Identification, characterization and expression of A-mating type genes in monokaryons and dikaryons of the edible mushroom Mycoleptodonoides aitchisonii (Bunaharitate)

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ABSTRACT

Identifying the mating-type in Mycoleptodonoides aitchisonii is important for enhancing breeding and cultivation of this edible mushroom. To clarify the molecular mechanisms of the bipolar mating system in M. aitchisonii, the homeodomain protein gene 2 (Mahd2) was characterized. A genomic DNA fragment of Mahd2 in M. aitchisonii 50005-18 strain was 1,851 bp long and encoded a protein of 614 amino acids. Transcriptional analysis revealed that the expression of Mahd2 was higher in monokaryotic strains that produced clamp cells than in those that did not. The highest relative expression level of Mahd2 was observed in monokaryon TUFC 50005, which was capable of forming a true clamp. These results suggested that the formation of clamp cells is regulated by A-mating type homeodomain proteins, and the frequency of clamp cell formation might be promoted by high expression of the Mahd2 gene.

Keywords: bipolar, clamp cell, gene expression, homeodomain protein, Mahd2

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1. Introduction

The sexuality of most cultivated edible fungi has been characterized. The majority of edible basidiomycete mushrooms are heterothallic. Sexuality is heterothallic when each spore receives a single post-meiotic nucleus and an incompatibility system prevails. There are two types of incompatibility systems: (1) the unifactorial system, in which sexuality is controlled by a single mating type (MAT) locus and only two mating types segregate in meiosis; and (2) the bifactorial system, in which sexuality is controlled by two unlinked mating types, commonly called the A and B loci. These systems are termed bipolar and tetrapolar, respectively. In the tetrapolar system, when two MAT loci are unlinked, four mating types can be generated by meiosis among the haploid progeny (Whitehouse, 1949; Au et al., 2014; Coelho, Bakkeren, Sun, Hood, & Giraud, 2017). In several higher basidiomycetes, bipolarity has been traced to the loss of mating specificity of the MAT-B locus. Where MAT-B is complete and present, only the MAT-A locus segregates in a mating type-specific manner (Aimi, Yoshida, Ishikawa, Bao, & Kitamoto, 2005; James, 2007). Recently, MAT-A and MAT-B loci were identified as the homeodomain (HD), and pheromone (Phe) and pheromone receptor (Prc) loci, respectively (Kües, 2015; Maia et al., 2015).

In both Schizophyllum commune and Coprinopsis cinerea, a difference in one of the A or B subloci (α or β) is sufficient to activate the respective pathway of A- and B-controlled development. The B genes regulate reciprocal nuclear exchange and nuclear migration in both mates, while the A genes control the development of clamps involved in the formation of dikaryotic hyphal compartments. This includes the initial pairing of haploid nuclei with different A and B specificities and the synchronous division of the nuclear pair in association with the initial development of the clamp cell, i.e., hook formation. Different B genes are then needed for fusion of the hook cell to the subapical cell, which completes formation of the clamp connection (Koltin, Stamberg, & Lemke, 1972; Casselton & Olesnicky, 1998; Kües, 2000; Fowler, Mitton, Rees, & Raper, 2004). Under suitable environmental conditions and the regulation of both A and B mating loci, the dikaryon will develop fruiting bodies and produce basidiospores after meiosis (Kües, 2000).

In our previous report, we determined that Mycoleptodonoides aitchisonii has the ability to produce fruiting bodies and clamp cells
in monokaryon strain isolates (Riffiani, Wada, Shimomura, Yamaguchi, & Aimil, 2019a). We demonstrated that dikaryotization was not required for clamp cell formation, fruiting body formation, and meiosis in this mushroom. This result indicates that mating is not essential for mushroom development.

We examined the polarity of mating types in *M. aitchisonii* as well as the genetic linkage between mating type locus and the ability to form monokaryotic clamp cells using classical genetic analysis. In addition, we sought to clarify the mechanisms underlying monokaryotic clamp cell formation in *M. aitchisonii*. Mating compatibility could be divided into two groups and indicates that *M. aitchisonii* is a bipolar mushroom. No genetic linkage was observed between the phenotype capable of forming monokaryotic clamp cells and mating type, indicating that monokaryotic clamp formation was not linked to the mating type locus (Riffiani et al., 2019b).

The reason why monokaryons can form clamp cells is not clear. In the present study, we employed a molecular approach to examine the mating type system of *M. aitchisonii*. Our goal was to further characterize the A locus and gene structure of *M. aitchisonii* and develop a framework for understanding *Mahd2* gene expression in monokaryons with and without true clamp connection.

2. Materials and methods

2.1. Strains and culture conditions

The strain used in these experiments, TUFC50005, was deposited at the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University (TUFC), Japan. Monokaryon strains [50005-1 to 50005-20 (F1)] were derived from dikaryotic strain TUFC 50005 (parent), and 86 strains (BRW-2 to BRW-177) were isolated from the basidiospores of 50005-7 × 50005-18 as F2. The strains were maintained on 2% malt agar slants at room temperature. Among the monokaryotic isolates, 50005-18 as a F1 derived from TUFC 50005 was used for characterization and expression of homeodomain protein genes.

2.2. Mycelium preparation, DNA and RNA extraction techniques

Mycelium for DNA extraction was prepared by growing isolates in minimum media. To prepare genomic DNA, three agar blocks (5 × 5 × 5 mm) were added into 20 mL minimal medium [1.5 g/L *NH₄*HPO₄, 1 g/L KH₂PO₄, 20 g/L glucose, 25 mg/mL thiamine hydrochloride, pH 5.5]. The mycelium was grown at 25 °C without shaking for 2 wk and then harvested by filtration; the mycelium was then frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Genomic DNA was extracted from the lyophilized mycelium according to the method described by Dellaporta et al. (1983). Genomic DNA was extracted using an improved cetyltrimethylammonium bromide (CTAB) method as follows: freeze-dried mycelia (100 mg) was ground into a powder using a mortar and pestle and mixed with 700 µL CTAB buffer [2% CTAB, 1.4 M NaCl, 100 mM Tris HC1 (pH 8.0), 20 mM EDTA (pH 8.0)] preheated at 65 °C. An equal volume of chloroform: isooamy alcohol (24:1) was added to the retained supernatant fraction. After gentle mixing, the suspension was kept at room temperature for 20 min and then centrifuged at 1,000 rpm for 10 min. The supernatant was transferred to a new microtube containing a two-thirds volume of cold (600 µL) isopropanol and, after gentle shaking, the mixture was centrifuged (15,000 rpm for 5 min). The DNA pellet was washed twice with 1 mL 75% (v/v) ethanol and air-dried. After dissolving the pellet in 50 mL TE buffer, RNA was removed by adding 1 mL RNase solution (10 mg/mL) and incubating at 37 °C for 1 h. For high DNA purity and concentration, several tubes containing DNA were mixed and purified using a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. The quality of DNA extracts was confirmed by polymerase chain reaction (PCR) amplification of ribosomal DNA internal transcribed spacers (ITS) using the primers described in Table 1. RNA was extracted using a MagExtractor™ Kit (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. cDNA was synthesized using total RNA as a template using ReverTra Ace® qPCR RT Master Mix with a gDNA Remover Kit (Toyobo).

2.3. Genome sequencing and isolation of A locus alleles

Whole genomic sequences of monokaryon *M. aitchisonii* 50005-18 were determined using the Illumina HiSeq 2000 paired-end technology provided by Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). This sequencing run yielded 43,044,484 bp high-quality filtered reads with 100-bp paired-end sequencing. The genome was assembled using Velvet assembler (hash length, 85 bp) (Zerbino & Birney, 2008). The final assembly contained 3,342 contigs with a total size 39,963,208 bp, and an N₅₀ length of 224,401 bp. According to Fernandez-Fueyo et al. (2012), the deduced amino acid sequence of known HD2 mating type protein genes from public databases [a2-1 (EMD41907) from *Gelatoporia subvermispora*], HD protein gene was assigned as *Mahd2*-18 using tBlastn software.

| Table 1. PCR primers used in this study |
|---------------------------------------|
| **Primer**                | **Sequence**                        |
| HD2C_Maspi1R_F1  | 5' - ATGACGACATGGTTGGATGGAGA-3' |
| HD2C_Maspi1R_R1  | 5' - GGAACGCGCGAAGTGTAGTGTTG-3' |
| ITS 1       | 5' - TCCGTAGGGACACCTGGG-3'         |
| ITS 4       | 5' - CCTCCGGCTTATGAGTCG-3'         |
| M1_M4      | 5' - GGTCTTCCCAGTACCACAGC-3'       |
| M1_M1M     | 5' - CCAAGTCAGCAGTTGTA-3'          |
| HD2_1R_F0   | 5' - ACACACGGTAGTGTATGATTTGA-3'    |
| HD2_1R_F1   | 5' - GGATGGAAGCTCAGTGAACCCAT-3'    |
| HD2_1R_J1* | 5' - CGTACCGAGCCATG-3'             |
| HD2_1R_F0   | 5' - ACACACGGTAGTGTATGATTTGA-3'    |
| HD2_1R_F1   | 5' - GGATGGAAGCTCAGTGAACCCAT-3'    |
| Mal1_RACE2  | 5' - CCGTGACGCGATGACCCAGGAGA-3'    |
| HD2_1R_J2   | 5' - GCATTCTACTGCGAAAATTCCT-3'     |
| HD2_1R_J2   | 5' - ATGATGTGGTCCAAAATTCACC-3'     |
| HD2_1R_J2   | 5' - GAGTGGCAATGTGTTACCT-3'        |
| Actin_18F   | 5' - AGTACGCAGCTCTCGTTATGCACA-3'   |
| Actin_18R   | 5' - AGCCCTCATGCGGATAGG-3'         |

**Applications**

- Amplification of *Mahd2* of 50005-18
- Universal fungal barcode sequence
- Oligo dT-Adaptor
- S-Race
- Reverse transcription for S-Race
- 5'-Race
- 5'-Race of *Mahd2*
- Real time PCR for amplification of *Mahd2*
- Real time PCR for amplification actin

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(Altschul et al., 1997) on scaffold 760. Primer pairs for amplification of Mahd2 50005-18 were designed based on predicted HD2 sequences using GENETYX 13 software (Genetyx, Tokyo, Japan).

### 2.4. PCR conditions

Amplification of the homeodomain gene (Mahd2) was conducted using the specific primers (Table 1). The PCR reaction mixture contained: 8 μL dNTPs, 1.0 μL primer 1 (100 μM), 1.0 μL primer 2 (100 μM), 0.5 μL (1 unit) DNA Taq polymerase, 1 μL DNA, 10 μL 10× Taq buffer, and 78.5 μL H₂O for a final volume of 100 μL. DNA amplification was performed with a T100TM Thermal Cycler (Bio-Rad, Tokyo, Japan) using the followed program: 3 min hot start at 95 °C, followed by 35 cycles consisting of denaturation (45 s at 95 °C), annealing (30 s at 58 °C), extension (60 s at 72 °C), and a final extension step (5 min at 72 °C). PCR products were analyzed using 1.5% agarose gel electrophoresis run with TAE buffer (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) and then stained with ethidium bromide.

### 2.5. Reverse transcription-PCR (RT-PCR)

Total RNA from the dikaryotic M. aitchisonii strain was used as a template for RT-PCR. Amplification of full-length cDNA by RT-PCR was carried out with ReverTra Ace qPCR RT Master Mix with a gDNA Remover Kit (Toyobo) using total and 3'-rapid amplification of cDNA ends (3'-RACE). 3'-RACE PCR was performed using the PrimeScript™ RT PCR kit (Takara Bio). 5'-RACE PCR was performed using a 5'-Full RACE Core Set (Takara Bio). All reverse transcription reactions and PCR were carried out according to the manufacturer’s instructions. The amplified fragments generated using these methods were subcloned into a pMD20 T-vector (Takara Bio) and sequenced.

### 2.6. Real-time PCR assay

Actin was used as the housekeeping gene. Partial actin, and Mahd2-18 genes in M. aitchisonii were cloned using the generated PCR primers. Primers for Mahd2-18 and actin were designed according to their cDNA sequences using GENETYX 13 software. Amplification of genomic DNA was prevented by designing primers for exon-exon junctions. All primers were tested to ensure the amplification of single bands with no primer-dimers. Plasmids with the inserted target gene (Mahd2-18) and housekeeping gene (actin1) were extracted according to the method modified by Birnboim (1983).

A standard curve was constructed using five ten-fold dilutions of the plasmid. Real-time PCR was performed using the KOD SYBR qPCR Mix Kit (Toyobo). Thermal cycling conditions were as follows: 90 °C for 30 s to activate the thermostable DNA polymerase, 61 °C for 20 min for reverse transcription, 95 °C for 30 s pre-denaturation, and then 40 cycles of 95 °C for 15 s, 60 °C for 1 s and 74 °C for 30 s. Each run was completed with melting curve analysis to confirm the specificity of amplification and the absence of primer-dimers. Data analysis was performed according to the manufacturer’s instructions.

To estimate the difference of expression level of homeodomain gene in monokaryotic strain can make clamp cell comparing with the no clamps cell strain, we use statistical analyses using IBM SPSS software version 25 (IBM Japan, Ltd., Tokyo, Japan) with Tukey HSD multiple comparison test. All effects were considered significant at P < 0.05.

### 2.7. Frequency analysis of clamp cell formation

Frequency analysis of true clamp-cell formation in 10 d-old mycelia on TM7 medium was performed under a microscope. The frequency of clamp formation at the septa that formed between the subterminal cell and the third cell of a mycelium was determined by the following formula: Clamp formation frequency (%) = (Number of septa with clamps/number of septa observed) × 100 (Riffiani et al., 2019b).

### 3. Results

#### 3.1. Nucleotide sequence of homeodomain Mahd2 50005-18 protein of M. aitchisonii

The nucleotide sequences of A mating type loci Mahd2-18 from M. aitchisonii were amplified and sequenced. The nucleotide sequences of the A mating-type locus and its flanking regions from M. aitchisonii 50005-18 have been deposited in the DDBJ database under the accession numbers LC532152. The open reading frame of Mahd2-18 gene from ATG to the stop codon was 1,851 bp. The 3 introns of the Mahd2-18 gene interrupted the coding sequence, which is comprised of 4 exons (Fig. 1). The upstream region was analyzed, and a putative TATA box at position 57 bp before the start codon was identified. A predicted transcription termination sequence (poly-A signal) for Mahd2-18 was found 113 bp after the stop codon.

The PSORTII program (Nakai & Hortonn, 1999) predicted that the Mahd2-18 protein contains 3 nuclear localization signals: PT-KRRVP at 382, PFPRRTK at 434, and PRPRTRPG at 436 bp. The Mahd2-18 gene encodes a protein of 614 amino acids with a predicted molecular mass of 69.93 kDa. Mahd2-18 gene had the highest homology (48.78% identity) to Pholiota microspora A3-hox1 and A3-hox2 homeodomain proteins (complete CDS, accession number AB435543.1), and had 48.48% identity with Agaricus bisporus var. bisporus H97 homeodomain type 2 mating protein (accession number XM_006454908.1). The MOTIF program predicted that the protein sequence of Mahd2-18 (position 154–210, 59 amino acids in length, e-value 3E-17) is a DNA binding domain involved in the transcriptional regulation of key eukaryotic developmental processes, and may bind to DNA as monomers or homo- and/or heterodimers in a sequence-specific manner (Fig. 1).

#### 3.2. Characterization of A locus in M. aitchisonii

In order to address why monokaryotic strains form clamp cells, we investigated the mating system of M. aitchisonii including gene structure and segregation. The total genome of M. aitchisonii was amplified and sequenced. BLAST searches revealed that the A locus in M. aitchisonii is located between mitochondrial intermediate peptidase (MIP) and beta-flanking gene (β-fg). Comparative genomics revealed that gene content is generally conserved around the HD locus in basidiomycetes. MIP has been employed as a marker for the isolation of HD loci from non-model species (James, Kües, Rehner, & Vilgalys, 2004a ; Coelho et al., 2017). While β-fg codes for a putatively secreted protein that appears to be fungi specific and can be found in basidiomycetes and ascomycetes (Ohm et al., 2010; Kües, James, & Heitman, 2011 ; van Peer et al., 2011).

HD1 and HD2 domains are both highly conserved among basidiomycetes including M. aitchisonii. The two genes are linked and divergently transcribed (Casselton & Olesnicky, 1998; James, Srivillai, Kües, & Vilgalys, 2006). However, HD1 might be absent in the...
A-mating type locus of 50005-18 strain (Fig. 2). Four of the genes around the A locus (including β-fg, mip, Glydh, Up11) have homologous genes around the A locus of the bipolar mushrooms Pholiotina microspore (Yi, Mukaiyama, Tachikawa, Shimomura, & Aimi, 2010), Panerococha chrysosporium (James, Lee, & van Diepen, 2011) and Pleurotus djamar (James et al., 2004b).

The mip gene and glycine dehydrogenase gene (GLYDH), which are conserved in the A mating type region, appear to have been present in the ancestor of these bipolar Polyporales species, as previously suggested (James et al., 2004b, 2006). Other genes of known function that display conserved synteny between M. aitchisonii and other homobasidiomycetes (e.g., Coprinellus disseminate) are RNA polymerase II (RPB2) and glycine dehydrogenase gene (GLYDH) (James et al., 2006). The PBI gene, which encodes a protein for para-amino benzoic acid synthase, is located upstream of the mating type region, appear to have been present in the ancestor of these bipolar monokaryotic strains (James, Lee, & van Diepen, 2011) and M. aitchisonii, and other homobasidiomycetes (e.g., Coprinellus disseminate). These genes are present in the 50005-18 strain around the homeodomain locus (including Aeb, 2002). Ten genes showing similarity to those in the EMBL database were identified in 50005-18 strain around the homeodomain locus of the bipolar mushrooms Pholiota and Coprinellus disseminate, and other homobasidiomycetes (e.g., Coprinellus disseminate).

**3.3. Mating type of monokaryotic strains**

Based on the result of HD2 gene mapping, we designed specific primers to amplify the homeodomain gene HD2. Primers HD2C_Maspi18F1 and HD2C_Maspi18R1 amplified a 567 bp product for Maspi18F1 and HD2C_Maspi18R1 amplified a 567 bp product for the homeodomain gene HD2. Primers HD2C_Maspi18F1 and HD2C_Maspi18R1 amplified a 567 bp product for 3.3. Mating type of monokaryotic strains

Based on the result of HD2 gene mapping, we designed specific primers to amplify the homeodomain gene HD2. Primers HD2C_Maspi18F1 and HD2C_Maspi18R1 amplified a 567 bp product for Mahd2-18 (Table 1; Fig. 3). These primers can be used to identify a...
Monokaryotic clamp cell formation and mating type segregation among monospore isolates derived from dikaryotic strains (50005-7 × 50005-18) are shown in Table 3. The 20 strains could be divided into 2 incompatibility groups depending on the different size of the PCR product. Group 1 comprised strains 50005-2, 6, 9, 10, 12, 15, 16, 18, and 19, in which the PCR product was 567 bp in length. Group 2 comprised strains 50005-1, 7, 8, 11, 13, 14, 17, and 20, in which no band was observed. The mating type of Groups 1 and 2 was A1 (60%) and A2 (40%), respectively. Next, we confirmed these results by analyzing 86 F2 progenies derived from the fruiting body of the dikaryotic stock (50005-7 × 50005-18). The result showed that the 86 monokaryons could be separated into two
Table 3. Monokaryotic clamp cell formation and mating type segregation among monospore isolates derived from dikaryotic strains (50005-7 × 50005-18).

| Mating type | Size of PCR product (hd2) | A1 | A2 |
|-------------|---------------------------|----|----|
| Ability of monokaryotic clamp cell formation | A1: 567 bp length (A1) & A2: No band (A2) | 25, 41, 52, 58, 60, 88, 143, 154, 165 | 31, 51, 63, 65, 59, 64, 177, 175 |
| A1 | No clamp | 2, 3, 4, 5, 6, 7, 8, 9, 12, 13, 14, 18, 19, 20, 21, 22, 24, 29, 30, 33, 37, 38, 39, 40, 42, 47, 48, 49, 53, 57, 70, 71, 74, 75, 77, 79, 106, 113, 122, 137, 126, 128, 30, 131, 132, 134 |
| A2 | No clamp | 10, 11, 15, 16, 17, 23, 26, 36, 43, 45, 46, 50, 55, 62, 67, 69, 81, 107, 108, 109, 110, 112, 129, 145 |

*, Strain numbers BRW is abbreviated.

groups, A1 (567-bp amplified product, 62.8%) and A2 groups (no band, 37.2%). Moreover, nine A1 strains and eight A2 strains produced true clamp among 86 monospore isolates. Thus, monokaryotic clamp cell formation had no relationships with mating type (Table 3). Recent study (Riffiani et al., 2019b) and the segregation data in Table 3 seem to indicate that other gene is involved in clamp cell formation. However, previously, higher expression level of the HD genes promoted formation of true clamp and pseudoclamp cell in Pholiota microspora (Yi et al., 2010), but the clamp cell formation was controlled with only HD genes (Aimi et al., 2005). Therefore, only HD genes directly may be control clamp cell formation in M. aitchisonii, but presence a factor promote expression of Mahd2 should be suggested.

The Mahd2-18 primer set (primers HD2C_Maspi18F1 and HD2C_Maspi18R1) was developed for the molecular A-mating type assay and enabled quick determination of mating type (Fig. 4). The frequency of clamp cell formation among all monokaryotic strains were few. The formation of clamp cell is a local event in the mycelium colony, and the frequency of clamp cell formation among all monokaryotic is very lower than that of clamp cell in dikaryon (50005-7 × 50005-18). However, total RNA must be prepared from the whole colony. Extraction of total RNA from only clamp cell is technically impossible, and then most of the total RNA was extracted from cells that did not form clamp cells. Therefore, there is no significant correlation between expression of the Mahd2 and the frequency of clamp cell formation among all monokaryons.

### 3.4. Gene expression of HD2-18 in monokaryon and dikaryon strains

We previously demonstrated that a monokaryotic strain (50005-4) formed fruiting bodies and exhibited clamp cell formation (Riffiani et al., 2019a). The efficiency of clamp cell formation is controlled by the expression level of homeodomain protein genes; thus, we amplified a partial cDNA encoding fragment of Mahd2-18 using the primer pair HD2-18F-RT/HD2-18R-RT (Table 1). In order to predict the role of HD2 to drive true clamp cell formation, 10 monokaryotic strains that do or do not produce true clamp cells were examined by qRT-PCR. The results indicated that the relative expression level of Mahd2 in monokaryotic strains that produce clamp cells is higher than monokaryotic strains that do not. Mahd2 transcription in monokaryotic strain 50005-4, which can produce true clamp cells, was the highest among all monokaryotic strains (Fig. 4). The frequency of clamp cell formation in monokaryotic strain 50005-4 and 50005-18 were higher among all monokaryotic strains (Table 4). However, the dikaryon (50005-7 × 50005-18) showed much higher HD2 expression and frequency of clamp cell formation than the monokaryons (Fig. 4). Thus, significant correlation between expression of the Mahd2 and frequency of clamp cell formation in monokaryons and dikaryon was observed. However, differences of HD2 expression and frequency of clamp cell formation among all monokaryotic strains were few. The formation of clamp cell is a local event in the mycelium colony, and the frequency of clamp cell formation among all monokaryotic is very lower than that of clamp cell in dikaryon (50005-7 × 50005-18). However, total RNA must be prepared from the whole colony. Extraction of total RNA from only clamp cell is technically impossible, and then most of the total RNA was extracted from cells that did not form clamp cells. Therefore, there is no significant correlation between expression of the Mahd2 and the frequency of clamp cell formation among all monokaryons.

### 4. Discussion

Based on the results of the mating type test, which employed molecular approach, we propose that M. aitchisonii has a bipolar mating system, as only two mating types segregate in meiosis. Moreover, recombination mating type strains might not be generated after meiosis, indicating that there may only be a single mating type locus in M. aitchisonii. It is suggested to be easier to score the mating type using a round of PCR amplification of mating type genes prior to test mating (Kothe, 2001). For such breeding programs, it will be important to determine compatible mating types; hence, expanding our knowledge of mating-type genes can facilitate such efforts. In particular, for the introduction of recessive markers, inbred strains are a necessity.

In the present study, the gene encoding HD1 in 50005-18 strain was absent upstream of the HD2 protein gene. This finding highlights a significant difference in the structure of the A-mating type locus from other mushrooms. Analysis of A mating type proteins in Coprinopsis cinerea by Asante-Owusu et al. (1996) suggested the presence of HD1 and HD2 heterodimers. HD2 contributes an essential DNA binding domain, and HD1 provides a transcriptional activation domain. HD1 proteins contribute a potential activation domain present in an essential C-terminal sequence, which has been shown to activate transcription of a reporter gene in Saccharomyces cerevisiae. This indicates that the HD2 protein should play an important role during clamp cell formation. HD1 can be deleted from Coprinopsis cinerea without impairing function (Asante-Owusu, Banham, Böhner, Mellor, & Casselton, 1996). Moreover, heterodimerization of a pair of compatible homoeodomain 1 (HD1) and 2 (HD2) proteins were required for nuclear localization including penetration of nuclear membrane (Spit, Hyland, Mellor, & Casselton, 1998). However, in M. aitchisonii, monokaryon can produce true clamp. Even though HD1 was absent in A-mating type locus of 50005-18, the strain was able to produce clamp cells. Therefore, HD2 might be formed as a homodimer to penetrate the nuclear membrane.

Three predicted nuclear localization signals (NLS) in Mahd2-18 were determined by PSORTII analysis (Nakai & Horton, 1999). NLS on the HD2 protein permit the heterodimer to localize to the nucleus. Once in the nucleus, the heterodimer binds specific
**Table 4.** Frequency of clamp cell formation

| Strain                  | Nuclear phase | Total number of septa | Number of septa with clamp cells | Frequency (%) |
|-------------------------|---------------|-----------------------|----------------------------------|---------------|
| 50005-7 × 50005-18<sup>a</sup> | Dikaryon      | 48                    | 26                               | 54            |
| 50005-1                  | Monokaryon    | 22                    | 1                                | 4.6           |
| 50005-2                  | Monokaryon    | 24                    | 1                                | 4.2           |
| 50005-3                  | Monokaryon    | 30                    | 1<sup>b</sup>                    | 3.3           |
| 50005-4<sup>a</sup>      | Monokaryon    | 18                    | 1                                | 5.6           |
| 50005-6                  | Monokaryon    | 46                    | 0                                | 0             |
| 50005-7<sup>ab</sup>     | Monokaryon    | 26                    | 1                                | 3.9           |
| 50005-10                 | Monokaryon    | 52                    | 0                                | 0             |
| 50005-12                 | Monokaryon    | 49                    | 0                                | 0             |
| 50005-18<sup>b</sup>     | Monokaryon    | 18                    | 1                                | 5.6           |
| 50005-20                 | Monokaryon    | 31                    | 1<sup>b</sup>                    | 3.2           |

<sup>a</sup>: Riffiani et al. (2019b)

<sup>b</sup>: Observed as pseudoclamp

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Fig. 4. Relative *Mahd2* expression in the dikaryon and in monokaryotic strains capable or incapable of producing clamp cells. Total RNA was measured on day 8 by qRT-PCR in duplicate. Different letters indicate statistically significant differences (one-way ANOVA with Tukey’s HSD multiple comparison test, *P* < 0.05).
operator sites to bring about A-regulated changes in gene transcription (Wen et al., 1995).

The homeodomain found in many proteins regulate gene expression (Gehring, 1987). RT-PCR analysis revealed that HD2 in the dikaryon strain was preferentially expressed (10-fold increase) compared to monokaryon strains. This phenomenon relates to our previous study (Riffiani et al., 2019b), in which the frequency of clamp formation in dikaryons was proposed to be greater than 50%.

The difference of expression level among monokaryons might not be significant, because the expression level of hd2 gene is significantly lower than dikaryon (Fig. 4.). In previous our study, frequency of clamp cell formation was strongly affected by expression level of homeodomain protein gene in bipolar mushroom (Yi et al., 2010). Therefore, if expression level of hd2 gene will increase, pseudoclamp might mature to true clamp. From this hypothesis, the pseudoclamp is a step before producing true clamp, and I think it is meaningless to distinguish this difference between pseudoclamp and true clamp. The most important finding in this paper are, (1) monokaryon can produce true clamp, and (2) single homeodomain protein control clamp cell formation.

Using a DNA-mediated transformation system, in which a single homeodomain protein (A3-box1 or A3-box2) from the A3 monokaryon strain of Photobota microspora was transformed into the A4 monokaryon strain, the transformants produced many pseudoclamp but very few clamps (Yi et al., 2010). When two homeodomain protein genes (A3-box1 or A3-box2) were transformed together into the A4 monokaryon, the ratio of clamps increased to approximately 50%. Based on these results, it can be concluded that complete clamp formation is controlled by the expression level of homeodomain protein genes, and that altered expression of A mating type genes is sufficient to drive true clamp formation (Yi et al., 2010).

Dikaryons of both Coprinopsis cinerea and Coprinus bilanatus have characteristic clamp connections between individual cells. Clamp cell formation normally occurs after fusion of monokaryons carrying different A factors. However, clamp cells can also be induced in monokaryons by introducing one or more different A factor genes by transformation (Mutasa et al., 1990; Kues et al., 1992). The entire A42 factor of Coprinopsis cinerea and individual genes were introduced into a monokaryon of Coprinus bilanatus and, where expressed, resulted in altered colony morphology associated with the development of clamp cells (Challen et al., 1993). It is of particular interest that only Coprinopsis cinerea HD2 genes can elicit A-regulated development in Coprinus bilanatus. These results indicate that clamp cell formation in monokaryons is regulated by the A- mating type and is related to the high expression of homeodomain proteins.

One possible hypothesis for monokaryotic clamp cell formation is that high concentrations of pheromones or products secreted by autoregulation might induce homeodomain protein expression, resulting in promotion of clamp cell formation. In future studies, we will need to confirm the effect of artificially synthesized pheromone peptides or products secreted by autoregulation on clamp cell formation.

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