Study of fruiting body formation and clamp cell formation in the monokaryon of the edible mushroom *Mycoleptodonoides aitchisonii* (Bunaharitake)

(食用きのこ*Mycoleptodonoidesaitchisonii*（ブナハリタケ）のモノカリオンにおける子実体形成とクランプ細胞形成に関する研究)

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A Dissertation for Doctoral Degree

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Chapter I

General Introduction

Two types of sexual reproduction systems are found in basidiomycetes mushrooms: heterothallic and homothallic. Heterothallic mating occurs between two separate monokaryons with compatible mating types, which is required for the formation of clamp cells and complete fruiting bodies. In typical heterothallic mushrooms, the life cycle generally starts from the germination of the haploid basidiospores and haploid mycelium, which is usually called a monokaryon (Fig. 1.1). This form has a simple septa and contains one or two identical haploid nuclei in the cells of its hyphae. When two monokaryon having compatible mating types meet in nature, their cells can be fused to produce dikaryon, and the two monokaryons reciprocally exchange nuclei. Each monokaryon donates a nuclei to the other mycelium, which incorporates the nuclei into its cytoplasm. These nuclei divide (Kües and Liu, 2000) and are actively transported through the entire mycelium (Gladfelter and Berman, 2009) until the mycelium is completely fertilized (Buller, 1930). Unlike other eukaryote kingdoms in which nuclei fuse immediately after fertilization, the two haploid nuclei, or the so-called dikaryon, in basidiomycetes remain separate for an extended period (Anderson and Kohn, 2007). This secondary mycelium is characterized by two distinct haploid nuclei per hyphal cell—one from each parental monokaryon. The secondary

mycelium grows by cell division to clamp cell formation, and the dikaryon efficiently produces fruiting bodies. Meiosis and basidiospore formation occur during fruiting body formation to produce four meiotic products wrapped separately into individual basidiospores.

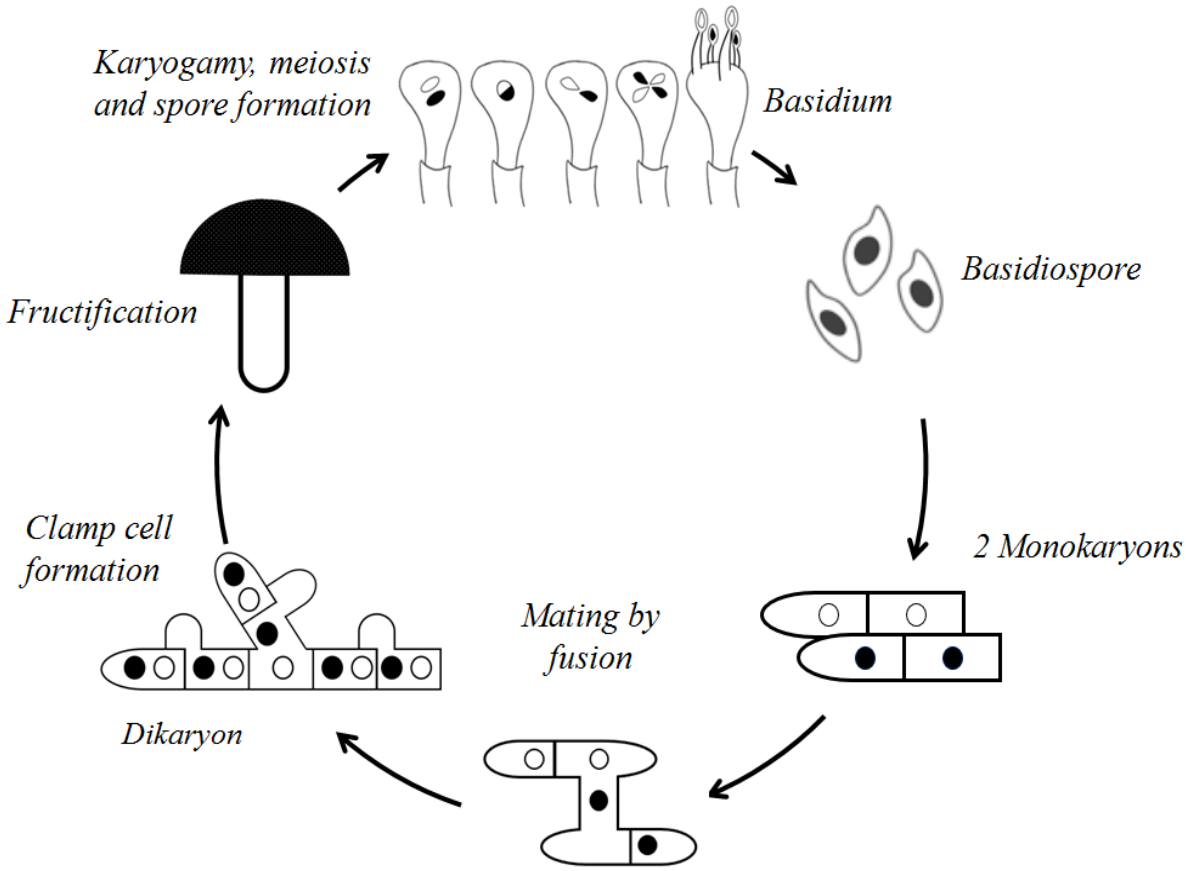

Fig. 1-1 Lifecycle of basidiomycetes(Nieuwenhuis et al., 2013)

A meiotically produced haploid spore germinates to form a mycelium. The mycelium grows as a monokaryon, which contains a single nucleus in each hyphal compartment. When two compatible monokaryons meet, they fuse and reciprocally exchange nuclei. The nuclei divide and migrate into the other's mycelium. When the entire mycelium is dikaryotized, vegetative growth continues by cell division with clamp connections. A dikaryon can produce fruiting bodies, mushrooms, on which basidia are found. A basidium is a specialized cell in which the two haploid nuclei fuse and form a diploid nucleus that immediately starts meiosis. The four meiotic products are wrapped separately into individual spores, and these spores disperse through the air.

In the typical lifecycle of the mushroom, however, monokaryotic fruiting body formation was previously reported in *Schizophyllum commune* (Leslie and Leonard, 1979), *Sistotrema brinkmanii* (Ullrich, 1973) and *Coprinopsis cinerea* (Uno and Ishikawa, 1971). Therefore, it is unclear whether dikaryotizaton is necessary for the formation of clamp cells and/or complete fruiting bodies because clamp cell formation normally occurs after fusion of monokaryon carrying different *A* factors.

1-1Mating system in Basidiomycota

Among the mushrooms belonging to Basidomycota, 90% of the species are heterothallic (self-incompatible), and 10% are homothallic (self-compatible) species (Whitehouse, 1949). Of the heterothallic species, about 61% have two unlinked mating-type loci, called *A* and *B* mating types. The remaining 39% have a single mating-type locus, called an *A* mating type (Casselton, 1978, Heitman at al., 2007). Following are detailed descriptions of the mechanisms controlling homothallism and heterothallism.

1-1-2 Homothallism

The homothallic pattern of sexuality in basidiomycetes may be one of two types, primary or secondary.

a. Primary homothallism

Primary homothallism, which has been established with certainty in only a few forms, does not appear to involve incompatibility factors. A self-fertile mycelium directly develops from a single spore with a single post-meiotic nucleus, and the presence of incompatibility factors cannot be detected (Fig. 1-2a). The fertile mycelium is homokaryotic; that is, it has genetically identical nuclei. It may be dikaryotic, with or without clamps, but more commonly it is multikaryotic without clamps. Karyogamy and meiosis occur regularly in the basidia of the fruit body, but the heterokaryotic phase is absent in the life cycle of primary homothallic forms. A self-fertile mycelium develops directly from a single spore with a single post-meiotic nucleus, and the incompatibility factors can not be detected (Raper, 1966).

b. Secondary homothallism

Secondary homothallism has a distinctly different mechanism. It involves incompatibility factors and is determined by the mechanism of nuclear distribution. Basidia of secondary homothallic forms usually only bear two spores each. Two compatible post-meiotic nuclei migrate into each basidiospore, and the self-fertile mycelium that develops directly from a single spore is intrinsically heteroallelic for the involved incompatibility factor or factors (Fig. 1-2b, upper right). The fertile mycelium is heterokaryotic, with two genetically different nuclear types. It is usually dikaryotic with clamps but

may be dikaryotic without clamps or multikaryotic without clamps. The homokaryotic phase is absent in the life cycle of secondary homothallic forms. Secondary homothallic forms may be either unifactorial or bifactorial, and their functional distinctness from heterothallic forms is due only to the manner in which post-meiotic nuclei are regularly distributed in the basidiospores (Rapper, 1966).

1-2-2 Heterothallism

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 genetic factor, *A*, with multiple specificities (alleles), and (2) the bifactorial system in which sexuality is controlled by two unlinked genetic factors, *A* and *B*, each with multiple alleles. These systems are also termed bipolar and tetrapolar, respectively, based on patterns of fertility observed in mating between siblings in the two systems (Rapper, 1966).

a. Unifactorial

In unifactorial forms, the single-*A* factor controls compatibility as it isrelated to the entire sexual cycle (Metzenberg and Glas, 1990). In a mating, differences in *A* factor alleles (*A*) permit completion of the entire cycle. This typically involves the migration of invading nuclei into and throughout the resident

mycelium, which is a reciprocal process in both mates through which the establishment and propagation of the dikaryon is accompanied by the formation of clamp connections between cells (true clamps) and ultimately the formation of fruit bodies on the dikaryon. The *A* factor appears to be a single genetic unit and, in a given cross, its two loci, e.g., *A1* and *A2*, segregate in a 1:1 ratio (Table 1-1). The mycelia that develop from the four spores constitute two mating-type groups and interact in a bipolar pattern when mated in all pairings. Bipolar pattern mating interactions in all paired combinations are indicated for the four progenies derived from the four spores of a basidium (a tetrad) from a cross involving incompatibility types *A1* X *A2* (Raper, 1966).

Table 1-1 Bipolar pattern mating interactions

Incompatibility Type	*A1*	*A1*	*A2*	*A2*
A1	—	—	+	+
A1	—	—	+	+
A2	+	+	—	—
A2	+	+	—	—

—, incompatibility and no interaction; +, compatibility and complete interaction to produce a fertile dikaryon with true clamps

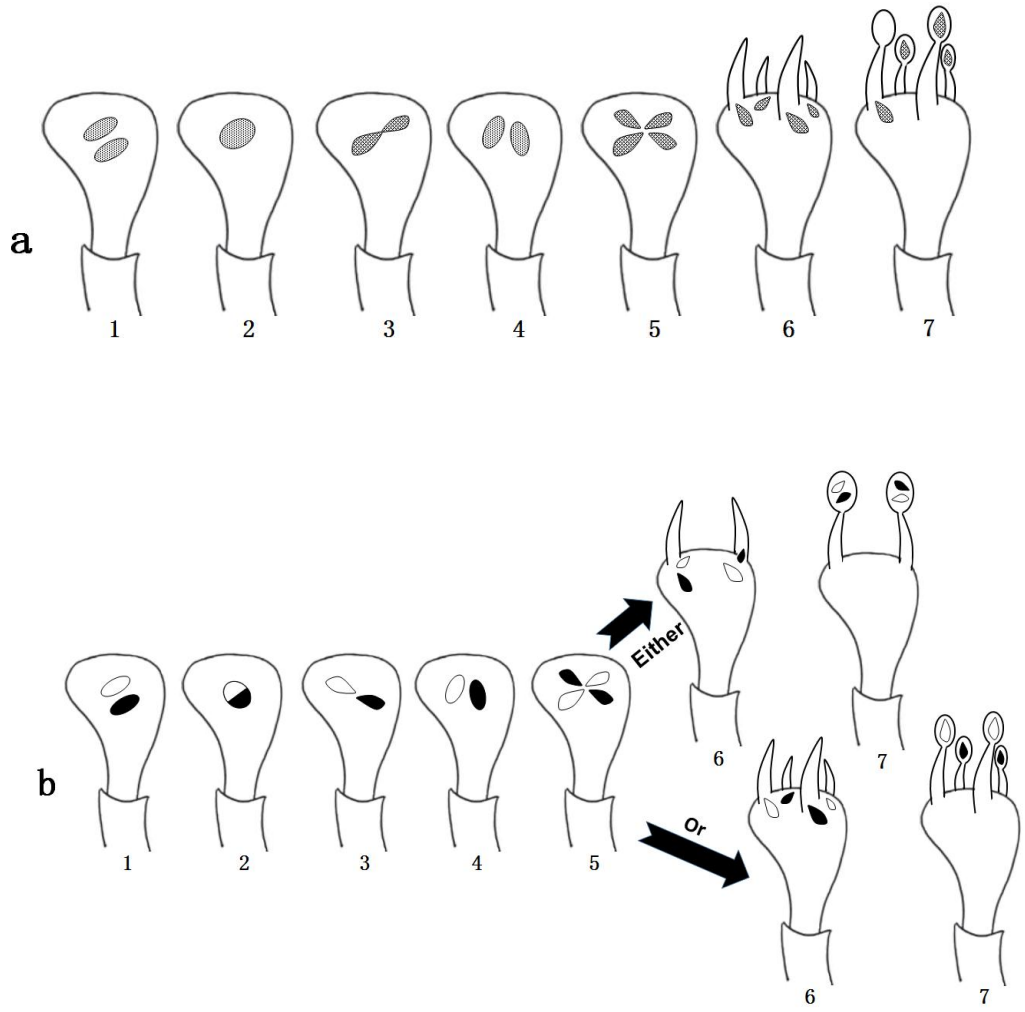

Fig.1-2. Establishment of sexual patterns in basidiomycetes. Karyogamy (1 and 2), meiosis (3, 4 and 5), formation of sterigmata and spores and distribution of post meiotic nuclei to the spores on the basidium (6 and 7) are illustrated for three different patterns: (a) primary homothallism with no incompatibility factors (stippled nuclei), (b, upper right) unifactorial secondary homothallism with meiotic segregation of two alleles of a single incompatibility factor (black and white nuclei) and the inclusion of two compatible post meiotic nuclei in each of two basidiospores, and (b, lower right) unifactorial heterothallism with meiotic segregation of two alleles of a single incompatibility factor (black and white nuclei) and the inclusion of a single post meiotic nucleus in each of four basidiospores (Raper, 1966).

b. Bifactorial

In bifactorial forms, *A* and *B* factors have been shown to control distinct but coordinated parts of the sexual cycle (Heitman et al., 2007). In mating, the *A* factor controls nuclear pairing and clamp formation, and the *B* factor controls nuclear migration and clamp fusion. Mating interactions in all paired combinations are indicated for four progenies representing a four-type tetrad from a cross with the incompatibility types *A1 B1* X *A2 B2* (Table 1-2) (Raper, 1966, Billiardet al., 2012).

Table 1-2 Tetrapolar pattern mating interactions

Incompatibility Type	*A1B1*	*A2B1*	*A1B2*	*A2B2*
A1B1	—	—	—	+
A2B1	—	—	+	—
A1B2	—	+	—	—
A2B2	+	—	—	—

—, incompatibility; +, full compatibility in which a no restricted fertile dikaryon with true clamps is formed

1-3 Clamp cell formation

In tetrapolar mushrooms, both *A* and *B* mating-type genesare required for clamp formation. The *B* locus regulates reciprocal nuclear migration via

hyphal fusion through broken septa from one haploid hypha to the other. The *A* locus controls the pairing of the haploid nuclei from the parental strains and the formation and septation of clamp cells, as well as the mitotic division of the paired nuclei. The growth of the hook toward and subsequent fusion to the subapical cell are controlled by the *B* gene and allow the movement of the nucleus from the hook into the subapical cell to complete clamp formation (Fig. 1-3) (Casselton and Olesnicky, 1998; Raper, 1966).

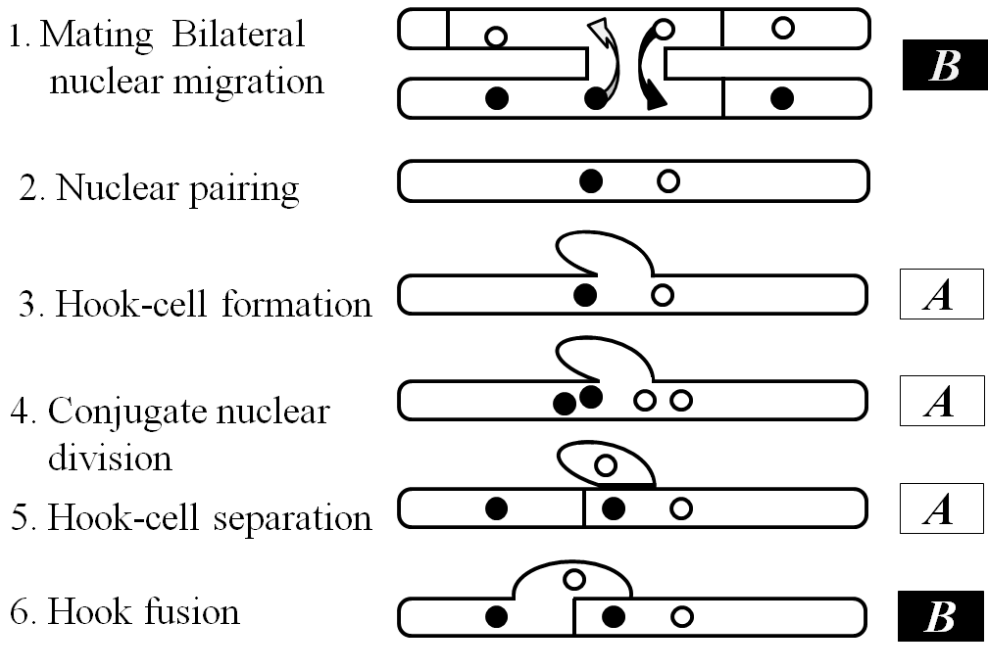

Fig. 1-3 Respective pathway of *A*- and *B*-controlled clamp cell development in tetrapolar mushrooms.

Under suitable environmental conditions and the regulation of both *A* and *B* mating loci, the dikaryon will develop fruiting bodies with meiotic basidiospores (Kües, 2000).

1-4 *A*-mating type loci

A standard sub-locus encompasses a divergently transcribed pair of homeodomain1 (HD1) and homeodomain2 (HD2) genes that form a functional group that is flanked by the mitochondrial intermediate peptidase (MIP) gene on one side and the β-flanking gene on the other (James et al., 2004a; Kües et al., 2011). In the case of multiple HD subloci, several of these functional groups will be positioned between the MIP gene and the b-flanking gene (Brown and Casselton, 2001; James, 2007; Kües et al., 2011). A successful mating brings together different allelic forms of these genes that in turn triggers part of the developmental sequence required for sexual reproduction. The fusion protein retains most of the HD2 sequence but only the C-terminal part of the HD1 protein. Only the HD2 homeodomain motif is included as a potential DNA binding domain fused to the essential C-terminal region of the HD1 protein, which is a normal HD1-HD2 protein complex that may be the major activation domain (Fig.1-4). HD1 and HD2 proteins from different alleles of the same sub-locus can heterodimerize with each other to form a functional transcription factor. HD1 and HD2 proteins from the same locus, however, fail to heterodimerize (Banham et al., 1995; Kües et al., 1994).

HD1 proteins contain two more nuclear localization signals and an activation domain but only weakly bind DNA (Tymon et al. 1992, Asante-Owusu et al., 1996), whereas HD2 proteins lack these domains but have strong DNA binding properties (Spit et al., 1998).

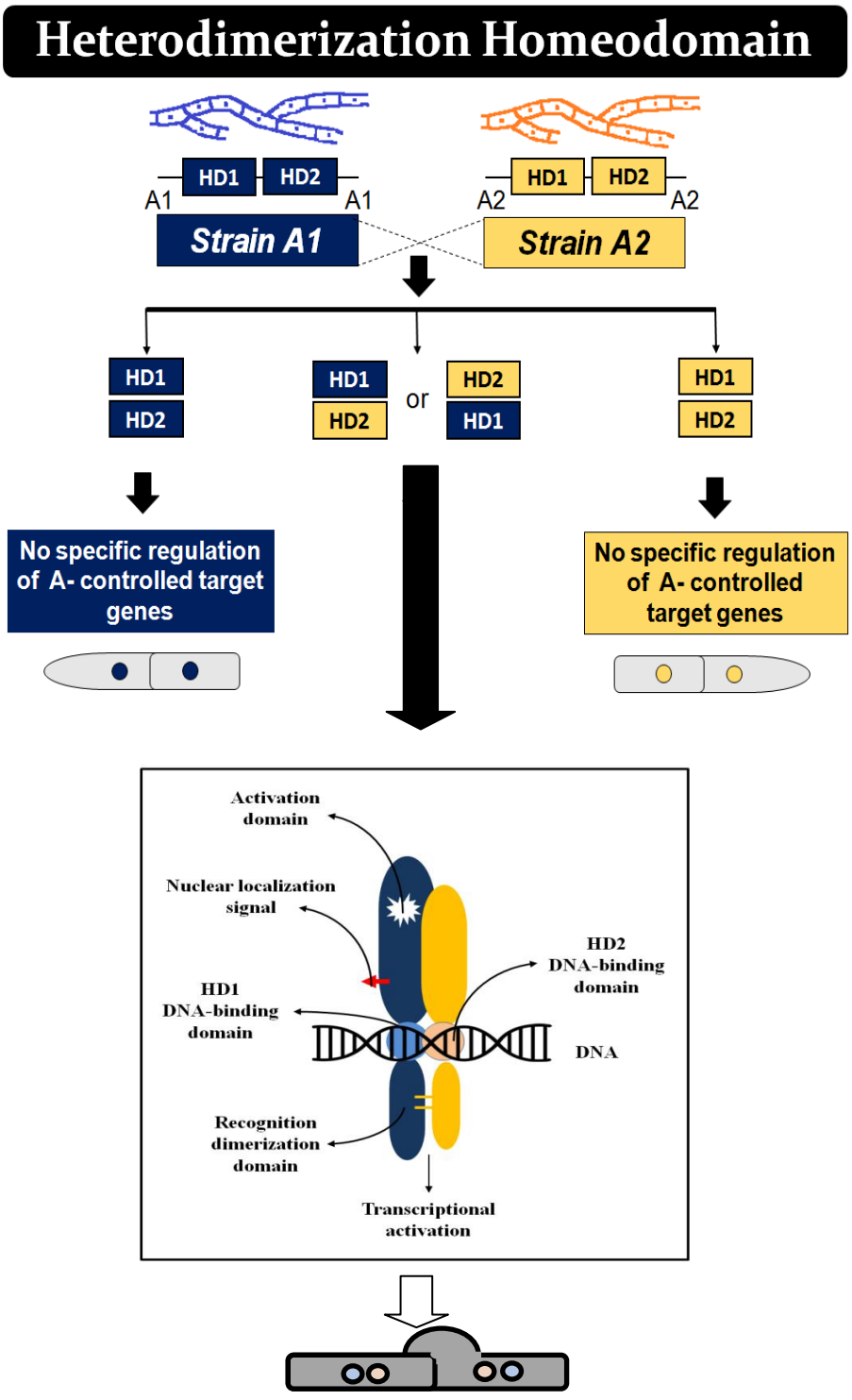

Fig. 1-4 A typical A locus contain two divergently transcribed genes encoding homeodomain transcription factor types HD1 and HD2. Heterodimer HD1/HD2 has two gene products and is only active in inducing *A*-regulated development, resulting in dikaryon formation in matings with different *B* genes between the two strains if the two proteins have different allelic specificity, e.g., from strain *A1* and *A2*, respectively (Brown and Casselton, 2001; Osiewacz,2002).

Both HD1 and HD2 proteins possess N-terminal dimerization motifs that facilitate their interactions. Interaction of an HD1 protein with a compatible HD2 protein generates a heterodimer that serves as a transcription factor for the *A* pathway (Banham et al., 1995). The separation of different functional domains onto two proteins represents an elegant strategy to ensure that the mating-dependent developmental pathway is activated only after fusion between compatible mates (Kües et al. 1994).

1-5 Homeodomain

Homeodomain (HD) proteins are transcription factors (TFs) that are present in the entire eukaryote kingdom, from unicellular organisms to plants, fungi and animals (Burglin and Affolter, 2016). HD TFs represent about 15%–30% of all TFs in plants and animals and act in all tissues of the embryo and adult organism to regulate processes as diverse as axis patterning (Pick and Heffer, 2012) or metabolic responses (Wu et al., 1996). HD refers to the DNA-binding region, which is typically 60 amino acids long and contains a helix-turn-helix (HTH) motif. Owing to this feature, HD proteins are identified as DNA binding TFs; that is, they are TFs that recognize specific DNA sequences to access target genes in the genome and to control their expression.

1-6 Purpose of this study

Mushroom *Mycoleptodonoides aitchisonii* is of culinary interest and belongs to the Climacodontaceae in Polyporales. It has been widely cultivated on logs of broadleaf trees from summer to fall in Asia. The white or straw colour fruiting body has no pileus,and the basidiocarp is sessile. The surface is smooth and the edges are tooth-shaped. The cap is fan shaped or spatulate, and the size is approximately 3x3 cm to 8x10 cm. The mycelium consists of two needle-shaped mycelia of approximately 3-10 mm in length, and the sporesare approximately 2 to 2.5x6.5 μm. *M. aitchisonii* has pharmaceutical activities including immunomodulation, lipid-lowering effects and antibacterial effects (Okuyama et al., 2004).

Choi et al.(2014) reported an endoplasmic reticulum stress-protecting compound isolated from *M. aitchisonii* that showed protective activity against endoplasmic reticulum stress-dependent cell death. Volatile compound 1-phenyl-3-pentanone produced by *M. aitchisonii* was tested for antifungal activity against fungal growth and spore germination of plant-pathogenic fungi (Nishino et al., 2013). *M. aitchisonii* has been reported to have anti-asthmatic effects based on studies in an ovalbumin-induced asthma mouse model. Administration of *M.aitchisonii* howed dose-dependent suppression of white blood cells, eosinophils and immunoglobulin (Ig)E resulting from ovalbumin-induced asthma in BALB/c mice (Lee et al., 2018). In order to achieve successful breeding and artificial cultivation of *M. aitchisonii* for potential use

as a nutritional food and an important source of biological compounds, it is essential to understand its life cycle to understand how monokaryotic mycelia can produce clamp connections.

Here, we describe monokaryotic clamp cell formation, fruiting body formation and meiosis in *M. aitchisonii*. A single dikaryotic *M. aitchisonii* strain, TUFC50005, which exhibits a wide spectrum of monokaryotic fruiting, and 20 monokaryons derived from TUFC50005 were used. Most strains of monokaryons formed primordia, or young fruiting body-like structures, but only one of the 20 monokaryons, strain TUFC50005-4, formed a complete fruiting body, even though it had only one nucleus and produced only two basidiospores after meiosis. In Chapter 2, we demonstrate that dikaryotization is not required for clamp cell formation, fruiting body formation and meiosis in this mushroom. This is the first report to show that mating and nuclear fusion are not essential for development in this mushroom.

Although basidiomycetes mushrooms normally form fruiting bodies in the dikaryotic state, monokaryons have long been known to also form fruiting bodies under certain conditions, such as nutritional depletion (Verrinder and Lu, 1984). The reason monokaryotic strains can form clamp cells and fruiting bodies remains to be clarified. In Chapters 3 and 4, we present our investigation of the mating system in *M. aitchisonii* using classic and molecular approaches, functional gene mapping, gene segregation, and determination of *A*-mating type expression levels. Here, we briefly introduced *M. aitchisonii*, its

mating system in the context of the homobasidiomycetes and reviewedresearch findings for the*A*-mating type related to clamp cell formation in monokaryon and dikaryon of edible mushroom *M. aitchisonii* (Bunaharitake).

In Chapter 3, we use classical genetic analysis to examine the polarity of mating types in *M. aitchisonii* as well as the genetic linkages between mating-type loci and the ability to form monokayotic clamp cells. In addition, we seek to clarify the mechanisms underlying monokaryotic clamp cell formation in *M. aitchisonii*. Mating compatibility of this species falls into two groups, indicating that *M. aitchisonii* is a bipolar mushroom. Moreover, recombinant mating type strain might not be generated after meiosis, indicating that there may only be a single mating-type locus in *M. aitchisonii*. No genetic linkages were observed between phenotypes capable of forming monokaryotic clamp cells and mating type, indicating that monokaryotic clamp formation was not linked to the mating-type locus.

Further, the reason why monokaryons can form clamp cells is not clear. In the present study, we have examined the mating type of *M. aitchisonii* using molecular techniques. We designed specific primers for HD2, and we describe the characterization of genomic DNA and cDNA sequencesfor the HD2 gene from *M. aitchisonii*. We also analysed the expression of the HD2 gene in monokaryon, whichhas a true clamp connection and no clamp connection.

Chapter 2

Monokariotic fruiting body and clamp cell formation in *Mycoleptodonoides aitchisonii* (Bunaharitake)

2-1 Abstract

The ability to produce monokaryotic fruiting bodies and clamp cells in culture was examined in monokaryotic strain isolated from several dikaryotic parental strains of the edible mushroom, *Mycoleptodonoides aitchisonii* (Bunaharitake). We describe a single dikaryotic *M. aitchisonii strain*, TUFC50005, and 20 monokaryons derived from it, which exhibited a wide spectrum of monokaryotic fruiting types. Most strains formed primordia, or young fruiting body-like structures, but only one of the 20 monokaryons, strain TUFC50005-4, formed a fruiting body, even though it had only one nucleus and produced only two spores after meiosis. We demonstrated that dikariotization was not required for clamp cell formation, fruiting body formation, or meiosis, in this mushroom.

2-2 Introduction

Two types of sexual reproduction systems exist in basidiomycete mushrooms: heterothallic and homothallic systems. The heterothallic system is better known as it is employed by many familiar mushrooms, such as *Lentinula edodes*, *Pleurotus ostreatus*, and *Flammulina velutipes* (Wang et al., 2016).

The term heterothallic refers to mating between two separate monokaryons carrying different compatible nuclei that are required for the formation of true clamp cell and complete fruiting bodies. Conversely, the heterothalic system is characterized by the presence of pseudo-homothallic or secondarily homothallic species. These fungi develop a self-fertile mycelium that has the ability to generate heterokaryotic spores containing mating-compatible nuclei.

The homothallic system is a self-fertile system in that two genetically identical nuclei can fuse and undergo complete fruiting body formation and meiosis (Grognet and Silar, 2015). In homothallic mushrooms, clamp cell formation is typically not observed. However, in both systems, fruiting body formation is characterized by the fusion of two of two different nuclei or the same nucleus fusing together and undergoing meiosis.

Monokaryotic fruiting body was previously reported in *Schizophyllum commune* (Leslie & Leonard, 1979). Ullrich (1973) observed that 27% of *Sistotrema brinkmanii* formed monokaryotic fruiting bodies, but only 6% of *Coprinus cinereus* did so (Uno and Ishikawa, 1971). However, it is unclear whether mating and nuclear fusion are really necessary for the formation of true clamp cells and/or complete fruiting bodies. We previously induced monokaryotic clamp cell formation by introducing a compatible homeodomain protein gene into *Pholiota microspora* using a recombinant DNA technique (Yi et al. 2010). In that study, the expression levels of the homeodomain protein was affected by the frequency of clamp cell formation and not gene dosage. It

is therefore possible that nuclear fusion is not essential for clamp cell or fruiting body formation.

Mycoleptodonoides aitchisonii belongs to the family Climacodontaceae, Polyporales, and has been widely found on dead broad-leaved trees from summer to fall in Asia. Its fruiting bodies are effused-reflexed with fan- or spatula-shaped caps. The species exhibits pharmaceutical properties, including immunodulation, lipid-lowering effects and antibacterial effects (Chandrasekaran and Shin, 2012). Here, we report monokayotic clamp cell formation, fruiting body formation and meiosis in *M. aitchisonii*. This is the first reports to show that mating and nuclear fusion are not essential for mushroom development of this species.

2-3 Materials and methods

2-3-1 Strains

Eighteen *Mycoleptodonoides aitchisonii* strains (TUFC10099, TUFC11027, TUFC12764, TUFC13505, TUFC13506, TUFC30296, TUFC30297, TUFC30298, TUFC30563, TUFC30627, TUFC31439, TUFC33001, TUFC33266, TUFC33541, TUFC33593, TUFC50005, TUFC100880, TUFC100881) deposited at the Fungus/Mushroom Resource and Research Center, Faculty of Agriculture, Tottori University (TUFC), Japan were used in this study. The strains were maintained on 2% malt agar slants.

2-3-2 Cultivation of fruiting bodies

The general substrate formulation consisted of sawdust and rice bran at a volumetric ratio of 9:1 (v/v). The moisture content of the substrate was adjusted to 65%. All ingredients were combined and mixed, and 600g of prepared substrate was bagged into high-density polyethylene bags (18x8x28 cm, SE-25ES, Sakato San-gyo Co. Ltd., Minakami-machi, Tone-gun, Gunma, Japan) sealed with a heat sealer, sterilized by autoclaving (121^0C, 60 min), cooled, and then inoculated with mycelia. The substrate was incubated at 25^0C in the dark for 60 d to allow the substrate to become fully colonized by the mushroom mycelia, a process hereafter referred to as “spawn running”. After spawn running, the fully colonized substrate was irradiated with fluores-cent lamp at 200 lux for 1 wk at 25 C (stage 1). The substrate was then incubated at 15^0C at 85% relative humidity under fluorescent lamp at 200 lux for 1 mo until fruiting started. To induce fruiting, each culture bag was cut using a sterile cutter with a cross-shaped pattern, and incubation was continued until fruiting bodies matured.

2-3-3 Isolation of basidiospore isolates

Mature fruiting bodies of strain TUFC50005 were removed with a knife and pinned to the lids of petri dishes using sterilized Vaseline. After 10-15 min, enough spores had fallen on the agar, 16 single spores were picked up by using micromanipulator, each spores were transferred to individual agar plate.

Mycelium germinated from single basidiospore were maintained as 50005-1-50005-20 strain. Other strains were isolated from the agar plate incubated at 25 C for 3 d in the dark after spores had fallen. Single germinated mycelia were picked and placed on a 2% malt agar slant with a sterile steel needle. More than 200 strains were isolated in this way.

2-3-4 Microscopy

A slide culture method was performed to observe mycelium using a 0.2% malt agar and 0.2% Tween 80 thin-layer slide and the mycelium was observed using a microscope (Nikon Eclipse 80i, Nikon Corp., Tokyo, Japan) equipped with a camera (Nikon DS-L2). The mycelia on the 0.2% malt agar were stained with 4 ppm 4',6-diamidino-2-phenylindole (DAPI) and 2 ppm calcoflour-white. Observations after staining with DAPI and calcoflour-white were performed immediately under a fluorescence microscope (Nikon Eclipse 50i, Tokyo, Japan) with a UV excitation apparatus (Nikon C-SHG1, Tokyo, Japan).

For scanning electron microscopy (SEM) the hymenium stipules of fruiting bodies were pre-fixed overnight in a 0.1 M phosphate buffer containing 2.5% glutaraldehyde (pH 7.2), washed with 50 mM phosphate buffer, and then post-fixed for 2 h in 0.1 M phosphate buffer containing 1.0% osmium tetroxide (pH 7.2). After washing with distilled water, the specimens were dehydrated with a graded series of ethanol, and then immersed in t-butyl alcohol Specimens were frozen at -15 °C and then freeze-dried (VED-21, Vacuum

Device, Ibaraki, Japan). The dried specimens were mounted on aluminum sample stubs and coated with platinum using an ion sputtering system (EM-1010, Hitachi Science Systems, Tokyo, Japan) and then examined under a field emission SEM (SU8020, Hitachi High-Technologies, Tokyo, Japan) at 15 kV.

2-4 Results

2-4-1 Morphological and cytological characterization of fruiting bodies

In order to understand the life cycle and mating system of *M. aitchisonii*, 18 strains deposited at TUFC were cultured on sawdust medium to produce fruiting bodies.We examined, only six strains (TUFC10099, TUFC12764, TUFC13505, TUFC33001, TUFC50005 and TUFC100881) produced fruiting bodies. In this study, the formation of fruiting bodies was confirmed from 6 strains. The remaining isolates did not produce fruiting body. Both of strain TUFC50005 and TUFC10088, normal fruiting bodies were formed at a relatively high frequency (Fig. 2-1A), in contrast, strains TUFC12764, TUFC13505, TUFC33001, normal fruiting bodies were formed with low frequency. Only deformed fruiting bodies were formed from TUFC10099. In TUFC50005 and TUFC100881, true clamp cells were appeared in the high frequency. However, in TUFC10099, TUFC11027, TUFC13506, TUFC30297, TUFC30627, TUFC31439, TUFC33593, TUFC100880, clamp cell was not appeared. Among fruiting body formed strains such as TUFC12764, TUFC13505 and TUFC33001 were formed clamp cell relatively lower

frequency than TUFC50005. TUFC12764, TUFC13505, TUFC30296, and TUFC33001, were the frequency of true clamp cell formation was lower than that in dikaryotic strain TUFC50005, but true clamp cells were appeared. Our findings show that true clamp cells formed strains could form fruiting body, and the strains which produce true clamp cells in the high frequency could form normal shape fruiting body among the stocked strain. Strain TUFC50005 was therefore used in this study. Four spores were observed on each basidium in polypores of the fruiting body (Fig. 2-1B). Dikaryotic strain TUFC50005 was capable of producing both dikariotic cells with clamp cells (Fig. 2-1C). Single nuclei were observed in each basidiospore (Fig. 2-1D).

2-4-2 Clamp cell formation in monospore isolates

Clamp cells bearing two nuclei in each cell of the dikaryotic parent strain TUFC50005 were clearly observed under the fluorescence microscope after staining with DAPI and calcoflour-white (Fig. 2-2A). Clamp cell formation was examined in 189 monospore isolated strains under a light microscope including 16 strain isolated by micromanipulation under the microscope. Surprisingly, the frequency of clamp cell formation was lower than that in dikaryotic strain TUFC50005; however, pseudoclamp cells and true clamp cells were observed among the monospore isolates (Fig. 2-2B and C). Although most clamp cells were pseudoclamp cells and the frequency of appearance of true clamp cells was low, 83 monokariotic strains had the ability to form

pseudoclamp cells and 11 strain had the ability to form true clamp cells among 189 monospore-isolated strains (data not shown).

2-4-3 Monokariotic fruiting

In order to characterize the life cycle of this mushroom, 20 monospore isolates (strains 50005-1 to 50005-20) were isolated from the fruiting body of the dikaryotic strain TUFC50005. These isolates were cultivated on sawdust medium to induce fruiting body formation. Although most of the strains (50005-3, 50005-6, 50005-7, 50005-9, 50005-11, 50005-12, 50005-14) formed primordia (i.e. young fruiting body-like structures), development stopped and browning occurred (Fig. 2-3A and 2-3B). However, strain TUFC50005-4 produced monokariotic fruiting bodies with both normal and abnormal morphologies (Fig 2-3C and 2-3D). This mushroom strain, the strain TUFC50005-4 produced a monokariotic fruiting body.

However, monokariotic strain TUFC50005-4 produced 2 spores on 65.7% of basidia, although dikariotic strain TUFC50005 produced 4 spores on 96.7% of basidia (Table 2-1). These results showed that monokariotic strain TUFC50005-4 has the ability to produce fruiting bodies, even though this strain has only one nucleus and can only produce two spores after meiosis. As shown Fig. 2-4, in the monokaryotic fruiting body of TUFC50005-4, the number of basidia is reduced by half. The characteristics of 20 monospore isolates (strain 50005-1 to 50005-20) from fruiting body of dikaryotic strain

TUFC 50005 are shown in Table 2-2. Among 20 monospore isolates, 3 strain (50005-2; 50005-8, 50005-13) can form true clamp cell connection but cannot form fruiting bodies. In contrast, true clamp cells were not appeared in strain 50005-6 and strain 50005-11 but they can formed fruiting body with abnormal morphology. Strain 50005-7, 50005-9 and 50005-14 can form true clamp cell or pseudoclamp cell, however, after primordia formation, fruiting body formation was terminated. These results suggested that there is not relationship between the production of true clamp cell connection and the formation of fruiting bodies.

2-5 Discussion

Generally, only dikaryon can produce fruiting bodies. The frequency of true clamp cell and fruiting body formation, and the efficiency of normal fruiting body production, were both affected by dikaryotization. Therefore, *M. aitchisonii* essentially has a heterothallic life cycle and mating system. However, surprisingly, we also demonstrated that dikariotization is not required for clamp cell formation or fruiting body formation in this mushroom. We previously showed that clamp cell formation is dependent upon high expression levels of homeodomain protein genes, which alter the expression of *A*-mating-type genes. In addition, the efficienfy of clamp cell formation was also affected by gene dosage and promoter activity (Yi et al., 2010). The *A* locus controls clamp cell formation: in the common-A heterokaryon, neither

clamp nor pseudoclamp cells are never formed. Rather, only when the A factor is heteroallelic are true clamp cells or pseudoclamp cells formed (Furtado, 1966). It was therefore considered that *M. aitchisonii* is heterothallic, and thus similar to typical heterothallic mushrooms such as *Lentinula edodes*. In contrast to *M. aitchisonii*, the monokaryon TUFC50005-4 derived from strain TUFC50005 was capable of forming numerous pseudoclamp cells and just a few true clamp connections. These results indicate that mating type genes, such as the homeodomain protein genes, pheromone genes, and pheromone receptor genes (Coelho, Bakkeren, Sun, Hood, & Giraud, 2017; Kothe, 1999) may promote or inhibit expression of each developmental gene, but essentially, development can occur without mating type. Therefore, efficient fruiting body formation might occour as the results of dikaryotization and maintenance of dikaryon by production of clamp cell formation regulated with *A* mating-type homeodomain protein. Although these monokaryotic fruiting bodies are abnormally shaped, most form basidia. However, the basidia bear two basidiospores, rather than the four basidiospores that are typically borne by dikaryotic fruiting bodies. In *Polyporus ciliates*, the monokaryotic fruiting mechanism does not involve sexual reproduction. Indeed, plasmogamy, karyogamy or meiosis are not required; rather, monokaryotic fruiting is strictly a vegetative phenomenon in which the fruiting body bears basidia that contain only two basidiospores (Stahl & Esser, 1976). In the monokaryotic fruiting body 50005-4, the number of basidia is drastically reduced. These basidia are

also two-spored, unlike the typical four-spored basidia of dikaryotic fruiting bodies. The reason why monokaryons can form clamp cells is not clear. In future studies, we intend to investigate the mating system in *M. aitchisonii* (Bunaharitake), including aspects related to gene structure and segregation

Table 2-1 Number of spores on the basidia of dikaryotic and monokaryotic fruiting bodies

Strain No.	Number of spores on the basidium {ratio among total basidium (%)}				Total number (total ratio)
	Single	Double	Triple	Quadruple	
50005 (dikarion)	0 (0)	0 (0)	10 (3.3)	292 (96.7)	302 (100)
50005-4 (monokarion)	7 (1.9)	237 (65.7)	52 (14.4)	65 (18.0)	361 (100)

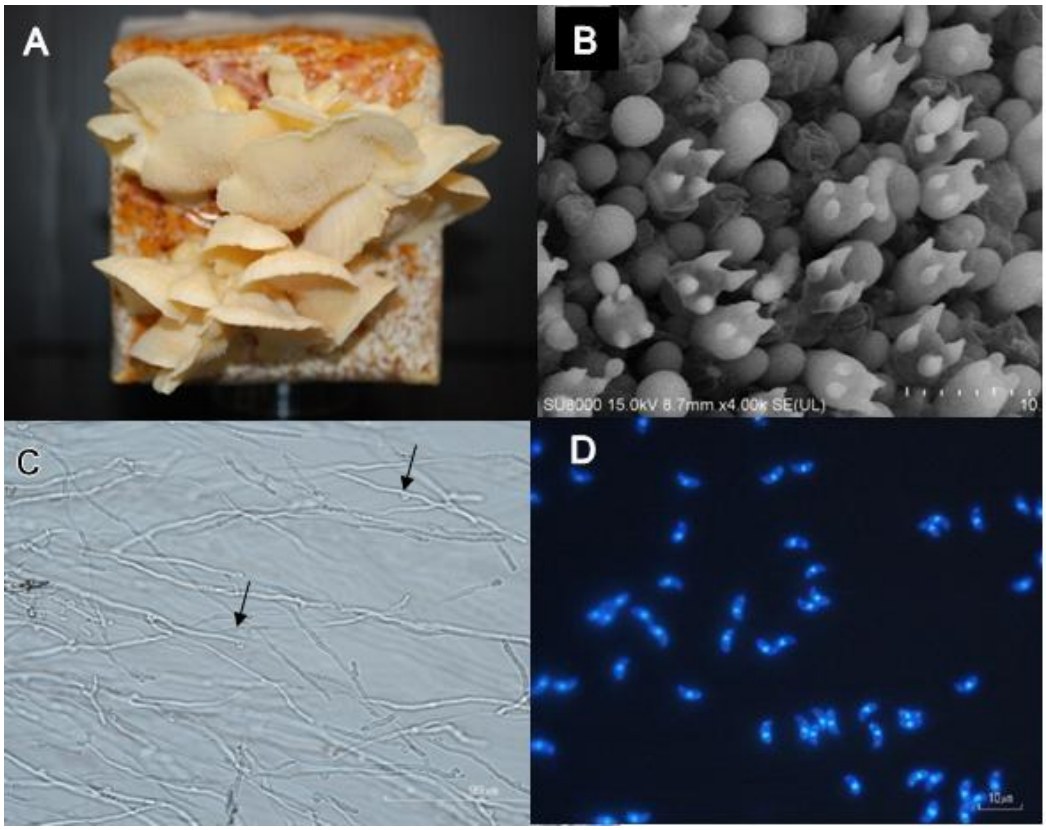

Fig. 2-1 Morphological characteristics of *Mycoleptodonoides aitchisonii*, strain TUFC50005. Panel A: Fruiting body of *M. aitchisonii* strain TUFC50005 cultured on sawdust media; Panel B: SEM image of basidia, Scale bar represents 10 µm; Panel C: Secondary mycelia of strain TUFC50005 under a light microscope; Arrows indicate true clamp connections. Scale bar represents 99 µm; Panel D: Basidiospores stained with DAPI under a fluorescence microscope, *Bars*: 10 µm.

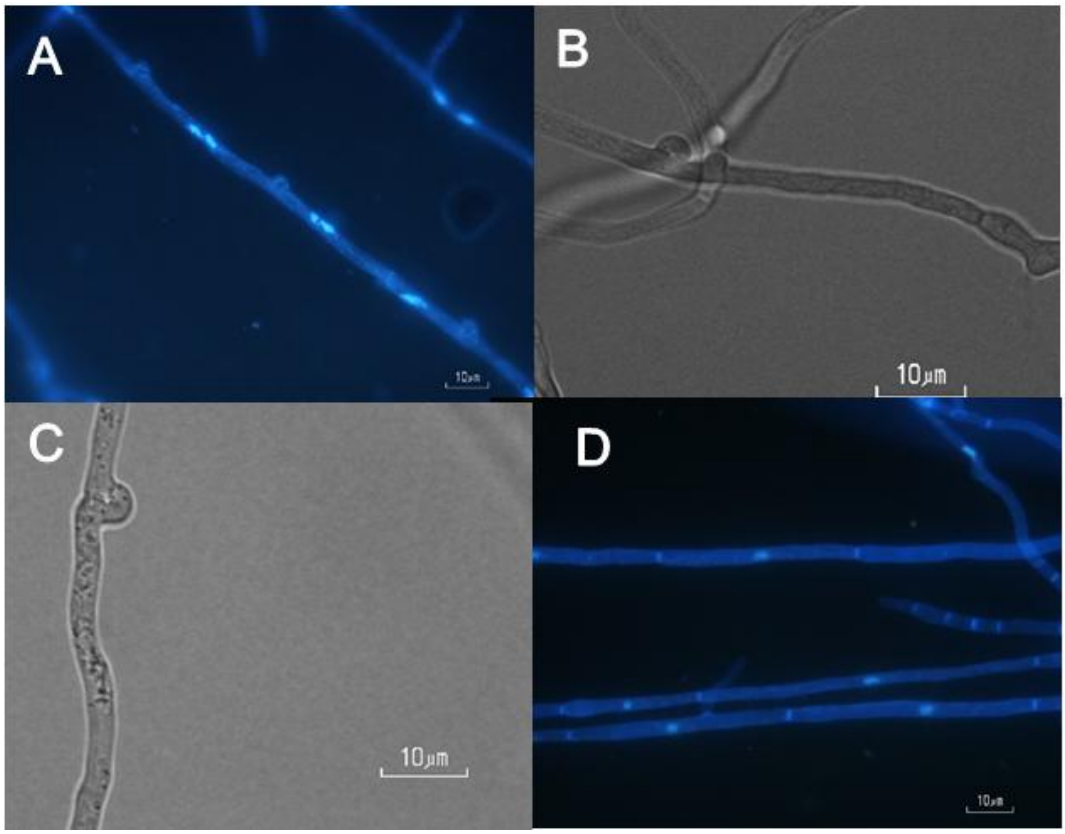

Fig. 2-2 Clamp connection of *Mycoleptodonoides aitchisonii*, strain TUFC50005. Panel A: Mycelium of strain TUFC50005 stained with DAPI and calcoflour-white under a fluorescence microscope; Panel B: pseudoclamp of *M. aitchisonii*, strain TUFC50005-180; Panel C: True clamp cell of *M. aitchisonii*, strain TUFC50005-85; Panel D: Mycelium of strain TUFC50005-4 stained with DAPI and calcoflour-white under a fluorescence microscope, *Bars*: 10 µm.

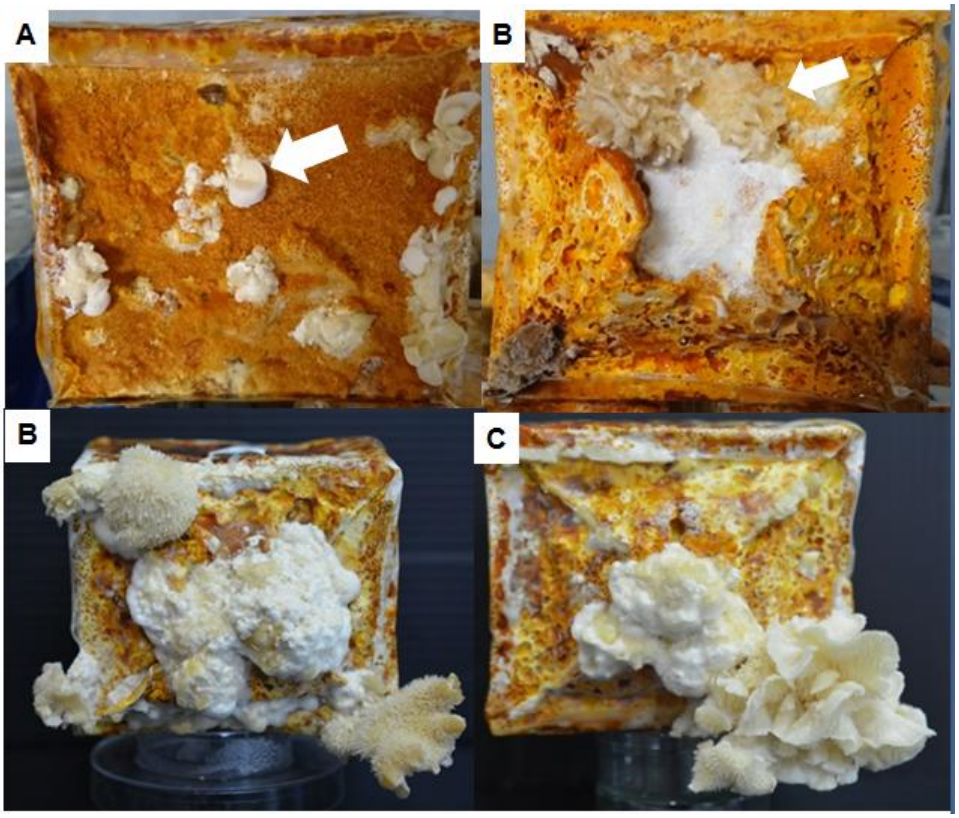

Fig. 2-3 Sawdust culture of monospore isolates of strain TUFC50005; Panel A: Primordium of strain TUFC50005-12 (arrow); Panel B: browning of young fruiting body of strain TUFC50005-3 (arrow); Panel C, fruiting body of strain TUFC50005-4 exhibiting abnormal morphology; Panel D, normal fruiting body of strain TUFC50005-4.

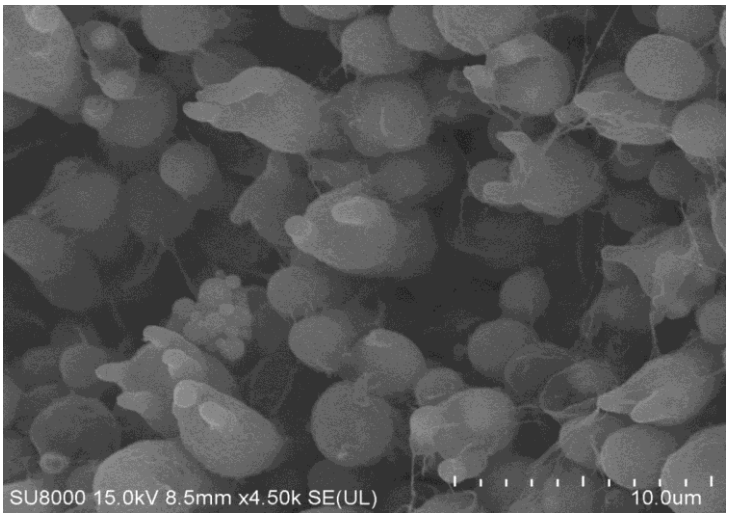

Fig.2- 4. SEM image of basidium in fruiting body of 50005-4 strain, scale bar represent 10µm.

Table. 2-2 Characteristics of 20 monospore isolates from fruiting body of dikaryotic strain TUFC50005

Strain Number	clamp cell formation		Fruiting body formation
	true clamp	pseudoclamp	
50005-1	+	-	-
50005-2	+	+	-
50005-3	±	-	+ (with abnormal morphologies)
50005-4	+	+	+ (with both normal and abnormal morphologies)
50005-5	-	-	-
50005-6	-	-	+ (with abnormal morphologies)
50005-7	+	-	- (after primordia formation, fruiting body formation was terminated)
50005-8	+	+	-
50005-9	-	+	- (after primordia formation, fruiting body formation was terminated)
50005-10	-	-	-
50005-11	-	-	+ (with abnormal morphologies)
50005-12	-	-	+ (with abnormal morphologies)
50005-13	+	-	-
50005-14	+	-	- (after primordia formation, fruiting body formation was terminated)
50005-15	+	-	-
50005-16	-	-	-
50005-17	+	-	-
50005-18	+	-	-
50005-19	±	-	-
50005-20	±	-	-

+, observed; - not observed; ±, observed but very few.

Chapter 3

Mating type in *Mycoleptodonoides aitchisonii* is not genetically associated with the monokaryotic clamp cell formation phenotype

3-1 Abstract

Mycoleptodonoides aitchisonii is a wood-rooting edible mushroom. In response to previous reports that some basidiospore isolates of this mushroom can form complete fruiting bodies and true clamp cells, the frequency of true clamp cell formation was compared between the dikaryotic and the monokaryotic strains. Compared to monokaryotic strains, true clamp cells were observed with greater frequency in dikaryotic strains. Mating incompatibility groups were examined among basidiospore isolates from dikaryotic strain TUFC50005 (P) and TUFC50005-7 × TUFC50005-18 (F1), which were derived from strain TUFC50005. Mating compatibility could be divided into two groups indicating that *M. aitchisonii* is a bipolar mushroom. Moreover, recombinant mating type strain might not be generated after meiosis, indicating that there may only be a single mating-type locus in *M. aitchisonii*. 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.

3-2 Introduction

In basidiomycete mushrooms, mating compatibility is controlled by one or two sets of multiallelic genes in what are referred to as bipolar or tetrapolar mating systems, respectively (Whitehouse, 1949). These so-called mating-type genes regulate nuclear pairing, clamp formation and investigations of mating compatibility increase our understanding of the life cycle of individual species (Fowler et al, 2004; Iwasa, 1998). Bipolar mating systems are controlled by a single mating-type locus and only two mating types are produced by meiosis. The tetrapolar mating system is based on two unlinked mating types, commonly referred to as *A* and *B* loci. In tetrapolar systems, when the two mating type loci are unlinked, four mating types can be generated after meiosis among the haploid progeny (Au et al, 2013; Coelho et al. 2017). In several higher basidiomycetes, bipolarity has been traced to the loss of mating specificity of the *B* locus. However, when *B* is complete and present, only the *A* locus segregates in a mating-type specific manner (Aimi et al. 2005; James et al. 2006).

The edible mushroom, *Mycoleptodonoides aitchisonii* (Bunaharitake) belongs to the family Climacodontaceae and is commonly found on dead broad-leaved trees during summer to fall in Asia. The fruiting bodies are effused-reflexed with fan- or spatula-shaped caps. The species exhibits pharmaceutical properties, including immunomodulation, lipid-lowering effects and antibacterial effects (Chandrasekaran et al. 2012). Recently, the ability to

produce monokaryotic fruiting bodies and clamp cells was reported in strains isolated from basidiospores (Riffiani et al. 2019). In that report, a single dikaryotic *M. aitchisonii* strain, TUFC50005, and 20 monokaryons derived from a basidiospore of dikaryotic strain TUFC50005, exhibited a wide spectrum of fruiting body developmental stages. While most strains formed primordia, or young fruiting body-like structures, one of the monokaryons, strain TUFC50005-4, formed a complete fruiting body, even though it had only one nucleus and produced only two spores after meiosis. We previously demonstrated that dikaryotization was not required for clamp cell and fruiting body formation (Riffiani et al. 2019).

In the present study, we examined the polarity of mating types in *M. aitchisonii* as well as the genetic linkage between the mating-type locus and the ability to form monokayotic clamp cells by classical genetic analysis. In addition, we sought to clarify the mechanisms underlying monokaryotic clamp cell formation in *M. aitchisonii.*

3-3 Materials and Methods

3-3-1 Strains and culture conditions

The strain used in these experiments, *M. aitchisonii* TUFC50005, was obtained from the Fungus/Mushroom Resource and Research Center at the Faculty of Agriculture, Tottori University, Japan (TUFC). Basidiospore isolates

50005-1 to 50005-20 were isolated from fruiting bodies of TUFC50005. All of the *M. aitchisonii* strains were maintained on 2% malt agar slants at room temperature.

3-3-2 Cultivation of fruit bodies

The sawdust medium for the cultivation of fruiting bodies consisted of sawdust and rice bran at a ratio of 9:1 (v/v). Water was added to the sawdust-rice bran medium so that the moisture content of the substrate was 65%. Then, 600 g of substrate was bagged into high-density polyethylene bags (18 × 8 × 28 cm, SE-25ES, Sakato Sangyo Co. Ltd., Japan), sealed with a heat sealer, sterilized by autoclaving (121°C, 60 min), cooled, and then inoculated with an agar plug containing mycelia. The inoculated substrate was incubated at 25°C in the dark for 60 days to facilitate colonization of the substrate by the fungus (hereafter referred to as “spawn running”). Upon completion of spawn running, the fully colonized substrate was irradiated with visible light at 200 lux for a week at 25°C (Stage 1). Samples were then incubated at 15°C and a relative humidity of 85% under visible light at 200 lux for 1 month until the initiation of fruiting. To induce fruiting, each culture bag was cut in a cross-shaped pattern using a sterile knife, and incubation was continued until the fruiting bodies matured.

3-3-3 Isolation of basidiospore isolates

To prepare a spore suspensions, 1 ml of sterilized water was pipetted onto spore prints in petri dishes which were then vigorously shaken. The concentration of spores, which was determined using a hemocytometer under a microscope, was adjusted to approximately 1×10^4 to 1×10^6 cells/ml. Then, 0.1 ml of suspension was mixed with 2 ml of soft agar (0.7%) melted at 50°C in a test tube and then poured onto a 2% malt extract agar plate to prepare a double-layer agar culture. After incubating the culture at 25°C for 5-7 days, the single colonies that appeared on the plate were transferred to 2% malt agar slants. These slants were kept at 25°C before being used for crossing experiments.

3-3-4 Crossing

The two monokaryotic stocks were placed 1 cm apart at the center of a 2% malt agar plate. After incubation for 3 weeks at 25°C, the mycelia on the 2% malt agar plate at the contact zone between the two parental monokaryons were observed under a microscope to check for the formation of clamp connections as evidence of dikaryotization. A slide culture method was performed to clearly observe clamp connections using TM7 medium. The TM7 medium comprised 0.2% (w/v) malt agar with 0.2% (w/v) Tween 80 on a thin-layer slide and 2% (w/v) agar, with pH adjusted to 7.0 with 1 M NaOH (Shimomura et al. 2012). Inoculated mycelia were incubated at 25°C for 10 days and clamp cells were

observed under a microscope (Nikon Eclipse 80i, Nikon Corp., Tokyo, Japan) equipped with a camera (Nikon DS-L2).

3-3-5 Frequency analysis of clamp cell formation

Frequency analysis of true clamp-cell formation in 10 day-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 % (Shimomura et al. 2012).

3-3-6 Statistical analysis

The chi-square goodness of fit test is useful for comparing a theoretical model to observed data (McHugh, et al. 2013). At first, we attempted to confirm how many progeny in first and second generation strains were carrying the *A1* and *A2* at equal frequencies. To determine the relationship between the mating type and monokaryotic clamp cell formation we used the chi-square test of independence.

The chi-square goodness of fit test was estimated using the following equation:

$$x^2 = \sum_{j=1}^{k} \frac{(O-E)^2}{E}$$

where x^2= Chi-square goodness of fit, O = Observed value, E = expected value, k = number of categories or groupings.

The chi-square test of independence was estimated using the following equation:

$$x^2 = \sum_{i=1}^{k} \sum_{j=1}^{c} \frac{(oij - Eij)^2}{Eij}$$

where x^2 = chi-square test of independence, $O_{i.j}$ = observed value of two nominal variables,

$E_{i.j}$ = expected value of two nominal variables

3-4 Results

3-4-1 Frequency of true clamp cell formation

In a previous study (Riffiani et al. 2019), we described true clamp cell formation in monokaryons that had been isolated from a basidiospore. In this study, to check the mating compatibility after crossing, we compared the frequency of clamp cell formation between dikaryons produced by crossing compatible monokaryons and monokaryons alone (Fig. 3-1A and B, respectively). The frequency of clamp formation in dikaryons was greater than

50%, i.e., more than 50% of septa had clamp cells. On the other hand, the maximum frequency of clamp formation in monokaryons was 5.9%, and almost strains were less than 10% (Table 3-1). The difference in frequencies was thus very clear, and we considered the frequency of clamp cell formation can be used to distinguish between dikaryotic cells (dikaryons) and clamp cells producing monokaryons.

3-4-2 Incompatibility groups among first-generation (F1) basidiospore isolates produced from strain TUFC50005

Basidiospore isolates from strain TUFC50005 (50005-1 to 50005-20) were crossed with each other and clamp cell formation was investigated under a microscope. These strains were designated F1 progeny of strain TUFC50005 and the results are shown in Table 3-2**.** The 20 strains could be clearly divided into two incompatibility groups depending on the production of true clamp cells at high frequency after crossing each other. Group 1 comprised strains 50005-1, 7, 8, 11, 13, 14, 17 and 20, and Group 2 comprised strains 50005-2, 3, 4, 5, 6, 9, 10, 12, 15, 16, 18 and 19. Based on this grouping, we considered that *M. aitchisonii* is a bipolar mushroom. The mating types of Group I and II were *A2* and *A1*, respectively.

3-4-3 Incompatibility among second generation (F2) basidiospore isolates from dikaryotic strain 50005-7 × 50005-18

To identify subloci of the mating locus, a fruiting body was cultivated using the crossed strain 50005-7 × 50005-18, and then 86 strains (BRW-2 - BRW-177) were isolated from the basidiospore of 50005-7 × 50005-18 and crossed with each other. These strains were designated as F2 progeny. Microscopic examination of clamp cell formation revealed that the 86 strains were clearly divided into two incompatibility groups based on the production of true clamp connections at high frequencies after crossing (Table 3-3). This phenomenon corresponded with the incompatibility group of basidiospore isolates of TUFC50005, indicating that this mushroom is bipolar. Moreover, among the 86 isolates, none of the strains were compatible or incompatible with strains belonging to either of the compatibility groups. Since the recombinant mating type strain might not be generated after meiosis, the mating type loci of *M. aitchisonii* might not carry the subloci.

3-4-4 Genetic linkage mating type and phenotype of monokaryotic clamp cell formation

Basidiospore isolates 50005-7 and strains 50005-18 from TUFC50005 produced true and pseudo-clamps in this and previous studies (Riffiani et al. 2019). To examine whether the bipolar *A* incompatibility factor of *M. aitchisonii*

was related to monokaryotic clamp cell formation, we examined basidiospore isolates from strain TUFC50005 (50005-1 - 50005-20) and 50005-7 × 50005-18 (BRW-2 - BRW-177). The relationships between the true-clamp formation phenotype and mating type among basidiospore isolates are summarized in Table 3-2 and 3-4. Strains 50005-7 and 50005-18 were the F1 progeny of strain TUFC50005; thus, strain TUFC50005 was parental (P). Moreover, both 50005-7 and 50005-18 strains had the ability to produce clamp cells when they were monokaryons. Segregation analysis of mating type *A* in F2 progeny showed that segregation was significantly different from expected values.

We therefore conducted the chi-square goodness of fit test for the first and second generations to compare the observed sample distribution with the expected probability distribution. To determine the relationship between the mating type and phenotype of monokaryotic clamp cell formation, the chi-square test of independence was conducted (Table 3-5 and 3-6). Based on the chi-square goodness of fit analysis, the first generation showed a p-value greater than the significance level ($p = 0.180 > 0.05$), indicating that the data follow a distribution of certain proportions and that the progeny carry *A1* and *A2* in relatively equal frequencies. The progeny of the cross between F1 strains not in accurate compatible 50% of its. The relative distribution of *A1* and *A2* in F1 was approximately 60% and 40%, respectively. In bipolar mating systems, the progeny of a cross between a strain carrying, for example, *A1* and *A2* will carry

the mating types of the two parental types in equal frequencies. Consequently, the spores from any strain in the F1 generatio will be compatible with 50% of its siblings. However, in the second generation, the *p*-value was less than the significance level ($p = 0.024 < 0.05$), indicating that data did not follow a distribution of certain proportions i.e., the progeny did not carry *A1* and *A2* in equal frequencies. If the *A* factor was comprised of two loci, then the inbreeding potential would increase to a level determined by the frequency of recombination between the loci. Thus, the chi-square test of independence showed that there is no relationship between the *A* mating type locus and the phenotype of monokaryotic clamp cell formation. Moreover, in this study some of the progeny of strain 50005-7 × 50005-18, in which monokaryotic clamp formation was not observed, basidiospore isolates were able to produce monokaryotic clamp cells. Therefore, monokaryotic clamp cell formation is not only affected by the mating type locus, but also by several other loci.

3-5 Discussion

Based on the results of polarity tests, we propose that *M. aitchisonii* is a bipolar mating system because only two mating types segregate in meiosis and mating incompatibility is controlled by a single mating type locus. In bipolar species, this locus can have two or multiple alleles; for example, approximately 25% of basidiomycete species have a bipolar (unifunctional) mating system

(Bakkeren, 1994). In the bipolar incompatibility system, only 50% of the F1 produced by random crossing are fertile. As the number of recombinant in a population increases, so does the probability that non-sister mating will be compatible. Based on the chi-square goodness of fit test in the F1 generation with 20 monokaryons, the p-value was greater than the significance level (p = 0.180 > 0.05), indicating that the data follow a distribution of certain proportions, i.e., that the progeny carry *A1* and *A2* in relatively equal frequencies (1:1). However, in the F2 generation with approximately 86 monokaryons, the progeny did not carry *A1* and *A2* in equal frequencies.

In a hypothetical cross between strains carrying *A1* α1-β1 and *A2 α2-β2*, recombinant spores carrying *A3 α1-β2* and *A4 α2-β1* would be formed and these recombinants would be compatible with all of their siblings except those carrying the identical specify. If the two loci were unlinked the inbreeding potential would be 75%, but if the loci were linked, the inbreeding potential would be between 50 and 75% depending on the distance between the loci (Koltin, 1972).

However, we did not observe the recombinant in the progeny tests using *M. aitchisonii.* It is possible that we need more monospores to detect the recombinant, as in our previous work on *Pholiota nameko* (Ratanatragooldacha et al. 2002). In that study on the incompatibility factors and mating characteristics of spore isolates in the bipolar mushroom, *Pholiota nameko*, only

one of 300 monospore isolates carried *Ar1* (in addition to the two parental mating types of isolates i.e., 121 of *A2* and 152 of *A4*). Further, it was also found that 26 modified isolates were unable to produce a dikaryon with both parental monokaryons. These modified isolates were the product of recombination during sexual reproduction. Isolates carrying the new *A* incompatibility factor resulted from successful meiotic recombination in sexual reproduction. (Ratanatragooldacha et al. 2002).

The results of the chi-square test for independence between mating type and monokaryotic clamp connection formation showed that the two groups are weakly related ($p > 0.05$). In other words, these two groups are high capability independent. No relationship was observed between the *A*-mating type gene and monokaryotic clamp connection in the F1 and F2 generations. The frequency of true clamp formation was thus not controlled by genotype of mating type gene. In the bipolar basidiomycete *Pholiota microspora*, a pair of homeodomain protein genes located at the *A* mating-type locus regulates mating compatibility (Yi et al. 2010). We previously induced monokaryotic clamp cell formation by introducing a compatible homeodomain protein gene into *P. microspora* using a recombinant DNA technique (Yi et al. 2010). In that study, the expression levels of the homeodomain protein were affected by the frequency of clamp cell formation, gene dosage and activity promoter. True clamp formation is controlled by the expression level of homeodomain protein genes and that

altered expression of *A* mating type is sufficient to drive true clamp formation. It is therefore possible that dikaryotization is not essential for clamp cell or fruiting body formation and that monokaryotic clamp connection is controlled by the expression level of the mating-type gene.

Although both of the monokaryotic parental 50005-7 and 50005-18 strains can produce monokaryotic clamp connections, more than 50% of the resulting progeny cannot make clamp connections. However, it is possible that some of the monokaryotic clamp connections cannot be observed because the frequency clamp connection is very low. Alternately, another gene related to clamp connection formation may control HD2 expression, which location of the gene in different alleles. Thus, *A* mating-type loci may have only one HD1/HD2 gene pair with several different alleles, as is the case in *Pholiota nameko* (Yi, 2010), *Pleurotus* djamor (James, et al. 2004a), and *Laccaria bicolor* (Niculita, 2008), or two HD1/HD2 subloci, as in *Flammulina velutipes* (van Peer, 2011), or even more than three subloci, as in *Schizophyllum* commune (Ohm, 2010).

Table 3-1 Frequency of clamp cell formation

Strain	Nuclear phase	Total number of septa	Number of septa with clamp cells	Frequency (%)
50005	Dikaryon	68	40	58.8
50005-4	Monokaryon	18	1	5.5
50005-7	Monokaryon	26	1	3.8
50005-18	Monokaryon	17	1	5.9
50005-7 × 50005-4	Dikaryon	18	12	66.7
50005-7× 50005-18	Dikaryon	48	26	54.2

TUFC was abbreviated in each strain number

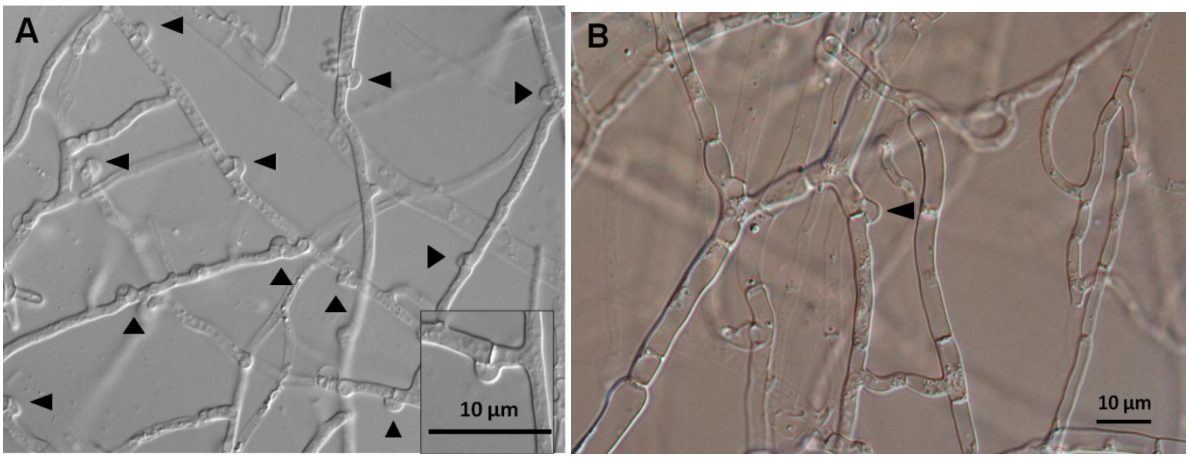

Fig 3-1 Visualisation of (A) dikaryotic clamp cell connections (arrows) and (B) monokaryotic clamp cell formation (arrow) under a light microscope.

Table 3-2 Incompatibility groups among basidiospore isolates derived from strain TUFC50005

		A1												*A2*							
		2*	3*	4*	5	6	9	10	12	15*	16	18*	19*	1*	7*	8*	11	13*	14*	17*	20*
A2	1*	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	7*	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	8*	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	11	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	13*	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	14*	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	17*	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
	20*	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
A1	2*	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	3*	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	4*	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	5	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	6	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	9	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	10	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	12	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	15*	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	16	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	18*	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
	19*	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+

Strain number 50005– is abbreviated

+: clamp cells observed in large numbers

−: clamp cells were not observed

*monokaryotic true clamp cell formation was observed

Table 3-3. Incompatibility groups among basidiospore isolates derived from strains 50005-7 × 50005-18

A1	*A2*
2, 3, 4, 6, 7, 8, 9, 12, 13, 14, 18, 19, 20, 21, 22, 24, 25, 29, 30, 33, 37, 38, 39, 40, 41, 42, 47, 48, 49, 52, 53, 57, 58, 60, 70, 71, 74, 75, 77, 79, 88, 106, 113, 122, 125, 126, 128, 130, 131, 132, 134, 143, 154, 165	10, 11, 15, 16, 17, 23, 26, 31, 36, 43, 45, 46,50, 51, 55, 59, 62, 63, 64, 65, 67, 69, 81,107, 108, 109, 110, 125, 129, 145, 175, 177

Strain number BRW– is abbreviated

Table 3-4. Monokaryotic clamp cell formation among monospore isolates derived from dikaryotic strains (50005-7 × 50005-18)

Clamp Cell formation	*A1*	*A2*
True clamp	58, 60, 25, 41, 52, 88, 143, 154, 165	31, 63, 65, 51, 59, 64, 177, 175
No clamp	2, 3, 4, 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, 125, 126, 128, 130, 131, 132, 134	10, 11, 15, 16, 17, 23, 26, 36, 43, 45, 46, 50, 55, 62, 67, 69, 81, 107, 108, 109, 110, 125, 129, 145

Strain numbers BRW– is abbreviated

Table 3-5 Chi-squared analysis of the first generation (F1)

Chi-squared			
Test of goodness-of-fit[x]		Test of independence[w]	
X^2	1.8	X^2	2.967
p	0.180	p	0.085

[X]Variables are the number of observed (13 : 7) and expected (1:1) mating types

[w]Variables are mating type and monokaryotic clamp cell phenotype

Table 3-6 Chi-squared analysis of the second generation (F2)

Chi-squared			
Test of goodness[x]		Test of independence[w]	
X^2	5.069	X^2	0.880
p	0.024	p	0.348

[X]Variables are the number of observed (54 : 32) and expected (1:1) mating types

[w]Variables are mating type and monokaryotic clamp cell phenotype

Chapter 4

Identification of *A*-Mating Type genes and their expression in Basidiomycete *Mycoleptodonoides aitchisonii*

4-1 Abstract

To understand the molecular mechanisms of the bipolar mating system in *M. aitchisonii*, the homeodomain protein gene 1 (*Mahd1*) and 2 (*Mahd2*) has been characterized. A genomic DNA fragment of *Mahd1* in *M. aitchisonii* 50005-7 strain is 1922 bp long and encoded 640 amino acids. On the other hand genomic fragment of *Mahd2* in 50005-18 (Maspi 18) strain is 1851 bp long and encoded 614 amino acids. Transcriptional analyses of the *Mahd1* and *Mahd2* showed that expression of the *Mahd2* was higher in a monokaryotic strain which can produce clamp cells than monokaryon which could not produce clamp cells. The highest relative expression level of *Mahd2* was shown in monokaryon TUFC 50005-4 which capable of forming true clamp. These results suggested that the formation of clamp cells regulated with *A* mating-type homeodomain protein and frequency of the clamp cell formation might be promoted by high expression of the *Mahd2* gene.

4-2 Introduction

Sexuality has been characterized for most of the cultivated edible fungi. In basidiomycetous mushroom, almost edible mushrooms are heterothallic. Sexuality is heterothallic when each spore receives a single post-meiotic nucleus and an

incompatibility system prevails. There are two types incompatibility systems: (1) the unifactorial system in which sexuality is controlled by single mating-type (*MAT*) locus and only two mating-type segregates in meiosis and (2) the bifactorial system in which sexuality is controlled by two unlinked mating-type, commonly called the *A* and *B* loci. These systems are termed bipolar and tetrapolar, respectively. In tetrapolar, when two *MAT* loci are unlinked, four mating types can be generated by meiosis among the haploid progeny (Coelho et al 2017; Au et al, 2013, Whitehouse, 1949). In several higher basidiomycetes, bipolarity has been traced to the loss of mating specificity of *Mat-B* locus. Whereas *Mat-B i*s complete and present, only the *Mat-A l*ocus segregates in a mating-type specific manner (Aimi et al. 2005: James et. 2007). Recently, *MAT-A* and *MAT-B* loci were named homeodomain (*HD*) and pheromone (*Phe*) and pheromone receptor (*Prc*) loci, respectively (Kües 2015; Maia et al. 2015).

In both *Schizophyllum commune* and *Coprinopsis cinerea*, the 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 formation of dikaryotic hyphal compartments. This includes the initial pairing of the 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, the hook formation. Different *B* genes are then needed for the fusion of the hook cell to the subapical cell, which

completes the clamp connection formation (Fowler, et al 2004, Koltin et al 1972, Casselton and Olesnicky, 1998, Kües, 2000). Under suitable environmental conditions and the regulation of both *A* and *B* mating locus, the dikaryon will develop the fruiting bodies and produce basidiospores after meiosis (Kües, 2000).

The basic model of the HD locus encompasses a divergently transcribed pair of homeodomain1 *(HD1*) and homeodomain2 (*HD2)* genes that form a functional group, flanked by the mitochondrial intermediate peptidase (*MIP*) gene on one side, and the β-flanking gene on the other (James et al. 2004a; Kües et al. 2011). In the case of multiple HD subloci, several of these functional groups will be positioned between the *MIP* gene and the *β--flanking* gene (Brown and Casselton 2001; James 2007; Kües et al. 2011).

HD1 and HD2 protein from different alleles of a same-sub locus can heterodimerize with each other in order to form a functional transcription factor. The HD1 and HD2 protein from the same locus however, fail to heterodimerize (Kües et al. 1994, Ohm et al. 2010, Tymon et al. 1992). Transcription factors are sequence-specific DNA-binding proteins with a variety of functions: some are thought to help fold the DNA molecule into distinct domains; others assist in the initiation of DNA replication, and control transcription of many gene. Binding affinity to DNA sequences, the concentration of transcription factors and the presence of cofactors are important determinants in gene activation. Transcription factors can be classified according to the three-dimensional structure of their DNA-binding domains. More than 80% of all transcription factors are

characterized by zinc finger, helix-turn-helix, helix-loop-helix, leucine zipper, and winged-helix motifs. The homeodomain is typically 60 amino acids long and contains a helix-turn-helix (HTH) motif (Banerjee-Basu and Baxevanis, 2001).

Here we report on the characterization of the mating system in *M. aitchisonii* base genotype identification. In our previous report, we found that *M. aitchisonii* has ability to produce monokaryotic fruiting bodies and clamp cells in monokaryotic strain isolate. The reason why monokaryons can form clamp cells is not clear. In the present study, we have examined the mating type of *M. aitchisonii* using a molecular basis. We designed specific primers for HD2, in which we describe the characterization of genomic DNA and cDNA sequence of *HD2* gene from *M. aitchisonii*. We also analysed the expression of the *HD2* gene in monokaryon which had true clamp connection and no clamp connection.

4-3 MATERIALS AND METHODS

4-3-1 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 from 50005-1 to 50005-20 (F1) which derived from strain TUFC 50005 as parental strain and then 86 strain (BRW-2- BRW-177) were isolated from the basidiospore of 50005-7 x50005-18 as an F2. The strains were maintained on 2% malt agar slants at room temperature.

Among the monokaryotic isolates, 50005-7 and 50005-18 were used for characterization and expression of the homeodomain protein gene.

4-3-2 Mycelium preparation, DNA and RNA extraction techniques

Mycelium for DNA extraction was prepared by growing isolates in minimum media. For the preparation of genomic DNA three square agar blocks (5 x 5 x 5 mm) into 20 ml minimal medium (1.5 g L^{-1} $(NH_4)2HPO_4$, 1 g L^{-1} KH_2PO_4, 20 1 g L^{-1} glucose, 25 mg ml-1 thiamine hydrochloride, pH 5.5 liquid medium. The mycelium was grown at 25°C without shaking for 2 weeks and then harvested by filtration, frozen in liquid nitrogen, and ground to a fine powder using mortar a pestle. Genomic DNA was extracted from lyophilized mycelium according to the method described by Dellaporta et al.(1983). Genomic DNA was extracted from fungal mycelium using an improved cetyltrimethylammonium bromide (CTAB) 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 NaC1, 100 mM Tris HC1 (pH 8.0), 20 mM EDTA (pH 8.0)] preheated at 650C. An equal volume of chloroform: isoamyl alcohol (24:1) added to the retained supernatant fraction. After gentle mixing, the suspension was kept at room temperature for 20 min and then centrifuged 1000 rpm for 10 min. The supernatant was transferred to a new microtube containing 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 two times 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 37^0C for 1 h. To get high purify and high concentration of DNA, several tubes contain DNA mix and purification using DNeasy Plant Mini Kit (Qiagen) base on manufactory instruction. The Quality of DNA extracts was tested by polymerase chain reaction (PCR) amplification of the ribosomal DNA internal transcribed spacer (ITS) using the primers at Table 4-1. 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 with ReverTra Ace® qPCR RT Master Mix with gDNA Remover Kit (Toyobo). PCR was carried out using Takara Ex Taq® polymerase (Takara Bio, Japan).

4-3-3 Genome Sequencing and Isolation of *A* locus alleles

The complete nucleotide sequence of the genomic DNA of monokaryotic Maspi 7 and Maspi 18 were determined using Illumina HiSeq 2000 paired-end technology provided by Hokkaido System Science Co., Ltd (Sapporo, Hokkaido, Japan). The final assembly for monokaryotic Maspi 7 contained 7377 total sequences (40.997.064 bp) and Maspi 18 contained 3342 sequences (40.997.064 bp). Scaffold sequence 760 of monokaryon Maspi 18 and scaffold 5381 of monokaryon Maspi 7 were identified to contain both homeodomain HD1 and HD2, respectively, using TBLAST (Altschul et al. 1997). The primer pairs for

amplification of HD2 of Maspi 7 and Maspi 18 were designed based on prediction HD2 sequences using GENETYX software.

4-3-4 PCR condition

Amplification Homeodomain gene (HD2) using the specific primer (Table 4-1). Reaction mixture for PCR contained In PCR buffer: 8 µL dNTPs 1.0µL primer1 (100 µM), 1.0 µL primer 2 (100 µM), 0.5 µL (1 unit) DNA Taq polymerase, 1 µL DNA, 10 µL 10x *Taq* buffer, 78.5 µL H2O in final volume 100 µl. DNA amplification was performed with T100TM Thermal Cycler Bio-Rad (Japan) by 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 at 72°C for 5 min. PCR product were analysed in 1.5% agarose gel electrophoresis run with TAE buffer (40 mM Tris-acetate pH 8,1 mM EDTA) and stained with ethidium bromide.

4-3-5 Analysis of nucleotide and Phylogenetic analysis.

Nucleotide and protein sequences data were analysed using GENETYX ver. 13 Software (GENETYX, Tokyo, Japan) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Subcellular localization of the homeodomain was predicted by the PSORT II program, which available on the internet (https://psort.hgc.jp/form2.htm). Protein motif in HD2-18 sequence was identified using the web-based MOTIF search program

(https://www.genome.jp/tools/motif/). In order to analyse phylogenetic relationships of A-mating type locus, a phylogenetic tree based on the amino acid sequences of the HD2, were constructed. Phylogenetic analysis was constructed by MEGA 5.05 software using the neighbour-joining method with a bootstrap value of 1000 replicates. DNA and amino acid sequences were aligned with Expasy software (https://www.expasy.org/), multiple sequence alignment was aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). To predict and analyse the protein structure of homeodomain binding DNA were using the Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). In order to confirm the homologues genes, the BLASTN program (https://blast.ncbi.nlm.nih.gov/home) was used.

4-3-6 Reverse transcription-PCR (RT-PCR)

Total RNA from the dikaryotic *M. aitchisonii* strain was used as a template for all RT-PCR. Amplification of full-length cDNA by RT-PCR was carried out with a ReverTra Ace qPCR RT Master Mix with gDNA Remover Kit (Toyobo) using total and 3'-rapid amplification of cDNA ends (3'-RACE). 3'-RACE PCR was performed using the PrimeCript TM RT PCR kit. 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 the pMD20 T-vector (Takara Bio) and sequenced.

4-3-7 Real-Time PCR assay

We used the actin as the housekeeping gene. A partial actin, HD218, HD17 gene in *M. aitchisonii* was clone by generate PCR primer. Primer for HD218, HD17 and actin were design according to their cDNA sequences using GENETYX 13 software (Genetyx, Tokyo Japan). Amplification of genomic DNA was prevented by designing primer for exon-exon junction. All primers were tested to ensure the amplification of single bands with no primer-dimers. Plasmid with the inserted target gene (HD218 and HD17) and housekeeping gene (actin) were extracted according to the method modified by Birboim (1983).

The Standard curve was constructed using five ten-fold dilutions of a plasmid. Real time PCR was performed using the KOD SYBR qPCR Mix kit (Toyobo). Thermocycling was carried out using Bio-Rad Real Time Cycler. Each reaction was run twice. The cycling parameter was 90^0C for 30s to activate thermostable DNA polymerase, 61^0C for 20 min to reverse transcription, 95^0C for 30s pre-denaturation, and then 40 cycles of 95^0C for 15s, 60^0C for 1 s and 74^0C for 30 s. Each run was completed with a melting curve analysis to confirm the specificity of amplification and an absent of primer-dimers. Data analysis was performed according to the manufacturer's instructions**.**

4-3-8 Statistical analysis

The chi-square goodness of fit test is useful for comparing a theoretical model to observed data (McHugh, 2013). We attempted to confirm how many

progenies in first and second-generation strains were carrying the *A1* and *A2* at equal frequencies.

The chi-square goodness of fit test was estimated using the following equation:

$$x^2 = \sum_{j=1}^{k} \frac{(O - E)^2}{E}$$

where x^2= Chi-square goodness of fit, O = Observed value, E = expected value, k = number of categories or groupings.

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 $P < 0.05$.

4-4 Result

4-4-1 Amplification internal transcribed spacer region (ITS) in strain TUFC 50005, derived strain (filial 1) Monokaryon 50005-1 until 50005-20, and monokaryon derived Maspi 7 x Maspi 18

For the fungi and oomycetes, the internal transcribed spacer region in the ribosomal RNA (rRNA) operon has been recognized as the formal DNA barcoding region (Porter and, Golding, 2011). The full ITS region in fungi has an average length of 500 and 600 base pairs (bp) for Ascomycetes and Basidiomycetes,

respectively, and an average length of 600 bp across all fungal lineages. All the monokaryon can amplify the ITS region with length 600 bp that indicated the good quality of DNA extraction (Fig 4-1).

4-4-2 Nucleotide sequence of homeodomain (HD2) 50005-18 and HD1 50005-7 characterization of protein *M. aitchisonii*

In order to determine the nucleotide sequence of the *A* mating type locus HD2- 50005-18 and HD1-50005-7 from *M. aitchisonii* were amplified and sequenced. The open frame region of HD2-18 from ATG to the stop codon is 1851 bp. On the other hand the open frame region of HD1-7 is 1922 bp. The location of exons and introns were determined from the nucleotide sequences of PCR products amplified by 3'- Race and 5'- RACE PCR. All the introns started with GT and ended with AG. The 3 introns of *HD2-18* gene interrupt the coding sequence, which comprised of 4 exons (Fig. 4-2). The upstream region was analysed, there found a putative TATA box at position 57 bp before start codon. Prediction termination transcription sequences poly-A signal HD2-18 was found 113 bp after the stop codon. The 5 introns of *HD1-7* gene interrupt the coding sequence, which comprised of 6 exons (Fig. 4-3).

4-4-3 Characteristics of protein sequence HD2-18 of *M. aitchisonii*

The PSORTII program (Nakai and Horton, 1999) predicted that HD2-18 protein contains 3 nuclear localization signal PTKRRVP at 382, PFPRRTR at 434,

PRRTRPG at 436. The HD2-18 gene encodes of 614 amino acids with the predicted molecular mass of 69.93 Kilodaltons. HD2-18 had the highest homology (48.78% identity) to the *Pholiota A3*-hox2, *A3*-hox1 genes for homeodomain protein, a homeodomain protein, 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). The MOTIF program predicted that protein sequence of HD2-18 (position 154-210, length 59 amino acids, e-value 3E-17) is the DNA binding domains involved in the transcriptional regulation of key eukaryotic developmental processes, may bind to DNA as monomers or as homo- and/or heterodimers, in a sequence-specific manner (Fig 4-2). Summarize of nucleotide sequence and characteristics of protein of homeodomain (HD2) was shown in Fig 4-4.

4-4-4 Characteristics of protein sequence HD1-7 of *M. aitchisonii*

The PSORTII program (Nakai and Horton, 1999) predicted that HD1-7 protein contains 3 nuclear localization signal PIAGRKR at 150, RPRK at 174, RKRR at 252. The HD1-7 gene encodes of 640 amino acids with the predicted molecular mass of 71,85 Kilodaltons. HD1-17 had the highest homology (33% identity) homeodomain 1 mating type protein of *Pleurotus tuoliensis* (accession number AER51795). The MOTIF program predicted that protein sequence of HD1-7 (position 153-232, length 80 amino acids, e-value 4.7E-04). This domain is C-terminal to the homeodomain transcription factor region. Summarize of

nucleotide sequence and characteristics of protein of homeodomain (HD1-7) was shown in Fig 4-5.

4-4-5 Phylogenetic relationship of HD2-18 protein

Phylogenetic relationships of *A*-mating type protein base on the amino acid sequence of HD2 region from basidiomycetous mushrooms were constructed using 12 amino sequences is shown in Fig 4-6. The homeodomain (HD2) from the basidiomycetes divided into 3 main clades. The phylogenetic tree indicated *M. aitchisonii* clustered together with *Ceriporiopsis subvermisopra*, and formed single clade with *Phanerochaeta chrysosporium*, *Trametes coccinea* and *Ganoderma sinensis* which the mating type system still unknown. Therefore, *M. aitchisonii* has same clade with *Rhizopogon roseolus* which has bipolar mating type, different clade with other bipolar strain like *Pholiota nameko* and *Coprinellus disseminatus.*

4-4-6 Multiple alignment analyses of amino acid sequences an encoded DNA binding domain

Multiple sequence alignment of the homeodomain protein from various basidiomycetes fungi were performed based on data from GeneBank database. The method for alignment used here based on sequence similarity scores. The similarity amino acid sequence encodes DNA binding domains *M. aitchisonii* and various basidiomycete fungi from GeneBank database are shown in Fig.4-7. In that figure, the sequences are arranged to show the conserved features for each various

basidiomycetes fungi. Several position amino acids that are absolutely conserved within the homeodomain include Phe, Leu, Ser, Pro, Ala, Trp, Asn, and Arg.

4-4-7 Prediction structure of homeodomain HD1-7 and HD2-18

The Pyre2 program predicted that a structure of DNA binding motif in HD2-18 protein is a helix-turn-helix (HTH) motif. The structure consists of two α helices joined by a short strand of amino acid. The recognition and binding to DNA by helix turn helix proteins is done by the two α helices, one occupying the N-terminal end of the motif, the other at the C-terminus. 143 residues (28% of the sequence) have been modelled with 99.9% confidence by the single highest scoring template (Fig 4-8.A). On the other hand, the structure of HD1-7 is transport protein (dimerization domain), 35 residues (9% of the sequence) have been modelled with 65.3% confidence by the single highest scoring template (Fig 4-8.B).

The colours indicate the predicted confidence of the model along the sequence. Regions modelled of Phyre2 are always coloured blue to indicate minimum confidence. Other colours are inherited from the confidence in the template(s) used to model that region (Kelley et al, 2015, Banerjee-Basu and Baxevanis, 2001).

4-4-8 Genomic DNA structure of the *A*- mating type locus

From Two genomic DNA sequences of mating compatible strain Maspi 7 (50005-7) and Maspi 18 (50005-18), derived from parental dikaryotic TUFC50005 strain, homeodomain protein genes and were identified. The region homeodomain protein genes are shown as red boxes (Fig.4-9). Eight genes with similarity to genes in EMBL data base were identified in Maspi 18 (50005-18) strain around homeodomain protein gene (Table 4-2). Presence of at least single pair of HD1 and HD2 protein genes were in the *A* mating-type locus of other basidiomycetous mushroom (Yi et al. 2009). In order to confirm the presence of *HD1* around the *HD2* gene, nucleotide sequence of downstream region (2370 bp) of HD2 was analysed using BLASTX and BLASTN program to discover homologues genes in the DDBJ/EMBL/GenBank database. Surprisingly, there was no putative conserved domain have been detected in both protein and nucleotide sequence data bases. This phenomenon indicates *HD1* might be absent in *A* mating-type locus at Maspi 18 strain. The other two genes commonly present around *HD2* gene are found in Maspi 18 strain; the mitochondrial intermediate peptidase gene (*mip*), which is conserved in the *A* mating-type region (James et al. 2004a, 2004b) and glycine dehydrogenase (*glydh*) (James et al. 2006). We also used GeneBank Similarity searches to find homologues gene in other mushrooms. Four gene around *A* locus (including *β-fg, mip, Glydh, Up11*) have homologues genes around *A* locus in the bipolar mushroom *Pholiota nameko* (Yi et al. 2010) and *Coprinellus disseminatus* (James et al. 2006). Other genes of known function

displaying conserved synteny between *M. aitchisonii* and other homobasidiomycetes (*e.i C. disseminatus*) are RNA polymerase II (*RPB2*) and glycine dehydrogenase (*GLYDH*) (James et al. 2006). *PB1* gene, encoding for para-amino benzoic acid synthase as seen upstream from the mating-type gene in *M. aitchisonii* have homologues genes around *A* locus with *Coprinus bilanatus* (Kües et al. 2001; James et al. 2002).

In contrast, a Maspi 7 strain *A* mating type locus, HD1 and HD2 protein was transcribed in the opposite direction (Fig. 4-9). In order to confirm the structure of *HD1* in Maspi 7 strain, a 1922 bp nucleotide was amplified and sequenced. There was a conserved domain have been detecting in both protein and nucleotide sequence databases. Analysis shown, *HD1 7* structure homolog with C- terminal domain of Homeodomain (E-value**:** 3.28e-10^{-6}). Although four other genes (*ADE5*, *pab1*, *Up11* and *RPB2*) in Maspi 7 strain were not discovered around the *A* locus (Table 4-3).

4-4-9 Mating type in monokaryotic strains

Base on the result of the mapping gene of HD2, we design a specific primer to amplify the homeodomain gene HD2. Primers HD2C_Maspi18F1 and HD2C_Maspi18R1 amplified a product, HD2-18, of 567 bp. Primers HD2C_Maspi7F1 and HD2C_Maspi7R1 amplified a product, HD2-7, of 763 bp (Table 4-1 and Fig. 4-10 A). These primers can be used as a molecular marker to determine mating type of *M. aitchisonii*. Using PCR amplification mating

incompatibility groups were examined among basidiospore from dikaryotic strain TUFC50005 (P) and 50005-1 to 50005-20 which derived from strain TUFC50005. All the strain were analysed by comparing the size of a PCR product (Fig. 4-10 B). The 20 strain could be divided into 2 incompatibility groups depending on different sizes of PCR products. Group 1 comprised strains 50005-1, 7, 8, 11, 13, 14, 17, and 20 with a size of PCR product were 763 bp in length. Group 2 comprised strain 50005-2, 3, 4, 5, 6, 9, 10, 12, 15, 16, 18, and 19 with a size of PCR product were 567 bp in length. The mating type of Group I and 2 were *A2* (40%) and *A1* (605), respectively.

As a dikaryotic strain, TUFC50005 (P) carried a different mating-type gene, 567 bp and 763 bp in length, respectively. The mating-type parental gene segregate well in derived strain. Each of filial 1 carried 1 gene from a parent. Then we confirm again by analysis using 86 F2 progenies derived from the fruit-body of dikaryotic stock (Maspi 7 x Maspi 18). The result showed Table 4-4, total of 86 monokaryon which isolated from a dikaryotic fruiting body (Maspi 7 x Maspi 18), were separated into two groups. A1 (monokaryon can amplified 567 bp, 62.8%.) and A2 group (763 bp, 37.2%).

Based on the chi-square goodness of fit analysis, the first generation showed a p-value greater than the significance level ($p = 0.180 > 0.05$), indicating that the data follow a distribution of certain proportions and that the progeny carry *A1* and *A2* in relatively equal frequencies. However, in the second generation, the *p*-value was less than the significance level ($p = 0.024 < 0.05$), indicating that data did not

follow a distribution of certain proportions i.e., the progeny did not carry *A1* and *A2* in equal frequencies (Table 4-5).

4-4-10 Gene expression of HD2-18 and HD1-7 in monokaryon and dikaryon strain

We previously demonstrated monokaryotic strain (50005-4) formed monokaryotic fruiting body and clamp cell formation (Riffiani et al. 2019). To detect the efficiency of clamp cell formation is controlled by the expression level of homeodomain protein genes, we amplified partial cDNA encoding fragment of HD2-18 and HD1-7 using the primer pairs HD2_18F-RT/HD2_18R-RT and HD1_7F-RT/HD1_7R-RT (Table 4-1), respectively. The expected size of cDNA fragments of HD2 and HD1, which were 169 bp and 360 bp in length, respectively, appeared in each line (Fig 4-11). In order to predict the role of HD2 and HD1 to drive true clamp cell formation, 10 strain of monokaryotic strains which can produce true clamp cell and no clamp cell was examined by qRT-PCR. The result indicates that the relative expression level of HD1 (Fig. 4-12) and HD2 (Fig. 4-14) in a monokaryotic strain that can produce a clamp cell higher comparing with monokaryotic cannot produce clamp cell formation. Transcription of HD2 gene in monokaryotic strain 50005-4 which can produce true clamp cell shown the highest expression level of HD2 gene comparing others strain (Fig 4-14).

In the dikaryon expression level in HD1 and HD2 more higher comparing expression level in monokaryon (Fig. 4-13 and Fig. 4-15)

4-5 Discussion

In order to address why monokaryotic strain can form a clamp cell, we investigate the mating system in *M. aitchisonii* including gene structure and segregation. A total genome of *M. aitchisonii* was amplified and sequenced. *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. Two genes usually have a pattern, one encodes mitochondrial intermediate peptidase (*MIP*), which has served as a valuable marker for the isolation of HD loci from non-model species and the others is known as a beta-flanking gene (*β-fg*) for unknown protein (Coelho, 2017, James et. al, 2004a). Beta-flanking gene (*β-fg*) coding for a putatively secreted protein appears to be fungal specific and occurs in Basidiomycetes and Ascomycetes (Kües et al, 2011: Ohm et al., 2010; var Peer et al. 2011). In the present study, gene encoding HD1 in Maspi 18 strain was absent in upstream of HD2 protein gene. This finding was significant difference in structure of *A* mating-type locus from another mushroom.

In analysis of *A* mating-type protein in *C. cinereus,* Asante-Owusu et al. (1996) suggested that in the HD1 and HD2 heterodimer, the HD2 contributes an essential DNA binding domain and HD1 provides a transcriptional activation

domain. It means that HD2 protein should play in important role during the clamp cell formation. Here we found the unique structure of *A* mating-type locus, there is only one HD2 protein genes in the *A*-mating type locus in Maspi 18 strain. But in Maspi 7 strain single pair of HD1 and HD2 protein gene were found om *A*-mating type locus.

Two of the set primer HD2 Maspi 7 (Primers HD2C_Maspi7F1 and HD2C_Maspi7R1) and HD2 Maspi 18 (primers HD2C_Maspi18F1 and HD2C_Maspi18R1) develop accordingly for molecular *A* mating-type assay and contribute a quick determination of mating incapability. This method will be more rapid and efficient to determine the mating- type by around PCR amplification as compared with a traditional mating test. Comparing the size of a PCR product, i.e. *A1* (567 bp in length) and *A2* (763 bp) is simpler straightforward than examining morphological changes during a mating test to determine mating-types. In our previous study, we determine mating-type in F1 and F2 strain using the classical method. Both of method shown the same result, that *M. aitchisonii* has a bipolar mating-type because only two mating types segregate in meiosis and controlled by single mating- type locus. These markers can be used to distinguish monokaryon and dikaryon and more effective than microscopic observations.

The *A* mating type genes, the subject of this report, encode two dissimilar subunits of a heterodimeric transcription factor. The two subunits are distinguished as homeodomain proteins HD1 and HD2 on the basis of conserved but distinctly different homeodomain motifs (Kües et al. 1992). After mating, HD1 and HD2

proteins heterodimerize to generate a dikaryon-specific transcriptional regulator that triggers the initial steps in sexual development.

Pyhre analysis (Kelly et al. 2015) analysis predicted the structure of DNA-binding motifs HD2-18 *M. aitchisonii* is a helix-turn-helix DNA motif which is a major structural motif capable of binding DNA. It binds to the major groove of DNA through a series of hydrogen bonds and various Van der Waals interactions with exposed bases. The 180 bp homeobox region encodes a helix- turn-helix DNA binding motif. A DNA-binding domain (DBD) is an independently folded protein domain that contains at least one motif that recognizes double-or single-stranded DNA (Brennan and Matthews, 1989).

N-terminal two helices of the homeodomain are antiparallel, and the longer C-terminal helix is roughly perpendicular to the axes established by the first two. It is this third helix that interacts directly with DNA via several hydrogen bonds and hydrophobic interactions, as well as indirect interactions via water molecules, which occur between specific side chains and the exposed bases within the major groove of the DNA (Schofield, 1987).

The predict structure of HD1-7 is transport protein (heterodimerization domain). HD1 proteins contribute a potential activation domain present in an essential C-terminal sequence that has been shown to activate transcription of a reporter gene in *S. Cerevisiae*. The HD1 homeodomain can be deleted from the *C. cinereus* HD1 protein without causing impaired function (Asante-Owasu et al. 1996). These observations lead to the proposal the two components of the

heterodimer contribute different functional domains on DNA, the HD2 protein provides the critical binding activity and the HD1 protein contributes the activation domain (Asante-Owasu et al. 1996).

Three predicted nuclear localization signals (NLS) are present in HD1-7 and HD2-18 by PSORTII analysis (Nakai and Horton, 1999). NLS on the HD1 protein permit the heterodimer to localize to the nucleus. Once in the nucleus, the heterodimer binds specific operator sites to bring about *A*-regulated changes in gene transcription (Wen et al, 1995).

The similarity amino acid sequence encodes HD2-18 DNA binding domains *M. aitchisonii* and various basidiomycete fungi. Although the absolute sequence similarity varies different groups, two position (L=Leu and F=Phe) and five positions (W=Trp, F=Phe, N= 51, R=Arg) are most always conserved. Tryptophan (Trp), Phenylalanine (Phe), Asparagine (Asn) and Arginine (Arg) has major implication for DNA binding and overall stability of the tertiary structure of the homeodomain (Banerjee-Basu and Baxevanis, 2001).

Homeodomain found in many proteins that regulate gene expression (Gehring, 1987)

RT-PCR analysis revealed that HD1 and HD2 in the dikaryon strain was preferentially expressed 10 times more higher than monokaryon strains. This phenomena related with our previous study in chapter 3 that propose the frequency of clamp formation in dikaryons was greater than 50% while maximum frequency

of clamp formation in monokaryon was 5.9 %. HD1 and HD2 expression were higher in a monokaryotic strain which can produce monokaryotic clamp cells than monokaryotic non-clamp cell. The highest relative expression level of HD2 was shown in monokaryon 50005-4 which capable of forming pseudoclamp and few true clamps. These results indicate that a production of clamp cell formation regulated with *A* mating- type homeodomain protein. In *Pholiota nameko,* using a DNA-mediated transformation system when a single homeodomain protein (A3-hox 1 or A3-hox2) from the A3 monokaryon strain was transformed into the A4 monokaryon strain, the transformants produced many pseudo-clamps but very few clamps. When two homeodomain protein genes (A3-hox 1 or A3-hox2) were transformed together into the A4 monokaryon, the ratio of clamps increased to approximately 50%. Based on these results 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 enough to drive true clamp formation (Yi et al. 2010).

In the dikaryons of both *C. cinereus* and *C. bilanatus* have characteristic clamp connections between each cell. The formation of the clamp cell normally occurs after fusion of monokaryons carrying different *A* factors, but clamp cells can also be induced in a monokaryon by introducing one or more different *A* factor genes by transformation (Mutasa et al. 1990; Kües et al. 1992). The entire *A42* factor of *C. cinereus* and individual genes were introduced into a monokaryon of *C. bilanatus* and, where expressed, resulted in a change in colony morphology

associated with the development of clamp cells (Challen et al. 1993). It is particularly interesting to observe that only the *C. cinereus* HD2 genes can elicit *A*-regulated development in *C. bilanatus*. Both HD1 and HD2 genes encode proteins with homeodomains, indicating that they are transcription factors that bind DNA and there is substantial genetic evidence that the active regulator is a heteromeric complex of both types of protein (Kües and Casselton, 1992a, b).

The fact that *C. cinereus* HD2 proteins are active *in C. bilanatus* shows that the HD2 homeodomains are sufficiently conserved to detect the same DNA target site. The homeodomain sequences of HD1 proteins are known to be less strongly conserved between species (Kües and Casselton 1992a, b) so these may not have the same target sequence. However, as shown in animal systems, homeodomain sequences may vary by as much as 50% but still bind the same target site (Hayashi and Scott 1990). So, we concluded the reason why monokaryon in *M. aitchisonii* can make form clamp cell-related high expression level of homeodomain genes.

Table 4-1 PCR primers used in this study

Primer	Sequence	Applications
HD2C_Maspi7F1	5'- GCCGCTGATCCATTATCGCTTAGG3’	Amplification of HD2 of Maspi 7
HD2C_Maspi7R1	5 - TTCCCATTGCTTCTCCTCGTCTTC -3’	
HD2C_Maspi18F1	5'- ATGCAGCATGGTTGGATGGAGA -3’	Amplification of HD2 of Maspi 18
HD2C_Maspi18R1	5' -GGACACGACGAAGTTGATGTTGGT-3’	
HD_7F1	5' -CGACGAGACTGGGGATTAGCATAC-3’	Amplification of HD1of Maspi 7
HD_7R1	5' -AGTGTTCGGCCGGTATACGACAG-3’	
ITS 1	5' -TCCGTAGGTGAACCTGCGG-3’	Universal fungal barcode sequence
ITS 4	5' -TCCTCCGCTTATTGATATGC-3’	
HD1_7FJ1	5'- CCCAAGGAAAGCACTTAAACGCGTC-3’	3'-Race HD1 Maspi 7
HD1_7FJ2	5'-AAGGAAAGCACTTAAACGCGTCC-3’	3'- Race HD1 Maspi 7
M13M4	5'-GTTTTCCCAGTCACGAC-3’	Oligo dT-Adaptor
HD2_7FJ1	5'-ATAAGCAAATCAGTGTTTGGAATCG-3’	3'- Race HD2 Maspi 7
HD2_7FJ2	5'-GCAATGGGAAGGACGCACCAATCC-3’	3'- Race HD2 Maspi 7
M13M1M	5'-CCAGTCACGAC GTTGTA-3’	3-'Race
HD2_18FJ0	5'-ACAAACAGGTTCATGTATGGTTCCA-3’	3'- Race HD2 Maspi 18
HD2_18FJ1	5'-GCGATGGACTCAGTGATAACACATT-3’	3'- Race HD2 Maspi 18
HD2_18F-RT	5'-ATGTATGGTTCCAAAATCACC-3’	Real time Pcr for amplification of $HD_2$18
HD2_18R-RT	5'-GATGGCAATGTGTTATCACT-3’	
HD2_18RJ1*_P	5'-CGATGGCAATGTGTTAT-3’	Reverse transcrip 5'Race
HD2_18FJ0	5'-ACAAACAGGTTCATGTATGGTTCCA-3’	5'Race $HD_2$18
HD2_18R5_1	5'-GTCCAGACGCGAAGGGAAAGCATT5'-3	
Ma18_RACE2	5'-CCGTAGCCGATACAGGAGAGA-3	
HD2_18R5_2	GCATTGGACTCGAAAAAATTCTCT	
HD1_7F-RT	5'-CTACCTTCTCACCGTCTCCT-3’	Real time Pcr for amplification of $HD_1$7
HD1_7R-RT	5'-GGATGCTGTTGTATCGATCGTT-3’	
Actin_18F	AGTAGCAGCTCTCGTTATCGACA	Real time PCR for amplification actin
Actin_18R	AGCCTCATCGCCGATAGG	

Table 4-2 Gene homologs identified in the HD2-18 strain *A* mating-type and its flanking region

Gene	Position	Possible function	Reference
para-aminobenzoic acid synthetase (Pb1)	393276..395722	unknow	James et al.2006
Uncharacterized protein (UP11)	568650..570874	unknow	
Aminoimidazole ribonucleotide synthetase (ADE5)	379589..382416	unknow	
Beta-flanking protein	585909..586876	unknow	Ri et al. 2009
DNA-dependent RNA polymerase II second largest subunit (RPB2	626949..629537	Catalyse the transcription of DNA into precursors of mRNA, snRNA and microRNA.	
Glycine cleavage system protein (Glydh)	554247..557964	Glycine dehydrogenase activity, lysase activity	Ri et al. 2009
HD2 mating type protein	**575398..577402**	**Trancription factor activity**	**Wan et al, 2013Aimi et al. 2005, James et al. 2006**
Mitochondrial Intermediate Peptidase	571239..573724	Hydrolase activity, MIP activity, zinc ion binding	Ri et al. 2009

Table 4-3 Gene homologs identified in the HD2-7 strain *A* mating-type and its flanking region

Gene	Position	Possible function	Reference
Glycine cleavage system protein (Glydh)	8813..12310	Glycine dehydrogenase activity, lysase activity	Ri et al. 2009
Mitochondrial Intermediate Peptidase	25712-28070	Hydrolase activity, MIP activity, zinc ion binding	Ri et al. 2009
HD2 mating type protein	**64249..65530**	**Trancription factor activity**	**Wan et al, 2013Aimi et al. 2005, James et al. 2006**
HD1 mating type protein	**61860-62932**	**Trancription factor activity**	**Wan et al, 201Aimi et al. 2005, James et al. 2006**
Beta-flanking protein	63574-64286	unknow	Ri et al. 2009

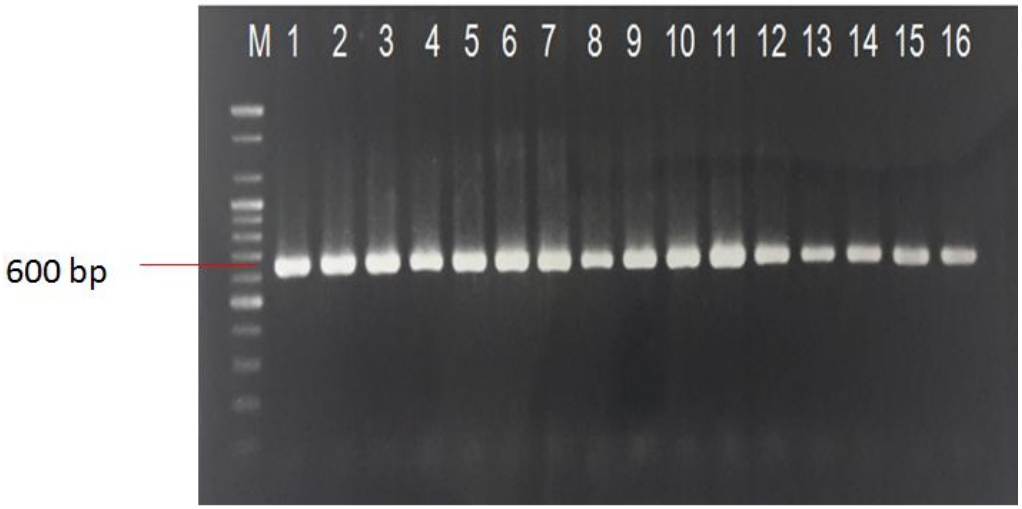

Fig. 4-1 Extracted monokaryon DNA of *M. aitchisonii* was amplified with primer pair for ITS region to check the quality of DNA.

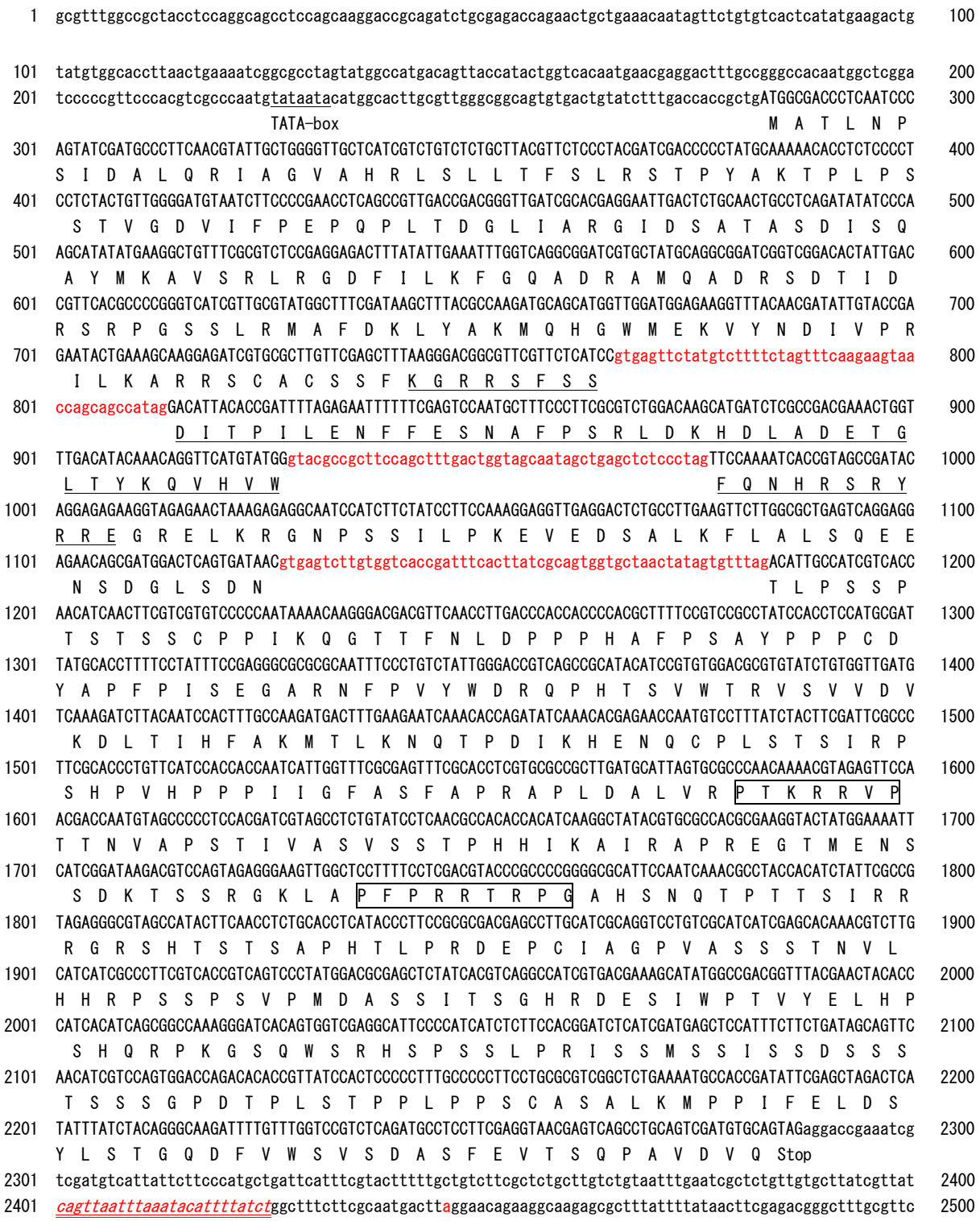

Fig 4-2 Nucleotide and asam amino sequences of HD2-18 strain. Capital letters indicate exon sequence and amino acid sequence are shown below nucleotide sequence. Introns, 5'-upstream and 3'-downstream are shown in lowercase letters. The consensus amino acids of homeodomain (DNA binding domains) is underlined. 3 nuclear localizations signal (NLS) are boxed. Prediction transcription sequences poly-A signal are shown in double underlined letter.

Fig 4-3 Nucleotide and asam amino sequences of HD1-7 strain. Capital letters indicate exon sequence and amino acid sequence are shown below nucleotide sequence. Introns, 5'-upstream and 3'-downstream are shown in lowercase letters. The consensus amino acids of C-terminal domain of homeodomain 1 is underlined. 3 nuclear localizations signal (NLS) are boxed.

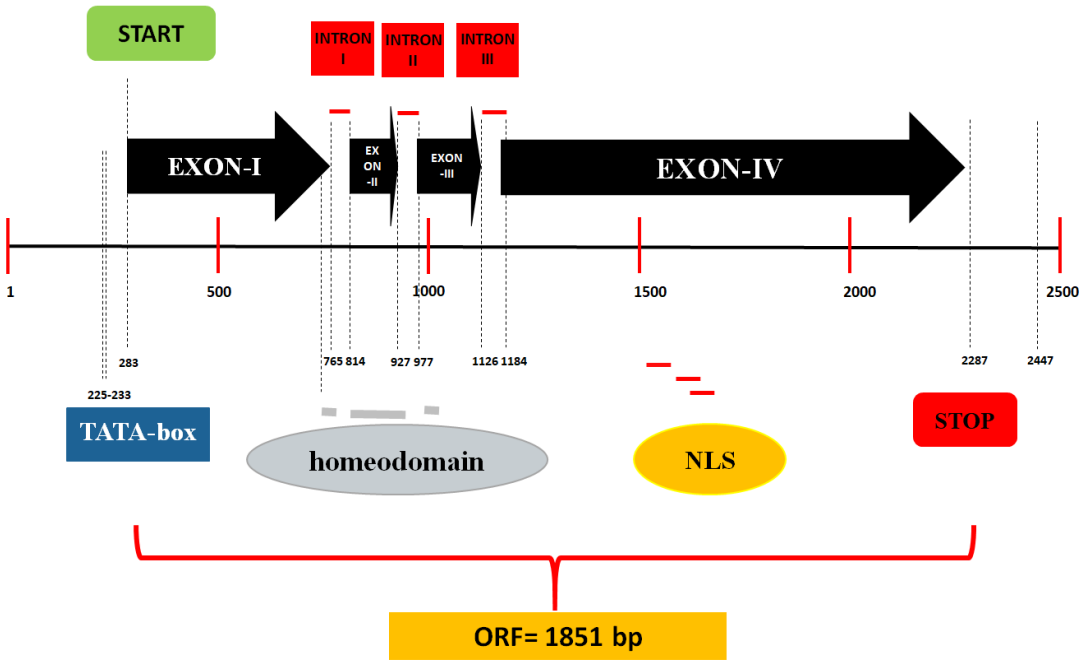

Fig 4-4 Schematic diagram of nucleotide and amino acid sequences of HD2-18 from *M. aitchisonii*

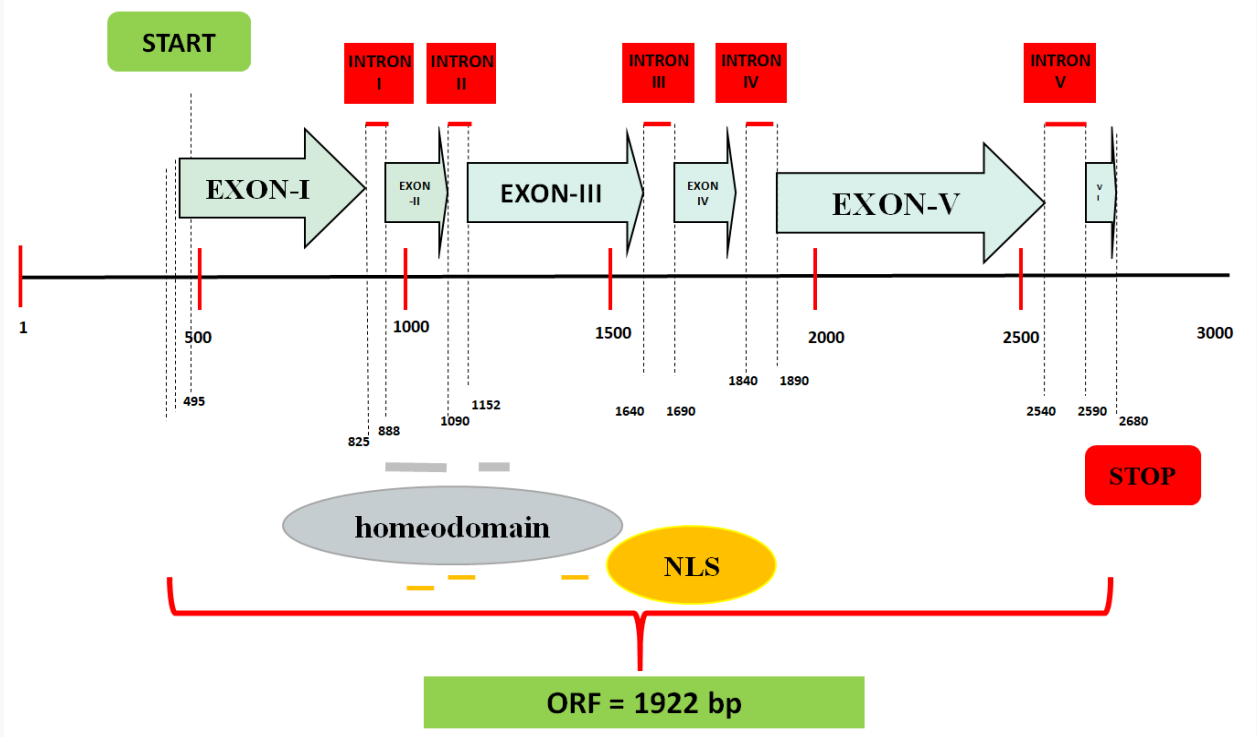

Fig 4-5 Schematic diagram of nucleotide and amino acid sequences of HD1-7 from *M. aitchisonii*

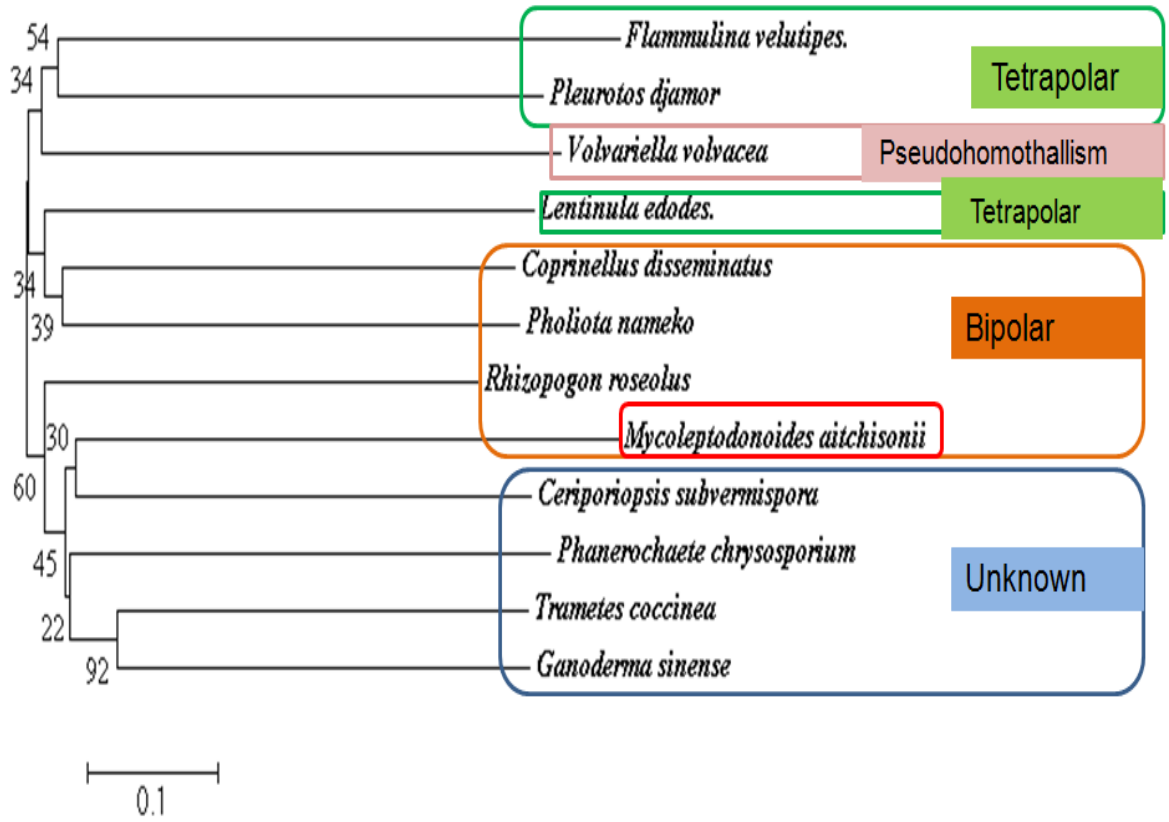

Fig. 4-6 Phylogeny tree constructed from the amino acid sequence alignments of homeodomain mating type protein homologs of *M. aitchisonii* and various basidiomycete fungi. Phylogenetic tree constructed by the maximum parsimony method. Accession number and names of protein sequences of HD protein in this study is as follows; *Flammulina velutipes* (ALT22411), Pleurotus *djamor* (AY462111.1, HD), *Volvariella volvacea* (AEO99208.1 HD2), *Lentinula edodes* (AEN14465), *Coprinellus disseminatus* (AAZ20167.1 MAT-A), *Pholiota* (BAH22599.1, HD2), *Rhizopogon roseolus* (BAL45603.2, HD2), *Ceriporiopsis subvermispora* (EMD41907), *Phanerochaete chrysosporium* (E7DAH1), *Trametes coccinea* (A0A1Y2IQ65), *Ganoderma sinense* (ZZ0214-1A0A2G8SDV9).

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Mycoleptodonoides aitchisonii   ---CSSFKGRR FSSDITP LENFFESNAFPSRLDKHD ADETGLTYKQVHVWFQNHRSR  206
Lentinula edodes                QTSIPAYEAPVPFNNEYTPILETYFQYDPYPTSRDRQII AERSGMTRRQIEVWFQNHRRT  213
Phanerochaete chrysosporium     SGSCGKGTSRGAFTQEVTSVLEHAFAQNAYPTRLEKESLAEITNMEYRQVNVWFQNRRTR  206
Rhizopogon roseolus             -DKPRTSSCTPKFNHEYIPLLEHFFAENAFPTQADKAFLAKKSAMTYRQIHVWFQNRRNR  199
Ceriporiopsis subvermispora     -GQPETSGSRRPFTSAVVAVLEAFFVENAFPTRDEKHELAAETHMDYRQIHVWFQNRRNR  214
Trametes coccinea               -NPKGSVQARPAFNHSAIPTLEQFFSKNPFPSRLEKFELASTCKMEYRQIHVWFQNRRSR  212
Ganoderma sinense               PSATEFIERKRPFNASAIPILEQFFEQNAFPSRLEKIELASKCDMDFKQIHIWFQNRRTR  195
Flammulina velutipes            -ASTIPSTSTAQFNHAFIPILTSYFNWNPFPPAQDRKMLADKGSMTERQIEVWFQNRRAL  197
Pleurotus djamor                -ADQVKPAAKTSFNADFVPFLEKYFEFNAYPSAADRSLMARKSMMTPRQIEVWFQNHRNR  197
Volvariella volvaceae           ---SSHDRKRKAFNHHYSHLLERYFEQNAYPSAADRRHLAQKTVMSPRQIEVWFQNHRSR  196
Coprinellus disseminatus        -VTRRTEKQKIPFNHESLPLLETYFEYNAYPSAQDRALLAKKSMMCPRQIEVWFQNHRRR  204
Pholiota nomeko                 -KRNSQDTKKPPFNSEYTPLLERYFQSNAYPSRPDRLLLATKSSMTERQIEVWFQNHRNR  206
                                          *.       *   *  : :*   ::  :*    :  :*:.:****:*

Mycoleptodonoides aitchisonii   YRREGR----------ELKRGNPSSIL--P--------------------------------  224
Lentinula edodes                ARQSGI----------TLPAKRPPGAIAPP-GLDVPFAVGQGQGGGQGQGHGQQYQGEGQG  262
Phanerochaete chrysosporium     SKRSGH----------GVKRGHVFHRVHRV---------------------------------  226
Rhizopogon roseolus             MKKEGQ----------VLKKKPAAEGAIRP-----L---------------------------  220
Ceriporiopsis subvermispora     SREKGK----------AVKNGVQA-QL--P---------------------------------  231
Trametes coccinea               LRKEGK----------ELKKPERKGVL--P---------------------------------  230
Ganoderma sinense               LRKEGK----------EPKRSQNNALL-------------------------------------  212
Flammulina velutipes            SRKRTGLALRKQDFKRCLNRPPPDDVL-------------------------------------  224
Pleurotus djamor                ARKEGK----------CLPRLRLAEEL--PKDLCL-----------------------------  220
Volvariella volvaceae           ARKEGK----------ILKRPYQS-SD--L----L-----------------------------  214
Coprinellus disseminatus        TKKEGR----------RLRRLSQD-PL--PHELDF-----------------------------  226
Pholiota nomeko                 SKKEGI----------LLKRISPH-QL--PSAESM-----------------------------  228
                                :.
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Fig. 4-7 The similarity amino acid sequence homeodomain *M. aitchisonii* and various basidiomycete fungi indicate by an *asteriks*.

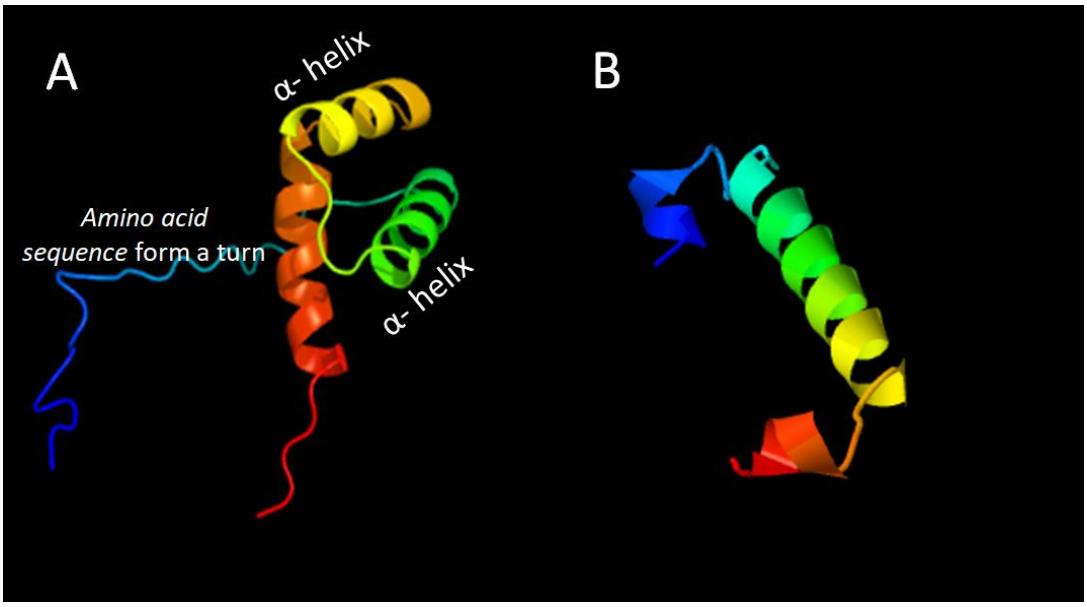


Fig. 4-8 Structure of helix-turn-helix (HTH) motif of DNA binding domain at HD2-18 (A) and transport protein (heterodimerization) at HD1-7 (B) by Phyre2 analyses**.** Coloured by rainbow N ⟶ C terminus. C terminus binds to the major groove of DNA, N terminal help to position the complex.

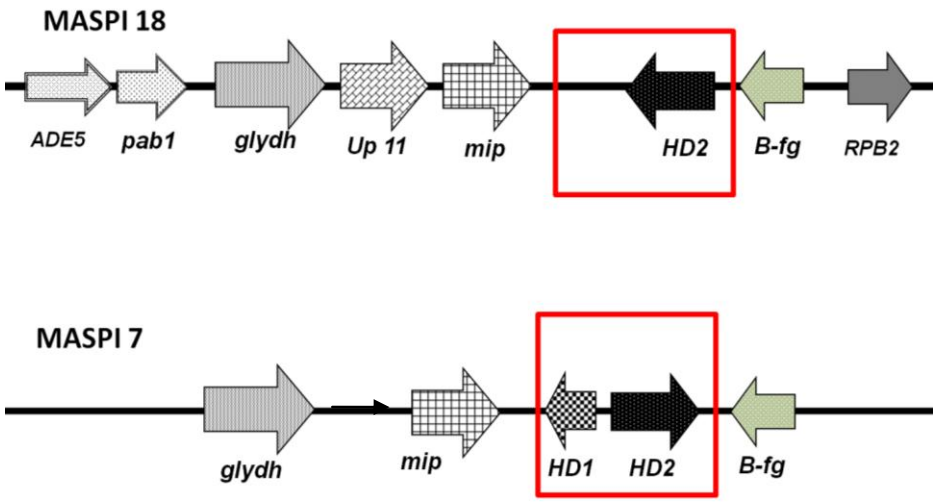
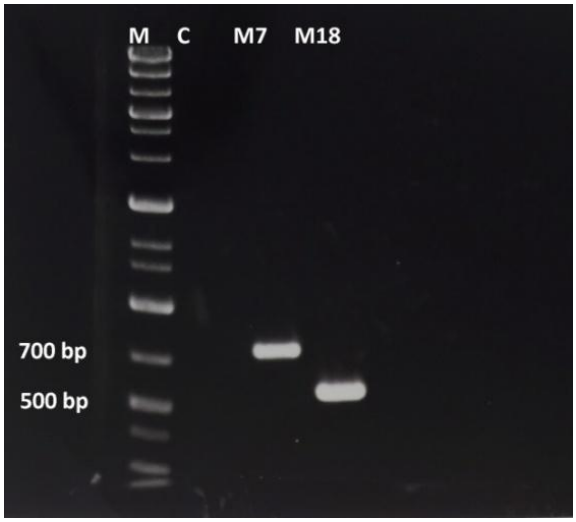


Fig. 4-9 Predicted of *A* mating locus Maspi 7 and Maspi 18 strain. Homeodomain protein genes are shown as black boxes (➡).

A



B

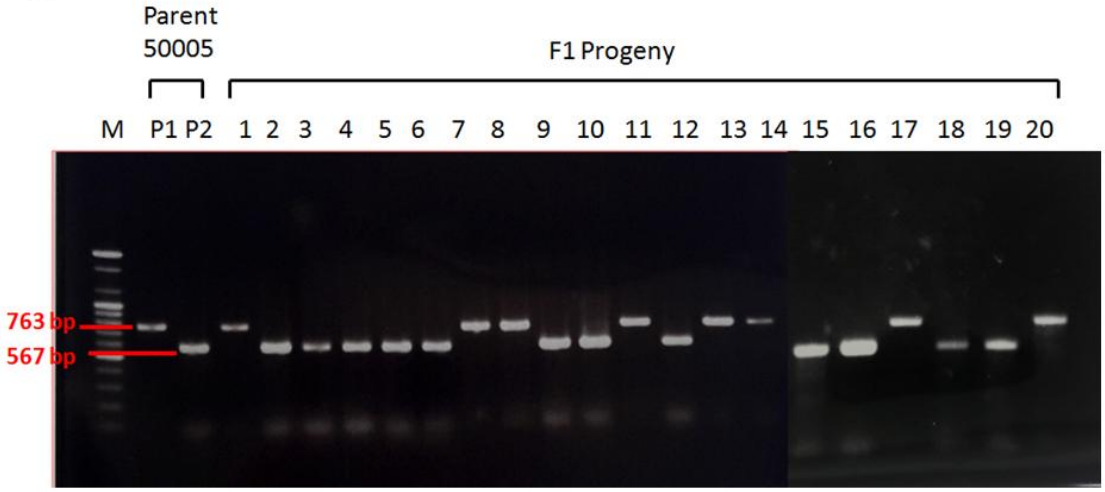


Fig 4-10 Amplification of homeodomain HD2 Maspi 7 and Maspi 18 in a 1.5 % agarose gel stained with ethidium bromide (EtBr). Panel A, amplification of HD2, line 1 is marker 2 kb, line 2 is control, line 3 HD2 Maspi 7 (763 bp), line 4 HD2 Maspi 18 (567 bp). Panel B, inheritance of homeodomain (HD2) from *M. aitchisonii* strain TUFC50005 (parent) among F1 progeny monokaryon 50005-1 until 50005-20.

Table 4-4 Monokaryotic clamp cell formation and mating type segregation among monospore isolates derived from dikaryotic strains (50005-7 × 50005-18)

Clamp Cell formation	***A1***	***A2***
	Size of PCR product (HD2)	
	567 bp length (HD2-18)	**763 bp length (HD2-7)**
True clamp	25, 41, 52, 58, 60, 88, 143, 154, 165	31, 51, 63, 65, 59, 64, 177, 175
No clamp	2, 3, 4, 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, 130, 131, 132, 134	10, 11, 15, 16, 17, 23, 26, 36, 43, 45, 46, 50, 55, 62, 67, 69, 81, 107, 108, 109, 110, 125, 129, 145

Strain numbers BRW– is abbreviated.

Table 4- 5. Chi-squared analysis of the first generation (F1) and second generation (F2)

	Chi-squared- Test of goodness-of-fit[x]	
	F1	F2
X^2	1.8	5.069
p	0.180	0.024

[x]Variables are the number of observed F1(13 : 7), F2 (54 : 32) and expected (1:1) mating types.

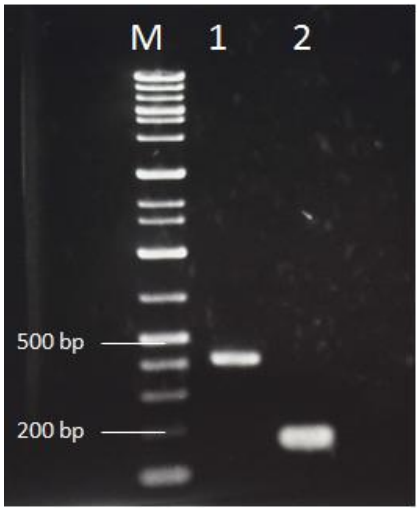


Fig. 4-11 Expression of HD2 and HD1 from *M. aitchisonii* analyzed by RT-PCR. The each of RT-PCR product were separated in 1.5% agarose gel. The size markers correspond to 2 kb DNA ladder. Line 1; HD1-7, Line HD2-18.

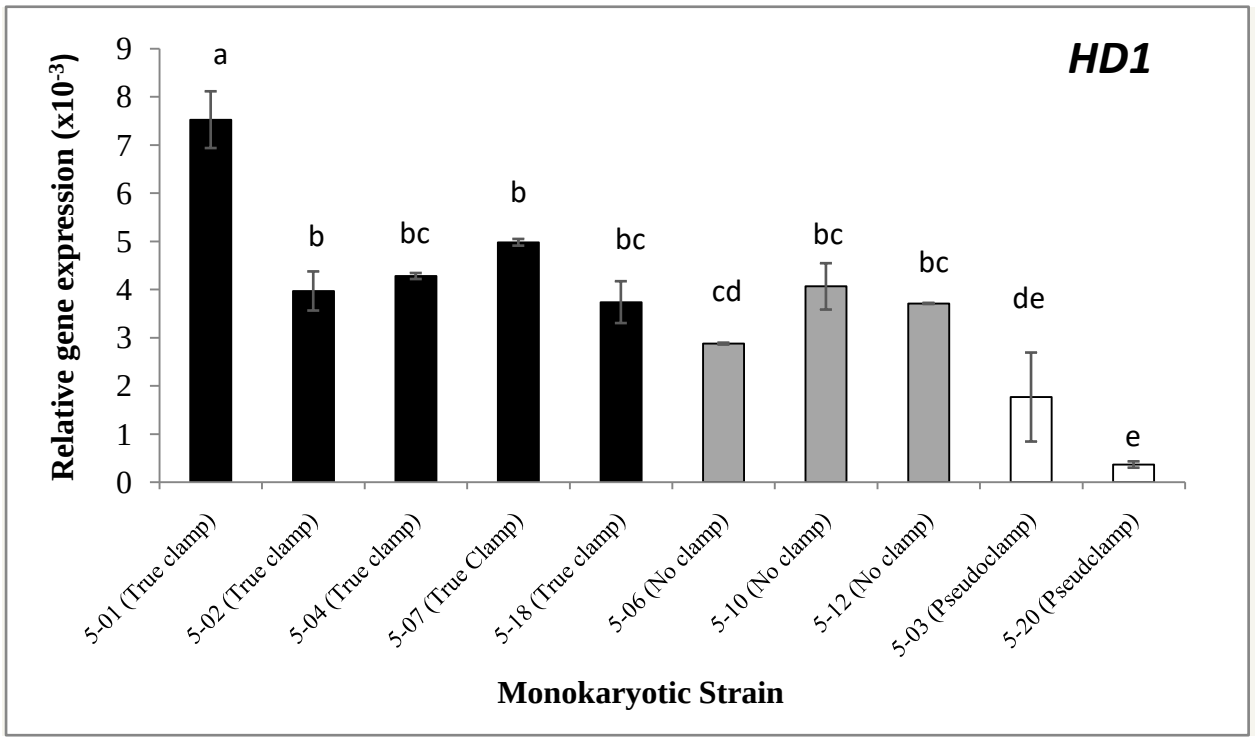


Fig. 4-12 Relative expression of HD1 in monokaryotic strain which can produce clamp cell and no clamp cell. Total RNA was measurement on day 8 by qRT-PCR in duplicate with variation denoted by standard error bars. Letters means statistically significant differences (one-way ANOVA with Tukey HSD multiple comparison test, $P < 0.05$.

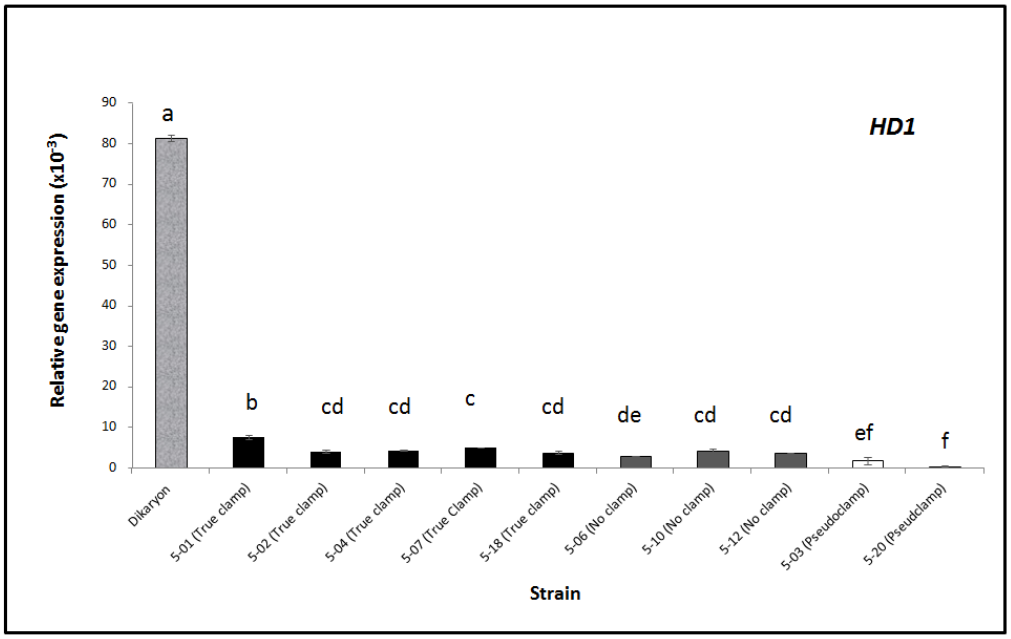


Fig. 4-13 Relative expression of HD2 in dikaryon and monokaryotic strain which can produce clamp cell and no clamp cell. Total RNA was measurement on day 8 by qRT-PCR in duplicate with variation denoted by standard error bars. Letters means statistically significant differences (one-way ANOVA with Tukey HSD multiple comparison test, $P < 0.05$).

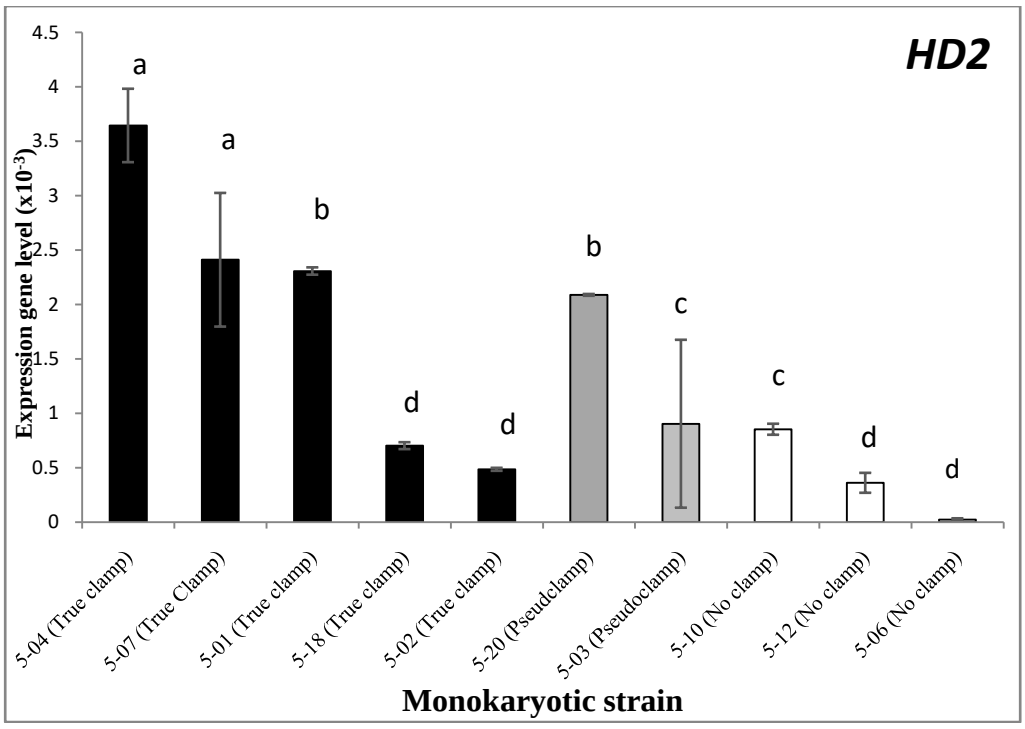


Fig 4-14 Relative expression of HD2 in monokaryotic strain which can produce clamp cell and no clamp cell. Total RNA was measurement on day 8 by qRT-PCR in duplicate with variation denoted by standard error bars. Letters means statistically significant differences (one-way ANOVA with Tukey HSD multiple comparison test, $P < 0.05$).

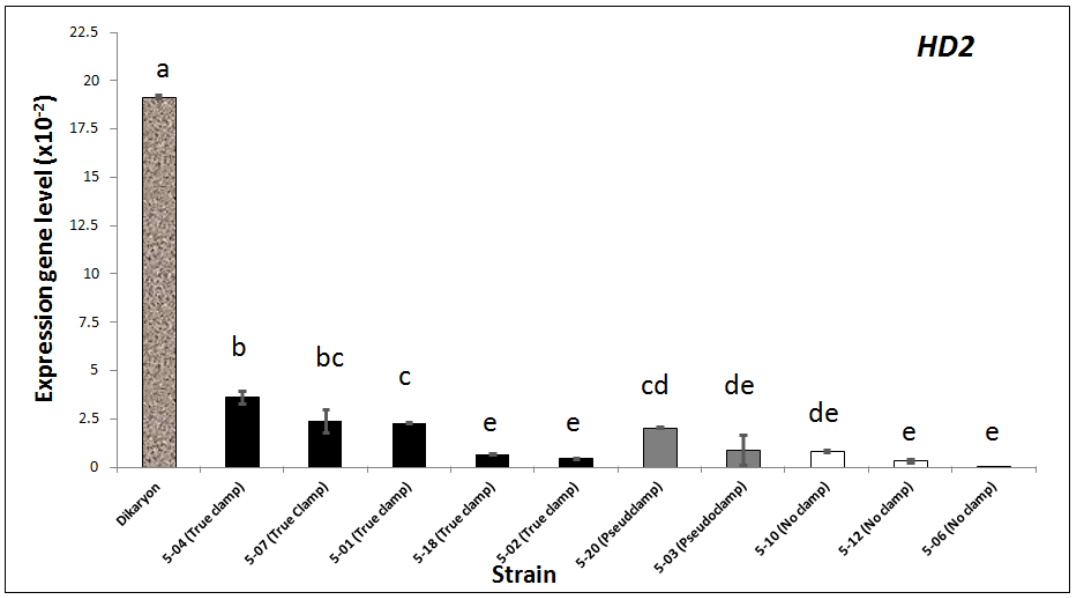


Fig. 4-15 Relative expression of HD2 in dikaryon and monokaryotic strain which can produce clamp cell and no clamp cell. Total RNA was measurement on day 8 by qRT-PCR in duplicate with variation denoted by standard error bars. Letters means statistically significant differences (one-way ANOVA with Tukey HSD multiple comparison test, $P < 0.05$).

Chapter 5

Discussions and Conclusions

During sexual development, both heterothallic Ascomycetes and Basidiomycetes are in a dikaryotic state. Basidiomycetes have a uniquely long dikaryotic state during which the dikaryotic mycelia can grow indefinitely. Nuclei in the dikaryotic hyphae of basidiomycetes divide and migrate in a coordinated manner, and the formation and fusion of the clamp connection ensures that both nuclei are faithfully transmitted as the hyphae extend. Nuclear fusion and meiosis occur in the basidia. Unlike Ascomycetes in which heterothallism is generally bipolar and controlled by the nonalleclic single mating type, heterothallism in basidiomycetes is often tetrapolar and controlled by two structurally complex unlinked loci that are typically multiallelic. The production of fruiting bodies is dependent on the interaction of morphogenetic genes, incompatibility factors and environmental conditions (Casselton and Kües, 1994).

Studies on the formation of fruiting bodies in higher basidiomycetes have produced basic information on the control of sexual morphogenesis. Sexual morphogenesis in these species results in the formation of a dikaryon, which produces fruiting bodies. Although the formation of fruiting bodies is not limited to the dikaryotic mycelium, fruiting structures may be induced in

the monokaryotic mycelium of several basidiomycetes species under specific conditions (Raper and Krongelb, 1958, Niederpruem et al., 1977).

Esser and Stahl (1973) reported that approximately 60% of all the single-spore, monokaryotic isolates of *Polyporus ciliatus*, *P. brumalis* and *Agrocybe aegerita* behave as monokaryotic fruiters. In *P. ciliatus*, monokaryotic fruiting does not involve sexual reproduction, and none of plasmogamy, karyogamy or meiosis are required. Monokaryotic fruiting is strictly a vegetative phenomenon considered to be mitotic and asexual. In *P. ciliatus*, monokaryotic mycelia and fruiting stages were confirmed to remain truly monokaryotic for the whole life cycle, i.e., mycelial and fruiting stage hyphae were reported to never form clamp connections, and they only ever contained one nucleus per cell (Stahl and Esser, 1976).

The capability to produce monokaryotic fruiting bodies and clamp cells in culture was, surprisingly, examined in a monokaryotic strain isolated from several dikaryotic parental strains of the edible mushroom, *M. aitchisonii* (Bunaharitake). In our analysis presented in Chapter 2, we found that most strains formed primordia, or young fruiting body-like structures, but only one of the monokaryons, strain 50005-4, formed a fruiting body, even though it had only one nucleus and produced only two spores after meiosis. Although most clamp cell connections were pseudo clamps and the frequency of appearance of true clamps was low, most monokaryotic strains had the capability to form true clamp cells. From these results, two hypotheses were

generated. First, dikariotization is not required for clamp cell formation, fruiting body formation or meiosis in *M. aitchisonii*. Second, nuclear fusion and mating are not essential for mushroom development. The reason why monokaryons can form clamp connections and monokaryotic fruiting bodiesis not clear.

Several mechanisms for the formation of monokaryotic fruiting body have been described. The monokaryotic mycelia of mutant strain, *fis*^{c}, of *Coprinus macrorhizus*, which can form monokaryotic fruiting bodies underlight conditions, failed to form any fruiting bodies when held in the dark, indicating that light may also be effective for inducing cAMP synthesis by adenylate cyclase and that the increase in cAMP-binding activity may be responsible for the formation of fruiting bodies. Cyclic AMP has been reported to affect cell division and morphology in mammalian cells (Uno et al., 1974). In *Schizophyllum commune*, injury induced the monokaryotic fruiting body (Leslie, 1979). Here, we describe that mechanism in some basidiomycetes.

Monokaryotic fruiting is mitotic, asexual and a vegetative phenomenon, but based on our findings in *M. aitchisonii*, it has been shown to be a modified form of sexual reproduction. Further, several reports have indicated that chemical substances can initiate the process of monokaryotic fruiting. The inducing factor of monokaryotic fruiting bodies was isolated from fruiting bodies of the same species and also from unrelated species of fungi (Leonard and Dick, 1968). Although the inducing factor was partially purified in some

cases, chemical identification and underlying control mechanisms by which the factor induces fruiting remain to be clarified. On the other hand, it has been demonstrated that expression of monokaryotic fruiting is controlled by genetic factors (Leonard and Raper, 1969). Further investigation of the mechanism of monokaryotic clamp cell and fruiting body formation is needed.

Two types of monokaryotic fruiting have been described in *Coprinus cinereus*, a tetrapolar heterothallic homobasidiomycete (Uno and Ishikawa, 1971). First, the *fis*$^{+}$ strain produces monokaryotic fruiting bodies under inductive conditions of growth on media containing cell-free extractsalong with fruiting-inducing substance (FIS) from fruiting bodies of *C. macrorhizus* or *Lentinus edodes.* Crude extracts were prepared from dikaryotic fruiting bodies of *C. macrorhizus* obtained from a cross between two wild type strains, 708-7 A_7B_8 and 708-15 A_8B_7, and from *Lentinus edodes*. Sixteen wild type strains of *C. macrorhizus* were inoculated on slant media containing prepared crude extracts or water as a control. Strain 708-15 A_8B_7 produced monokaryotic fruiting bodies in all cultures supplied with crude extracts, although this strain produced monokaryotic fruiting bodies spontaneously at low frequency in the absence of crude extract. Strain708-15 was identified by marker symbol *fis*$^{+}$. Other strains, which produced no monokaryotic fruiting bodies in the presence of crude extracts, were identified by marker symbol *fis*$^{-}$. To determine whether the character for FIS-susceptibility is inherited, strain 708-15 *fis*$^{+}A_8B_7$ was crossed with strain 506-23 *fis*$^{-}A_6B_6$. In this cross, 17% of

progeny were *fis*$^{+}$, indicating that more than one gene may be responsible for this character. Since *fis*$^{-}$ A_8B_7 isolates were obtained from this cross, the *fis*$^{+}$ loci may not be identical to the mating-type loci. Two fractions of FIS were obtained from Sephadex G-25 chromatography. One contained protein and the other had a chemical nature similar toadenosine-3', 5'-monophosphate or adenosine-3'-monophosphate, which are active in inducing monokaryotic fruiting.

In the second type of monokaryotic fruiting, *fis*^{C} strains constitutively produced monokaryotic fruiting bodies. During the monokaryotic fruiting process in *fis*^{C} strains, young basidia have only one haploid nucleus, which divides mitotically into two daughter nuclei that then fuse to form a diploid nucleus. The fused nucleus then divides, producing four basidiospores on the basidium. Synaptonemal complexes and typical meiotic chromosome behaviours were observed, suggesting that monokaryotic fruiting processes can be meiotic sexual processes (Miyake and Ishikawa, 1980). Basidium development was further confirmed by fluorescence microscopy, revealing a shift in the relative DNA content in basidia in the monokaryotic basidiocarp of the *fis*^{C}strain, which is consistent with the meiotic nature of the fruiting process (Oishi et al.,1982). In these studies, the meiotic product originated from the single nucleus. Whether the monokaryotic strain can fuse with another strain with asimilar mating type to exchange DNA and produce genetically distinct progeny is not known. Monokaryotic *M. aitchisonii* strain

50005-4 produced two spores in 65.7% of the basidia, while dikaryotic strain 50005 produced four spores in 96.7% of the basidia. In the monokaryotic fruiting body of 50005-4, the number of basidia was drastically reduced. These basidia were also of the two-spore type, unlike the typical four-spore basidia of dikaryotic fruiting bodies. These results show that monokaryotic strain 50005-4 can produce fruiting bodies, even though this strain has only one nucleus and can only produce two spores after meiosis. However, the reason for the number of basidia being reduced by half in the monokaryotic fruiting body of 50005-4 has not yet been clarified, and future studies will be needed to address the nuclear behavior of the monokaryotic fruiting body process.

Another mechanism of monokaryotic fruiting having a bipolar mating system and unusually large mating-type locusis found in *Cryptococcus neoformans*, which is the most common causative fungal agent of meningitis (Lengeler et al, 2002). *C. neoformans* is a heterothallic fungus with the capability to self-pollinate in the absence of the opposite mating type. The traditional mating process of this heterothallic fungus involves haploid cells of α and a mating types (Kwon-Chung, 1975), and equal proportions of α and a basidiospores with four chains are produced (Hull et al., 2005). Haploid *C. neoformans* α strain can also undergo a transition from yeast to filamentous growth and sporulation through monokaryotic fruiting (Erke, 1976). Fruiting occurs under the same conditions that promote traditional mating between two strains with opposite α and a mating types (Kwon-Chung, 1977): nitrogen

starvation, desiccation, darkness and the presence of mating pheromone. During monokaryotic fruiting, haploid nuclei diploidize, either before filamentation, during filamentation or in the basidia immediately preceding sporulation. Diploidization can occur between cells of the same mating type.

The observation of monokaryotic fruiting suggests that mating-type genes, while essential for establishing and maintaining compatible heterokaryotic status, may not be essential for meiosis and fruiting body morphogenesis (Leslie and Leonard, 1979). Homothallic species may originate from heterothallic predecessors, and the homothallic life style may have a selective advantage under certain ecological pressures (Perkins, 1991). This hypothesis is consistent with repeated occurrence of homothallism within numerous genera and the fact that many heterothallic fungi achieve homothallism in the form of pseudo-homothallism by mating-type switching or packaging two compatible nuclei into one spore. This hypothesis is supported by the prevalence of monokaryotic fruiting bodiesof TUFC50005-4, which produce two spores in the basidia, such that the number of basidia is reduced by half, although dikaryotic strain TUFC50005 produces four spores. Thus, we suggest that sexual reproduction systems of *M. aitchisonii*s witch from the heterothallic system to the homothallism system (Fig. 5- 1).

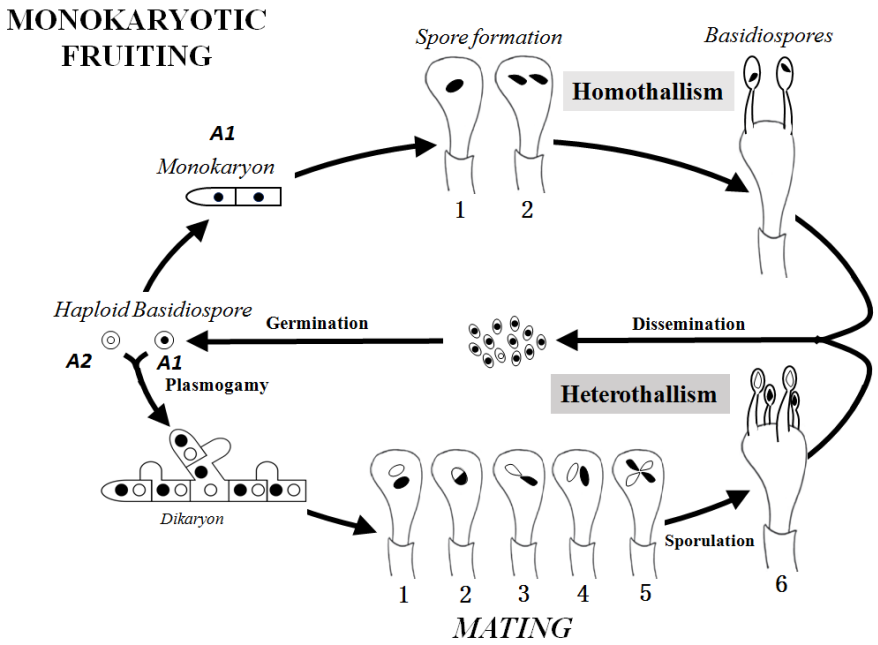


Fig.5-1. Predicted sexual cycle of *M. aitchisonii*. During mating (the heterothallic life cycle) *A1* and *A2* cells undergo cell-cell fusion and produce dikaryotic hyphae. At the stage of basidium development, the two of parental nuclei fuse and undergo meiosis to produce four meiotic products. During monokaryotic fruiting (homothallic life cycle), cells of one mating type, e.g., *A1* become diploid *A1*/*A1* cells, either by endoreplication or by cell fusion followed by nuclear fusion between two *A1* cells. At the stage of basidium development, meiosis occurs and haploid basidiospores of one mating type are produced in two basidium (Heitman et al., 2006).

Monokaryotic fruiting may be widespread in fungi and has been demonstrated to occur in monokaryotic hyphae in the absence of a dikaryotic phase in some heterothallic basidiomycetes. For example, fruiting bodies have been found on the monokaryotic mycelia of *Endophora Ludoviciana* (Biggs, 1938), *Schizophyllum commune* (Raper and Krongelb, 1958) and *Fomes cajanderi*, although the basidia of these heterothallic species normally form on

dikaryotic mycelia during classical mating with compatible mating partners. Thus, in Chapter 3, we investigated the mating system of*M. aitchisonii* using classical methods.

Examination of the polarity of mating types in *M. aitchisonii* as well as the genetic linkage between the mating-type locus and the ability to form monokaryotic clamp cells by classical genetic analysis generated two hypotheses. First, based on the results of polarity tests, we propose that *M. aitchisonii* is a bipolar mating system because *A1* and *A2* are found in relatively equal frequencies (1:1) in the progeny of the first and second generations. Only two mating types segregate in meiosis, and mating incompatibility is controlled by a single mating type locus. Second, no genetic linkages were observed between the phenotypes capable of forming monokaryotic clamp cells and mating type, indicating that monokaryotic clamp formation is not linked to mating-type locus. Moreover, the possibility of not producing a recombinant mating type strain after meiosis indicates that there may only be a single mating-type locus in *M. aitchisonii*. However, in the F2 generation with approximately 86 monokaryons, progeny did not carry *A1* and *A2* in equal frequencies. In a hypothetical cross between strains carrying *A1* α1-β1 and *A2 α2-β2*, recombinant spores carrying *A3 α1-β2* and *A4 α2-β1* would be formed, and these recombinants would be compatible with all of their siblings, except those carrying the identical combination. However, we did not find any recombinants in this study. This finding might be due to

one of nuclei being more dominant than the other, which is a phenomena similar to that found in *Pholiota nameko*.

P.nameko has a bipolar incompatibility factor. In *P. nameko* monokaryotized mycelia can easily be isolated from the peripheral growing zone in a dikaryotic colony. Hyphal monokaryotization is of great interest as it is the probable cause of instability in conjugate nuclear division. In this operational hypothesis, one nucleus in the dikaryotic cell divides slightly earlier than the other, producing a leading nucleus that controls the second, or following nucleus. Inferior control in this system may cause the monokaryotization of dikaryotic cells. Since the monokaryon with the leading nucleus consistently persists in monokaryotization, it may contribute genetically to other traits that dominate expression of the subsequent monokaryon (Matsuda et al., 1985). In two generations, *M. aitchisonii* monokaryotic strain 50005-18 (*A1*) became the leading nucleus with monokaryotic strain 50005-7 (*A2*) as the following nucleus based on a progeny ratio (*A1*:*A2*) of 13:7 in the F1 generation and 54:32 in the F2 generation.

In Chapter 4, we analysed the molecular and expression levels of homeodomain genes in monokaryon shaving either a true clamp connection or no clamp connection to examine the mating system of *M. aitchisonii.* We made four findings that contribute to this field. First, by using specific primers to amplify HD2 from basidiospores, the mating type of *M. aitchisonii* could be determined based on the size of the PCR products, and F1 and F2 strains could

be divided into two incompatibility groups. Natural selection for outbreeding in basidiomycetes is governed by mating-type genes, and amplifying mating-type genes by PCR offers a way to score the mating system in *M. aitchisonii* prior to carrying out test matings (Kothe, 2001). Expanding the availability of mating-type genes can support breeding programs and expand the availability of inbred strains needed for the introduction of recessive markers.

Second, the HD2 gene was characterized. The open frame of *Mahd2* from ATG to the stop codon is 1851 bp, encoding 614 amino acids over three introns interrupted by four exons. *Mahd1* from ATG to the stop codon is 1922 bp, encoding 640 amino acids over five introns interrupted the coding sequence which comprised of 6 exons. The gene encoding *Mahd1* in the Maspi 18 strain was absent upstream of the HD2 protein gene. HD2 proteins contain the fully conserved homeodomain sequence, which appears to be essential for DNA-binding. In contrast, HD1 class proteins may not be critical or correct DNA recognition. Deletions or sequence alterations in the homeodomain of HD1 proteins did not abolish protein function in the regulating clamp cell formation *in vivo*, indicating that indeed the HD1 homeodomain is not essential for dikaryon development (Asante-Owusu et al., 1996). Members of the HD2 class have a sequence motif that more closely resembles the consensus (Börglin, 1994). Thus, heterodimerization is likely essential for function, and we predict that the HD2 protein cannot enter the nucleus without first associating with an HD1 protein; although the HD1 protein can enter the

nucleus without its HD2 partner, once there it lacks the specificity to recognize their joint target site on DNA (Spit et al., 1998).

Third, the DNA-binding region in HD2-18 *M. aitchisonii* was found to be 59 amino acids long and contain a helix-turn-helix (HTH) motif. HD1-7 has a potential activation domain at the C terminus as transport protein structure (heterodimerization domain). Owing to this feature, HD proteins are DNA binding transcription factors that recognize specific DNA sequences to access target genes in the genome and to control their expression.

Fourth, transcriptional analyses of *Mahd1* and *Mahd2* showed that expression of *Mahd1* and *Mahd2* was higher in a monokaryotic strain that can produce clamp cells than in monokaryons that could not produce clamp cells. The highest relative expression level of *Mahd2* was shown in monokaryon 50005-4, which is capable of forming true clamp. These results suggest that the formation of clamp cells is regulated with *A* mating-type homeodomain proteins, and the frequency of clamp cell formation might be promoted by increased expression of the *Mahd2* gene.

Our findings show that strains with true clamp cells could form fruiting bodies, and strains that produce true clamp cells in the high frequency could form normal shape fruiting bodies in the stock strain. These results indicate that mating type genes, such as homeodomain protein genes, pheromone genes and pheromone receptor genes may promote or inhibit expression of each

developmentalgene, but essentially, development can occur irrespective of mating type.

Strains 50005-7 (MASPI-7) and 50005-18 (Maspi-18) have identical pheromone and pheromone receptor gene sequences. The pheromone is not involved in discriminating between self and non-self. However, pheromone signaling might be involved in quorum sensing. In other words, one of the hypotheses for monokaryotic clamp cell formation is that a high concentration of pheromone or secreted autoregulatory products might induce homeodomain protein gene expression, which in turn may promote clamp cell formation. Therefore, further study is needed to confirm the effect of artificially synthesized pheromone peptide or autoregulatory secreted products in clamp cell formation.

Abstract

Study of fruiting body formation and clamp cell formation in the monokaryon of the edible mushroom *Mycoleptodonoides aitchisonii* (Bunaharitake)

Two types of sexual reproduction systems exist in basidiomycete mushrooms: heterothallic and homothallic systems. The term heterothallic refers to mating between two separate monokaryons carrying compatible mating type that are required for the formation of clamp cells and complete fruiting bodies. In typical heterothallic mushroom, its life cycle generally starts from germination of the haploid basidiospore and haploid mycelium which is usually called as monokaryon, can be produced. When two monokaryon carrying compatible mating type meet in nature, their cells can be fused and produce dikaryon. They grow by cell divisions followed by clamp cell formation. The dikaryon can produce fruiting bodies, efficiently. During fruiting body formation, meiosis and basidiospore formation have occurred. The four meiotic products are wrapped separately into individual basidiospores. Those are typical lifecycle of mushroom, however, monokaryotic fruiting body formation was previously reported in *Schizophyllum commune, Sistotrema brinkmanii*, and *Coprinopsis cinerea*. Therefore, it is possible that dikaryotizaton is not necessary for the formation of clamp cells and/or complete fruiting bodies. Here, we describe monokayotic clamp cell formation, fruiting body formation and meiosis in *Mycoleptodonoides aitchisonii*. *Mycoleptodonoides aitchisonii* belongs to the family Climacodontaceae and has been widely found on dead broad-leaved trees from summer to fall in Asia. Its fruiting bodies are effused-reflexed with fan-or spatula-shaped caps. The species exhibits pharmaceutical properties, including immunodulation, lipid-lowering effects and antibacterial effects.

A dikaryotic *M. aitchisonii* strain, TUFC50005, and 20 monokaryons derived from the TUFC 50005 strain, which exhibited a wide spectrum of monokaryotic fruiting. Most strains formed primordia, or young fruiting body-like structures, but only one of the monokaryons, strain TUFC50005-4, formed a complete fruiting body, even though it had only one nucleus and produced only two basidiospores after meiosis. Our findings show that true clamp cells formed strains could form fruiting body, and the strains which produce true clamp cells in the high frequency could form normal shape fruiting body among the stocked strain. These results indicate that mating type genes, such as the homeodomain protein genes, pheromone genes, and pheromone receptor genes may promote or inhibit expression of each

developmental gene, but essentially, development can occur without mating type.We demonstrated that dikaryotization was not required for clamp cell formation, fruiting body formation, and meiosis in this mushroom. This is one of the first reports to show that mating and nuclear fusion are not essential for mushroom development.

There are two different mating systems in heterothallic basidiomycetes, i.e., bipolar and tetrapolar mating systems. It is assumed that about three-forth of mushroom fungi may contain tetrapolar system, and the remaining may involve bipolar mating system. Bipolar mating systems are controlled by a single mating-type locus and only two mating types are produced by meiosis. The tetrapolar mating system is based on two unlinked mating types, commonly referred to as *A* and *B* loci. These so-called mating-type genes regulate nuclear pairing and clamp formation. In tetrapolar systems, when the two mating type loci are unlinked, four mating types can be generated after meiosis among the haploid progeny.

In response to previous reports that some basidiospore isolates of this mushroom can form complete fruiting bodies and true clamp cells, the frequency of true clamp cell formation was compared between the dikaryotic and the monokaryotic strains. Compared to monokaryotic strains, true clamp cells were observed with greater frequency in dikaryotic strains. Mating incompatibility groups were examined among basidiospore isolates from dikaryotic strain TUFC50005 (P) and 50005-7 ×50005-18 (F1), which were derived from strain TUFC50005. Mating compatibility could be divided into two groups indicating that *M. aitchisonii* is a bipolar mushroom. Moreover, recombinant mating type strain might not be generated after meiosis, indicating that there may only be a single mating-type locus in *M. aitchisonii*. 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.

The genetic structure of mating genes in the tetrapolar basidiomycetes has been well understood based on the researches on the model organism *C. cinereus* and *S. commune*. However, there are few insights into the bipolar mushrooms. The study on the mating type genes of *M. aitchisonii* will help us to understand the molecular mechanisms of the bipolar mating system in mushrooms. We analyzed mating system *M. aitchisonii* base on structure of the gene and expression level of homeodomain gene in monokaryon which had true clamp

cell and no clamp cell. Spesific primers for amplification of HD2 can be used as a molecular marker to determine mating type by comparing the size of their PCR products of among basidiospore isolates of *M. aitchisonii*. Mating type of all the strain were analysed. The strain from F1 and F2 could be divided into 2 incompatibility groups depending on different sizes of PCR products.

Gene structure of the bipolar mating system in *M. aitchisonii*, the homeodomain protein gene *Mahd1* and *Mahd2* has been characterized. A genomic DNA fragment of *Mahd2* is 1851 bp long and encoded 614 amino acids with the predicted molecular mass of 69.93 Kilodaltons. The coding area of *Mahd1* from ATG to stop codon is 1922 bp long and encoded 640 amino acids with the predicted molecular mass of 71.85 Kilodaltons.

The location of exons and introns were determined from the nucleotide sequences of PCR products amplified by 3'-Race and 5'-RACE PCR. All the introns started with GT and ended with AG. The 3 introns of *Mahd2* gene interrupt the coding sequence, which comprised of 4 exons. The 5 introns of *Mahd1* gene interrupt the coding sequence which comprised of 6 exons. The PSORT II program predicted there are three nuclear localization signals (NLS) in *Mahd2* (PTKRRVP, PFPRRTR and PRRTRPG) and *Mahd1* (PIAGRKR, RPRK, RKRR).

Gene encoding *Mahd1* in Maspi 18 strain was absent in upstream of HD2 protein gene. HD2 proteins are those that contain the fully conserved homeodomain sequence which appears to be essential for DNA-binding. In contrast, HD1 class proteins may be dispensable for correct DNA recognition. Deletions or sequence alterations in the homeodomain of HD1 proteins did not abolish protein function in regulating clamp cell formation in vivo, indicating that indeed the HD1 homeodomain is not essential for dikaryon development. Members of the HD2 class have a sequence motif that more closely resembles the consensus. Thus, heterodimerization is likely to be essential for function—we predict that the HD2 protein cannot enter the nucleus without first associating with an HD1 protein, and although the HD1 protein can enter the nucleus without its HD2 partner, once there it lacks the specificity to recognize their joint target site on DNA.

DNA-binding region was found in *M. aitchisonii* with 59 amino acids long and contains a helix-turn-helix (HTH) motif. HD1-7 has a potential activation domain at the C terminus as

transport protein structure (heterodimerization domain). Because of this feature, HD proteins are identified as DNA binding transcription factors, recognize specific DNA sequences to access their target genes in the genome, and to control their expression.

Transcriptional analyses of the *Mahd1* and *Mahd2* showed that expression of the *Mahd1* and *Mahd2* was higher in a monokaryotic strain which can produce clamp cells than monokaryon which could not produce clamp cells. The highest relative expression level of *Mahd2* was shown in monokaryon TUFC 50005-4 which capable of forming true clamp cells. These results suggested that the formation of clamp cells regulated with *A* mating-type homeodomain protein and frequency of the clamp cell formation might be promoted by high expression of the *Mahd2* gene.

和文要旨

食用きのこ *Mycoleptodonoides aitchisonii*（ブナハリタケ）のモノカリオンにおける子実体形成とクランプ細胞形成に関する研究

担子菌キノコには、ヘテロタリックとホモタリックの 2 つのタイプの有性生殖システムが存在する。ヘテロタリックという用語は、クランプ細胞と完全な子実体の形成に和合性のある交配型をもつ 2 つの異なるモノカリオン間の交配が必要であることを指している。典型的なヘテロタリックなきのこでは、そのライフサイクルは一般に半数体の担子胞子の発芽により、通常はモノカリオンと呼ばれる半数体の菌糸体が生じる。和合性のある交配型をもつ 2 つのモノカリオンが自然に出会うと、それらの細胞は融合してダイカリオンを形成する。それらは、クランプ細胞を形成しながら、細胞分裂によって成長していき、そのダイカリオンは子実体を効率的に発生します。子実体の形成中には、減数分裂に続き、担子胞子が形成され、その後、 4 つに減数分裂した核は、個々の担子胞子に別々に運ばれていく。以上は、きのこの典型的なライフサイクルであるが、モノカリオンによる子実体の形成は、以前、*Schizophyllum commune*, *Sistotrema brinkmanii*, 及び *Coprinopsis cinerea* において報告されている。従って、クランプ細胞　及び/または、完全な子実体の形成には、２核化が必要でない可能性がある。本論文では、*Mycoleptodonoides aitchisonii* のモノカリオンにおけるクランプ細胞形成、子実体形成および減数分裂について示す。 *M. aitchisonii* は、Climacodontaceae に属し、アジアの夏から秋にかけて広葉樹の枯れ木で広く発見される。その子実体は, 半背着生で且つ 漏斗形あるいはへら型の傘を有する。この種は、免疫調節、脂質低下効果、抗菌効果などの薬理作用が報告されている。

M. aitchisonii TUFC50005　二核菌株、およびその TUFC50005 株に由来する 20 株のモノカリオンは、子実体形成能を示した。ほとんどの株は原基、または若い子実体のような構造で発達が止まったが、モノカリオンである TUFC50005-4 株のみが完全な子実体を形成した。我々の発見は、真のクランプ細胞を形成することができる株が子実体を形成し、高頻度で真のクランプ細胞を産生する株が正常な形状の子実

体を形成することを示しいる。これらの結果は、ホメオドメインタンパク質遺伝子、フェロモン遺伝子、フェロモン受容体遺伝子などの交配型遺伝子が各発生遺伝子の発現を促進または阻害する可能性があることを示しているが、本質的には、交配なしで子実体を発生することができる可能性があり、このきのこのクランプ細胞形成、子実体形成、および減数分裂には二核化が必要ないことを実証した。これは、交配と核融合が子実体の発達に必須ではないことを示す最初の報告の 1 つである。

ヘテロタリズムの担子菌には 2 つの異なる交配システム、すなわち、二極性および四極性交配システムがあり、約 4 分の 3 のきのこが四極性システム、残りが二極性交配システムであると想定されている。二極性交配システムは、単一の交配型遺伝子座によって制御され、減数分裂によって生成される交配型は 2 つだけである。四極性交配システムは、通常 *A* および *B* 遺伝子座と呼ばれる連鎖しない 2 つの交配型遺伝子に基づいており、これらの交配型遺伝子は、核のペアリングとクランプ細胞形成を調節している。四極性システムでは、2 つの交配型遺伝子座がリンクしていないため、減数分裂後に半数体子孫の間で 4 つの交配型が生成される。

このきのこのいくつかの担子胞子分離株は完全な子実体と真のクランプ細胞を形成できるという以前の報告に続いて、真のクランプ細胞形成の頻度をダイカリオンと単核性株の間で比較した。モノカリオンと比較して、真のクランプ細胞はダイカリオンでより高頻度で観察された。 TUFC50005 株に由来するダイカリオン株 TUFC50005（P）及び TUFC50005-7×TUFC50005-18（F1）由来の担子胞子分離株間で交配不和合性グループを調べた。交配の和合性グループを 2 つのグループに分けることができ、*M. aitchisonii* が二極性きのこであることを示した。さらに、減数分裂後に交配型に関する組換え体が検出できなかったことから、*M. aitchisonii* は単一の交配型遺伝子座で制御されている可能性があることを示した。モノカリオンにおいて、クランプ細胞を形成することができる表現型と交配型との間に遺伝的連鎖は観察されず、モノカリオンによるクランプ細胞形成に関わる遺伝子座が交配型遺伝子座と連鎖していないことを示している。

四極性担子菌の交配型遺伝子の構造は、モデル生物である *C. cinereus* 及び *S. commune* に関する研究において、よく理解されている。ただし、二極性きのこに関する報告はほとんどない。 *M. aitchisonii* の交配型遺伝子に関する研究は、二極性きのこの交配システムの分子メカニズムの理解に役立つと考えられる。*M. aitchisonii* の交配システムについて、真のクランプ細胞形成とクランプ細胞のないモノカリオンのホメオドメイン遺伝子構造と発現レベルについて分析した。交配型特異的 HD2 プライマーは、*M. aitchisonii* の交配型を決定する分子マーカーとして使用した。 得られた PCR 産物のサイズを比較することにより、すべての株の交配型を分析した。F1 および F2 の株は、PCR 産物のサイズに応じて 2 つの不和合性グループに分けることができます。

ホメオドメインタンパク質遺伝子 2（*Mahd2*）の遺伝子構造により、 *M. aitchisonii* 、の二極性交配システムの特徴付けた。 *M. aitchisonii* 50005-18（Maspi 18）株の *Mahd2* の ORF は、1851 bp の長さで、614 アミノ酸をコードし、ＭＡＨＤ2 の推定分子量は 69.93 Kda であった。エクソンおよびイントロンの位置は、3'-ＲＡＣＥおよび 5'-RACE PCR によって増幅した PCR 産物のヌクレオチド配列から決定した。すべてのイントロンは GT で始まり、AG で終わっていた。 *Mahd2* の 3 つのイントロンは、4 つのエクソンを分断していた。 BLAST 検索プログラムにより、ＭＡＨＤ2 タンパク質が、他の担子菌の HD2 タンパク質に保存されたホメオドメインモチーフを含むことを明らかにした。 PSORT II プログラムでは、3 つの核局在化シグナル（NLS）PTKRRVP、PFPRRTR および PRRTRPG の存在を予測しました。

Maspi 18 株の *Mahd1* をコードする遺伝子は、HD2 タンパク質遺伝子の上流には存在しなかった。 HD2 タンパク質は、DNA 結合に不可欠と思われるホメオドメイン配列を含むタンパク質である。対照的に、HD1 クラスのタンパク質は正しい DNA 認識に関与している可能性がある。 HD1 タンパク質のホメオドメインの欠失または配列の変化により、*in vivo* でのクランプ細胞形成の調節におけるタンパク質機能を失うことはなかった。これは、実際に HD1 ホメオドメインがダイカリオンの形成に必須ではないことを示している。 HD2 クラスのタンパク質には、コンセンサス配列に非常によく似たモチーフがあります。したがって、ヘテロ二量体化は機能に不可

欠である可能性が高いと思われる。HD2 タンパク質は最初に HD1 タンパク質と結合しないと核に入ることができないが、HD1 タンパク質は HD2 パートナーなしで核に入ることができ、その後、DNA の共同標的部位を認識する。

59 アミノ酸の DNA 結合領域は *M. aitchisonii* においても見つかり、ヘリックスターンヘリックス（HTH）モチーフが含まれていた。この機能により、HD タンパク質は DNA 結合転写因子として認識され、特定の DNA 配列を認識してゲノム内のターゲット遺伝子にアクセスし、その発現を制御する。 *Mahd2* の転写解析により、クランプ細胞を産生できないモノカリオンよりもクランプ細胞を産生できるモノカリオンの方が *Mahd2* の発現が高いことを示した。 真のクランプを形成できるモノカリオン TUFC 50005-4 株において、*Mahd2* の最高の相対発現レベルが観察できた。これらの結果は、*A* 交配型ホメオドメインタンパク質で調節されたクランプ細胞の形成とクランプ細胞形成の頻度が、*Mahd2* 遺伝子の高発現によって促進される可能性があることを示唆している。

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List Publications

1. Riffiani, R., Wada T., Shimomura N., Yamaguchi, T., Aimi, T. 2019. Monokariotic fruiting body and clamp cell formation in *Mycoleptodonoides aitchisonii* (Bunaharitake)

   Mycoscience, 60, 151-155 https://doi.org/10.1016/j.myc.2019.01.004 .

   (The coressponding content in chapter 2)

2. Riffiani, R., Wada T., Chen, F.C., Shimomura N., Yamaguchi T. and Aimi, T.

   Mating type in *Mycoleptodonoides aitchisonii* is not genetically associated with the monokaryotic clamp cell formation phenotype

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