Analysis of the Polarity of the Jelly Basidiomycete *Auricularia auricula*

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Abstract: *Auricularia auricula* (L. ex Hook) Underw is an important jelly edible phragmo-basidiomycetidae, the polarity of which has long been disputed. In this study, double fluorescent staining of nuclei and septa was utilized to observe clamp connections in order to identify the dikaryotic mycelia. Some monokaryons and dikaryons derived from mating cultures were subjected to fruiting tests. All the results indicated that *A. auricula* exhibits bipolar heterothallism. It was interesting that some monokaryons belonging to parental mating-type (H_2 or J_3) were unable to cross when mating cultures occurred in the crossing combinations. In some mating combinations, dimorphic colonies occurred when mycelia from the region of conjunction were subcultured on to a fresh agar plates. Some mycelia in the dimorphic colony were dikaryotic, and the others were monokaryotic. There were nine combinations forming dimorphic colonies among a total 45 combinations in the fourth crossing group. If combinations with a characteristic dimorphic colony were designated as incompatible, the compatible mating ratio was 37.5%, far below the 50% compatibility ratio of a bipolar basidiomycete. The reasons leading to the dispute over the polarity of *A. auricula* are discussed. Slow nuclear migration and difficulty in judging monkaryon against dikaryon could result in the conclusion that *A. auricula* exhibited tetrapolar heterothallism. The barrier between pheromone and pheromone receptor is likely to cause slow nuclear migration and incompatibility between two different mating-type monkaryons.

Key words: *Auricularia auricula*, nuclear fluorescent staining, septum fluorescent staining, polarity, dimorphic colony

1 Introduction

Both bipolar and tetrapolar mating systems are found in heterothallic basidiomycetes. The model species of tetrapolar mating systems are *Schizophllum commune, Coprinus cinereus*, and the smut fungus, *Ustilago maydis*, and their mating-type factors and genes have been systematically studied.[1-3] Mating-type factors of bipolar basidiomycete *Ustilago hordei* and *Pholiota nameko* have also been reported.[4, 5] Recently, a notable hypothesis suggesting that biopolar heterothallism might have derived from a tetrapolar system was presented based on comparisons of the genomic structure of the mating-type loci of *Cryptococcus neoformans*.[6]
The compatible ratio between mating monokaryons is the basis for determining judgment, and clamp connections are always the major marker in identifying monokaryons from dikaryons. It is often difficult to accurately recognize dikaryotic mycelia since clamp connections seldom form, and because of the false clamp connections. Nowadays, the method of distinguishing monokaryons from dikaryons is based on fluorescent staining of nuclei and this technique has been widely used in fungal studies.[7-10] Burnett[11] and Luo Xinchang[8] reported that *Auricularia auricula* was a biopolar basidiomycete, but Zhang Hong[9] reached a different conclusion. Duncan [12] reported that *A. polytrica* had a tetrapolar mating system.
The fluorescent dye, Calcofluor white M2R, is extremely useful for staining fungal cell walls and septa. [13] When this dye was applied in plant pathogenic fungi, the number of nuclei within a single mycelial cell could be observed distinctly.[14]
A. auricula is a typical edible jelly fungus belonging to the Phragmobasidiomycetes and has been cultivated in China for over one thousand years. The fruit body of *A. auricula* has a delicious taste, is highly nutritious, and

has important medical functions.[15, 16]

2 Materials and Methods

2.1 Strains and reagents

Strains He-1 and Ju-1 of *A. auricula* are cultivated strains and were supplied by the Experimental Spawn Center of Huazhong Agricultural University, Wuhan. Strains H_2 and J_3 are monokaryons originating from protoplast reproduction of strains He-1 and Ju-1 respectively, and strain H_2J_3 is a dikaryon arising from the crossing of strains H_2 and J_3. Fresh fruit bodies of H_2J_3 were collected to obtain F1 generation monokaryotic isolates. All isolates were confirmed as monokaryons by fluorescence microscopy.
Fluorescent dyes Hoechst33258, DAPI(4,6-diamidino-2-phenylindole) and Calcofluor white M2R were obtained from the Sigma Chemical Company. Stock solutions of the three dyes were prepared before utilization. Sodium hydrogen phosphate-citric acid buffer (pH 7.2), phosphoric acid buffer (pH 6.8, 0.1M) and ddH_2O were respectively used to dilute stock solutions to different concentrations.[7-10]

2.2 Isolation and determination of monokaryons

Basidiospores were taken fresh fruit bodies of cultivated H_2J_3. The number of nuclei per cell, and the presence of clamp connections in monosporic isolates, was determined by fluorescent staining of nuclei and septa.

2.3 Double fluorescence staining

Different dye concentrations were added to the surface of glass coverslips on which fungal mycelia had grown from the culture medium. Calcofluor white M2R[13] was used to stain septa, and Hoechst33258 and DAPI for staining nuclei. The various buffer types, buffer concentrations, staining times and mycelia ages were optimized for the fluorescent staining.

2.4 Determination and examination of the polarity

Sixty F_1 monospore cultures, designated strains No.1 to No.60, were divided into six groups, and mating was carried out by pairing 10 × 10. Mycelia from the conjunctional regions between the two monokaryons were inoculated on to another culture dish. Two sterilized cover glasses were inserted into the medium about 1.5cm away from the inoculum, and the plates were incubated at 25°C until the fungal hyphae had grown on to the coverslip. The coverslips were then stained using two different fluorescent dyes and observed for clamp connection formation. According to results above, the compatibility of two monokaryons could be identified.
A pair of compatible monokaryons drawn from each group were mated with the two parental monokaryons, H_2 and J_3, in order to determinate the polarity of the F_1 generation monokaryons. All the test monokaryons were then divided into two opposite mating stocks (H_2 and J_3). Eight monokaryons were selected at random from each mating stock and mated with each other to determine if the above polarity test was correct. Furthermore, twenty mating monokaryons and ten dikaryons derived from monokaryon crossing were cultivated

3 Results

3.1 Double fluorescent staining conditions and dikaryon identification

Mycelial cell walls and septa projected blue fluorescence when Calcofluor white M2R was used at 5μg/ml.

Nuclei stained by Hoechst33258 at 20μg/ml also projected blue fluorescence, the intensity of which increased with increasing concentrations up to 50μg/ml. Light blue fluorescence ejected by nuclei could not be observed when DAPI were utilized at three test concentrations.
The optimal double staining protocol was as follows: (1) stain with Hoechst33258 at 50μg/ml for 4 min, then wash with distilled water; (2) stain with Calcofluor white M2R at 2.5μg/ml for 1 min, then wash with distilled water. Not only septa but also the number of nuclei per cell could be clearly observed in mycelia stained using the above protocol (Figure 1). Clamp connections could also be seen distinctly after dikaryotic mycelia were stained using the same protocol (Figure 2) although the frequency of clamp connections was low.

Figure1. Double fluorescence staining for septa and nuclei of monokaryons

Figure 2. Double fluorescence staining for septa and nuclei of dikaryons

3.2 Polarity and mating-type identification

The numbers of monokaryotic and dikaryotic mycelia were calculated and the ratio of monokaryon to dikaryon found to be 131:139 in all the strains of six groups tested (Table 1). The modified χ_a^2 coefficient of total number was 0.18; however, $P_{(1,0.05)}$ was 4.84. The χ_a^2 is less than $P_{(1,0.05)}$ so that the hypothesis of ratio 1:1 is reasonable, and *A. auricula* belongs to bipolar heterothallism.

Table 1. The ratios of monokaryotic phase and dikaryotic phases in crossed mycelia of all the strains tested

Group	I	II	III	IV	V	VI	Total number
Ratio monokaryon: dikaryon	21:24	21:24	22:23	21:24	24:21	22:23	131:139
χ_a^2	0.09	0.09	0	0.09	0.09	0	0.18

Six pairs of compatible monokaryons from six separate group (5 x 8; 12 x 13; 25 x 27; 36 x 40, 52 x 54; 61 x 62) were combined with parental monokaryons into a test group for determining the mating-type of sixty monokaryons. Results showed that the ratio of the two opposite mating-type was 32:28 among the sixty monokaryons. After cultivation for 122 days, only four of twenty monokaryons, could form clustered jelly primordium the surface of which could not open, and were chrysanthemum-shape and light coloured. Others could not form primordia. Nevertheless, all the tested dikaryons could form black or yellow brown primordia and developed into normal jelly fruit bodies after growth for 72 days.

3.3 Appearance of dimorphic colonies in the mating type test

In the test above, two exceptional mating combinations (35 x 38 and 54 x 60)were found. However, monospore isolates belonging to the different mating-type (35,54 to H_2; 38,60 to J_3) could not mate and form dikaryotic mycelia. The same cases occurred when eight monokaryons from the H_2 mating-type stock were mated with another eight the from J_3 mating-type stock. Dikaryotic mycelia could not be observed in the combinations 25 x 52, 54 x 43 and 55 x 46. When mycelia from the conjunctional region between two monokaryons were transferred to another plate, mycelia of some combinations were identified as monokaryotic if cultured for 10 days, but as dikaryotic if grown for 20 days. Sometimes, different mycelial modality appeared in the same colony: some parts of the mycelium were monokaryotic, whereas other parts were dikaryotic (Figure 3). Nine abnormal domorphic colonies were observed among the 45 combinations in the fourth test group. If these combinations were designated as incompatible, the ratio of monokaryon to dikaryon would be 30:15 and the total compatible ratio only 37.35%.

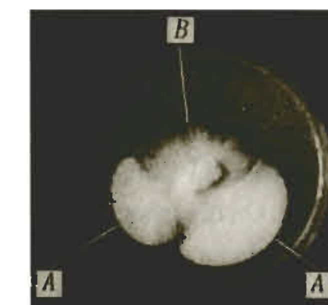

Figure 3. Dimorphic colonies on media following the crossing of mycelia from pairing cultures of monokaryons
A: dikaryotic mycelia; B: monokaryotic mycelia.

4 Discussion

4.1 Methods for identifying polarity

In previous studies of *A. auricula*, observing the presence of clamp connections and fluorescent staining of nuclei were employed to identify the mating response. Since clamp connections were difficult to observe because of the thin mycelia, and fluorescent staining of nuclei was unable to determine the number of nuclei per cell, the results of studies aimed at determining polarity often deviated. Zhang Hong[9] speculated that the reason for this deviation existed because some basidiospores with different mating-types collected from dry fruit bodies had difficulty geminating. In this study, basidiospores used to the mating tests were collected from fresh fruit bodies. All the results from double fluorescent staining, clamp connection determinations and fruiting tests showed that *A. auricula* was a bipolar heterothallic basidiomycete.
It was found that some mycelia were identified as monokaryotic after 10 days culture, whereas these same mycelia were identified as dikaryotic after growth for more than twenty days. Differences in the regions of nuclear migration, and exchange between compatible monokaryotic mycelia when these came into contact, could be expected.[11] It was unusual that some mycelia from conjunctional regions between two monokaryons could develop abnormal dimorphic colonies in which some mycelia were monokaryotic but others were dikaryotic. The conclusion drawn from this phenomenon was that the period of nuclei migration and exchange was probably longer in some combinations. For a long time, rapid bi-directional nuclei migration was popularly accepted. So, diversity in both velocity and region of nuclei exchange was likely to result in deviations during mating

tests, further influencing attempts to determine the polarity of *A. auricula*.

4.2 Structure of the bipolar or tetrapolar mating-type factor

The mating-type loci in the pathogen *U. hordei* contain two distinct gene-complexes, a and b . Since these two gene-complexes are not only genetically linked but also physically linked, recombination is suppressed within the mating-type locus.[4, 17]

Supatra et al.[5] reported that only one monospore was compatible with both parental strains, while 99 monospores were incompatible with both parents in 300 F_1 monospore isolates of the bipolar mushroom, *Pholiota nameko*. A hypothesis was provided suggesting that the mating-type factor of *P. nameko* included two subunits, A_α and A_β, and the genetic distance of those two subunits was about 0.3 centimorgan. There are three possible cases of recombination on a single mating-type factor during meiosis in *P. nameko*. If recombination occurs outside of the mating-type factor on the chromosome, production of two types of monospore isolates carrying either one of the parental mating-type factors would be expected. However, if the combination occurs between two subunits, recombinant isolates may develop a new mating-type factor that is compatible with both parental mating-type factors. On the other hand, if the combination occurs in the intra- A_α or intra- A_β subunit of the mating-type factor, recombinant isolates are unable to mate with the two parental strains.

According to popular opinion, the bipolar mating-type factor consists of two distinct subunits, A_α and A_β. If the genetic distance between the two subunits is sufficiently large enough to cause the recombination ratio to rise towards 50%, the mating phenotype is tetrapolar. On the other hand, if the two subunits are tightly linked so that crosses are impossible, the mating phenotype is bipolar. However, if the recombination ratio is in the middle of 0 and 50%, this could be called false tetrapolarity. This phenomenon might popularly exist among basidiomycetes.

4.3 Polarity of *A. auricula*

Based on the abnormal mating of some monkaryons and the fan-shape colonies observed in our study, there may be some repression/depression factors encoded by genes within the mating-type factor, which may control the pheromone/pheromone receptor response pathway. The repression factors are produced by mycelia under normal growth. If depression factors act slowly, dikaryons can be observed after long-term culture of mated mycelia. However, if the depression factors are ineffective, abnormal phenomenon whereby compatible monokaryons do not mate with each other may occur. At present, we cannot be certain that there are subunits similar to *P. nameko* lying at the mating-type locus of *A. auricula*. Structural differences in the mating-type factor of different strains can obviously lead to conflicting conclusions made during previous studies on the polarity of *A. auricula*.

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