Nuclear Behavior and Mating System Analysis in the Bipolar Mushroom, *Pholiota nameko*

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Abstract: *Pholiota nameko* is a wood-rotting edible mushroom that carries a bipolar *A* incompatibility factor gene. Linkage analysis of the multiple allelomorphic *A* factor gene revealed that this bipolar *A* incompatibility factor gene consists of two functional subunits, *Aα* and *Aβ*, which appear to be located on the same chromosome within 0.3 centi-Morgan. The genes for the homeodomain protein (*hox1*) and the pheromone receptor (*rcb1*) in *P. nameko* were characterized. In order to reveal the structure of A compatibility factor gene, linkage analysis mapped *hox1* to the *A* mating type locus, but *rcb1* was mapped to the other linkage group and was not linked to the *A* mating type locus. Therefore, these results strongly suggest that mating incompatibility in the bipolar mushroom may be regulated by homeodomain protein genes and may not be affected by pheromone receptor genes.

Key words: Mating system analysis, *Pholiota nameko*, bipolar A incompatibility factor gene, homeodomain protein gene (*hox1*), pheromone receptor gene (*rcb1*)

1 Introduction

The mating system of basidiomycetes, such as basidiomycetous yeasts and mushrooms, is controlled by incompatibility factor genes. These regulate the formation of the dikaryon between complementary pairs of monokaryons carrying different incompatibility factors. The incompatibility factors of basidiomycetous yeasts function as allelomorphic genes in their sexual reproduction.[1, 2] In contrast, the incompatibility factors of basidiomycetous mushrooms consist of one or two sets of multiple allelomorphic genes called bipolar and tetrapolar incompatibility factors, respectively.[3] Mushrooms such as *Coprinus cinereus*,[4] *Flammulina velutipes*,[5] *Lentinula edodes*[6] and *Schizophyllum commune*[7] have been shown to carry tetrapolar incompatibility factors. In the tetrapolar mushrooms, both *A* and B factors are constructed of two subunits (*α* and *β*) that are located on different chromosomes.[7- 10]

In tetrapolar basidiomycetes such as *Coprinus cinereus*, two compatibility factors, usually designated as factors *A* and *B*, determine compatibility.[11] The *A* locus consists of homeodomein protein genes, which regulate the pairing of nuclei within the dikaryotic tip cell and coordinate nuclear division and clamp cell formation. The *B* locus consists of pheromone and pheromone receptor genes that coordinates clamp cell fusion. Moreover, both *A* and *B* factors control the maintenance of the dikaryon.[12, 13]

Pholiota nameko is a wood-rotting basidiomycete that produces an economically important edible mushroom called "Nameko".[14] Due to recent advances in bottle cultivation technology in Japan, this mushroom is now available in domestic markets throughout the year. However, the commercial spawns of this mushroom may have unstable genetic characteristics that lead to formation of monokaryotic hyphal cells from dikaryotic mycelia. This phenomenon can often result in very poor production or a near total lack of fruiting when using inferior strains.[15]

It is well known that *P. nameko* carries a bipolar *A* incompatibility factor gene.[16] However, little is known about the makeup and function of incompatibility factors in bipolar basidiomycetous mushrooms. Moreover, the genomic organization of the *A* incompatibility factor gene in *P. nameko* has never been reported.

Therefore, in this report, we describe the genetic makeup of the bipolar *A* incompatibility factor gene. We also describe the cloning and genetic mapping of the homologue genes in *P. nameko* by linkage analysis and show that they are homologues of *A* and *B* factors of *C. cinereus*. The present study is part of our attempt to understand the structure and function of incompatibility factors in the bipolar mushroom, *P. nameko*.

2 Materials and Methods

2.1 Test organisms

Monokaryotic lines of *Pholiota nameko* were obtained by monospore isolation from the fruit bodies of various wild strains as detailed in our previous report.[15, 17] The incompatibility factors of these monokaryotic stocks were determined by crossing them with tester stocks. Thirteen monokaryons with were assigned as *A1* through *A6* according to their type of bipolar incompatibility factor (Table 1). Monokaryotic and auxotrophic mutants of *P. nameko*, such as NA4-580 (*A1, pab1*) and NGW16-9 (*A4, pdx1*), were also used in this study.

Table 1. Monokaryotic stocks of *P. nameko*

Stock No.	Incompatibility factor
NX-1	*A1*
NX-15	*A2*
NX-20	*A2*
NX-2	*A2*
NGW-12	*A3*
NX-3	*A3*
NGW-19	*A4*
NGW-20	*A4*
NX-4	*A4*
NF-7	*A5*
NF-8	*A5*
NX-5	*A5*
NX-6	*A6*

2.2 Method for producing dikaryotic hybrid stocks

The dikaryotic stocks used for linkage analysis of the incompatibility factor of this mushroom were prepared by reciprocal crossing of two compatible monokaryotic stocks. The inoculation for crossing was carried out by planting two different monokaryotic stocks 4 mm apart in the center of a potato dextrose agar (PDA) plate (Nissui Seiyaku, Tokyo, Japan). After incubation for 7 to 10 days at 25°C, the colony on the PDA plate at the contact zone between two parental monokaryons was inspected under a microscope for the formation of clamp connections as evidence for dikaryotization. Positively identified hybrid dikaryotic mycelia were transplanted onto PDA slants for the preparation of hybrid stocks.

2.3 Cultivation of fruit bodies of dikaryotic hybrid stocks

Cultivation of fruit bodies in the tester mushrooms was carried out on a sawdust substrate. The substrate was prepared by mixing beech (*Fagus sieboldi*) sawdust and rice bran at a volumetric ratio of 5:1 and adjusting the moisture content to approximately 65%. Approximately 40 ml of the substrate was placed into a 100-ml Erlenmeyer flask, plugged with silicon-polarized rubber (Shinétsu Chemical, Tokyo, Japan), and autoclaved at 121

to 123°C for 15 min. After cooling the flask to room temperature, the mycelium fragment from the stock culture was inoculated on the surface of the sawdust substrate and then incubated for approximately 30 days at 25°C in the dark. After completing spawn running, the culture was then incubated at 10°C under continuous fluorescent light (200 lux) for the formation of fruit bodies. The mature pilei of the fruit bodies were cut off with a knife and placed in a Petri dish to obtain spore prints.

2.4 Isolation of basidiospore-derived monokaryotic stocks

Five ml of sterilized water was pipetted onto the spore prints in the Petri dishes and then vigorously shaken to prepare the spore suspension. The spore density in the suspensions was determined by counting with a hemocytometer under a microscope. The suspension was then diluted to approximately 1×10^4 to 1×10^6 cells/ml. Next, 0.1 ml of the suspension was mixed in a test tube with 2 ml of melted PDA soft agar (0.7%) medium at 50°C and then poured onto a PDA plate to prepare a double layer agar culture. After incubating the culture at 25°C for one week, the colonies that appeared were isolated and transferred onto PDA slants. These slants were incubated for approximately 7 days at 25°C before using in the crossing experiments.

2.5 Determination of the incompatibility factor of monospore isolates

The mating type of mycelium of the monospore isolate was determined by crossing them with the tester monokaryotic stocks of known mating types. The isolates were crossed with tester strains that had the same incompatibility factor composition as each of the two parental monokaryotic stocks. Isolates showing incompatibility with a parental monokaryon tester but compatible with either of the tester monokaryons were considered to have the same nuclear type as the tester. If the tester monokaryotic isolates could not produce clamp connections with parental monokaryons, the isolates were further examined in crossing tests by using different monokaryotic tester stocks carrying the same incompatibility factors (see Table 1). If the tester monokaryons showed incompatibility characteristics different from those of the parental monokaryons, it was identiffied as a recombinant carrying the new *A* incompatibility factor. The new incompatibility factor produced by recombination was designated *Ar* with a numerical suffix.

2.6 DNA preparation

Potato dextrose broth (potato extract containing 2% sucrose) was used in fungal cultures for the preparation of DNA. Genomic DNA from lyophilized the mycelium of the *P. nameko* parent strain, and monospore isolates were prepared with a Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Tokyo, Japan) according to the manufacturer's instructions.

2.7 Polymerase chain reaction (PCR)

Specific oligonucleotide primer pairs were designed to amplify the whole genomic clones of *hox1* (Hox1F1, 5'-TGACTCTGCAGATGGAAGCTGATG-3'; and Hox1R1, 5'-AGCAGAGCTAGCCAAATTACACGA-3') and *rcb1* (Rcb1F1, 5'-AGATGGCAGCGCGCACCAGACA-3'; and Rcb1R1, 5'-CATCGGCTGCTACCCGGTAGTC-3') based on the nucleotide sequence of DNA fragments obtained by several PCR methods (data not shown).PCR was carried out in a 100 µl volume reaction mixture containing 1 x Ex Taq buffer, 100 ng of extracted genomic DNA, 100 pmol of each primers, each dNTP at a concentration of 0.2 mM, and 2.5 U of Ex Taq polymerase. The reaction was performed for 30 cycles, and the following cycling profile was used. The first denaturing step was at 94°C for 1 min, and then the PCR cycles were 30 s of denaturation at 94°C, 5 min of annealing and extension at 68°C. The amplified DNA fragments for *hox1* and

*rcb*1 were purified with a Microcon-100® filter (Millipore, Bedford, MA, USA) and used as templates for direct sequencing.

2.8 Southern hybridization and restriction fragment length polymorphism (RFLP) analysis

Genomic DNAs were isolated from *P. nameko* parent strains and monospore isolates from the fruit body of the NA4-580×NGW16-9 strain. These were digested with *EcoRI*, after which the fragments were separated by agarose gel electrophoresis and blotted onto a nylon membrane (Hybond-N+, Amersham Biosciences). The blot was probed with DNA fragments that had been labeled with a DIG-High Prime DNA Labeling and Detection Kit (Roche Diagnostics, Tokyo, Japan). Probe labeling, hybridization, and detection were carried out according to the manufacturers' recommendations.

3 Results and Discussion

3.1 Linkage analysis of the A incompatibility factor gene composition

To determine whether the bipolar *A* incompatibility factor gene of *P. nameko* is composed of subunits similar to those of the tetrapolar mushrooms, we conducted linkage analysis of monospore isolates from three different hybrid dikaryotic stocks. Two monokaryons carrying different mating factors were crossed. The resulting basidiospore-derived monokaryotic isolates were backcrossed with both the parental monokaryons to determine the genotypes of their incompatibility factors.

The results of the linkage analysis of the incompatibility factor in a hybrid stock, NX-2 (*A2*) ×NGW-20 (*A4*), are shown in Table 2a. Of 300 monospore isolates, one carried *Ar1* in addition to two parental mating types, 121 were *A2*, and 152 were *A4*. However, 26 modified isolates were unable to produce a dikaryon against both parental monokaryons. We assume that these modified isolates were due to unequal crossing-over during the process of sexual reproduction. Further, an isolate carrying the new *A* incompatibility factor (I-8, *Ar1*) was the product of successful meiotic recombination in sexual reproduction. In the case of the NGW-12 (*A3*) ×NGW-19 (*A4*) hybrid, 223 of 300 isolates produced dikaryons against either one of the parental monokaryons, and one isolate (II-127, *Ar2*) produced dikaryons against both parental monokaryons.

However, 76 isolates did not produce dikaryons against the two parental monokaryons. This result contrasts with the apparently small number of isolates carrying the *A3* factor as compared with the expected segregation ratio (1:1) between the two types of parental mating factors, *A3* and *A4* (Table 2b). Similarly, we found an unusually small number of A2-factored monospore isolates among the 300 monospore isolates from the NA-15 (A2) × NGW-19 (A4) hybrid, and we also detected an isolate (III-17) carrying *Ar3* (Table 2c). The formation of *Ar* monokaryons by sexual reproduction suggests that the bipolar A incompatibility factor gene of P. nameko is made up of two functional subunits (e.g., *Aα2* and *Aß2*) in the parental monokaryon NX-2 (*A2*), which may be adjacent to each other on the same chromosome. The average apparent genetic distance between the two subunits of the *A* incompatibility factor on the first chromosome was 0.3 centi-Morgan (cM) (Fig. 1). Giasson et al. [18] have estimated that 0.6 cM corresponds to approximately 100 kbp of DNA in *S. commune,* suggesting that the two subunits are within 50 kbp of each other.

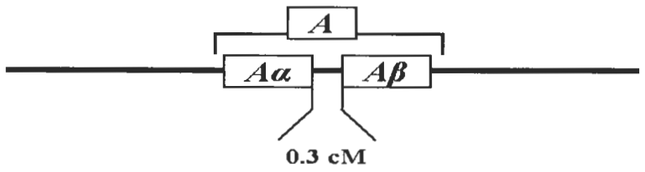


Fig 1. Genetic distance between *Aα* and *Aß* in *P. nameko*

Table 2. The results of a backcrossing test of monospore isolates from three different hybrids against the corresponding parental monokaryons

a. NX-2 (*A2*) × NGW-20 (*A4*)

Tester monokaryon		Number of spore isolates
NX-2 (*A2*)	NGW-20 (*A4*)	
+	+	1
+	-	152
-	+	121
-	-	26

b. NGW-12 (*A3*) × NGW-19 (*A4*)

Tester monokaryon		Number of spore isolates
NGW-12 (*A3*)	NGW-19 (*A4*)	
+	+	1
+	-	142
-	+	81
-	-	76

c. NA-15 (*A2*) × NGW-19 (*A4*)

Tester monokaryon		Number of spore isolates
NA-15 (*A2*)	NGW-19 (*A4*)	
+	+	1
+	-	154
-	+	46
-	-	99

3.2 Crossing characteristics of three monospore isolates carrying different Ar incompatibility factors

We next examined the crossing characteristics between each of three *Ar* isolates and several different monokaryotic tester stocks carrying *A1* through *A6*. As shown in Table 3, two recombinant isolates, I-8 and II-127, produced dikaryons against all tester stocks. The isolate III-17 was able to mate against all of the testers except for NX2 and NA-20, both of which had the *A2* factor. However, this isolate could produce a dikaryon against NA-15, which contained the *A2* factor. This suggested that the isolate had an essentially new type of mating characteristic, although its incompatibility factor gene was partially defective in the process of recombination. Consequently, these three isolates were the products of a recombination between the two subunits of the *A* incompatibility factor gene in this bipolar mushroom.

Table 4 shows the reciprocal crossing among the three *Ar* isolates. These isolates all produced dikaryons with the other isolates. Therefore, I-8 may have a subunit composition in combination with *A2* and *A4* factors, specifically, either *Aα2Aß4* or *Aα4Aß2*. Similarly, II-127 and III-17 might have A incompatibility factor compositions of *Aα3Aß4* or *Aα4Aß3* and *Aα4Aß2* or *Aα2Aß4*, respectively.

3.3 Structure of the homeodomain protein gene (hox1) in *P. nameko*

A schematic diagram of the genes encoding the homeodomain protein of *P. nameko* is shown in Fig. 2. The length of coding regions (from ATG to stop codon) is 2105 bp in *hox1*. This gene encodes a protein of 599 amino acids and that Hox1 contains a 31-amino acid homeodomain. The deduced protein sequence of Hox1 has

28% amino acid identity and 43% similarity with the Aß1 (B52) protein of *C. cinereus* (accession number AF126790 in DDBJ/EMBL/GenBank database) and 26% identity and 41% similarity with the HD1 protein of *Pleurotus diamor* (accession number AY462112). The PSORTII program[19] predicted that Hox1 contains two nuclear localization signals (KRRR and KRKR). Therefore, this protein may bind to DNA and regulate the expression of the genes related to incompatibility

Table 3. Crossing of the incompatibility factor gene recombinant isolates from three different hybrids against the tester monokaryons with different incompatibility factors

Tester stock	Recombinant stock		
	I-8 (NX-2 × NGW-20)	II-127 (NGW-12 × NGW-19)	III-17 (NA-15 × NGW-19)
NX-1 (*A1*)	+	+	+
NX-2 (*A2*)	+	+	-
NA-20 (*A2*)	+	+	-
NA-15 (*A2*)	+	+	+
NX-3 (*A3*)	+	+	+
NX-4 (*A4*)	+	+	+
NX-5 (*A5*)	+	+	+
NF-7 (*A5*)	+	+	+
NF-8 (*A5*)	+	+	+
NX-6 (*A6*)	+	+	+

3.4 Structure of the pheromone receptor gene (*rcb1*) in *P. nameko*

A schematic diagram of the genes encoding the pheromone receptor protein of *P. nameko* is shown in Fig. 3. The length of the coding regions (from ATG to the stop codon) is 1455 bp in *rcb1*. This gene encodes a protein of 389 amino acids. The deduced protein sequence of Rcb1 has 61% amino acid identity and 74% similarity with the Rcb3 (B45) protein of *C. cinereus* (accession number AY393908) and 51% identity and 71% similarity with the Bar3 protein of *Schizophyllum commune* (accession number U76688). The SOSUI program (http://sosui.proteome.bio. tuat.ac.jp/sosuimenu0.html) predicted that Rcb1 is a membrane protein containing seven transmembrane helices. Therefore, based on these results, we suppose the Rcb1 protein appears to be a pheromone receptor protein belonging to the seven transmembrane G-protein-coupled receptor family reported by Gola and Kothe.[20]

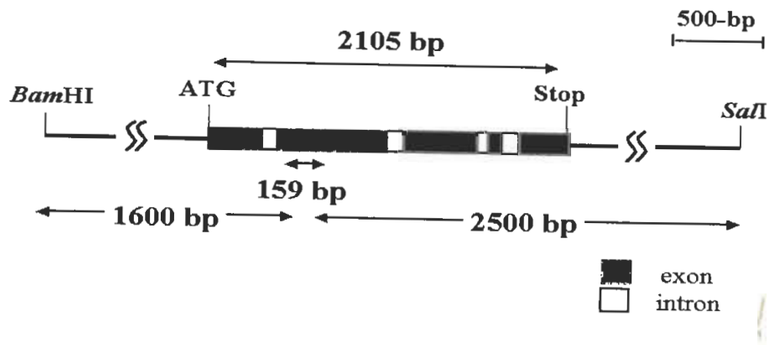


Fig 2. Schematic diagram of the *hox1* gene Solid boxes indicate positions of exons

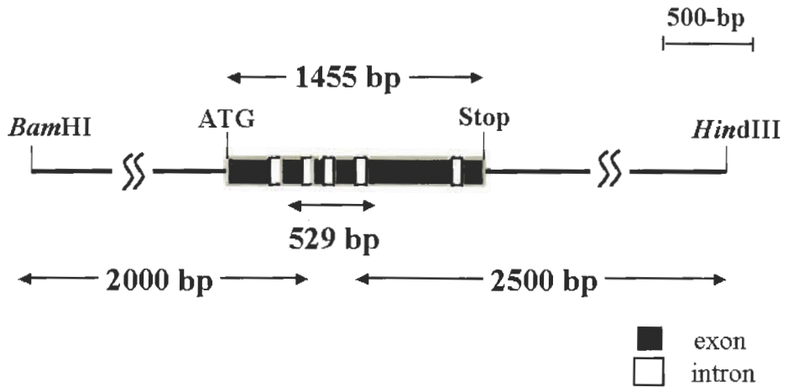


Fig 3. Schematic diagram of the *rcb1* gene
Solid boxes indicate positions of exons.

3.5 Linkage mapping

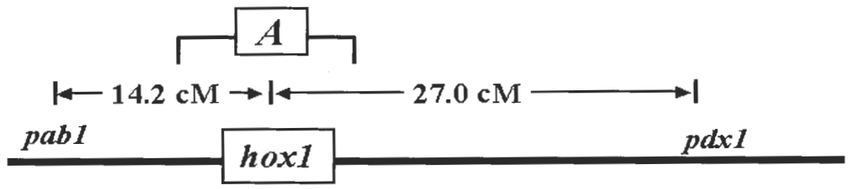
To carry out the linkage analysis, the genetic distances between two RFLP markers (*hox1* and *rcb1*), two auxotrophic markers (*pub1* [p-aminobenzoic acid requirement] and *pdx1* [pyridoxine requirement]), and an incompatibility factor (*A*-factor) were analyzed using more than 100 F1 progeny derived from the fruit body of dikaryotic stock (NA4-580 ×NGW16-9). The example of the RFLPs with *hox1* and *rcb1* as probe are shown in Fig. 3. The hybridization patterns of *hox1* in basidiospore-derived *A1* and *A4* strains were completely corresponded with that of *hox1* in NA4-580 (*A1*) and NGW16-9 (*A4*), respectively. However, The inheritance of *rcb1* did not correspond with that of mating type. The recombination ratio between *rcb1* and mating type (*A*-factor), *pub1, pdx1* and *hox1* were 45.9%, 55.6% 47.6% and 54.0%, respectively. Therefore, it is determined that the *rcb1* was not linked to linkage group I including mating type locus (*A*-factor).

The linkage map is shown in Fig. 4. The genetic distances between *hox1* and *pub1* or *pdx1* were 14.2 and 27.0 cM, respectively. The genetic distance between *hox1* and *A*-factor was not determined. Therefore, *hox1, pub1, pdx1*, and *A*-factor were mapped to linkage group I. Moreover, the map position of *hox1* genetically corresponded with the map position of the *A*-factor. Thus, *hox1* appears to be a component of the *A*-factor, which consists of homeodomain gene complexes in this bipolar mushroom.

rcb1 was not mapped to linkage group I and was not linked to the *A*-locus. This indicated that *rcb1*, which is a pheromone receptor gene, is not related to incompatibility. Therefore, in the bipolar mushroom *P. nameko,* incompatibility may be regulated by only homeodomain protein gene complexes. This phenomenon suggests that the mating system in bipolar and tetrapolar mushrooms is different. In contrast to *Ustilago hordei* that is a bipolar basidiomycetous smut fungus, its mating system is controlled by one mating type locus (*MAT*). However, the homeodomain protein, pheromone and pheromone receptor, which are controlled mating type are encoded within the *MAT* locus on the same chromosome in a bipolar basidiomycete *U. hordei*.[21, 22] Therefore, it may be proposed that the bipolar system in *P. nameko* is genuineness bipolar system, and that in *U. hordei* is seemed to be a bipolar system but essentially same as tetrapolar system.

In this study, *A*-factor in *P. nameko* is bipolar *A* incompatibility factor gene consists of two functional subunits, *A-alpha* and *A-beta*, which appear to be located on the same chromosome within 0.3 centi-Morgan.[17] However, in this study, it is not determined that relationship between *hox1* and the two functional subunits, *A-alpha* and *A-beta.* Moreover, if one of the two functional subunits is consistent with homeodomain protein gene complexes, what kind of gene or genes are components of the two subunits? Therefore, in further studies, we will determine the genomic DNA sequence around *hox1* in *P. nameko* to investigate the complete genomic structure of the bipolar *A*-locus.

◆ **Linkage group I**



◆ **Linkage group II**

Fig 4. Linkage map of *hox1* and *rcb1* in *P. nameko*

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