libraries were constructed using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) following the manufacturer's instructions. Paired-end (2 × 150 bp) sequencing of the DNA libraries was performed on the Illumina HiSeq X platform (Illumina, San Diego, CA, USA). Sequencing was completed by the Hangzhou Woosen Bio-technology Co. Ltd (Hangzhou, Zhejiang, China). The clean sequence reads were assembled with CLC 1Genomics Workbench (v. 6.5) with the default parameters. The ACW001-33 and ACP004-33 genomes were scanned with SciRoKo 3.4 software (Kofler et al., 2007) to obtain microsatellite sequences and to design primers based on microsatellite flanking sequences. The SRAP primers were synthesized as described elsewhere (Li and Quiros, 2001; Lin et al., 2003).

2.8. Preliminary mapping of color control locus

Using the DNA of ACW001-33 and ACP004-33 as templates, SRAP and SSR primers were designed to distinguish the genotypes of the two strains. Then, the genotypes of 87 monokaryotic strains of the mapping population were identified using the designed primers (those with the same genotype as the parent ACW001-33 were designated as “W”, and those with the same genotype as the parent ACP004-33 were designated as “P”. The SSR loci were named after the corresponding primers, and the SRAP loci were named after the corresponding primer combination plus the DNA fragment length). JoinMap 4.0 was used for linkage analysis of the markers, estimation of recombination frequencies, and determination of the linear order of loci (Van Ooijen and Voorrips, 2006). The likelihood of odds (LOD) score was 5.0. Finally, a genetic linkage map showing the color control locus and molecular marker loci was drawn.

2.9. Fine mapping of color control candidate genes

The molecular markers closely linked to the color control locus and the contigs harboring the molecular markers locating were found by genetic linkage mapping. Then, the contig sequences of ACP004-33 and ACW001-33 were compared. According to sequence differences between the ACW001-33 and ACP004-33 genotypes, fine-mapping primers were designed using Primer 3.0 software (Rozen and Skaletsky, 2000) to distinguish the two genotypes. The primers designed from the ACW001-33 sequence were named the FMW series, and those designed from the ACP004-33 sequence were named the FMP series.

The primers that were able to amplify clear, stable bands were screened for genotyping of the mapping population (as above, those with the same genotype as the parent ACW001-33 or ACP004-33 were designated as “W” or “P”, respectively.). Using JoinMap 4.0 software, encrypted linkage maps of the color control locus were separately constructed with the FMW series of primers and the FMP series of primers. These linkage maps were used for fine mapping. Genes in the finely mapped contig regions were predicted using AUGUSTUS (version 3.2.1) (Stanke and Waack, 2003) with *Auricularia auricula-judae* as the model species (Lu et al., 2017). Finally, the functions of the color control candidate genes in the finely mapped contigs region were identified.

2.10. Candidate gene function prediction and functional verification

The color control candidate genes were annotated against the NR and Pfam databases using BLASTX (cut-off value, 1e-5). The protein families of the putative color control genes were identified using online tools at InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search). Then, the genes encoding key enzymes in pigment synthesis were predicted. According to the flanking sequences of these key genes, primers were designed using Primer 3 software, and the 87 dikaryotic strains of the testcross population were analyzed by PCR. Finally, the amplified fragments were separated on polyacrylamide gels.

2.11. Experimental flowchart

(see Fig. 2).

3. Results

3.1. Analysis of parental mating system and determination of mating type of monokaryotic strains

In total, 108 ACW001 self-successful strains and 108 ACP004 self-successful strains were obtained by constructing selfing populations. We assumed that the mating type of ACW001-33 was A_{w1}, that of nine monokaryotic strains of ACW001 with successful mating was A_{w2}, and that of 11 strains with unsuccessful mating was A_{w1} (Supplementary Table 1). Similarly, we assumed that the mating type of ACP004-33 was A_{p1}, that of nine monokaryotic strains of ACP004 with successful mating was A_{p2}, and that of 11 strains with unsuccessful mating was A_{p1} (Supplementary Table 1). The separation ratio of the two parents' mating type was 1:1 (confirmed by chi-squared test), consistent with the Mendelian genetic law. This result showed that the parental strains have a unifactorial mating system. This conclusion was consistent with the findings of a previous study (Luo, 1988). Through the construction of the hybrid population, 100 pairs of hybrid combinations were successfully mated. The success rate of hybridization was 100 %, indicating that A_{w1}, A_{w2}, A_{p1}, and A_{p2} were different mating types.

3.2. Identification of color homozygosity of parents

All the fruiting bodies of 108 strains obtained from the ACW001 selfing population were white (Supplementary Table 2), indicating that ACW001 was a white homozygote for the color trait. The fruiting bodies of 108 strains from the ACP004 selfing population were purple without color separation (Supplementary Table 3), indicating that ACP004 was a purple homozygote for the color trait. All the fruiting bodies of 100 strains in the hybrid population were purple (Supplementary Table 4).

3.3. Determination of color genotype of mapping population

After identifying the parents as color homozygotes, the color genotypes of the mapping population were determined by test crossing. In the mapping population, all the monokaryotic strains were successfully mated with ACW001-9. Of the 87 members of the testcross population, 30 produced purple fruiting bodies and 57 produced white fruiting bodies (Supplementary Table 5). The color genotypes of 87 monokaryotic strains of the mapping population were obtained.

3.4. Preliminary location of color control locus

The whole genomes of the monokaryotic strains ACW001-33 and ACP004-33 were sequenced. The ACW001-33 and ACP004-33 genomes contained 4949 and 4096 SSR loci, respectively (Supplementary Table 6). After screening, we obtained 32 pairs of SRAP primers and 56 pairs of SSR primers that could distinguish between the ACW001-33 and ACP004-33 genotypes (Supplementary Table 7). These selected 88 pairs of primers were used to distinguish the genotypes of the mapping population, and a total of 257 loci (56 SSR loci and 201 SRAP loci) were obtained (Supplementary Table 8). By linkage analysis, a genetic linkage map of *A. cornea* with 190 loci including the color locus was established. The total length of the map was 1315.2 cM, and it contained 12 linkage groups (LGs) with an average spacing of 6.9 cM (Table 1). The color control locus was located on LG11 and was closely linked with three marker loci (P2322, W1385, and W1387) (Fig. 3). The P2322 locus was located on ACP004-33_contig802, while the W1385 and W1387 loci were located on ACW001-33_contig733.

3.5. Fine mapping of color control candidate genes

The lengths of ACW001-33_contig733 and ACP004-33_contig802 were 30,632 bp and 64,250 bp, respectively. ACW001-33_contig733 was homologous with the 24,180–54,812 bp region of ACP004-33_contig802 (similarity, 43.70 %) (Fig. 4B). Six DNA fragments showed >90 % similarity in the homologous region. Because the homologous sequences of ACW001-33_contig733 and ACP004-33_contig802 were quite different, the FMW-series and FMP-series primers were designed based on the homologous sequences of the two contigs. In total, 20 pairs of primers (Supplementary Table 9) were screened for genotyping of the mapping population (Supplementary Table 10).

Fine mapping of the primary location area with FMW-series primers indicated that the color control locus was closely linked with FMW-7, FMW-12, and FMW-13 (Fig. 4C), between the FMW-7 and FMW-13 loci (ACW001-33_contig733; 7201–15,600 bp). This interval harbored only one protein-encoding gene (ACW001_g8479) (Fig. 4E). Fine mapping of the primary location area with FMP-series primers indicated that the color control locus was closely linked with FMP-2 and FMP-5 (Fig. 4D), between the FMP-2 and P2322 loci (ACP004-33_contig802; 26,181–35,573 bp). This interval harbored three protein-encoding genes; ACP004_g11814, ACP004_g11815, and ACP004_g11816 (Fig. 4F).

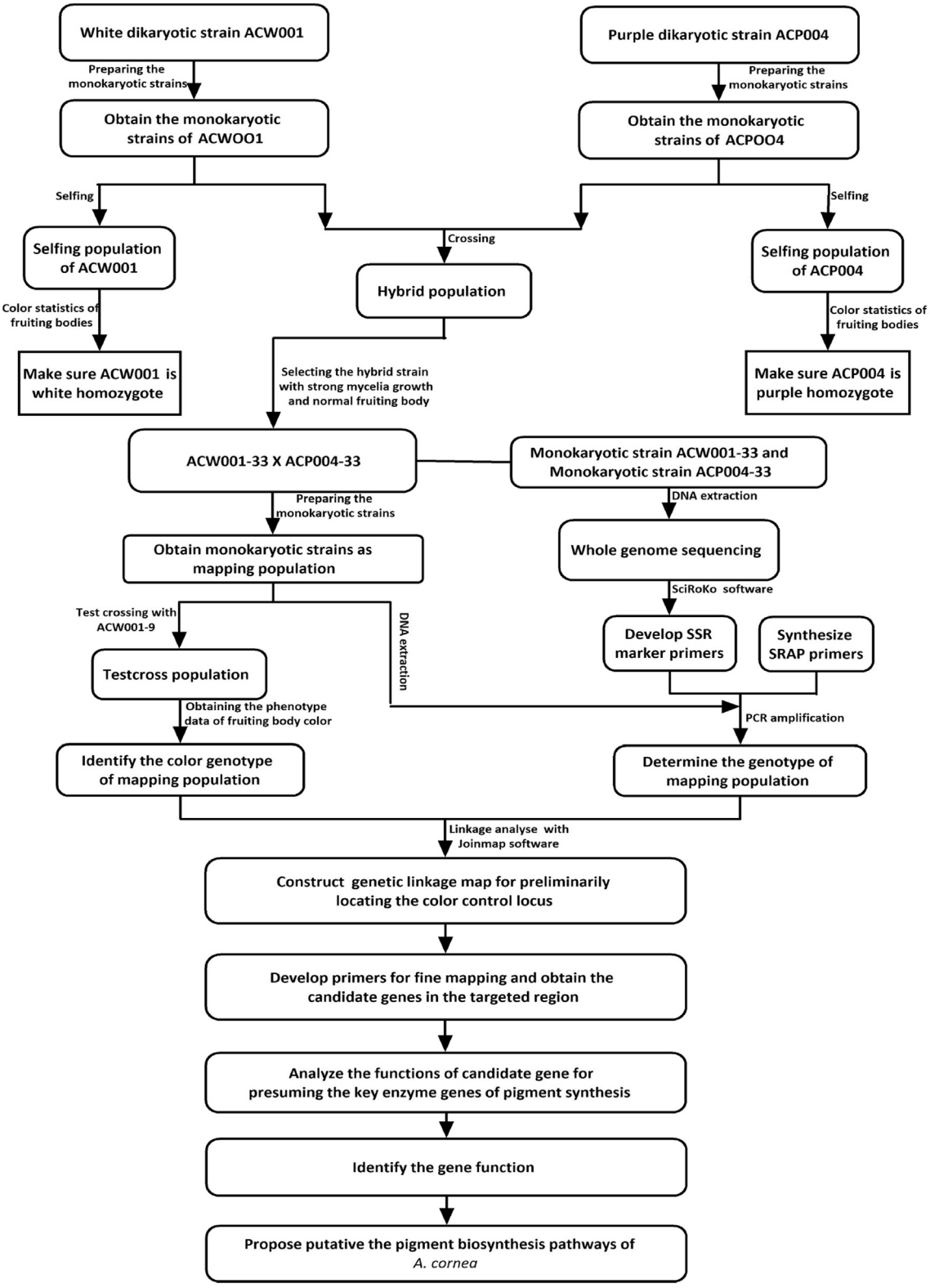

Fig. 2. Experimental flowchart.

3.6. Functional analysis and verification of color control candidate genes

The four candidate genes were compared with the NCBI and Pfam databases. ACW001_g8479 and ACP004_g11814 were proteins with unknown function. ACP004_g11815 and ACP004_g11816 belonged to the class I glutamine amidotransferase-like superfamily (SCOP52317) and both contained a GAT domain (PF03127). Proteins in this family function were glutamine amido transport (Table 2). Glutamine is an

Table 1
Genetic linkage map characteristics of *A. cornea*.

Linkage Group	Observed Length (cM)	Number of Markers	Average Marker Spacing (cM)
LG1	185.3	33	5.6
LG2	159.3	42	3.8
LG3	131.0	28	4.7
LG4	128.0	17	7.9
LG5	120.8	17	7.1
LG6	112.4	16	7.1
LG7	94.2	23	4.1
LG8	91.3	13	8.7
LG9	85.9	9	9.5
LG10	80.2	5	16
LG11	69.7	11	6.3
LG12	57.1	11	5.2
Total	1315.2	190	
Average	109.6	15.8	6.9

important component of the fungal pigment γ-glutamine-4-hydroxy-benzoate (GHB). Therefore, ACP004_g11815 and ACP004_g11816 were likely to encode key enzymes in the pigment synthesis pathway of *A. cornea*.

Primers were designed based on the flanking sequences of ACP004_g11815 and ACP004_g11816, and were used in PCR analyses of 87 dikaryotic strains in the testcross population. Bands of 990 bp and 1025 bp were amplified from the 30 strains with purple fruiting bodies, but not from the 57 strains with white fruiting bodies (Fig. 5).

4. Discussion

4.1. Identification of mating system and color homozygosity

In this experiment, a large number of genetic strains were required to study the color inheritance of *A. cornea*. It was important to control the mating reaction of monokaryotic strains to produce these genetic populations. However, most edible fungi do not have differentiated sexual organs at the mycelia stage. Therefore, it was important to clarify the mating system of the parents and the mating type of their monokaryotic strains to construct genetically distinct populations.

In another study on color inheritance in the fungus *F. velutipes*, F1 generations were crossed with yellow and white *F. velutipes* as parents. Analyses of monokaryotic strains of F1 generations backcrossed with two parents indicated that the color of *F. velutipes* was controlled by a pair of alleles (Xie et al., 2004). However, the results of another study indicated that the color of the *F. velutipes* fruiting body was controlled by multiple genes, based on analyses of selfing populations generated from white and yellow parents (Kong, 1996). These conflicting results may reflect the unclear genetic background of the parental strains, since the parents were not verified as being homozygous for the color trait. In the present study, therefore, we first constructed parental selfing populations to verify that the two parents were homozygous for the color trait. This laid the foundation for the accuracy of further experiments and analyses.

4.2. Determination of color genotype

In genetic research on plants, the gamete genotypes of the hybrid F1 population can be determined by backcrossing with the recessive parent. In *A. cornea*, a fungus with a bipolar heterothallism mating system, half of the mating-type genes in the gametes of the hybrid strain ACW001-33×ACP004-33 were the same as that of the recessive parent ACW001-33. Therefore, the color genotype of the gametes cannot be determined by backcrossing with the recessive parental monokaryotic strain. In this study, ACW001-9 was selected for testcrossing with the mapping population because its mating type differed from those of ACW001-33 and ACP004-33. The color genotypes of the mapping population were detected by fruiting tests.

4.3. Genetic linkage map

Genetic linkage maps have been constructed for several edible fungi, including *A. bisporus* (Kerrigan et al., 1993; Gao et al., 2015), *Lentinula edodes* (Terashima et al., 2002), *A. auricula-judae* (Lu et al., 2017), and *Pleurotus ostreatus* (Larraya et al., 2002). Each had 11–13 linkage groups, similar to the number obtained in this study for *A. cornea*. In those studies, genetic linkage maps were constructed to map quantitative trait loci for mycelial growth rate, yield, and sensitivity to mechanical damage. Few genetic maps have been used to map qualitative traits of edible fungi.

In general, SSR markers are the first choice of molecular markers for constructing genetic linkage maps because they are abundant, cover the whole genome, are inherited codominantly, follow Mendel's law of heredity, do not require high-quality DNA, and are amplified with easy-to-use PCR technology. In addition, they produce few bands, which facilitates typing of genetic groups for statistical analyses (Varshney et al., 2005). In this study, the whole genome of *A. cornea* was sequenced and SSR markers were developed to construct a genetic linkage map. However, only 23 % of the primers could successfully distinguish between the genotypes of the two monokaryotic parents. Therefore, we added SRAP molecular markers to increase the number of loci and construct a high-quality genetic linkage map with wider coverage and higher density.

4.4. Fine mapping

After preliminary mapping of the color control locus to the corresponding contigs of ACW001-33 and ACP004-33, it was found that the homologous sequences in the two parental contigs were quite different. This differed from our expectation that the color difference would be caused by base mutations in a gene encoding a key enzyme in pigment synthesis. However, most fine mapping primers are able to genotype mapping population because of large differences in contigs sequences. In other species, as fine mapping narrows the target area of the genes of interest, there are fewer differential sequences that can distinguish between the parents. Thus, the target area is usually narrowed to an interval of 30–50 Kb (Liu et al., 2017). In this study, the target area was narrowed to an

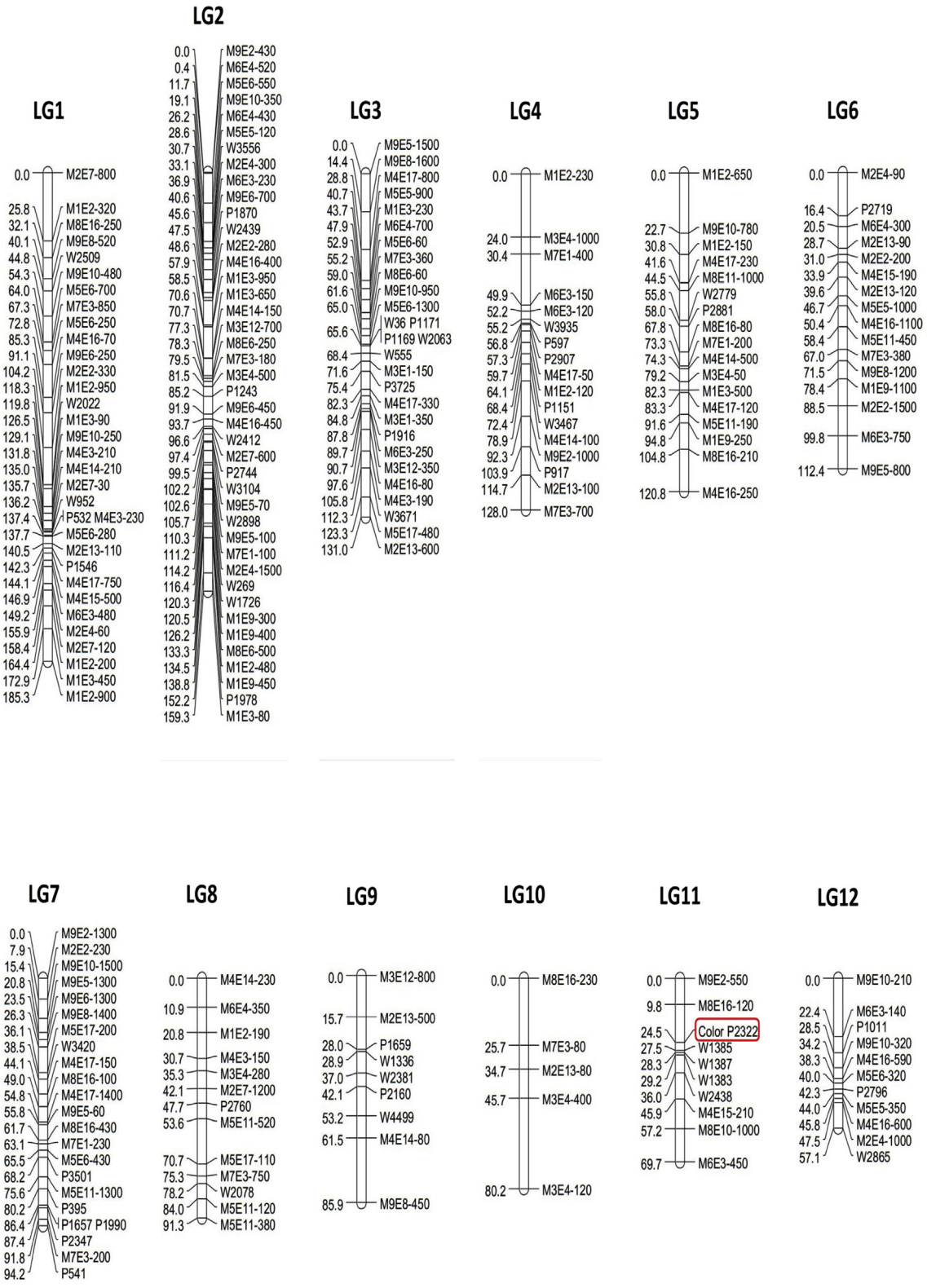

Fig. 3. Genetic linkage map and color control locus of *A. cornea*. Linkage groups (LG) are followed by group number. Genetic distances (cM) of markers are indicated at left of each LG, marker names are on right. SSR markers were named according to primer names (e.g. P2322). SRAP markers were named according to primer combination names and band length (e.g. M6E16-120).

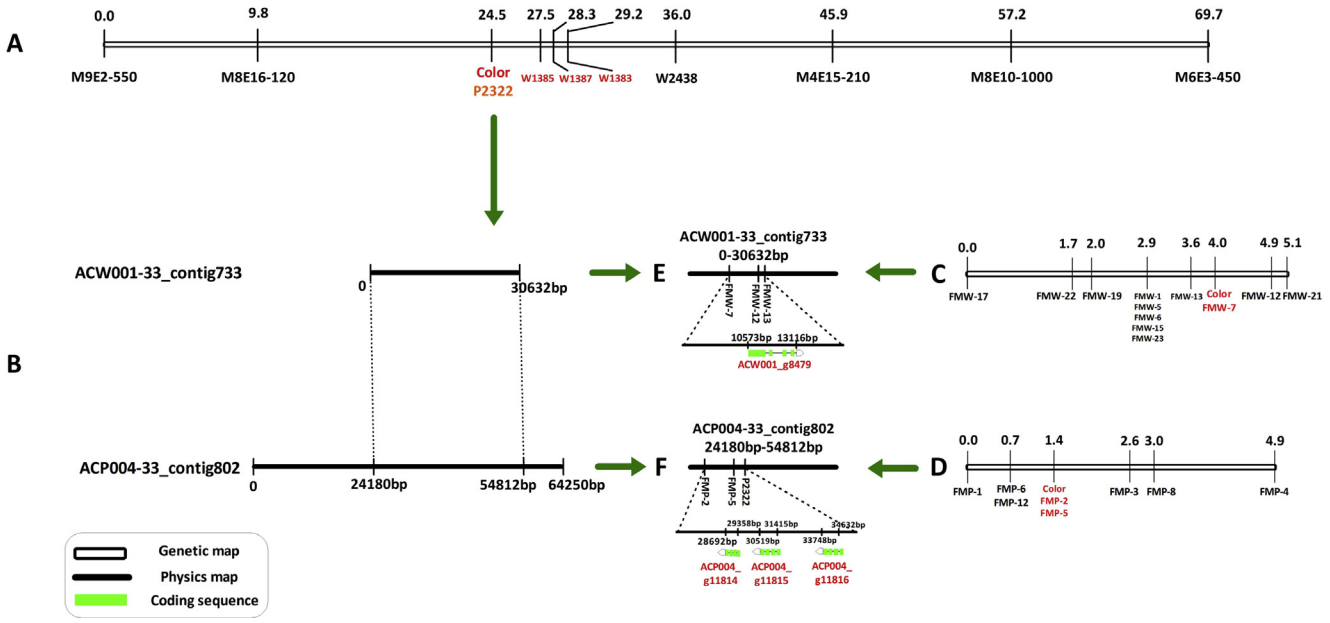

Fig. 4. Fine mapping of color control locus and candidate genes. (A) Preliminary location linkage map of color control locus; (B) Physical map of ACW001-33_contig733 and ACP004-33_contig802; (C) Fine-mapping linkage map of color control locus using FMW-series primers; (D) Fine-mapping linkage map of color control locus using FMP-series primers; (E) Location of color control locus in ACW001-33 genome and predicted functional genes in this region; (F) Location of color control locus in ACP004-33 genome and functional genes prediction in this region.

Table 2
Characteristics of candidate genes.

Candidate gene	Gene bank accession	Protein function	Domains	Number of exons	Gene length (bp)
ACW001_g8479	MK911860	unknown functional protein	None	4	2544
ACP004_g11814	MK911857	unknown functional protein	None	4	667
ACP004_g11815	MK911858	class I glutamine amidotransferase-like protein	GAT	4	897
ACP004_g11816	MK911859	class I glutamine amidotransferase-like protein	GAT	4	885

interval of 8.2–9.2 Kb by fine mapping. However, when the encrypted linkage map was constructed by calculating the recombination rate, it was found that the positions of different marker loci differed between the linkage map and the physical map. This may be due to the close proximity of the marker loci, because any minor recombination will lead to changes in the linkage analysis results.

4.5. Prediction of pigment synthesis pathway

According to their basic composition, fungal pigments mainly include terpenoids, acids, ketones, and some nitrogen-containing compounds. The pigments in large fungi of the same color often have similar composition, and therefore, similar pigment synthesis pathways (Gill and Steglich, 1987; Zhou and Liu, 2010). The

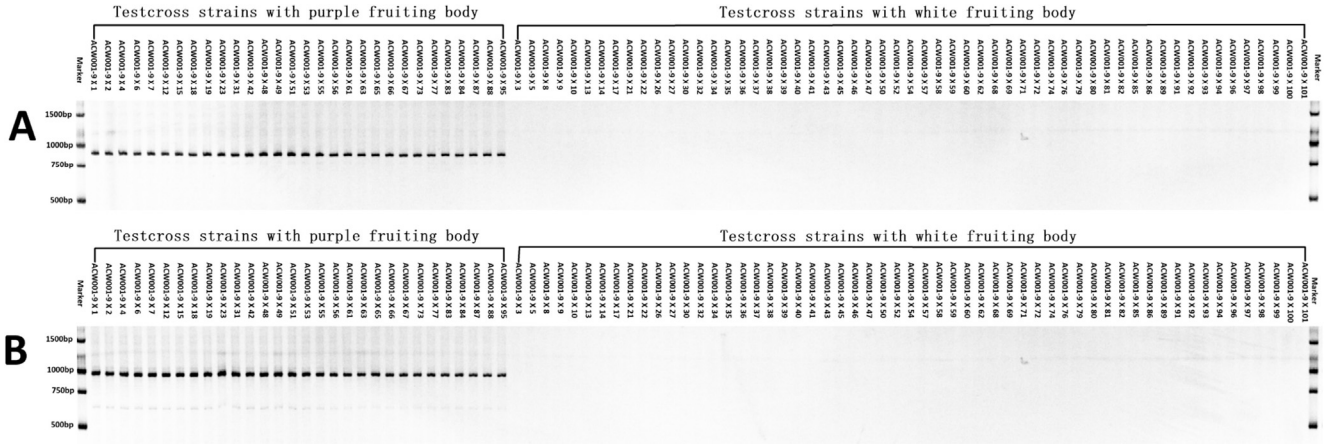

Fig. 5. Amplification products of tested populations on polyacrylamide gels. Marker: DL5000 (Takara); (A) Amplification products of ACP004_g11815; (B) Amplification products of ACP004_g11816.

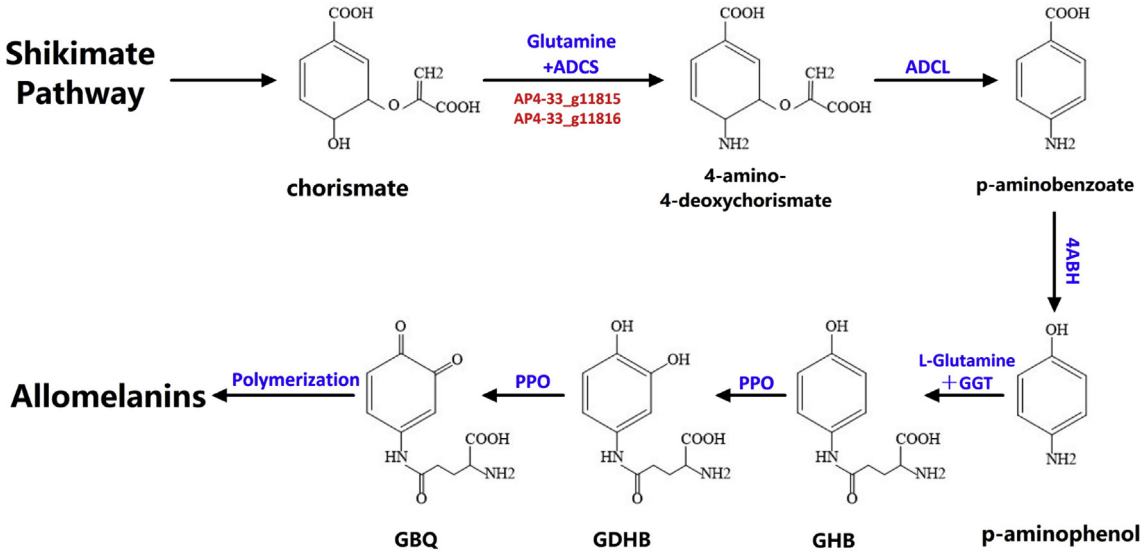

Fig. 6. Predicted pigment synthesis pathway in *A. cornea*. ADCS: 4-amino-4-deoxychorismate synthase; ADCL: 4-amino-4-deoxychorismate lyase; 4ABH: 4-aminobenzoate 1-monooxygenase; GGT: γ-Glutaminyltransferase; PPO: Polyphenol oxidase; GHB: γ-L-glutaminyl-4-hydroxybenzene; GDHB: γ-L-glutaminyl-3,4-dihydroxybenzene; GBQ: γ-L-glutaminyl-3,4-benzoquinone.

ventral surface of the normal fruiting body of *A. cornea* is purple, similar to the color of the gills and spore prints of *A. bisporus*, so their pigment composition and synthesis pathways are likely to be the same or similar. The function of the putative pigment synthesis enzymes identified in this study is to transfer glutamine amide groups. Therefore, we propose the following pigment synthesis pathway for *A. cornea*: chorismate is synthesized through the shikimic acid pathway. 4-amino-4-deoxychorismate is synthesized from chorismate and glutamine by 4-amino-4-deoxychorismate synthase, After elimination of pyruvic acid from 4-amino-4-deoxychorismate, the compound is aromatized to p-aminobenzoic acid by 4-amino-4-deoxychorismate lyase (Massiere and Badet-Denisot, 1998; James et al., 2002). Then, para-aminobenzoic acid is oxidatively decarboxylated by 4-aminobenzoate 1-monooxygenase (4ABH) to form aminophenol (Weijn et al., 2013). Aminophenol combines with L-glutamine γ-L-glutaminyl-4-hydroxybenzene (GHB) in a reaction catalyzed by γ-glutaminyltransferase (GGT) (Jolivet et al., 1999). The newly formed GHB first forms the unstable compound γ-L-glutaminyl-3,4-dihydroxybenzene (GDHB), which is oxidized to form the stable compound γ-L-glutaminyl-3,4-benzoquinone (GBQ) by polyphenol oxidase (PPO) (Boekelheide et al., 1979). Finally, GBQ polymerizes to form allomelanin (Belozerskaya et al., 2015) (Fig. 6). Therefore, the key enzyme in the synthesis pathway of *A. cornea* pigments is the glutamine-dependent amidotransferase that catalyzes the formation of p-aminobenzoic acid from chorismate. Its encoding genes in *A. cornea* are ACP004-33_g11815 and ACP004-33_g11816. The white fruiting body of ACW001 results from the blocked pigment synthesis pathway caused by the lack of these two key genes.

4.6. Application of map-based cloning technology to discover fungal functional genes

Since map-based cloning technology was first invented (Tanksley et al., 1995), this method has played a critical role in the discovery of plant functional genes and mutation sites (Jander et al., 2002; He et al., 2009). However, it had not been used in research on fungi. The main reasons for this were that: (1) Fungal evolution is lower than that of plants, their differentiation is simple, and few qualitative traits can be observed directly; (2) sequencing and research on fungal genomes lags behind that of field crops and model plants, and there is a lack of tools and databases for the development of molecular markers and functional gene annotation; (3) fungal chromosomes are small, and it is difficult to observe their chromosome numbers by optical methods (Xing et al., 2010). In addition, less re-sequencing data are available for fungi, making it difficult to assemble genome sequences to the chromosome level. Thus, the workload is greater at the initial positioning stage.

However, map-based cloning of fungi has the following advantages over plants: (1) Fungal genomes are small, usually between 2.5 and 81.5 Mb (Xing et al., 2010), with few redundant sequences, making gene location analyses more efficient and accurate; (2) fungal meiotic gametes can be cultured directly, so it is easy to obtain a mapping population similar to plant doubled haploid (DHL) lines (Zhang, 2003), and to control and target hybridization objects and processes; (3) under optimal conditions, fungi can complete their entire life cycle in a short time, thus shortening the experimental time. Therefore, with continuous progress in the development of sequencing methods and decreasing sequencing costs, fungal genome databases will continue to be enriched, and map-based cloning technology will become an important method for finding and studying excellent functional genes in fungi.

In locating genes controlling color in *A. cornea*, we found that the process for gene mapping in fungi using map-based cloning was very different from that in plants, especially in terms of mapping population construction and genotype determination. However, the methods for marker development, linkage analysis, and narrowing the location of genes of interest were essentially the same between fungi and plants. The specific steps and requirements are shown in Fig. 7.

5. Conclusion

To locate genes involved in controlling color and to elucidate the pigment synthetic pathway, we created different genetic

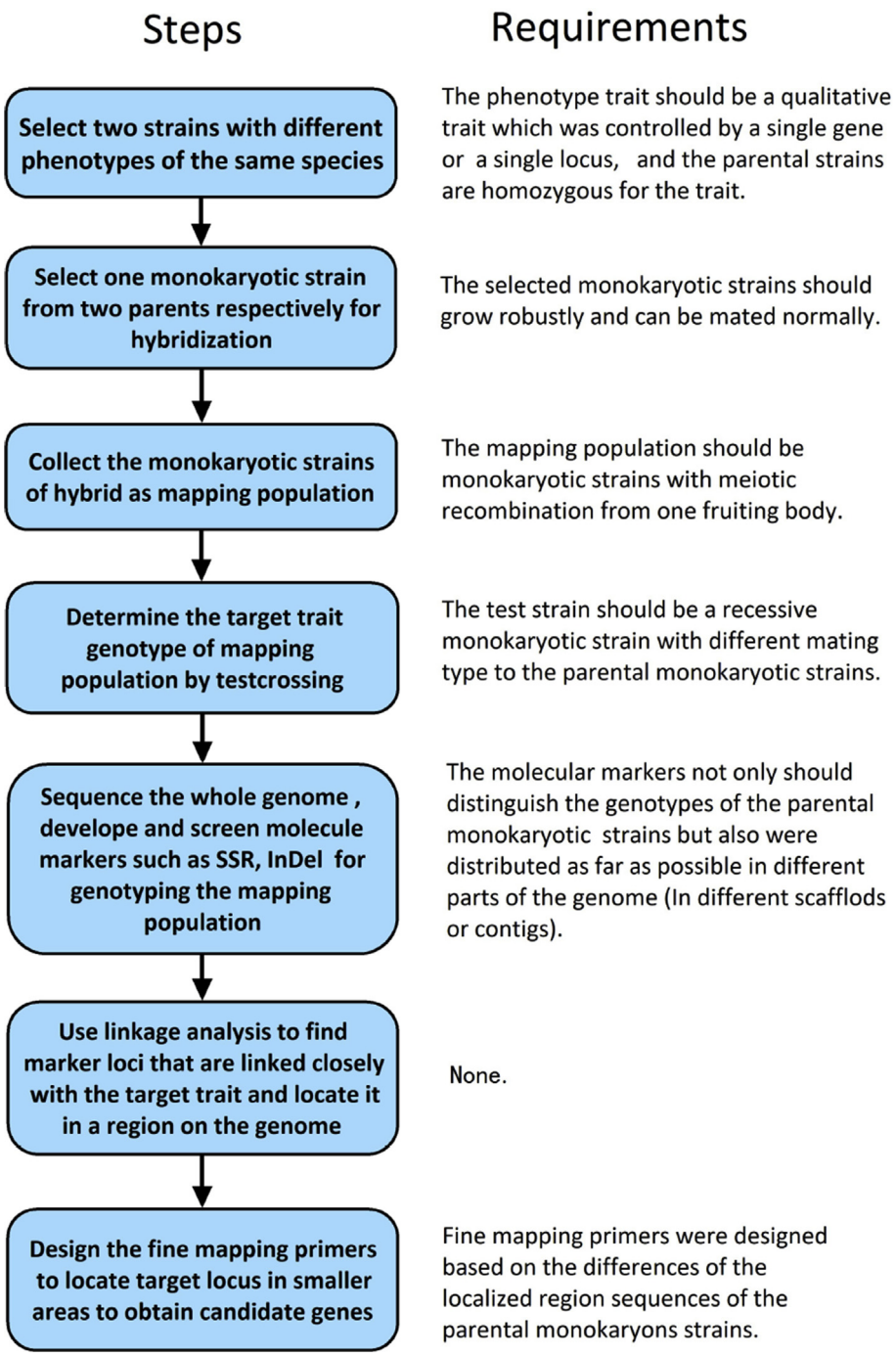

Fig. 7. The steps and requirements for fungi map-based cloning procedure.

populations. We sequenced the genomes of white and purple monokaryotic strains of *A. cornea* separately, and constructed a genetic linkage map and fine mapping maps. The results showed that the white fruiting body of the ACW001 strain may be related to the deletion of genes encoding key enzymes in pigment synthesis from the color control locus. This locus is located on ACP004-33_contig733, and harbors three protein-encoding genes; ACP004_g11814, ACP004_g11815, and ACP004_g11816. Among them, ACP004_g11815 and ACP004_g11816 are genes encoding glutamine amidotransferases, which catalyze the conversion of chorismate acid to 4-amino-4-deoxydeoxychorismate in the GHB pigment synthesis pathway. In this study, we obtained 87 monokaryotic strains in a mapping population with clearly distinguishable color genotypes, 108 dikaryotic strains in the white and purple selfing populations, 100 dikaryotic strains in a hybrid population, and 87 dikaryotic strains in a testcross population. These strains can be used for future research on the heredity and breeding of *A. cornea.* We have summarized the steps and requirements for the mapping and cloning of specific fungal genes by map-based cloning. The results of this study provide novel ideas and methods for the localization of fungal functional genes in further research.

Acknowledgments

This study was supported by Agriculture Research System of China (No. CARS-24). We thank Jennifer Smith, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.09.002.

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