



Evolution of the gene encoding mitochondrial intermediate peptidase and its cosegregation with the *A* mating-type locus of mushroom fungi

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Abstract

The high level of DNA polymorphism at the mating-type loci of mushroom fungi has made the cloning of mating-type genes difficult. As an alternative to strategies that employ sequence conservation, an approach utilizing conserved gene order could facilitate the cloning of *A* mating-type genes from mushroom fungi. It has been shown that a gene encoding a mitochondrial intermediate peptidase (*MIP*) is very close (<1 kbp) to the *A* mating-type locus of two model mushroom species. In this study, the cosegregation of *MIP* and the *A* mating-type locus was studied by genotyping progeny of seven additional mushroom species using PCR and genetic crosses. No evidence of any recombination between *MIP* and the *A* mating-type locus was detected among all seven species. Phylogenetic analysis of *MIP* sequences from diverse mushroom species agrees with the current organismal phylogeny, suggesting the sequences are generally orthologous.

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1. Introduction

In the homobasidiomycete fungi (mushrooms), a single species can have as many as thousands of different mating-types in natural populations. Most mushrooms possess a bifactorial mating system. In such fungi, the mating-type of an individual is determined by two unlinked loci, the *A* and *B* mating-type loci (Raper, 1966). Compatible mating in outcrossing mushroom fungi results when two homokaryotic (haploid) individuals that differ in mating-type at both *A* and *B* loci fuse hyphae and form a dikaryotic (functionally diploid) individual. Under suitable conditions, the dikaryon differentiates fruiting bodies upon which karyogamy, followed immediately by meiosis, and sporogenesis occurs.

The *A* and *B* mating-type loci of homobasidiomycetes have been intensively studied using both classical and

molecular genetic approaches. The *A* mating-type locus controls nuclear pairing in the dikaryon as well as the proper formation of the specialized hook cell, the clamp connection. The *B* mating-type locus is responsible for the reciprocal exchange and migration of nuclei that occurs between mating homokaryons (Casselton and Olesnick, 1998; Raper, 1983). In the two model mushroom species *Coprinopsis cinerea* (= *Coprinus cinereus*) and *Schizophyllum commune*, the *A* mating-type locus encodes one or more pairs of divergently transcribed homeodomain-like transcription factors (Hiscock and Kües, 1999; Pardo et al., 1996; Stankis et al., 1992). The *B* mating-type loci encode membrane spanning pheromone receptors, homologous to the *a*-factor receptor from yeast, and a few to several small lipopeptide pheromones (Casselton, 2002; Halsall et al., 2000; O'Shea et al., 1998; Vaillancourt et al., 1997; Wendland et al., 1995).

The mating-type loci can have as many as hundreds of different alleles or specificities (Raper, 1966). In large part, this variation can be explained by the structure of

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the mating-type loci in which each mating-type locus is comprised of two or more closely linked subloci (Halsall et al., 2000; Kües and Casselton, 1993). Recombination between subloci creates new combinations of alleles at the subloci; each combination confers a unique mating-type specificity. As a result of the high number of mating-types, any two haploid individuals that encounter each other in nature are likely to be compatible. The requirement for heterozygosity at the mating-type loci allows the maintenance of numerous mating-types due to balancing selection. The action of balancing selection extends the life of each mating-type allele so that its duration in the population is over a much longer time scale than an allele at a neutral locus (May et al., 1999). This, in turn, causes a large level of sequence divergence between mating-types due to their ancient divergence times (Badrane and May, 1999; Stankis et al., 1992). For example the range of DNA sequence similarity between *A* mating-type alleles of *C. cinerea* is 61.9–74.4% (Badrane and May, 1999). While interesting, this polymorphism has made studying mating-type very difficult. For example, mating-types do not cross-hybridize in Southern analyses (Halsall et al., 2000; Pardo et al., 1996), and the design of primers for PCR is difficult because of the lack of conserved regions. For this reason, molecular data on homobasidiomycete mating-type is almost entirely restricted to the model species *C. cinerea* and *S. commune*. Nonetheless, the *A* mating-type loci of *C. cinerea* and *S. commune* show a number of differences such as: the number of subloci, the amount of recombination between subloci, and the lengths of the mating-type proteins (Casselton and Olesnick, 1998; Hiscock and Kües, 1999). In order to have a better understanding of the canonical organization of the mushroom mating-type genes it is necessary to have data on multiple heterothallic species.

The *A* mating-type loci of *S. commune* and *C. cinerea* were first cloned using a long (~50 kb) chromosome walk from the *PABI* gene (Giasson et al., 1989; Mutasa et al., 1990). This approach, as well as other sophisticated approaches to cloning mushroom mating-type genes, relies on the functional identification of the genes by fungal transformation (May et al., 1991; O'Shea et al., 1998; Specht, 1995). A molecular approach that does not use transformation could have advantages in speed and utility in non-model organisms, e.g., those that cannot be transformed. One method that shows promise for the cloning of mating-type genes is the use of conserved gene arrangements for positional cloning. For example, the *A* mating-type loci of *C. cinerea* and *S. commune* show very close (<1 kb) physical linkage to the gene encoding a mitochondrial intermediate peptidase (*MIP*) (Casselton et al., 1995; Stankis et al., 1992). *MIP* appears to have no function in the mating process and instead has been shown to be responsible for processing proteins that are bound for the mitochondrion (Isaya et al., 1995).

Recently, the conservation of the *MIP* and *A* mating-type gene arrangement was exploited to clone the *A* mating-type locus from *Coprinopsis scobicola* (= *Coprinus bilanatus*; Kües et al., 2001). A probe for the *MIP* gene from *C. cinerea* was used to recover cosmid clones from a *C. scobicola* library. These cosmid clones contained both *MIP* and homologues of the *C. cinerea A* mating-type genes as determined using transformation and DNA sequence analysis (Kües et al., 2001). Unfortunately the *C. cinerea MIP* gene displayed no hybridization with other, more distantly related, homobasidiomycete species (*Agrocybe* and *Armillaria*), suggesting that the sequence evolution of *MIP* is not very conservative (U. Kües and C. Mohammed, unpublished results).

In this study, we explored linkage of *MIP* and the *A* mating-type locus in diverse mushroom species. A degenerate PCR approach was used to amplify and sequence the *MIP* gene from over 35 species. We then tested the cosegregation of *MIP* and *A* mating-type among progeny from seven species and found no evidence for recombination between the two loci. These data suggest that an approach utilizing conserved gene order could be useful for cloning *A* mating-type genes from non-model mushroom species.

2. Materials and methods

2.1. Specimens, strains, and growth conditions

Sequences for *MIP* were obtained from cultured mycelium as well as freshly collected fruitbodies (Table 1). Cultures were obtained by plating dilutions of basidiospores on 1.5% malt extract agar medium. Homokaryotic strains were obtained by subculturing singly germinated spores under a dissecting microscope. For DNA extraction, strains were grown in 2 ml of 1.5% malt extract broth or potato dextrose broth (Difco) in 10 ml screw-capped test tubes, under stationary growth, and in the dark. For long term storage, the strains are maintained on malt extract agar (MEA) slants at 4 °C and are available from the authors upon request.

2.2. Mating-type determination

A progeny set consisted of 12 or more homokaryotic strains derived from a single wild collected fruitbody (or in the case of *Pleurotus djamor* from a test cross between two wild homokaryotic strains). Before crossing experiments, all homokaryotic isolates were subcultured twice to insure genetic homogeneity. The mating-types of a set of progeny were determined by crossing at least 10 homokaryons in all possible combinations to establish the two (if unifactorial) or four (if bifactorial) mating-type

Table 1
List of species, strains, PCR primers, and GenBank accession numbers used to generate *MIP* sequences

Species	Strain/source	PCR primers	GenBank Accession No.
<i>Agaricus bisporus</i>	<i>Grocer</i> ^a	MIP2F–MIP1R	AY179564
<i>Amanita cokeri</i>	<i>RV5</i>	MIP1F–MIP1R	AY179565
<i>Amanita virosa</i>	<i>TJ00137</i>	MIP2F–MIP3R	AY179566
<i>Campanella subdendrophora</i>	<i>D8</i>	MIP1F–MIP1R	AY179567
<i>Coprinellus disseminatus</i>	<i>C345.1</i>	Cosmid ^b	AY179562
<i>Coprinellus heptemerus</i>	<i>C299</i>	MIP1F–MIP1R	AY179568
<i>Coprinopsis lagopus</i>	<i>TJ00195</i>	MIP1F–MIP1R	AY179569
<i>Coprinopsis narcotica</i>	<i>C249</i>	MIP1F–MIP1R	AY179570
<i>Coprinopsis scobicola</i>	<i>Cb.M8</i>	Cosmid	AY179561
<i>Crepidotus subfibrillosus</i>	<i>MCA371</i>	MIP1F–MIP2R	AY179571
<i>Cryptococcus neoformans</i>	<i>SGTC</i> ^c	NA ^d	NA
<i>Ganoderma resinaceum</i>	<i>379845</i>	MIP1F–MIP1R	AY179572
<i>Gyrodon merulioides</i>	<i>NCJ12</i>	MIP1F–MIP1R	AY179573
<i>Hygrocybe conica</i>	<i>SC28</i>	MIP2F–MIP3R	AY179574
<i>Hypholoma fasciculare</i>	<i>TJ00149</i>	MIP1F–MIP2R	AY179575
<i>Irpex lacteus</i>	<i>TJ00139</i>	MIP1F–MIP1R	AY179576
<i>Lactarius indigo</i>	<i>TJ00132</i>	MIP1F–MIP1R	AY179577
<i>Lentaria</i> sp.	<i>TJ00128</i>	MIP1F–MIP1R	AY179578
<i>Lenzites betulina</i>	<i>TJ00105</i>	MIP2F–MIP3R	AY179579
<i>Lepiota americana</i>	<i>TJ00190</i>	MIP1F–MIP2R	AY179580
<i>Leucoagaricus gongylophorus</i>	<i>SAR 000701-1</i>	MIP1F–MIP2R	AY338827
<i>Leucocoprinus luteus</i>	<i>TJ00198</i>	MIP1F–MIP2R	AY179581
<i>Marasmius rotula</i>	<i>VA595</i>	MIP1F–MIP1R	AY179582
<i>Marasmius siccus</i>	<i>TJ00148</i>	MIP2F–MIP3R	AY179583
<i>Mycena</i> sp. A	<i>TJ00146</i>	MIP1F–MIP1R	AY179584
<i>Mycena</i> sp. B	<i>TJ00188</i>	MIP1F–MIP1R	AY179585
<i>Nigroporus vinosus</i>	<i>TJ00145</i>	MIP1F–MIP1R	AY179586
<i>Phanerochaete chrysosporium</i>	<i>DOE-JGT</i> ^e	NA	NA
<i>Pleurotus cystidiosus</i>	<i>D419</i>	MIP1F–MIP2R	AY179587
<i>P. djamor</i>	<i>RV95/957.30</i>	Cosmid	AY179563
<i>P. ostreatus</i>	<i>D330</i>	MIP1F–MIP1R	AY179588
<i>Polyporus</i> sp.	<i>TJ00142</i>	MIP1F–MIP1R	AY179589
<i>Pulcherricium caeruleum</i>	<i>TJ00115</i>	MIP2F–MIP3R	AY179590
<i>Russula</i> sp.	<i>TJ00135</i>	MIP1F–MIP1R	AY179591
<i>Saccharomyces cerevisiae</i>	GenBank	NA	AAA21278
<i>Schizophyllum amplum</i>	<i>RGT970618/01</i>	SMEP1F–SMEP1R	AY179592
<i>S. commune</i>	GenBank	NA	P37932
<i>S. fasciatum</i>	<i>CBS267.60</i>	SMEP1F–SMEP1R	AY179593
<i>S. umbrinum</i>	<i>FL012.1</i>	SMEP1F–SMEP1R	AY179594
<i>Schizosaccharomyces pombe</i>	GenBank	NA	Q10415
<i>Stereum ostrea</i>	<i>TJ00126</i>	MIP1F–MIP1R	AY179595
<i>Suillus pictus</i>	<i>RV98/115</i>	MIP1F–MIP1R	AY179596
<i>Trametes versicolor</i>	<i>J114.1</i>	MIP1F–MIP1R	AY179597
<i>Trichaptum bifforme</i>	<i>TJ00170</i>	MIP1F–MIP2R	AY179598
<i>Xerocomus nigromaculatus</i>	<i>MCA513</i>	MIP1F–MIP1R	AY179599

Sources listed in italics indicate *MIP* sequences that were obtained from wild collected fruit bodies. “PCR primers” indicate which primers were used for the amplification of the given *MIP* gene fragment.

^a Sample obtained from local grocery store.

^b Sequence derived from a cosmid containing full-length sequence.

^c Assembly released March 28, 2003; *C. neoformans* Genome Project, Stanford Genome Technology Center, funded by the NIAID/NIH under cooperative agreement U01 AI47087, and The Institute for Genomic Research, funded by the NIAID/NIH under cooperative agreement U01 AI48594.

^d Not applicable.

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tester strains. Selected tester strains from each initial cross were subsequently used to assign mating-types to the remaining homokaryons of a progeny set. Assignment of incompatible reactions to common-*A* mating-

types was accomplished, when possible, using the formation of the characteristic “flat” interaction (Kües et al., 2002; Raper, 1966). Crosses were on nutrient media with 1.5% agar in 10 cm petri dishes. For

Coprinellus disseminatus, *Irpex lacteus*, and *P. djamor*, crosses were performed on half-strength Emerson's YpSs (Emerson, 1940). For *Hypholoma fasciculare*, *Lenzites betulina*, *Mycena* sp. A, and *Trichaptum biforme*, crosses were on 1.5% MEA. The inocula for the crosses were placed ~1 cm apart in the center of the petri dish and incubated at room temperature in the dark. Mating success was judged by examining the gross morphology of the paired strains after 1–3 weeks and by examining the growing margins of the paired strains under the light microscope for the presence of clamp connections that indicate dikaryotization has occurred.

2.3. DNA manipulations

DNA was extracted from basidiocarps and lyophilized mycelium of cultures following a CTAB miniprep protocol (Zolan and Pukkila, 1986). Amplification of partial *MIP* gene fragments from *Schizophyllum* spp. was accomplished using a pair of non-degenerate primers SMEP1F (5'-CGGCGGTCTCGAATGGCACGAAGT-3') and SMEP1R (5'-TCTTCCACCGCCCGACAGTCGCCAT-3'). Amplification of *MIP* from other homobasidiomycete species was accomplished using a set of several degenerate oligonucleotide primers. The sequences of the primers are: MIP1F (5'-CCATYCTMATGGARCACTTCTCA-3'), MIP2F (5'-TTCCAYGARATGGGNCAYGCCNAT-3'), MIP1R (5'-ACCTCYKCTTGTACYKYTCGCC-3'), MIP2R (5'-RTANCCRAANARRTGNCRAA-3'), and MIP3R (5'-TARTANGTSGCNCCRTANCC-3'). One pair of forward (F) and reverse (R) primers was used for each species. The degenerate nucleotide positions follow the IUPAC-IUB standard. The specific combinations of primers that were

found to be successful are indicated in Table 1. Amplification conditions were: ~1 ng template DNA, 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.5 μM each primer, and 0.625 U *Taq* polymerase in a 25 μl reaction. Thermal cycling parameters were an initial denaturation step at 94 °C for 3 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. Annealing temperatures were varied from between 42 and 55 °C in order to optimize the amplification of *MIP* PCR fragments and to reduce background amplification. Putative *MIP* fragments were purified on agarose gels using a QIAquick Gel Extraction Kit (Qiagen) and ligated into pCR2.1 (Invitrogen). Plasmids were propagated in *Escherichia coli* strains XLI-Blue (Stratagene) and TOP10 (Invitrogen). Plasmid templates for DNA sequencing were prepared using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced on both strands using universal forward and reverse M13 primers. Sequencing reactions utilized the BigDye sequencing kit (Applied Biosystems) and were analyzed on ABI377 or ABI3700 DNA sequencers. The segregation of *MIP* among the progeny sets of seven species was analyzed using PCR-RFLP or allele-specific PCR. New primers for PCR were often designed for more efficient amplification (see Table 2). Restriction digests of PCR products were conducted according to manufacturer's instructions (Promega; New England Biolabs).

2.4. Full-length *MIP* sequences

Sequences for the entire *MIP* coding region were also obtained from six species. For the species *C. scobicola*, *C. disseminatus*, and *P. djamor*, *MIP* genes were cloned

Table 2

Cosegregation of *MIP* and *A* mating-type in seven mushroom species. Next to each species name is given the strain number, progeny from which the segregation data were derived

Species	Cosegregants	Recombinants	Method	Primers
<i>Pleurotus djamor</i> (RV95/957.30 x ATCC38141.104)	99	0	Allele-specific PCR	MIP1F–MIP1R (see Materials and methods)
<i>Irpex lacteus</i> (TJ00/39 & TJ00/63)	33	0	PCR-RFLP (<i>Rsa</i> I)	MIP1F–MIP1R (see Materials and methods)
<i>Hypholoma fasciculare</i> (TJ00/49)	19	0	PCR-RFLP (<i>Sau</i> 3A)	MIP1F–MIP1R (see Materials and methods)
<i>Trichaptum biforme</i> (TJ00/104)	20	0	Allele-specific PCR	TMIP1F (5'-CTCACCCACTGTACTCAGTCTCTT-3')–TMIP1R (5'-TTGAGTTTGCCATGAAGTGC-3')
<i>Lenzites betulina</i> (TJ00/105)	21	0	Allele-specific PCR	LbMIP1F (5'-TGCATTGTACGTCCATGTGTT-3')–LbMIP1R (5'-TTTATACTGCGCGAGAG-3')
<i>Mycena</i> sp. A (TJ00/46)	36	0	PCR-RFLP (<i>Bfa</i> I)	MsMIP1F (5'-TTGACTTGGAGAGCACATCG-3')–MsMIP1R (5'-AAAGGATCCTCGAGAAGACA-3')
<i>Coprinellus disseminatus</i> (TJ00/38)	13	0	PCR-RFLP (<i>Bam</i> HI)	CdMIP-F (5'-CTGCGGGCAACTGGRAACAA-3')–CdMIP-R (5'-GAAGGACGTCTCTGGCACATA-3')

Cosegregants indicate the number of progeny in which no recombination between *MIP* and *A* mating-type occurred, and recombinants give the number of progeny displaying recombination between *MIP* and *A* mating-type. *A* mating-types were assigned based on pairings among progeny. Method indicates how progeny were genotyped at the *MIP* locus. Primers used for PCR are given in the last column. All tested species displayed bifactorial mating systems with the exception of *C. disseminatus*, which has a unifactorial mating system.

from a cosmid library (SuperCos-Pab1) screened for the *MIP* gene using a PCR based approach (Bottoli et al., 1999). The PCR primers used for screening the library were specific to each species and based on *MIP* sequences acquired using the degenerate PCR primers. These full-length *MIP* sequences were generated during the positional cloning of *A* mating-type genes from a cosmid library screened for *MIP* genes (the complete results will be published elsewhere). For *Leucoagaricus gongylophorus*, a mushroom symbiont of leafcutter ants, the full-length *MIP* gene was amplified and sequenced using the GenomeWalker Kit (Clontech). The initial *MIP* PCR fragment was obtained using degenerate PCR. For *Cryptococcus neoformans*, *Phanerochaete chrysosporium*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *S. commune*, the sequences were taken from either genome sequencing projects or from GenBank. The GenBank accession numbers of sequences used in this study are listed in Table 1.

2.5. Data analyses

The nucleotide sequences were translated into amino acid sequences following manual parsing of introns. Introns were identified by the inspection of the sequences for the consensus splice site (GTNNR-) and acceptor (-YAG) motifs using the translation of the nucleotide sequences as a guide for locating intron positions. Alignment of full-length *MIP* amino acid sequences was conducted using Clustal X (Thompson et al., 1997) with parameters: scoring matrix = Blosum30, pair wise gap opening = 10, pair wise gap extension = 0.1, multiple gap opening = 5, and multiple gap extension = 0.2. The alignment was adjusted manually by eye using the GeneDoc v2.6 editor (Nicholas and Nicholas, 1997). A sliding window plot of probability of amino acid identity over the alignment was calculated by hand using the observed amino acid frequencies for each aligned position (window size = 20; step size = 5). Gapped positions were considered a 21st character state for this analysis. Partial *MIP* DNA sequences were translated into amino acid sequences and aligned by eye with GeneDoc. The alignment of partial *MIP* sequences was 215 amino acids long. For phylogenetic analyses, a region of 18 amino acids in the alignment was excluded due to ambiguity in the alignment. Phylogenetic trees were generated using a maximum likelihood analysis as implemented in the program PROTML of the software package MOLPHY v2.3 (Adachi and Hasegawa, 1996). Tree searching employed 1000 heuristic quick add OTUs searches. The most likely of these trees was used for local rearrangement and statistical support was estimated using approximate bootstrap probabilities by the REL method (Hasegawa and Kishino, 1994). All likelihood models used the JTT-F substitution matrix.

3. Results

3.1. Variation within the *MIP* gene

Whole length *MIP* DNA sequences were obtained for six homobasidiomycete species (*C. scobicola*, *C. disseminatus*, *L. gongylophorus*, *P. chrysosporium*, *P. djamor*, *S. commune*), the heterobasidiomycete *C. neoformans*, and two ascomycete yeast species (*S. cerevisiae*, *S. pombe*). Following removal of the introns, the DNA sequences were translated into amino acids and aligned using ClustalX. The positions of three introns were conserved in all homobasidiomycete *MIP* sequences investigated. The *C. neoformans* *MIP* gene also contains three introns, but only two of these are shared with that of the homobasidiomycetes. The sliding window analysis of amino acid identities showed amino acid conservation to vary widely over the length of the molecule (Fig. 1). The conservation of homobasidiomycete protein sequences (68.2% pair wise identity) was greater, but of the same shape, than comparisons that included the ascomycete and *C. neoformans* sequences (Fig. 1). Complete conservation of the putative zinc-binding domain within the active site of *MIP*, L-F-H-E-M-G-H-A-M (amino acids 556–564 of *S. commune*; Isaya et al., 1995), was observed among all fungi with the exception of *C. neoformans* in which the second methionine is replaced by isoleucine. In addition, the two cysteines implicated in protein stability (C131 and C581 in *S. cerevisiae*) are completely conserved in the included fungi (Chew et al.,

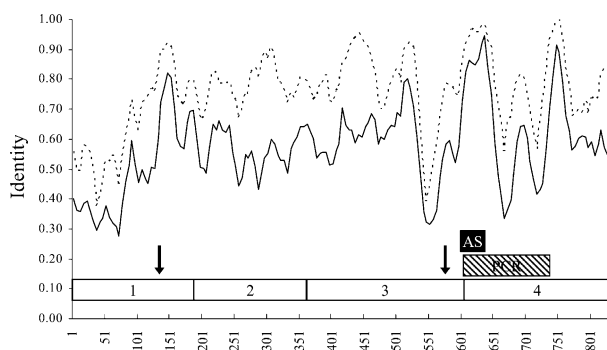


Fig. 1. Schematic of the exon structure of the *MIP* gene and the genetic variability along the *MIP* protein. Identity values were calculated for the full-length sequences (835 aligned amino acids) over a sliding window of size 20 amino acids with a step size of five amino acids. The solid line indicates the percent pair wise identity of all nine fungal sequences and the dotted line indicates the identity of only the homobasidiomycete sequences. The corresponding positions of the four exons found in the six homobasidiomycetes are indicated with boxes labeled 1–4. The conserved region that was targeted for DNA amplification of diverse homobasidiomycetes by degenerate PCR is indicated as a hatched bar above exons. The conserved amino acid sequence corresponding to the putative zinc-binding domain and active site (Isaya et al., 1995) is indicated with a black bar. Arrows indicate the two completely conserved cysteine residues that are important in protein stability (Chew et al., 1996).

1996). These results suggest the protein sequences analyzed in this study are likely to all be orthologous and function as mitochondrial intermediate peptidases.

3.2. Cloning *MIP* by degenerate PCR

Primers were designed to amplify partial *MIP* gene fragments from homobasidiomycetes by targeting the conserved regions of the protein, including the zinc-binding region (Fig. 1). Variable success in amplification of fungal DNA was observed. The most successful primer pairs were MIP1F–MIP1R and MIP2F–MIP3R. Using the battery of degenerate primers, *MIP* sequences were obtained from ~40% of homobasidiomycetes tested (Table 1). Groups which generally amplified well with the *MIP* primers were the polypores, boletes, Russulaceae, and Psathyrellaceae. Samples with low success in amplification were from the Phallales, Amanitaceae, Tricholomataceae, and Gasteromycetes. There was no more success in amplifying samples of one mating system over another. In addition, no success was found using any primer combinations with any heterobasidiomycete DNA (*Auricularia* spp., *C. neoformans*, *Septobasidium curtisii*, and *Tremella* spp.). The inability to amplify *MIP* from the majority of specimens attempted is probably due to poor quality DNA or multiple substitutions in the primer-binding regions.

The positive amplification products were gel purified, ligated into a plasmid, and then multiple sequences were typically obtained for each cloned PCR product. The multiple sequences from a single haploid strain were all identical. When multiple sequences from dikaryotic (presumably) fruiting bodies or cultures were obtained, the amount of variation was rather low and no more than two sequence types were obtained. For 13 of these dikaryotic samples the mean variation between the two copies was 2.3% at the nucleotide level (range 0.2–8.0%) and 0.7% at the amino acid level (range 0.0–3.3%). Such variation within a strain could be attributable to allelism or *Taq* polymerase error. As a comparison, the mean variation at *MIP* between haploid strains of *S. commune* collected from the eastern United States is 6% at the nucleotide level (range 0–9%) and 1% at the amino acid level (range 0–3%; T.Y. James, unpublished data). This, in turn, is much less than the variation observed between species.

3.3. *MIP* phylogeny

The partial sequences of the *MIP* gene fragments produced from subcloned PCR products were translated into amino acids and aligned to existing sequences by eye. The short (215 amino acids) region of mostly overlapping sequence was used for a phylogenetic analysis of protein sequences using maximum likelihood. The phylogeny produced from the *MIP* sequences

shows general agreement with the known organismal systematics of homobasidiomycetes (Fig. 2). Namely, the major clades of homobasidiomycetes group together, with monophyletic Euagarics (with the exception of *Hygrocybe conica*), Russuloid, Polyporoid, and Bolete clades (Hibbett and Thorn, 2001). In addition, all genera from which multiple species were sampled formed clades (e.g., *Marasmius*, *Pleurotus*, and *Schizophyllum*) with at least 80% bootstrap support. Most of the deeper phylogenetic nodes are poorly supported by bootstrap probabilities as would be expected considering the limited data in this short amino acid alignment.

3.4. Cosegregation of *MIP* and the *A* mating-type locus

For seven of the culturable, heterothallic species from which partial or complete *MIP* sequences were obtained (Table 2), progeny were obtained from individual fruiting bodies and crosses were performed to determine mating-types of the progeny. Taxa were selected to maximize the phylogenetic diversity of the chosen species. The bias towards bifactorial ($n = 6$) over unifactorial ($n = 1$) species was incidental and based on availability of homokaryotic progeny arrays. The cosegregation of *MIP* and the *A* mating-type of the seven species was tested by genotyping the progeny at the *MIP* locus using molecular methods that distinguished between the two *MIP* alleles within the parental dikaryon (see Fig. 3 for PCR-RFLP of *Mycena* sp. A). We tried to differentiate between the *A* and *B* mating-type loci using the formation of the characteristic “flat” mycelium that develops when two crossed homokaryons share an *A* mating-type allele but have different *B* mating-type alleles. The “flat” morphology was only consistently observed in *T. biforme*. The flat morphology of *T. biforme* displayed complete intermingling of the two strains, but the resulting heterokaryon had highly reduced aerial hyphal production, was slower growing, and had irregular branching hyphae. Such morphology is similar to that described for common *A* mating-type pairings in other homobasidiomycetes (Raper, 1966), and was observed in several, but not all, common *A* pairings of *L. betulina*, and *P. djamor*. Nonetheless, each of the six bifactorial progeny arrays displayed mating patterns that suggested the segregation of two mating-type loci. When compared with the segregation data for the *MIP* locus, one of the two mating-types demonstrated complete cosegregation with *MIP*. The mating-type locus linked to the *MIP* locus corresponded to the *A* mating-type as evidenced by the crossing data from *T. biforme*, *P. djamor*, and *L. betulina*. The *A* mating-type is also presumably linked to *MIP* in the remaining two bifactorial species, *Mycena* sp. A and *H. fasciculare*. Although the *A* and *B* loci could not be ultimately distinguished by the morphology of the crosses with these two species, both species displayed cosegregation of one mating-type

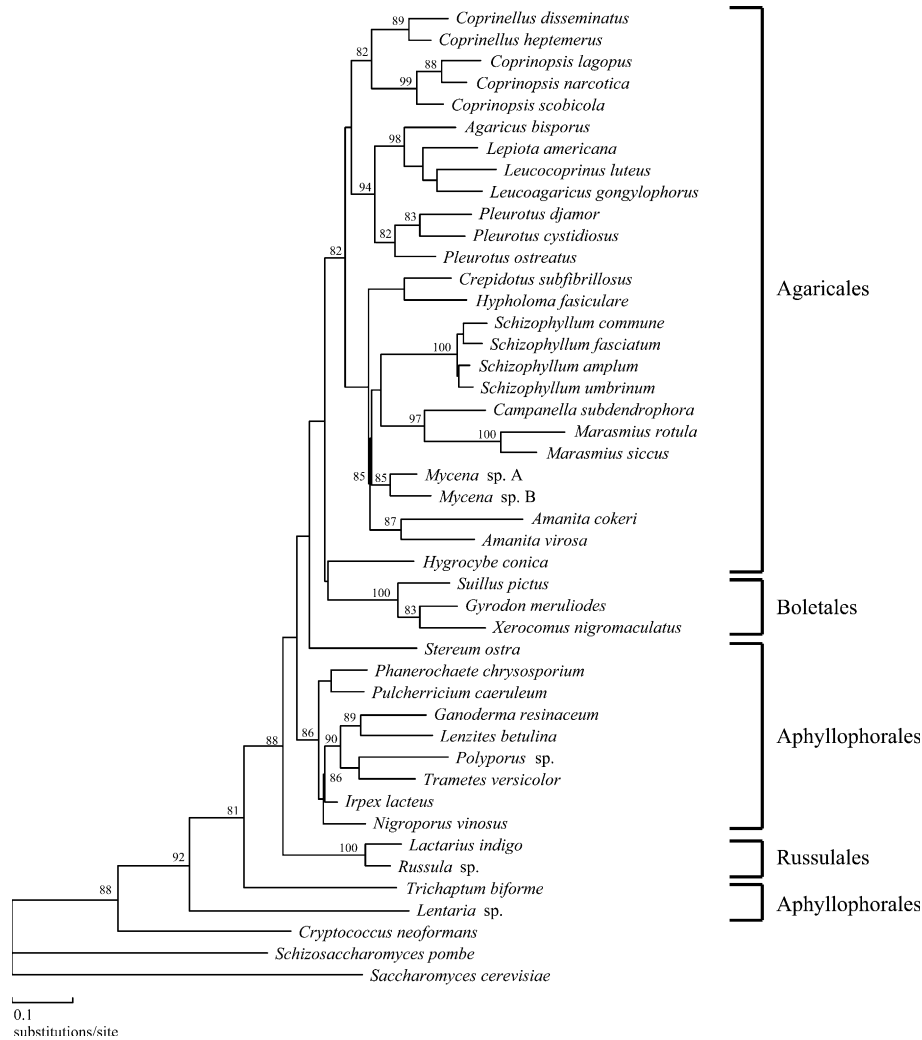


Fig. 2. Phylogeny of partial MIP sequences (based on hatched region in Fig. 1). Phylogenetic estimation used the maximum likelihood criterion as implemented in the program ProtML (Adachi and Hasegawa, 1996). Numbers above nodes indicate approximate bootstrap probabilities estimated by the RELL method. Only values above 80% are shown.

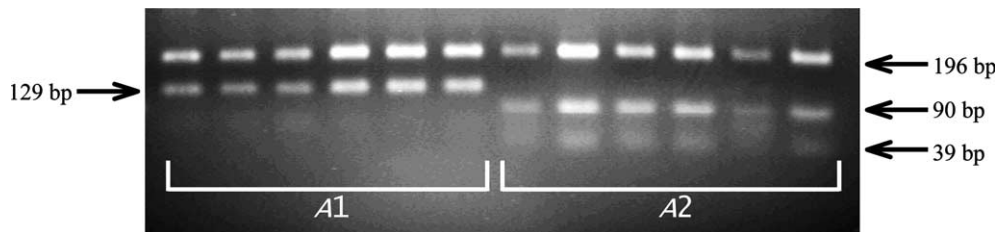


Fig. 3. Segregation of MIP among progeny array of *Mycena* sp. A ($n = 12$). Shown is an agarose gel of PCR amplicons digested with *Bfa*I. Initial amplicon of MIP allele linked to mating-type A1 is digested into two fragments of size 196 and 129 bp. Introduction of a novel *Bfa*I site into MIP allele linked to mating-type A2 results in digestion of 129 bp fragment into 90 and 39 bp fragments.

locus (putatively the *A* mating-type locus) with MIP. Finally, for the unifactorial species (*C. disseminatus*), the MIP locus appears to cosegregate with its single mating-type locus. Thus, the segregation data showed no evidence for any recombination between MIP and the *A* mating-type in each species tested, suggesting tight genetic linkage of these loci in the seven species (Table 2).

Irpex lacteus is a wood decaying homobasidiomycete that grows vigorously in culture, but unlike most of its relatives, *I. lacteus* does not produce clamp connections in culture or in wild collected fruiting bodies (Gilbertson and Ryvarden, 1986). As a result, the mating system of *I. lacteus* is unknown. Our preliminary results showed crosses among certain single-spore derived isolates

interacted antagonistically, while other crosses appeared to commingle and form a new mycelial type with greater production of aerial mycelium. No single-spore isolates of *I. lacteus* produced fruitbodies in culture, however several putative heterokaryons derived from crossing these single-spore isolates produced fruitbodies containing basidiospores. All of these data suggest an outcrossing mode of reproduction for *I. lacteus*. We analyzed the mating patterns and segregation of *MIP* in two progeny arrays of *I. lacteus* (field collections TJ00/39 and TJ00/63) and used these data to help understand its mating system. Both progeny arrays were each segregating for two *MIP* alleles suggesting that the fruiting bodies of *I. lacteus* are dikaryotic and that single-spore isolates are homokaryotic. Matings for the two progeny arrays were scored using gross morphology and found to conform to a bifactorial pattern for both progeny arrays. Furthermore, all of the crosses within a progeny array that displayed the vigorous, cottony growth attributed to dikaryotization were those between isolates that differed at the *MIP* locus. When the putative mating-type loci were compared with the segregation of *MIP*, there appeared to be a one-to-one correspondence with allele at *MIP* and putative *A* mating-type, suggesting cosegregation of these two loci in *I. lacteus* as in the other six homobasidiomycetes.

4. Discussion

The mitochondrial intermediate peptidase is a single-copy, nuclear encoded enzyme that functions in the mitochondrion to cleave protein precursors that are in transit to the mitochondrial matrix and inner membrane. *MIP* cleaves the leader peptide from the N-terminus of these precursors after they have been initially cleaved by the mitochondrial processing peptidase (Gakh et al., 2002; Isaya and Kalousek, 1995; Kalousek et al., 1988). This study has investigated *MIP* in the homobasidiomycete fungi, and has specifically examined the linkage of this gene to the homeodomain encoding mating-type genes (traditionally the *A* mating-type locus).

Although previous molecular investigations have not suggested any functional relationship between *MIP* and mating in the mushroom fungi (Casselton et al., 1995; Kües et al., 2001; Specht et al., 1992), the data presented here and the previous molecular investigations show very tight linkage of *MIP* to the *A* mating-type locus in these fungi. Since the seven fungal species for which the cosegregation of the two loci was studied (*C. disseminatus*, *H. fasciculare*, *I. lacteus*, *L. betulina*, *Mycena* sp. A, *P. djamor*, and *T. biforme*) together with *C. cinerea*, *C. scobicola*, and *S. commune* represent a large part of the diversity of this class of fungi, this linkage has been conserved over the course of hundreds of millions of

years of evolution (Berbee and Taylor, 2001). To our knowledge, it is only the homobasidiomycete fungi that show a linkage between *MIP* and mating-type. For example, in *S. cerevisiae*, *S. pombe*, and *C. neoformans*, the mating-type locus is on a different chromosome than *MIP* in each of these fungi (Cherry et al., 1997; Wood et al., 2002; A. Forche and T.Y. James, unpublished).

It is quite possible that the linkage between *MIP* and the *A* mating-type locus is an historical association that bears no functional significance. In fact, the very close proximity (<1 kb) of *MIP* and the *A* mating-type genes in *S. commune*, *C. cinerea*, and *C. scobicola* (Kües et al., 1992, 2001; Specht et al., 1992) would make random translocations that disrupt this linkage very rare. However, our unpublished population genetic studies in *S. commune* and *C. disseminatus* suggest that a low level of recombination does occur between *MIP* and the *A* mating-type because the *MIP* genotype of a given haploid strain provides no information regarding the strain's mating-type. In these studies, we sequenced *MIP* fragments from a population of haploid isolates of determined mating-type and did not find a one-to-one correspondence with an isolate's allele at the *MIP* locus and an isolate's *A* mating-type. Recombination between the loci suggests that *MIP* does not function in a mating-type-specific manner, but it is uncertain whether *MIP* has a general function in the mating process in the homobasidiomycetes, e.g., in the control of mitochondrial inheritance (Röhr et al., 1998). Evidence for the plausibility of this hypothesis derives from ΔMIP strains in *S. cerevisiae* which undergo loss of functional genomes, as evidenced by their inability to complement strains deficient in mtDNA replication or strains with mutations in mitochondrial genes (Branda and Isaya, 1995). However, such effects are presumably indirect and due to improper N-terminal processing of proteins involved in mitochondrial DNA replication and other mitochondrial-specific functions, such as respiration.

The phylogeny of the *MIP* amino acid sequences (Fig. 2) shows good agreement with our current understanding of mushroom systematics (Hibbett and Thorn, 2001). This in turn suggests that *MIP* has not undergone cycles of frequent duplication and loss that would create paralogy of the sampled gene sequences. In addition to maintenance of conserved gene order, if the positional cloning approach is to work, then *MIP* must exist as a single copy in the genome. The precedent of more than a single copy of *MIP* in a genome has been established by the presence of two distinct, but similar, copies in the latest release of the *C. neoformans* Genome Project (March 2003, <http://www-sequence.stanford.edu/group/C.neoformans/index.html>). Nonetheless, most current evidence points to a single gene copy in the genomes of most homobasidiomycetes. In this study, only a single copy of *MIP* has ever been amplified from a haploid strain, and when two copies have been isolated from the

same dikaryotic strain, they are very similar in amino acid sequence (>96%). Secondly, Southern analysis of *MIP* in *C. scobicola* (Kües et al., 2001) and *S. commune* (Specht et al., 1994) suggests only a single copy of *MIP* per haploid genome. Finally, database searches against near complete genomes reveal only a single *MIP* orthologue in all probed genome sequences (*Aspergillus nidulans*, *C. cinerea*, *Fusarium graminearum*, *Neurospora crassa*, *S. cerevisiae*, *S. pombe*, *Ustilago maydis*) with the exception of *C. neoformans*.

The probable tight linkage between *MIP* and the *A* mating-type genes in homobasidiomycetes can be exploited for positional cloning of these mating-type genes from novel species. *MIP* can be isolated through degenerate PCR or hybridization with heterologous probes. A set of degenerate PCR primers were developed, and these, while not universally applicable, could be again modified to more specifically target certain mushroom species or taxa. Approaches for positional cloning of mating-type genes from mushroom fungi could take several trajectories once *MIP* has been cloned. One approach could be to probe large insert libraries (e.g., BAC, cosmid, and phage) for *MIP* DNA. If the linkage has been maintained, an overlapping set of clones has a high probability of containing mating-type genes (Kües et al., 2001, Mutasa et al., 1990). Those clones can be identified by fungal transformation, scoring for a phenotype such as clamp connections (Giasson et al., 1989, Mutasa et al., 1990), or through DNA sequencing and identification of mating-type genes through database comparisons. Other methods that may be successful for isolating mating-type genes using a *MIP* anchor are short chromosomal walking methods such as inverse PCR (Ochman et al., 1988) and TAIL-PCR (Liu and Whittier, 1995).

The study of mating in mushroom species which are difficult to work with, such as those which are unculturable or whose spores fail to germinate could be accelerated through positional cloning methods. In fungi for which mating cannot be accomplished in the laboratory, the cloning of mating-type genes could provide information both on their mating system and population genetics. A conserved gene order approach that utilized DNA sequencing for identification of mating-type genes would also be applicable for species which are not suitable for genetic transformation. A further benefit of studying the *MIP* gene in non-model species is that its segregation may help distinguish the *A* from the *B* mating-type locus in progeny arrays which inconsistently produce “flat” mating-type reactions in pairings, under the assumption that linkage between the *A* mating-type and *MIP* has been conserved.

In summary, the data presented herein suggest a tight linkage between the *MIP* locus and the *A* mating-type locus of homobasidiomycetes has been conserved throughout much of their evolution. This conserved

linkage can be exploited to overcome the difficulties in cloning imposed by the hypervariability of the mating-type loci. We have successfully used the conserved gene order approach to clone the *A* mating-type gene homologues from *C. scobicola* (Kües et al., 2001). Mating genes were isolated by probing a cosmid library with a heterologous *MIP* probe from *C. cinerea*. The *A* mating-type genes of two additional mushroom species (*C. disseminatus* and *P. djamor*) have been isolated using the procedure proposed here. The results of these studies will be published elsewhere.

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