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The genetic structure and diversity of the *A* and *B* mating-type genes from the tropical oyster mushroom, *Pleurotus djamor*

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Abstract

In most heterothallic mushroom species, inbreeding is avoided by an incompatibility system determined by two loci each with multiple alleles (the *A* and *B* mating-type loci). In this study we investigated the genetic structure of the mating-type loci in the tropical oyster mushroom *Pleurotus djamor* using both positional cloning and degenerate PCR methods. DNA sequences from genomic regions cosegregating with the mating-type loci of *P. djamor* revealed homeodomain transcription factors (*A*) and pheromone receptors (*B*), suggesting the genetic basis for mating-type determination in *P. djamor* is the same as in the model mushroom species. Three pheromone receptors were detected in a single homokaryotic isolate of *P. djamor*. Only one pair of homeodomain genes was detected in the *A* mating-type region. It is hypothesized that the *A* mating-type locus of *P. djamor* is comprised of only one homeodomain pair, which may explain the lower number of *A* mating-type alleles relative to other mushroom species.
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Index Descriptors: Basidiomycota; Conserved gene order; Homeodomain; Mitochondrial intermediate peptidase; Pheromone receptor; Trans-specific

1. Introduction

The homobasidiomycetes, or mushroom fungi, have arguably the most complex mating system of all known organisms. Many species possess a mating system known as bifactorial (or tetrapolar) incompatibility, where two unlinked loci control the mating-type of an individual. Compatibility between mates occurs only when they differ in alleles at both of the incompatibility loci (the *A* and *B* mating-type loci). Mushroom incompatibility loci are also distinctive in that many species have over 100 mating-type alleles per incompatibility locus (Raper, 1966). Although, an estimated ~55–65% of mushroom species display the bifactorial mating system (Raper and Flexer, 1971; Whitehouse, 1949), our understanding of the genetic basis of mating-type control relies almost exclusively on knowledge from two model species, *Schizophyllum commune* and *Coprinopsis cinerea* (= *Coprinus cinereus*). In the present study, we use DNA sequence and genomic information from these model organisms as a roadmap for isolating and describing the mating-type gene organization in a non-model system, the tropical oyster mushroom *Pleurotus djamor*. Since the edible oyster mushrooms comprise ~25% of the world's production of cultivated fungi, knowledge about their mating-type loci could aid strain improvement through breeding (Kothe, 2001).

The mating-type loci of most bifactorial homobasidiomycetes have been shown to be composed of tightly linked subloci (Koltin, 1978; Raper, 1966). The subloci are considered functionally equivalent because compatibility at a given mating-type locus requires heterozygosity of only one of the two subloci. Much of the diversity in numbers of mushroom mating-type alleles is attributed to the permutation of all possible allelic combinations of the subloci through recombination. In laboratory crosses of *S. commune*, recombination

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between the two subloci of the *A* mating-type occurred at frequencies between 0.9 and 22.6%, depending on the mating-type alleles involved and the temperature at which the progeny were collected (Raper, 1966; Raper et al., 1960). In *C. cinerea*, recombination between *A* mating-type subloci is much lower (0.07%; Day, 1960). Each *A* mating-type sublocus encodes a pair of divergently transcribed homeodomain transcription factors (Kües et al., 1994a; May et al., 1991; Stankis et al., 1992). In *C. cinerea*, the *A* mating-type locus is composed of three subloci in an ~25 kbp cluster (May et al., 1991; Pardo et al., 1996). Likewise, the *C. cinerea* the *B* mating-type locus is also composed of three very tightly linked subloci that span ~20 kbp (Halsall et al., 2000). The genes responsible for *B* mating-type activity encode lipopeptide pheromones and G-protein-coupled pheromone receptors (O'Shea et al., 1998; Wendland et al., 1995).

It has been suggested from the limited survey information of mating-type alleles in various species that there are typically more *A* mating-type alleles than *B* mating-type alleles. For example, the number of estimated *A* alleles in *S. commune* is 288 versus the 81 estimated *B* alleles (Raper et al., 1958; Stamberg and Koltin, 1973), and the number of *A* alleles in *C. cinerea* is estimated at 164 versus 79 *B* alleles (Day, 1963). Mushrooms in the genus *Pleurotus* appear to deviate slightly from this pattern. Mating-type surveys of the two species *P. populinus* and *P. pulmonarius* (first reported as *P. ostreatus* and *P. sapidus*, respectively [see Anderson et al., 1973; Vilgalys et al., 1993]), revealed consistently more *B* alleles than *A* alleles (126 *A* and 354 *B* alleles estimated for *P. populinus* and 30 *A* and 90 *B* alleles estimated for *P. pulmonarius* [Anderson et al., 1991]). An additional unique property of the *Pleurotus A* mating-type is that with only a single exception, there has been no demonstration of intra-*A* mating-type recombination in *P. spondoleucus*, *P. ostreatus*, and *P. djamor* (Larraya et al., 1999; Liou, 2000; Takemaru, 1961). In the exceptional study of Eugenio and Anderson (1968), the recombination between *A* subloci was 0.5% and between *B* subloci was 4.5% among 643 progeny of *P. pulmonarius*. The *A* mating-type locus of *Pleurotus* has been associated with other interesting phenomena. Segregation bias favoring the inheritance of one *A* mating-type allele and a few linked AFLP markers was observed in *P. djamor* (Liou, 2000), and a correlation between the *A* mating-type locus and homokaryotic growth rate was observed in *P. ostreatus* (Larraya et al., 2001).

Hyperdiversity of DNA sequences at the mating-type loci (Badrane and May, 1999; Stankis et al., 1992) has made the cloning of mating-type genes from novel mushroom species difficult, as many methods of isolating genes rely on sequence similarity (e.g., PCR and DNA–DNA hybridization). To circumvent the problem of extreme diversity of mating-type gene sequences, we recently proposed a positional cloning approach that takes advantage of the conserved gene order surrounding the mating-type loci (James et al., 2004; Kües et al., 2001). By targeting closely linked genes that are more slowly diversifying and thus more easily isolated, regions of the genome proximate to mating-type genes can be isolated from genomic libraries with large average insert size. Our analyses of the gene encoding a mitochondrial intermediate peptidase (*MIP*) suggest that this gene has maintained a conserved gene order with the *A* mating-type locus in the majority of mushroom species (James et al., 2004). Similarly, the physical association of the *B* mating-type locus with the p21-activated kinase *CLA4* has been demonstrated in fungi as diverse as *Pneumocystis carini* (Smulian et al., 2001), *Cryptococcus neoformans* (Lengeler et al., 2002), and *Phanerochaete chrysosporium* (James, 2003). As a possible alternative to positional cloning of mating-type genes, direct amplification using degenerate PCR is an approach that has facilitated rapid sequencing and cloning of ascomycete mating-type genes (Arie et al., 1997).

To address hypotheses concerning the evolution and diversification of mating-type loci in the homobasidiomycetes, studies are needed beyond model species. In this paper, we demonstrate two methods for isolating the *A* and the *B* mating-type genes of the oyster mushroom *P. djamor*— positional cloning and degenerate PCR. A gene map for both of these chromosomal regions is presented. The mating-type loci contain homologues of mating-type genes from other model mushroom species, namely homeodomain transcription factors and pheromone receptors.

2. Materials and methods

2.1. Study species

Pleurotus djamor is a pantropical species of oyster mushroom with white-rot, wood decaying nutrition. Studies of mating compatibility suggest that *P. djamor* is globally intercompatible, and behaves like a single biological species (Liou, 2000; Nicholl and Petersen, 2000). Our lab has used the species to study the genetics of speciation and has created a genetic map based on amplified fragment length polymorphisms (AFLPs) segregating in a cross between a Malaysian strain (ATCC38141.104) and a Hawaiian strain (RV95/957.30; Liou, 2000). Cosegregation analyses have suggested that the positional cloning of the *A* mating-type genes from the species would be possible because the *A* mating-type locus is tightly linked to the mitochondrial intermediate peptidase gene (James et al., 2004).

2.2. Strains, culturing, and mating techniques

This study utilized 22 homokaryotic isolates of *P. djamor* (Table 1). Strains of additional *Pleurotus* species were also used for sequencing pheromone receptors: *P. ostreatus* strains D578.2, D330 (= FP 6507), D850; *P. cystidiosus* strains D419 (= FP 6467), D478 (= ATCC46391); *P. pulmonarius* strains D263 (= ATCC66382), D1979; *P. eryngii* strain D625; *P. populinus* strain D765; *Pleurotus purpureo-olivaceus* strain D2342; and *P. australis* strain D2332. Cultures were routinely maintained on half-strength Emerson's YpSS agar (Y/2; Emerson, 1940) or 1.5% malt extract agar (both with 1.5% agar) at room temperature. Crosses between homokaryotic cultures were performed by placing actively growing mycelia/agar plugs approximately 1 cm apart in the center of a 10 cm Petri dish of Y/2 agar. The crosses were considered positive if dikaryotic hyphae containing clamp connections could be observed under 100× magnification at the growing margin of either side of the interacting strains. The inference of incompatibility due to shared mating-type alleles was based on the use of full-sibling testers of both of the homokaryons that were initially observed to be incompatible. Assignment of repeated mating-types to the *A* or *B* locus was based on the distinctive "flat" phenotype of partially compatible crosses that share *A* mating-type alleles, but differ in *B* mating-types (Raper, 1966).

Table 1
Homokaryotic isolates used to survey mating-type diversity in *P. djamor*

Strain	Mating-types	Origin
ATCC38139.5	*A*1 *B*1	Malaysia
ATCC38139.24	*A*2 *B*2	Malaysia
ATCC38140.31	*A*3 *B*3	Malaysia
ATCC38140.32	*A*4 *B*4	Malaysia
ATCC38141.103	*A*5 *B*5	Malaysia
ATCC38141.104	*A*2 *B*6	Malaysia
GJW1396.101	*A*6 *B*7	Honolulu, Hawaii
GJW1398.101	*A*7 *B*8	Honolulu, Hawaii
GJW1398.108	*A*8 *B*9	Honolulu, Hawaii
L1.101	*A*9 *B*10	La Selva, Costa Rica
L1.106	*A*10 *B*11	La Selva, Costa Rica
RV95/133.101	*A*11 *B*12	Northern Territory, Australia
RV95/134.104	*A*12 *B*13	Northern Territory, Australia
RV95/135.101	*A*13 *B*14	Northern Territory, Australia
RV95/137.101	*A*14 *B*14	Northern Territory, Australia
RV95/52.101	*A*15 *B*15	Northern Territory, Australia
RV95/52.105	*A*16 *B*16	Northern Territory, Australia
RV95/53.102	*A*17 *B*17	Northern Territory, Australia
RV95/937.101	*A*18 *B*18	Morobe Province, Papua New Guinea
RV95/937.104	*A*2 *B*19	Morobe Province, Papua New Guinea
RV95/957.2	*A*19 *B*20	Oahu, Hawaii
RV95/957.30	*A*7 *B*21	Oahu, Hawaii

Strain nomenclature generally follows convention of field collection number followed by single-basidiospore isolate number after the period.

2.3. Cosmid library preparation and screening

Genomic DNA was prepared from lyophilized mycelium using the method of Zolan and Pukkila (1986). The genomic DNA from strain RV95/957.30 was used to make a genomic library in the cosmid vector SuperCos-Pab1 (Bottoli et al., 1999). This vector has an ampicillin resistance gene from *Escherichia coli*, the *p*-aminobenzoic acid synthetase gene from *C. cinerea*, and two *cos* sites for packaging into bacteriophage λ. Preparation of the library, including DNA digestion and ligation, followed the instruction manual for the SuperCos 1 Cosmid Vector Kit (Stratagene). The DNA was packaged into phage particles using Gigapack III XL packaging extract (Stratagene) and transfected into *E. coli* strain XL1-Blue MR (Stratagene). The library was amplified and stored in 15% glycerol at −80 °C.

Screening of the library was performed by direct PCR of *E. coli* cells using a pooling procedure similar to that of Bottoli et al. (1999). Briefly, the library was plated onto 50 LB agar plates containing ampicillin (Sambrook et al., 1989) at a density of ~100 colonies per plate. Following overnight growth, the colonies were scraped into ddH_2O, diluted 100-fold, and 10 μl of this suspension was used for PCR with gene specific primers. Positive colony pools were then re-plated onto LB + ampicillin agar and then subcultured using sterile toothpicks into ordered groups of 24 onto two duplicate LB + ampicillin plates. Each group of 24 colonies was scraped from one of the duplicate plates and suspended in 500 μl of ddH_2O. This mixture was diluted 10-fold and then subjected to PCR amplification. This procedure narrowed the focus to groups of 24 clones. Individual colonies of positive groups from the duplicate plates were then amplified by PCR. The positive PCR amplicons in this round indicated cosmid clones that contained the gene of interest.

The library was screened for two genes, *P. djamor MIP* and *CLA4*. The PCR primers for the *MIP* gene were PdMIP1F (5′-CGAGTACACCTTCGTCAGCA-3′) and PdMIP1R (5′-GGCATGGAAGACTTGACTCC-3′), and for the *CLA4* gene, the primers PdCLA4F (5′-CTTCGCACAGAAACCGAAAT-3′) and PdCLA4R (5′-GGTGCGTTGACGGACATAAT-3′) were used. PCR amplifications were performed in 25 μl volumes using Red Hot DNA Polymerase (ABgene) in a Primus 96 thermocycler (MWG Biotech). Thermal cycling parameters for PCR of bacterial cells were an initial denaturation step at 94 °C for 10 min followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 50 °C for 30 s, extension at 72 °C for 1 min, and a 7 min final extension at 72 °C.

2.4. Cosmid sequencing

Cosmid DNA was isolated from 2 to 4 ml of overnight liquid culture using the QIAprep Spin Miniprep

Kit or the QIAprep 96 Turbo Miniprep Kit (Qiagen). Two cosmids positive for the *MIP* gene and one cosmid containing *CLA4* were sequenced. Sequencing was accomplished using transposon-mediated primer insertion with the GeneJumper Kit (Invitrogen). The transposon contained a gene encoding kanamycin resistance allowing selection for transposed cosmids on LB agar containing ampicillin and kanamycin. The transposition reaction was transformed into *E. coli* strain GeneHogs (Invitrogen) using electroporation. Sequencing was performed from both ends of the transposon into the cosmid insert using the primers GJSeq-A3 (5′-GC CGATATCGTCGACAAGC-3′) and GJSeq-B2 (5′-CG GCGGCTTTGTTGAATA-3′). Sequencing reactions were performed with BigDye v.2 and electrophoresed on an ABI3700 DNA analyzer (Applied Biosystems). Assembly of sequences traces into contigs was performed with Sequencher v4.1 (Gene Codes). Approximately 5.2-fold coverage of the *MIP* cosmids and 4.7-fold coverage of the *CLA4* cosmid was obtained by sequencing. Gaps in the contigs were filled by primer walking using synthesized oligonucleotides (Operon).

2.5. Degenerate PCR

Degenerate PCR of *B* mating-type pheromone receptor homologues from homokaryotic and dikaryotic strains of *Pleurotus* spp. was accomplished using primers br1-F (5′-TGGCATMTNCARGCNTGGAA YTC-3′) and br1-1R (5′-GCGAGNRNCATNAGN CGNAKGTA-3′) targeting amino acids 26–212 of *S. commune* receptor BAR1 (GenBank Accession No. Q92275). We also used primers br2-F (5′-CCNC TNCCNTGGCAYTGGCGNGC-3′) and br2-2R (5′-ACGRRSAGCCATGWAGATSATKGG-3′) targeting amino acids 20–129 of *C. cinerea* receptor RCB1 (GenBank Accession No. CAA71964) to amplify a receptor sequence from strain RV95/957.30. Thermal cycling parameters for degenerate PCR were similar to bacterial PCR (see above) except the initial denaturation step was only 3 min. Approximately, 10 ng of genomic DNA was added to each reaction (final volume 25 μl). PCR amplicons were typically purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned into the plasmid pCR2.1-TOPO (Invitrogen), and 1–2 random subclones of each PCR product were sequenced. In two cases (strains ATCC38139.24 and RV95/137.101), the PCR amplicons were directly sequenced following post-amplification purification using a QIAquick PCR Purification Kit (Qiagen).

2.6. Cosegregation of the B mating-type locus, pheromone receptor gene homologues, and CLA4

The mapping population of *P. djamor* has been described (Liou, 2000; see above). Mating-types of 193 single spore progeny of this cross have been determined using pairing analyses (Liou, 2000). Cosegregation of *CLA4*, the putative pheromone receptors (*PDSTE3.1*, *PDSTE3.2*, and *PDSTE3.3*) and the *B* mating-type locus was studied by genotyping progeny from the mapping population. Genotyping of *CLA4* was performed by PCR amplification of DNA from 47 progeny using primers PdCLA4F and PdCLA4R followed by digestion of the amplicons using *Hae*III (New England Biolabs). The genotypes at the pheromone receptors were determined for a subset of 38 isolates by allele-specific amplification of the allele contributed by parental strain RV95/957.30. Primers for *PDSTE3.1* were Pdrcb2-F (5′-AGATTGCGGCTTACGACAGTAT-3′) and Pdrcb2-R (5′-CCCTCCTTTGATGTCATCTCTC-3′). Primers for *PDSTE3.2* were Pdrcb3-F (5′-CCACACTATCAC TAGCCGTCTG-3′) and Pdrcb3-R (5′-ATGATGAGC TTTGTCGCTAATG-3′). Primers for *PDSTE3.3* were Pdrcb1-F (5′-AGGGAACGTGATTCGTTTG-3′) and Pdrcb1-R (5′-TAGATCGTTCTACTCCGGCACT-3′).

2.7. Long PCR

Long distance PCR (Barnes, 1994) was accomplished using the enzyme *LA Taq* (Takara) following the manufacturer's instructions. Primers for amplification of the *A* mating-type locus were PdMIP1F and Pdlflank-F (5′-ACAGCCGATGTTTCCAAGAC-3′). The thermocycling parameters used were: initial denaturation at 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 4 min, and a final 10 min extension at 72 °C. Following amplification of the *A* mating-type locus region from strain RV95/134.104 using long PCR, the amplicon was gel purified with the QIAquick Gel Extraction Kit and ligated into the vector pCR2.1-TOPO. The resulting plasmid was sequenced using the GeneJumper kit containing the chloramphenicol resistance transposon. Plasmid sequencing from both ends of the transposon used the primers GJSeq-A3 and GJSeq-B4 (5′-CAACAGGGACACCAGGATTT-3′).

To determine the linkage relationships of the *STE3*-like pheromone receptors of the *B* mating-type locus (*PDSTE3.1-3*), five of the allele-specific pheromone receptor PCR primers (Pdrcb1-R, Pdrcb2-F, Pdrcb2-R, Pdrcb3-F, and Pdrcb3-R) were used in all compatible combinations in separate PCR reactions. The thermocycling parameters for the *B* locus long PCR amplifications were the same as the *A* locus PCR except the extension time for the 35 cycles was 6 min.

2.8. Data analyses

Identification of genes in the *MIP* and *CLA4* chromosomal regions was accomplished using BLASTX database searching of the GenBank database (Altschul et al., 1997) to find open reading frames (ORFs) with

significant matches to known genes. We chose $P < 10^{-10}$ as the threshold value for homologue assignment.

The graphical comparison of the genetic variation of two *A* mating-type locus alleles from *P. djamor* homokaryons RV95/957.30 and RV95/134.104 was accomplished using a sliding window analysis performed by DNAsp v3.53 (Rozas and Rozas, 1999). The sliding window graph calculated the average nucleotide diversity (π) within a window of size 200 nucleotides (nt) that was incremented in steps of 25 nt.

The alignment of *B* mating-type pheromone receptors of *Pleurotus* spp. to other homobasidiomycete pheromone receptors was accomplished by hand in the sequence editor GeneDoc v2.6 (http://www.cris.com/~Ketchup/genedoc.shtml). We also included the sequences of two mating-type pheromone receptors from *C. neoformans* as a potential outgroup. The total alignment length was 174 amino acids, spanning residues 31–204 of *S. commune* BAR1. The alignment of the pheromone receptors and resulting phylogeny can be found at the TreeBASE server (http://www.treebase.org/treebase/) under Accession No. S1062. The phylogeny of the receptors was inferred using the software package MOLPHY v2.3 (Adachi and Hasegawa, 1996). The program PROTML was used with the JTT (Jones et al., 1992) substitution matrix with the most likely tree found using 1000 heuristic searches. Support for nodes was estimated using the RELL method (Hasegawa and Kishino, 1994).

2.9. GenBank accession numbers

The *STE3* sequences for *P ostr* D578.2 and *P cyst* D419 have been deposited in GenBank under the Accession Nos. AY226012 and AY226008, respectively. The other *STE3* fragments amplified from *Pleurotus* spp. using degenerate PCR are deposited under Accession Nos. AY456962–AY456978. The *A* mating-type locus region from strain RV95/957.30 is deposited under AY462111 and the *B* locus region under AY462110. The *A* mating-type locus sequence for strain RV95/134.104 has been deposited under AY462112.

3. Results

3.1. Survey of mating-type variation in P. djamor

A sample of 22 homokaryotic isolates collected from diverse locations was used to estimate the number of mating-types throughout the species. These samples were crossed in all pairwise combinations and dikaryon formation was assayed by the presence of clamp connections. Initially, we used 27 homokaryotic isolates for the matrix of crosses. However, the mating behavior of certain strains demonstrated that not all strains were interfertile, perhaps due to genetic divergence between populations or mutation accumulation in long term culture as has been previously suggested (Liou, 2000). Strains with non-transitive mating behavior were excluded from further studies. The remaining 22 isolates appeared to cross with other isolates with no partial infertility and the production of very robust dikaryotic growth. One repeated *B* mating-type allele was found in two isolates (RV95/135.101 and RV95/137.101) collected from the same location in N. Australia. One *A* mating-type allele was repeated twice, and another *A* allele was repeated three times (Table 1). The low number of repeated mating-type alleles suggests that the number of alleles in the species is very high. Using Dobzhansky and Wright's (1941) formula, the species estimate for number of *A* mating-type alleles is 58, with a 95% confidence interval (Stevens, 1941) of 23–211. For the *B* locus, the estimated number of mating-type alleles is 231, with confidence interval of 42–9130.

3.2. Division of the A and B mating-type loci into subloci

Much of the generation of the numerous mating-type alleles of homobasidiomycetes is attributed to the presence of multiple subloci per mating-type locus and the recombination between them. We explored the genetic structure of the *A* and *B* mating-type loci in *P. djamor* to determine if they are composed of more than one sublocus. To create a genetic map of *P. djamor*, 193 progeny were genotyped at the mating-type loci by crossing progeny to a reference set of the four mating-types. Recombinant mating-type alleles would appear as progeny that can successfully mate and form a dikaryon with two of the four testers, whereas non-recombinant progeny can mate with only a single tester. There were zero non-parental *A* mating-type alleles and only two non-parental *B* mating-type alleles among the 193 progeny. Thus the *B* locus appears to be divided into more than one sublocus, while the *A* locus is composed of a single or very tightly linked subloci.

3.3. Structure and variation at the A mating-type region

Upon screening the *P. djamor* genomic library using PCR with *MIP* specific primers, several cosmid clones containing the *MIP* gene were isolated. Two cosmid inserts that overlapped by 952 bp (spanning 75.3 kb) were sequenced using transposon mediated primer insertion. The gene map of this region is shown in Fig. 1, and the position and characterization of these genes is given in Table 2. Thirteen genes with significant similarity to proteins in the GenBank database ($P < 10^{-10}$) were identified in this chromosomal region. Adjacent to the *MIP* gene was a pair of divergently transcribed homeodomain transcription factor genes *PDa1* and *PDa2* that displayed strong similarity to the two classes

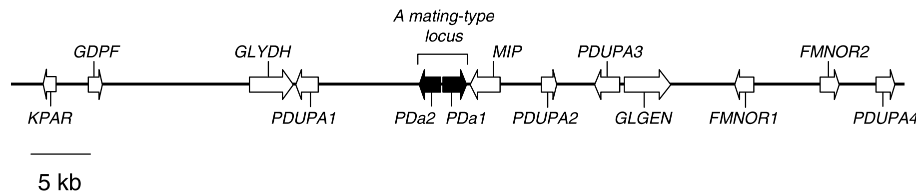

Fig. 1. Gene map of the *MIP A* mating-type locus chromosomal region in *P. djamor* strain RV95/957.30. ORFs with significant ($P < 10^{-10}$) matches to the GenBank database are shown. Genes are indicated as arrows, with orientation indicating the direction of transcription. The putative mating-type genes are indicated in black. See Table 2 for gene assignment and homology search results.

of mushroom *A* mating-type homeodomain genes, the HD1 and HD2 types (Kües et al., 1994b). The single pair of homeodomain genes represents the only genes in this region with similarity to known mating-type genes in other fungi. Other genes with known function in this region are glycine dehydrogenase (*GLYDH*), ketopantoate reductase (*KPAR*), and two NADPH dehydrogenases (*FMNOR1-2*). The *FMNOR* genes show a pattern of recent duplication because they display 85% DNA sequence identity between the eight exons and 79% amino acid identity.

If the putative *A* mating-type locus spanning the two homeodomain genes actually functions in mating-type determination, then our current knowledge of these proteins allows a prediction to be made regarding the magnitude of genetic variation between different mating-type alleles. Specifically, similarity between the predicted proteins encoded by allelic variants is generally very low and the N-terminal regions of the proteins are often hypervariable (Badrane and May, 1999; Kronstad and Leong, 1990). To ascertain whether the variation of the putative *A* mating-type genes agrees with their proposed role in mating-type determination, we sequenced an additional allele of the putative mating-type locus from another homokaryotic strain with a different *A* mating-type allele (RV95/134.104, mating-type allele *A12*). Long distance PCR was used to amplify a 5.9 kb fragment that spanned the entire putative *A* mating-type locus. Analysis of the amplicon sequence predicted a pair of divergently transcribed homeodomain genes as expected. These genes were translated into amino acid sequences and compared with the mating-type proteins of strain RV95/957.30 (Fig. 2). The amino acid identities between the two PDa1 and PDa2 protein variants were rather low (58 and 61%, respectively). The homeodomain region of the protein displayed the greatest sequence conservation (Fig. 2). The sliding window analysis of DNA sequence variation of the two mating-type alleles (Fig. 3) indicates that relative to the flanking regions, the variation at the putative *A* locus is higher specifically within the homeodomain genes. The analysis suggests for the HD1 gene, *PDa1*, that the 5′ region of the gene displays the highest genetic divergence between the two alleles. In contrast, the 5′ region of the HD2 gene, *PDa2*, is hypervariable, as is the region immediately downstream of the homeodomain. Finally, the genetic variation downstream of the *PDa2* gene abruptly drops to a very low level.

3.4. Analysis of the B pheromone receptors of the mating-type locus

We used two pairs of degenerate PCR primers to amplify pheromone receptor homologues from strain RV95/957.30. A fragment of size 780 bp was amplified by primers br1-F and br1-1R, and a smaller fragment (381 bp) was amplified by primers br2-F and br2-2R. These two fragments were subcloned, sequenced, and found to be similar to the *STE3* **a**-factor receptor class of pheromone receptors using BLAST searches (Altschul et al., 1997). These two gene regions were termed *PDSTE3.1* and *PDSTE3.2*.

We also tried to use conserved gene order between the kinase *CLA4* and the *B* mating-type locus to clone the *B* mating-type genes. The linkage relationship between *CLA4* and the *B* locus was determined by analyzing the cosegregation of these two loci among the progeny of the mapping population. Comparison of the *CLA4* genotypes with *B* mating-type alleles among 47 progeny demonstrated all parental-type combinations and no recombinant types suggesting these two loci are tightly linked (Fig. 4). We also tested the cosegregation of the *B* mating-type locus with the two putative pheromone receptor genes (*PDSTE3.1* and *PDSTE3.2*) using 38 of the same progeny. Allele-specific amplification of the allele contributed by parental strain RV95/957.30 demonstrated complete cosegregation of these putative receptor genes and the *B* mating-type locus with no evidence for recombination in this progeny array.

We then probed the *P. djamor* cosmid library for *CLA4* and identified several *CLA4* positive clones. One cosmid of 38.3 kb was chosen for complete sequencing using transposon mediated primer insertion. The gene map of the *CLA4/B* mating-type region is shown in Fig. 5 and the results of homology searches against the GenBank database are shown in Table 2. In general, the region had a low gene density. Only nine genes displayed significant similarity to known proteins; one of these (*PDSTE3.3*) was very similar to a pheromone receptor, the *B* mating-type protein RCB1 from *Coprinopsis*

Table 2
Table of gene homologues identified in the *P. djamor* mating-type regions

Gene	*P* value	Position	Homologue	Possible function
A Mating-type region (Fig. 1)				
KPAR	1×10^{-20}	(3934–2714)	COG1893: Ketopantoate reductase [*Pseudomonas syringae* pv. Syringae B728a] (ZP_00124108)	Thiamine/coenzyme A synthesis
GDPF	2×10^{-15}	(6481–7663)	Similar to *Homo sapiens* GDP-fucose transporter 1 [*Dictyostelium discoideum*] (AAO50954)	Sugar transport
GLYDH	10^{-169}	(20,102–23,843)	Putative glycine dehydrogenase (decarboxylating) [*Schizosaccharomyces pombe*] NP_592832	Glycine metabolism
PDUPA1	7×10^{-10}	(25,947–24,004)	Unknown protein F16M19.5 [*Arabidopsis thaliana*] (G96656)	Unknown
PDa2	2×10^{-17}	(36,204–34,389)	*A* Mating type protein [*C. cinerea*] (CAA56131)	Mating-type specific homeodomain transcription factor (HD2)
PDa1	6×10^{-22}	(36,389–38,435)	Mating-type protein β1 [*C. cinerea*] (AF126783)	Mating-type specific homeodomain transcription factor (HD1)
MIP	0.0	(41,234–38,711)	Mitochondrial intermediate peptidase, mitochondrial precursor (MIP) [*S. commune*] (P37932)	Cleavage of mitochondrial precursor proteins
PDUPA2	5×10^{-21}	(44,704–46,005)	Conserved hypothetical protein [*Neurospora crassa*] (CAD21156)	Unknown
PDUPA3	10^{-145}	(51,368–49,226)	Hypothetical protein [*N. crassa*] (XP_325455)	Predicted unusual protein kinase
GLGEN	4×10^{-36}	(51,744–55,643)	Related to glycogenin-2β [*N. crassa*] (CAD70922)	Lipopolysaccharide biosynthesis and glycogen synthesis
FMNOR1	2×10^{-68}	(62,555-60,958)	NADH:flavin oxidoreductase, old yellow enzyme family [*P. syringae* pv. syringae B728a] (ZP_00125795)	Mitochondrial electron transport system
FMNOR2	2×10^{-68}	(68,165–69,748)	NADH:flavin oxidoreductase, old yellow enzyme family [*Pseudomonas syringae* pv. syringae B728a] (ZP_00125795)	Mitochondrial electron transport system
PDUPA4	6×10^{-13}	(72,893–74,466)	Probable transcription factor tfiiib component [*S. pombe*] (T41239)	Transcription initiation
B Mating-type region (Fig. 5)				
PDSTE3.3	5×10^{-64}	(3962–1928)	Pheromone receptor [*C. cinerea*] (AAO17256)	Mating-type specific G-protein-coupled receptor
PDphb1[a]	0.001	(4699–4833)	pheromone phb1.1 [*C. cinerea*] (AAO17257)	Mating-type specific peptide pheromone
AKOR1	2×10^{-25}	(10,673–11,875)	Oxidoreductase aldo/keto reductase family [*Streptococcus pneumoniae*] (NP_345932)	NADPH-dependent oxidoreductase
AKOR2	3×10^{-19}	(12,259–13,807)	Putative reductase protein, YvgN [*Bacillus subtilis*] (CAA11712)	NADPH-dependent oxidoreductase
PDUPB1	2×10^{-17}	(14,814–14,060)	Hypothetical protein [*N. crassa*] (XP_331659)	Related to β-1,3 exoglucanase precursor
PDUPB2	3×10^{-29}	(17,30115,883)	Hypothetical protein F9K21.160 [*N. crassa*] (XP_324871)	Unknown
RPS19	2×10^{-12}	(30,978–31,429)	Mitochondrial ribosome small subunit component; Rsm19p [*Saccharomyces cerevisiae*] (NP_014435)	Translation, ribosomal structure, and biogenesis
CLA4	10^{-118}	(35,714–32,900)	STE20 [*C. neoformans* var. *neoformans*] (AAG48305)	Serine threonine/MAP kinase, cytoskeleton polarity, mating
CHODH	2×10^{-19}	(end–36,617)	4-Nitrobenzyl alcohol dehydrogenase NtnD [*Pseudomonas* sp. TW3] (AAC38361)	Choline dehydrogenase and related flavoproteins

P value indicates the probability of the match being due to chance in GenBank similarity searches. Under homologue, the species from which the lowest *P* value hit was obtained is given in brackets, followed by the GenBank accession number in parentheses.

[a] This *P* value represents the result of a BLASTp search for short nearly exact matches limited by "query, Basidiomycota." Gene was initially identified in a search for ORFs that ended in the typical prenylation signal-CAAX (Brown and Casselton, 2001).

cinerea (Table 2). *PDSTE3.3* was different in DNA sequence from both *PDSTE3.1* and *PDSTE3.2*, isolated from RV95/957.30 through degenerate PCR. In accordance with the possible function of *PDSTE3.3* as a G-protein-coupled receptor, the molecule was predicted by the software HMMTOP (Tusnády and Simon, 2001) to

```
A7  : MTDTPTRFENALHRIINVSESLRSKTKLDPQTSNTGCRSPISSQPL-SIDFPPPPNILPSLRSKGVSEQAAKKLTDIFNSKCHILQSLLRNKLHDIQSHSSLPIHHLLSSYAQKT
A12 : .A.I-...H...RQ.VE..R.IQA..T----KP.SLLDR.P..L..A........DVSQR...L.IT...VQ.IVET..A..GL..TVV.D..REM..Y.PPSL.P..C.SV..A

        120         *         140         *         160         *         180         *         200         *         220         *
A7  : YQEDILRFEDELHRVAALHLDKLQADQVKPAAKTSFNADFVPFLEKYFEFNAYPSAADRSLMARKSMMTPRQIEVWFQNHRNRARKEGKCLPRLRLAEELPKDLCLETLEERMSG
A12 : .L.NVNQM.I.VN...V..QQ.VKG.KL...T.IV...T..........Y........A....N....S.................Q....HPTDP..P....TS...-..D

            240         *         260         *         280         *         300         *         320         *         340
A7  : YIASETDKFSSDGSEVENNDSLNQGDEL--AIASEKKSLSANLLEP---PYTIPPPYVTPRTSFLHASVPECISVLSGAPLPWPRSPSISPTSPSKLRPLTQSQLDEFSDLFARL
A12 : LTQ..SE.SNAGE.DQ.DA.TIS.VEGVDLH.P.--.QA.LHV...SSSS.AF.A...A.PI...R.T.Q..T.TF..V......PQ.GPIST...N....K..V.K..E.....

          *         360         *         380         *         400         *         420         *         440         *         460
A7  : TVRDRSKKAVRGRATPLPLNHRHISDCAATCATTTVLCRGRHPSFVSTPNTPSSSLFAPLPLRERSSPDMEVRSPSVGPLDTASPSS---KRRKSPQLPRRVPGSSPSSHIRVQH
A12 : ....GPR.VA.RK...S.F.Q.CV.AR..V..............Y....CP......PSF.AT..R.SS---------QP..T....HSRRH.............A.R..T....

              *         480         *         500         *         520         *         540         *
A7  : MHHPYASPSRASSSSSSCRTPSSSSESLSTPRTPDNFNTLLPSQTSMTKSTSTPSYSPWTSCFNLPHLGSPSPSPFQSNPSWFPQDLFLHNSYGVSALC
A12 : I........E........................L..S..F...T.....I......A.Y.....F.....N...............P.PF......
```

Fig. 2. Alignment of HD2 homeodomain proteins (PDa2) from RV95/957.2 (*A*7) and RV95/134.104 (*A*12). Sequence for *A*7 is shown, and identical residues in RV95/134.104 are shown as a dot. The HD2 homeodomain motif (Kües et al., 1994b) is displayed as white lettering on black background.

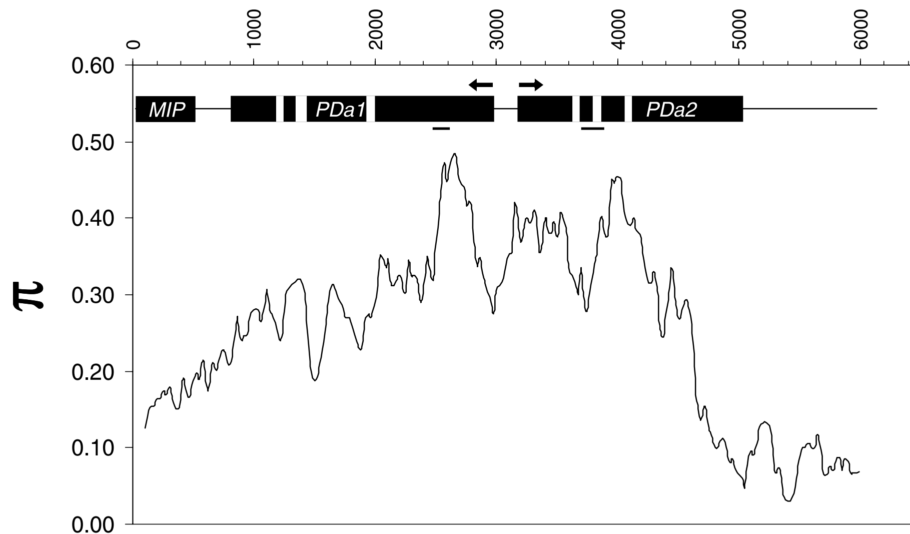

Fig. 3. Sliding window plot of nucleotide diversity (π) between two *A* mating-type alleles of *P. djamor* (*A*7 and *A*12). Coordinates of the abscissa refer to position in nt along the locus. Values of π were averaged over windows of size 200 nt and computed for every 25 nt. Higher values of π correspond with increased divergence between the two alleles. The position along the molecule is in schematic above the plot. Exons are shown as black boxes and introns are shown in white. Arrows indicate the direction of transcription. Underneath the exon/intron schematic the position of the homeodomain encoding region of the genes are shown as a bar.

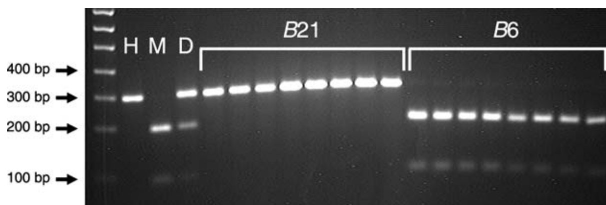

Fig. 4. Cosegregation of *CLA4* and the *B* mating-type locus in *P. djamor*. Pictured are PCR amplicons of *CLA4* digested with *Hae*III on an agarose gel stained with ethidium bromide. In the first lane is a molecular weight marker with sizes indicated to the left of the figure. Lane marked H, uncut *CLA4* amplicon of the Hawaiian parent (RV95/957.30) of the mapping population. Lane marked M, *CLA4* amplicon of the Malaysian parent (ATCC38141.104) of the mapping population that is digested into two smaller fragments. D, dikaryotic parent derived from crossing parents H and M. Lanes marked *B*21, homokaryotic progeny of the mapping population with the *B*21 allele inherited from the Hawaiian parent as determined by test pairings. Lanes marked *B*6, homokaryotic progeny with *B*6 allele inherited from Malaysian parent.

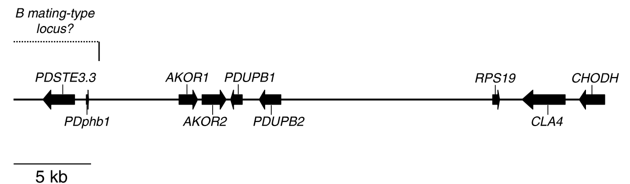

Fig. 5. Gene map of the *CLA4*/*B* mating-type chromosomal region in *P. djamor* strain RV95/957.30. Arrows indicate ORFs with significant ($P < 10^{-10}$) matches to the GenBank database. See Table 2 for gene assignment and homology search results. The gene *CHODH* is interrupted at one end of the contig.

be a seven-pass transmembrane spanning protein, as are all known proteins of the STE3 pheromone receptor class.

In the *B* mating-type loci of all homobasidiomycetes studied to date, each haplotype contains one or more

pheromone receptors as well as one to often several small pheromone genes (Brown and Casselton, 2001; Fowler et al., 2004). The encoded pheromone peptides have little structural similarity to one another. However, the described pheromones generally contain the motif "-CAAX" at the C-terminus (Fowler et al., 2004; Wendland et al., 1995), where C, cysteine; A, aliphatic residue; and X, one of several amino acids. We searched for short open reading frames (ORFs) in the vicinity of the *PDSTE3.3* pheromone receptor which contained this motif at the C-terminus. One such ORF of 135 nt was found which when compared with the existing GenBank database, showed 51% amino acid similarity to a pheromone protein (phb1.1) from *C. cinerea* (Fig. 6).

All three sequenced receptor genes (*PDSTE3.1-3*) are either part of or tightly linked to the *B* mating-type locus based on the cosegregation analyses. We used long distance PCR in an attempt to amplify the intergenic DNA regions between the pheromone receptor genes of the *B* locus. Primers specific to the three receptor genes (*PDSTE3.1-PDSTE3.3*) were designed and used to amplify the DNA regions as described in the materials. Only the primer pair Pdrcb2-F and Pdrcb3-F produced a positive PCR product of ~6 kb. This PCR amplicon confirms that *PDSTE3.1* and *PDSTE3.2* are linked at a distance of 3–4 kb. The ends of the PCR fragment were sequenced to verify that the amplification was specific.

Further evidence in support of the role of all three receptor gene products in determining mating-type derives from the analysis of the two homokaryons with putatively recombinant *B* mating-type alleles based on crossing data (ssi 15 and 178). Analysis of the inheritance of the *STE3*-like pheromone receptor genes in these two strains suggested they had undergone a recombination event between *PDSTE3.1*/*PDSTE3.2* and *PDSTE3.3*/*CLA4* to produce nonparental combinations of these genes.

3.5. Variation among B mating-type receptors in Pleurotus

We screened a small sample of isolates of *P. djamor* and other *Pleurotus* spp. for *B* receptors using the degenerate PCR primers br1-F and br1-1R. Roughly half of the *P. djamor* samples produced amplicons, and six of these were sequenced. Most (11 of 12) of the other strains of *Pleurotus* spp. produced one or two *STE3*-like amplicons. The putative pheromone receptor genes were translated into amino acid sequences and aligned with the three STE3-like sequences of isolate RV95/957.30 as well as all of the previously published homobasidiomycete pheromone receptors. Most of the *Pleurotus* STE3-like sequences recovered were highly divergent (mean 52%, range 14–99%). However, two of the alleles obtained from *P. djamor* strains L1.106 (Costa Rica) and RV95/53.102 (Australia) and two of the alleles from *P. ostreatus* strains D330 (Czech Republic) and D850 (California) differed by only one amino acid substitution.

A maximum likelihood phylogeny of the pheromone receptor genes, including the putative ones from *Pleurotus* spp., is shown in Fig. 7. The homobasidiomycete sequences can be loosely divided into two clusters with strong bootstrap support (groups 1 and 2). The sequences of *P. djamor PDSTE3.2* and *PDSTE3.3* are both in group 2. All of the sequences generated by PCR with primers br1-F and br1-1R are part of group 1, and the group 1 receptors can be subdivided into roughly three subgroups (A, B, and C). *P. djamor* sequences derived via PCR form a clade with other *Pleurotus* species in groups 1A and 1B. For each of the groups and subgroups, a sequence of a known mating-type gene (from *S. commune* or *C. cinerea*) can be found in a basal position in the group, suggesting that all of the STE3-like sequences of *Pleurotus* spp. derive from mating-type related proteins of other homobasidiomycetes. Finally, the group 1 receptor sequences are not monophyletic with respect to species, demonstrating trans-specific polymorphism as might be expected for loci under strong balancing selection (May et al., 1999).

4. Discussion

In this study, we cloned DNA sequences from the *A* and *B* mating-type loci of *P. djamor* using conserved gene order and degenerate PCR approaches. The homology of the genes in these sequenced regions to the mating-type genes from other basidiomycetes was assessed by amino acid sequence comparisons, conserved linkage to non-mating-type specific genes (*MIP* and *CLA4*), and the pattern of DNA polymorphism.

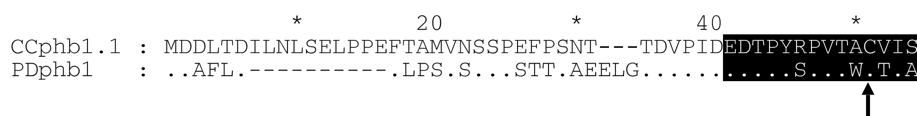

Fig. 6. Alignment of predicted peptide pheromone encoded by *PDphb1* with a mating-type pheromone of *C. cinerea* (CCphb1.1; GenBank Accession No. AAO17257). Residues in PDphb1 that are identical to CCphb1.1 are shown as a dot. The predicted mature 14 amino acid peptide based on Fowler et al. (2001) and Olesnicky et al. (1999) is shown with black background. The C-terminal cysteine that is subject to post-translational prenylation and carboxymethylation following the cleavage of the last three amino acids (Bölker and Kahmann, 1993) is indicated by the arrow.

Fig. 7. Phylogeny of homobasidiomycete STE3-like pheromone receptors. Labels give the species abbreviation in italics (*C cine*, *C. cinerea*; *C neof*, *C. neoformans*; *P aust*, *P. australis*; *P cyst*, *P. cystidiosus*; *P djam*, *P. djamor*; *P eryn*, *P. eryngii*; *P ostr*, *P. ostreatus*; *P popu*, *P. populinus*; *P pulm*, *P. pulmonarius*; *P purp*, *P. purpureo-olivaceus*; *S comm*, *S. commune*), followed by strain name or gene name. The three receptors sequences isolated from library strain RV95/957.30 are circled. Numbers above nodes show bootstrap probabilities for clades with greater than 80% support. GenBank accession numbers for published sequences are: *C cine* RCB1, CAA71964; *C cine* RCB2, CAA71963; *C cine* RCB2.42, AAF01419; *C cine* RCB3, CAA71962; *C cine* RCB3.42, AAF01420; *C neof* STE3a, AAN75156; *C neof* CPRα, AAF71292; *S comm* BAR1, Q92275; *S comm* BAR2, CAA62595; *S comm* BAR3, P56502; *S comm* BBR1, P78741; *S comm* BBR2, AAD35087.

The sequence data revealed only a single pair of homeodomain transcription factors in the *A* mating-type, rather than the two to three observed in the model mushroom species studied to date (Hiscock and Kües, 1999). In contrast, we provide data that the *B* mating-type locus is comprised of at least three pheromone receptor genes. Using a complete cross of 22 wild-collected homokaryotic *P. djamor* isolates, we recovered more *B* mating-type alleles than *A* alleles. In addition, we observed the generation of recombinant *B*, but not *A* mating-type alleles in a mapping population. Since recombination between the subloci of a mating-type locus is responsible for much of the diversity of mating-type alleles, these data are consistent with the estimated number of total alleles in the species. We hypothesize that the *A* mating-type locus may be a single, hyperdiverse locus.

4.1. Positional cloning of mating-type genes in homobasidiomycetes

This study verifies the utility of the positional cloning method for studying genes and DNA sequences of the mating-type loci of homobasidiomycetes (James et al., 2004; Kües et al., 2001). Our results suggest that the *MIP* gene is very tightly linked to the *A* mating-type locus (<1 kb; Fig. 1), while the physical distance between the *CLA4* kinase gene and *B* mating-type pheromone receptor gene *PDSTE3.1* is much greater (~30 kb;

Fig. 5). Searches for conserved gene order of *CLA4* and the *STE3*-like pheromone receptors in the unfinished genome sequences of two homobasidiomycete species, *P. chrysosporium* (http://genome.jgi-psf.org/whiterot1/whiterot1.home.html) and *C. cinerea* (http://www-genome.wi.mit.edu/annotation/fungi/coprinus_cinereus/), suggest that although these genes are syntenic, the distance between them is highly variable, ~6 and ~100 kb, respectively.

4.2. Genetic structure of the A mating-type locus

The canonical basidiomycete *A* mating-type locus is arranged as divergently transcribed pairs of genes, which encode for homeodomain proteins that dimerize with each other in heteroallelic matings (Brown and Casselton, 2001; Hiscock and Kües, 1999). We postulate a single locus structure for the *A* mating-type of *P. djamor* for two reasons. One, no recombinant *A* mating-types were recovered in the sample of 193 progeny of a mapping population. Two, the sequencing of *MIP* cosmid clones spanning over 75 kb revealed only a single pair of homeodomain encoding genes centrally located in the 75 kb of sequenced DNA (Fig. 1). Nonetheless, our numbers of sampled progeny in segregation analyses and numbers of sampled nucleotides in *MIP* cosmid sequencing are insufficient to rule out the possibility that the *A* mating-type has a multiple sublocus structure. The maximum distance, at $P < 0.05$, between the subloci of the *A* locus (if they do indeed exist but no recombinants were observed) is ~2 cM (Silver, 1995). Although it is difficult to relate this recombination distance to physical distance, a rough estimate on the relationship between the two was estimated for the vicinity of the *C. cinerea A* locus, where $1\,\text{cM} \approx 80\,\text{kb}$ (Lukens et al., 1996). In a comparison of mapping linkage length and genome size estimated by pulsed-field gel electrophoresis, Larraya et al. (1999) estimated $1\,\text{cM} \approx 25\,\text{kb}$ in *Pleurotus ostreatus*. A similar value ($1\,\text{cM} \sim 27.9\,\text{kb}$) was recently calculated for *C. cinerea* (Muraguchi et al., 2003).

The substructure of the *A* mating-type locus in the genus *Pleurotus* remains uncertain. Larraya et al. (2001) appear to conclude that the *A* locus of *P. ostreatus* has a single locus structure. However, results using a large sample of *P. pulmonarius* homokaryons uncovered recombinant *A* mating-type alleles (Eugenio and Anderson, 1968). The decisive experiment to determine the structure of the *A* locus in *Pleurotus* rests on a thorough genetic analysis of recombinant mating-types or on further genome sequencing, though it remains possible that the structure of the *A* mating-type is polymorphic within the genus. If the *A* mating-type of *Pleurotus* is comprised of only one nonrecombining locus, then this locus would have an unprecedented number of alleles (~50–100). The greatest number of alleles known for a single basidiomycete mating-type sublocus is *S. commune Aβ* with 32 variants (Stamberg and Koltin, 1973).

In the medically important basidiomycete yeast species, *C. neoformans*, the single mating-type locus contains many genes involved in mating and sporulation clustered into one nonrecombining region (Lengeler et al., 2002). Our analysis of the *P. djamor A* mating-type genomic region failed to uncover any genes with obvious function in mating other than the genes homologous to the homeodomain encoding mating-type genes of other mushroom species. Whether the genes linked to the *A* mating-type locus have, in fact, no mating function is yet to be determined. Genes in or near the *A* mating-type locus are, however, implicated in modulating homokaryotic growth rate in *P. ostreatus* (Larraya et al., 2001). While it is possible that the *A* mating-type genes themselves have an effect on growth rate since they are expressed in homokaryons (Richardson et al., 1993), it is more likely that the other genes in the *A* chromosomal region of *Pleurotus* are responsible for this effect. Many of the genes near the *A* locus of *P. djamor* are responsible for cellular energy metabolism, e.g., genes encoding glycogenin (*GLGEN*) and ketopantoate reductase (*KPAR*). It is conceivable that alleles at several of these genes could be responsible for the difference in growth rate of *P. ostreatus* homokaryons that Larraya et al. (2001) observed.

4.3. Genetic structure of the B mating-type locus

Our data implicate a complex structure for the *P. djamor B* mating-type locus that includes multiple subloci encoding for pheromone receptors and lipopeptide pheromones. This model is based on the occurrence of recombinant *B* mating-type alleles in a mapping population at a rate of ~1% and the recovery of three distinct pheromone receptors from the genome of a single haploid strain using degenerate PCR. Long PCR was used to establish the close proximity of two of these receptors (*PDSTE3.1* and *PDSTE3.2*), but it is unknown what physical distance separates this pair and the third receptor (*PDSTE3.3*). Furthermore, it is quite possible that there are additional receptors within the *B* locus that were not detected in this study. We have proposed that a gene encoding a putative mating-type pheromone (*PDphb1*) is in close proximity to one of the pheromone receptor genes (*PDSTE3.3*) on the sequenced cosmid. In the model mushroom species, each pheromone receptor is typically associated with multiple (2–8) pheromone genes (Fowler et al., 2004; Halsall et al., 2000). The low number of observed pheromone genes uncovered in this study is likely a product of the location of the pheromone receptor on the sequenced DNA fragment (it is near the end) and the difficulty in predicting these small (~50 amino acids), hypervariable proteins in silico.

4.4. DNA polymorphism at the mating-type loci

In the absence of functional data that could confirm the role of the genes sequenced in this study, we analyzed the genetic variation at these genes to demonstrate that they are likely to be mating-type specific. The putative *P. djamor* mating-type genes show evidence of balancing selection through the observation of high levels of sequence diversity between alleles (Fig. 2). Furthermore, the variation between the two sequenced alleles at the *A* mating-type locus showed substitutions to be elevated in the 5′ region of the homeodomain genes (the putative specificity determining regions) relative to the entire molecule and the flanking DNA regions (Fig. 3).

The polymorphism data for the *B* locus were obtained by PCR amplification of the conserved regions of the *B* mating-type genes, rather than the flanking regions. Therefore, there is no positional data which can be used to guarantee orthology of the sequenced DNA fragments. However, the sequenced gene fragments group into four clusters which may correspond with four paralogues (Fig. 7). If these groups correspond with mating-type subloci, the data argue for the inheritance of trans-specific mating-types through speciation events because within subgroups 1A and 1B the various *Pleurotus* spp. do not form monophyletic groups. Two lines of evidence suggest that these boundaries based on branch length and bootstrap support are artificial. Two alleles at the *C. cinerea* RCB2 locus (*C cine* RCB2 and *C cine* RCB2.42) do not belong to the same group (Fig. 7). Also, two of the genes from *P. djamor* strain RV95/957.30 (*PDSTE3.2* and *PDSTE3.3*) are both in group 2, though the genes are clearly paralogous since they are from the same haploid strain.

4.5. Future directions

This study provides a basis for understanding the organization and gene content of the mating-type loci in *P. djamor* as well as other *Pleurotus* spp. Further functional or gene organization studies would help clarify the homology or paralogy of the various *Pleurotus* pheromone receptor sequences in this study (Fig. 7). Future studies include expanding the genetic map of *P. djamor B* mating-type locus by restriction mapping or DNA sequencing so as to incorporate the other two receptors (*PDSTE3.1* and *PDSTE3.2*). Also, a more thorough search for recombinant *A* mating-type alleles will be necessary to confirm the single sublocus nature of this mating-type locus.

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