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SUMMARY OF DOCTORAL THESIS

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

（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）

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 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.

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 2 (*Mahd2*) has been characterized. A genomic DNA fragment of *Mahd2* in *M. aitchisonii* 50005-18 (Maspi 18) strain is 1851 bp long and encoded 614 amino acids with the predicted molecular mass of 69.93 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 HD2 gene interrupt the coding sequence, which comprised of 4 exons. The BLAST searching program identified that *Mahd2* protein contain the conserved homeodomain motifs which are similar with the HD2 motifs of other basidiomycetes, respectively. The PSORT II program predicted that there are three nuclear localization signals (NLS) PTKRRVP, PFPRRTR, and PRRTRPG.

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 can not 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. 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 *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 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.