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The chromosomal region containing *pab-1*, *mip*, and the *A* mating type locus of the secondarily homothallic homobasidiomycete *Coprinus bilanatus*

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Abstract In this paper we describe the cloning of the DNA region containing the *AI* mating type genes of the secondarily homothallic mushroom *Coprinus bilanatus* and compare its organization to that of heterothallic homobasidiomycetes. As in other species, the *C. bilanatus* *A* factor contains several different genes that encode two different types of homeodomain transcription factor (HD1 and HD2); and some of these genes are active in the heterologous host *C. cinereus*. The *HD1* and *HD2* genes are distributed over two closely linked subloci, *A α* and *A β* . A gene coding for a mitochondrial intermediate peptidase (*mip*) directly flanks the *A α* sublocus. The *pab-1* gene, required for para-aminobenzoic acid synthesis, is found 39 kb upstream of *mip*. The structural arrangement of this chromosomal region closely resembles the heterothallic *C. cinereus*. In contrast, the *A α* and *A β* subloci of *Schizophyllum commune* are further separated, with *pab-1* located between the two subloci, suggesting that a translocation event may have occurred during evolution.

Key words *A* Mating type genes · Homeodomain proteins · Mitochondrial intermediate peptidase gene · Para-aminobenzoic acid synthase gene

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Introduction

The life cycle of a typical homobasidiomycete alternates between two mycelial forms, the monokaryon and the dikaryon. The dikaryon arises from the fusion of two compatible monokaryons, an event controlled by the mating type loci. Many basidiomycetes have two unlinked mating type loci (*A* and *B*) that segregate randomly within sexual progeny. Most fascinating in terms of evolution and population genetics, the mating type loci in basidiomycetes have developed multiple specificities. Several mating type alleles (haplotypes) have been cloned from the heterothallic *Coprinus cinereus* and *Schizophyllum commune*. In both species, the *A* haplotypes contain pairs of functionally redundant, paralogous genes distributed between two closely linked subloci (*A α* and *A β*) previously identified from classical genetics. These genes encode two classes of homeodomain transcription factors (HD1 and HD2). Through mating, HD1 and HD2 products from allelic gene pairs are brought together to perform a compatible protein interaction. There are several HD1 and HD2 proteins present within dikaryotic cells and compatible protein combinations derived from different haplotypes need to be discriminated from incompatible interactions from the same haplotype. The specificity domains responsible for this discrimination have been localized to the highly variable N-terminals of the proteins (Casselton and Olesnick 1998; Hiscock and Kües 1999; Kües 2000).

A consequence of allelic discrimination is that the *A* genes are very dissimilar in DNA sequence. Alleles of the mating type genes typically have homologies of between 65–80% (Badrane and May 1999; Stankis et al. 1992); and paralogous genes have only 50% or less identity (Kües et al. 1994a; Shen et al. 1996). This low homology does not enable paralogous genes or the alleles of a given *A* mating type gene to cross-hybridize with each other, unlike the DNA regions directly linked to the mating type loci (Giasson et al. 1989; Kües et al. 1992, 1994c; May et al. 1991; Pardo et al. 1996; Specht et al. 1994).

In both *C. cinereus* and *S. commune* the *Ax* locus is flanked by a gene encoding a mitochondrial intermediate peptidase (*mip*), a specific metallo-endopeptidase (Casselton et al. 1995; Isaya et al. 1995). In *C. cinereus*, the *Ax* sublocus and the *Aβ* sublocus are separated by 7 kb of conserved non-coding DNA ('homologous hole'); and the *Aβ* locus is flanked by a short conserved gene of unknown function (*β-fg*; Kües et al. 1992, 1994c). In this study, we have used the *C. cinereus mip* gene (formerly termed *α-fg*; Kües et al. 1994a, c) to identify cosmids covering the *A* mating type region of *Coprinus bilanatus*, a two-spored, secondarily homothallic species with two multi-allelic mating type loci (Kemp 1974). *C. bilanatus* and *C. cinereus* belong to section Lanatuli, but phylogenetic analyses show the two species are not closely related (Hopple and Vilgalys 1999; Kühner and Romagnesi 1978). Nevertheless, *C. cinereus HD2* genes were previously shown to elicit *A* regulated clamp cell development when introduced into *C. bilanatus* monokaryons (Challen et al. 1993).

Materials and methods

Fungal strains, cultivation and transformation

C. bilanatus strains were grown at 28 °C on complete yeast extract or on the minimal medium of Raper et al. (1972) and *C. cinereus* strains were grown at 37 °C on YMG/T complete medium or on minimal medium with appropriate supplements (Granado et al. 1997). Genomic DNA was isolated from *C. bilanatus* strain *Cb1* (*A1 B1*; Elliott and Challen 1983). *C. bilanatus* strains R8 (*A2 B3 trp-2*) and S61 (*A3 B1 trp-2*; this study) and *C. cinereus* strains AT8 (*A43 B43 trp-3 ade-8*; Kües et al. 1992), PG78 (*A6 B42 pab-1 trp-1.1.1.6*; Granado et al. 1997), FA2222 (*A5 B6 acu-1 trp-1.1.1.6*) and LN118 (*A42 B42 ade-2 trp-1.1.1.6*; Mutasa et al. 1990) were used in transformation following published protocols (Burrows et al. 1990; Challen et al. 1994; Granado et al. 1997). For co-transformations, either 2 µg of vector pCBT2-S5 containing the *C. bilanatus trp2⁺* gene (Challen et al. 1994), 1 µg of pDB3 containing the *C. cinereus trp3⁺* gene (Burrows 1991), or 1 µg of pCc1001 containing the *C. cinereus trp1⁺* gene (Binninger et al. 1987) were combined with 0.3–2.0 µg of test cosmid DNAs. Phenotypes of transformants were determined from colony morphologies and by the presence of *A*-regulated clamp cells (Kües et al. 1992; Challen et al. 1993).

DNA techniques

Cosmid DNAs were prepared using the protocol of Little (1987) and fungal genomic DNA was prepared by the method of Zolan and Pukkila (1986). DNA manipulation and Southern blot analysis were performed by routine methods (Sambrook et al. 1989). Cosmid DNA was subcloned using pBluescript KS⁻ (Stratagene) and *Escherichia coli* hosts XL1-Blue or DH5α (Sambrook et al. 1989). Agarose gel-purified fragments or plasmid clones were labelled with [α -³²P]CTP using a nick-translation kit (BRL). Hybond-N-membranes (Amersham) were used for Southern blotting. Hybridization with homologous probes was carried out at 65 °C; and stringency was reduced (57 °C) for heterologous probes. Filters of pooled cosmid DNA or colony hybridization of microtitre-ordered *E. coli* clones were prepared as previously described (Bottoli et al. 1999). To sequence the *C. bilanatus Ax* sublocus (a 4.6-kb *EcoRV*–*SphI* fragment; GenBank accession number AF271164), shotgun sub-fragment libraries were generated in pUC119 or pZERO-2 (Invitrogen) following Zhou et al. (1988). Double-strand DNA

sequences were generated using dye terminator chemistries on ABI 373 or 377 DNA sequencers (Perkin Elmer). Sequences were edited and assembled using the Sequencher package (Gene Codes).

C. cinereus mating-type and *pab-1* plasmids

The following pBluescript KS⁻ subclones were used in hybridizations: (1) pHH5 and pHH7 containing the 5' and 3' ends of the *C. cinereus mip* gene on 2.4-kb and 1.1-kb *EcoRI* fragments, respectively, (2) pAMT6 containing a 4.0-kb *HindIII* fragment and pAMT7 containing a 1.9-kb *HindIII* fragment from the 7-kb conserved region ('homologous hole') separating the *C. cinereus Ax* and *Aβ* subloci, (3) pAMT1 containing a 3.0-kb *HindIII* fragment with the *HD1* gene *b1-1* which overlaps the 'homologous hole' at its 3' end, and (4) pUK6 containing *HD1* gene *d1-1* and gene *β-fg* on a 4.8-kb *SalI* fragment (Kües et al. 1992). pST17 contains the *C. cinereus pab-1* gene on a 5.6-kb *PstI* fragment (Granado et al. 1997; Mutasa et al. 1990) and was kindly provided by L.A. Casselton.

Cosmid cloning, mapping and characterization

The genomic library of *C. bilanatus* DNA of strain Cb.M8 (*A1 B1*) in cosmid LoristX has been previously described (Challen et al. 1994). The *C. cinereus mip* insert of pHH5 was used to probe Southern blots of the *EcoRI*-digested library, pooled in 46 lots of 96 different cosmids. Subsequently, individual cosmids were identified with the same probe in colony filter hybridization of the positive pools. Overlapping cosmids were detected by hybridization with a 2.7-kb *BamHI* fragment and a 1.8-kb *HindIII* that directly flanked the Lorist backbone in cosmid 28D4 and cosmid 38F10, respectively.

All cosmids were crudely mapped by comparing them in restriction digests using *BamHI* and *HindIII*, individually and in double-digests. DNA–DNA hybridizations further defined the order of fragments. The following *C. bilanatus* probes were used: the 1.8-kb Lorist flanking *HindIII* fragment of 38F10, cosmid C28H generated through *HindIII* restriction and religation of cosmid 28D4, an 11.5-kb *BamHI* fragment from cosmid 28D4 overlapping cosmid C28H by 3 kb, and the 7.5-kb *HindIII* fragment with the *mip* gene and its adjacent 3.7-kb and 7.7-kb *HindIII* fragments. Conserved genes were localized by hybridization with *C. cinereus* homologues. All probes were used against *BglII*, *Clal*, *EcoRI*, *PstI*, *SalI*, *XhoI* and *XbaI* single and double cosmid digests with *HindIII* to enable more precise mapping.

Results

Isolation of cosmids containing *C. bilanatus A* mating type DNA

The 3' end of the *C. cinereus mip* gene from pHH7 detected a 3.6-kb fragment in *EcoRI*-digested *C. bilanatus* genomic DNA, while the 5' end in pHH5 hybridized to the same 3.6-kb fragment and a 2.2-kb fragment. Both probes hybridized to a 10-kb band in *HindIII*-digested DNA (not shown), indicating the presence of a single *mip* gene in *C. bilanatus*. The pHH5 *mip* probe was further used to isolate three individual *C. bilanatus* cosmids (28D4, 38F10 and 45A8) from the Lorist library (Fig. 1A). Through transformation of various *C. bilanatus* and *C. cinereus* host strains (Table 1), we recovered transformants exhibiting the *A*-regulated clamp cell development. As in earlier experiments (Challen et al. 1993), the morphology of these transformants was changed from the normal condensed growth of monokaryons to the fluffy, less dense colony mycelia. These

observations indicate that the three *C. bilanatus* cosmids contain *A1* mating type DNA and that this haplotype contains genes active in the *A2* and *A3* backgrounds of *C. bilanatus* and a variety of *C. cinereus* mating type backgrounds.

Mapping the *C. bilanatus* *A1* mating type DNA and flanking *mip* and *pab-1* genes

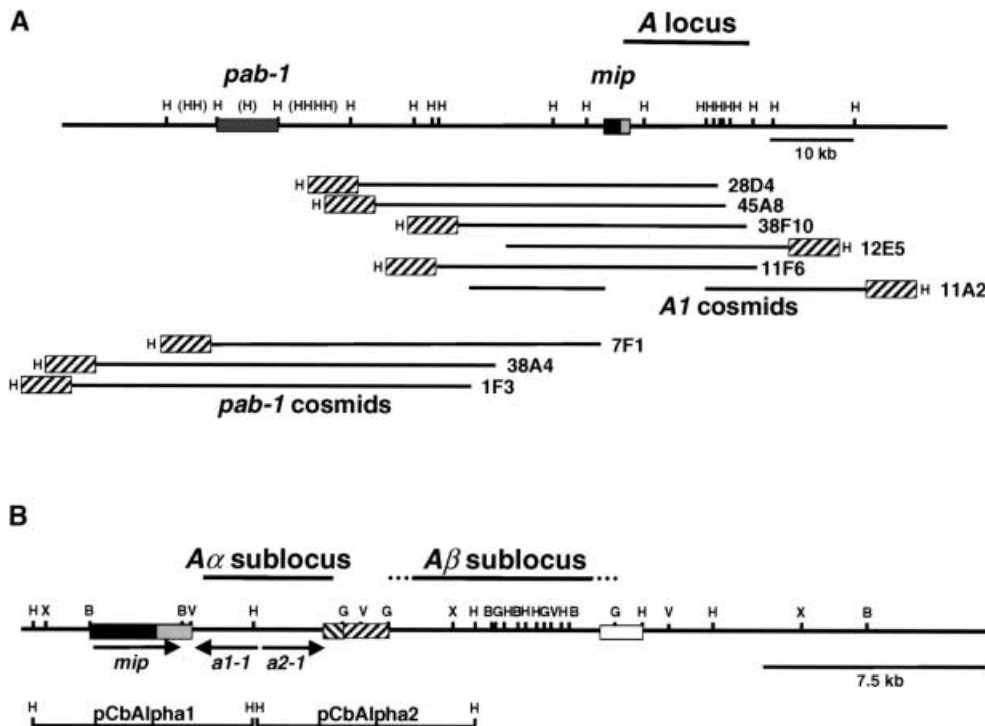
Further cosmids were identified by using the ends of cosmid inserts 38F10 and 28D4 as probes. Overlapping cosmids 1F3, 38A4, 7F1 and 7F5 (identical to 7F1) were detected with the 28D4 probe; cosmids 12E5, 11A2 and 11F6 overlap with the 38F10 probe (Fig. 1A). A physical map covering a chromosomal region of ca 100 kb was established from *Hind*III and *Bam*HI restriction digests of all cosmids and from DNA hybridization analyses (Fig. 1A). The *C. bilanatus* *mip* gene was identified in a 7.5-kb *Hind*III fragment through further hybridizations with pHH5 and was subcloned from cosmid 28D4 as pCbAalpha1. The location of *mip* and the direction of transcription (Fig. 1 A,B) were determined by hybridization with pHH5 and pHH7 and were confirmed by sequencing (T.Y. James, unpublished results).

Cosmids carrying sequences downstream of the *mip* gene (Fig. 1A) elicited *A*-regulated clamp cell development in *C. cinereus* (Table 1), indicating the presence of functional *A* mating type genes. In contrast, cosmids carrying only sequences upstream of *mip* (1F3, 38A4 and 7F1) did not and therefore are unlikely to carry *A* mating type genes. In both *C. cinereus* and *S. commune*, the *A α* mating type locus is closely linked to the *pab-1* gene which is necessary for the production of para-am-

inobenzoic acid (Paba; Giasson et al. 1989; Mutasa et al. 1990). The *C. bilanatus* cosmids 1F3, 38A4 and 7F1 complemented the *C. cinereus* *pab-1* auxotrophy, as determined through transformation of strain PG78. This indicates the linkage of *pab-1* and *mip* to the *A* mating type locus is conserved in *C. bilanatus*. Southern blot analysis of cosmids 1F3, 7F1 and 38A4 probed with the *C. cinereus* *pab-1* gene confirmed the position of *C. bilanatus* *pab-1* 39 kb upstream of *mip*, within two *Hind*III fragments, 1.3 kb and 3.8 kb in size (Fig. 1A).

Cosmid 28D4 contained 11.5 kb of sequence downstream of *mip*, and an active *A* mating type gene is likely

Fig. 1A, B Chromosomal organization of the *Coprinus bilanatus* DNA region containing *pab-1*, *mip* and the *A* mating type locus. **A** Cosmid mapping. *A1* cosmids induced *A*-regulated development in *C. bilanatus* and/or *C. cinereus* transformants; and *pab-1* cosmids conferred para-aminobenzoic acid prototrophy to *C. cinereus* monokaryon PG78. *H* *Hind*III sites, *brackets* indicate sites not localized to specific positions. *Striped squares* indicate the Lorist backbone and *H* at the side of the *squares* the vector's internal *Hind*III site. The localization of the *pab-1* gene is marked by a *grey box*. Regions hybridizing to the 5' end of *C. cinereus* *mip* are indicated by a *solid black box*, regions hybridizing to the 3' end of *C. cinereus* *mip* by a *light grey box*. **B** A more defined map of the *A* mating type region. The 5' and 3' ends of *mip* are marked as in **A**. Location and transcriptional direction of *a1-1* and *a2-1* in the *A α* sublocus were defined by sequencing. The likely borders defining the *A β* sublocus are indicated as follows: *downward from left-to-right striped box*: DNA region which hybridizes with the 4.0-kb *Hind*III fragment present in pAMT6, *upward from left-to-right striped box*: DNA region which hybridizes with the 3.0-kb *Hind*III fragment present in pAMT1, *white box* DNA region which hybridizes to the 4.8-kb *Sal*I fragment present in pUK6. The positions are indicated for the 7.5-kb *Hind*III fragment and the 7.7-kb *Hind*III fragment subcloned respectively in pCbAalpha1 and pCbAalpha2. Restriction sites are as follows: *B* *Bam*HI, *G* *Bgl*II, *H* *Hind*III, *V* *Eco*RV and *X* *Xba*I



to be located within this region. Interestingly, cosmid 11A2 had a large internal deletion which fortuitously covered nearly all of the 11.5-kb region including *mip* (Fig. 1A). Since cosmid 11A2 also conferred *A* mating type regulated development in *C. cinereus* transformants (Table 1), another *A* mating type gene must exist downstream of the sequences represented in cosmid 28D4 (Fig. 1A).

*Hind*III fragments from plasmids pAMT6, pAMT7 and pAMT1, spanning the *C. cinereus* ‘homologous hole’, were used to locate an analogous region in *C. bilanatus* (Fig. 1B). Only the pAMT6 and pAMT1 inserts hybridized to a region of a maximum length of 2.0–2.5 kb in the middle of a 7.7-kb *Hind*III fragment, about 5 kb downstream of the *mip* gene (Fig. 1B). Thus, it appears that although the border regions of the ‘homologous hole’ are present in *C. bilanatus*, internal parts either have been deleted or were never present in this species. In addition, a 4.8-kb *Sal*I fragment carrying the conserved *C. cinereus* β -*fg* gene and the *HD1* gene *d1-1* hybridized to *C. bilanatus* DNA ca 7 kb downstream from the regions hybridizing to the *C. cinereus* ‘homologous hole’ (Fig. 1B). Considered together, these hybridizations delimit the *A* mating factor of *C. bilanatus* to a region between *mip* and those sequences homologous to the *C. cinereus* β -*fg*/*d1-1* fragment. This indicates that the *A* mating type region of *C. bilanatus* is divided into *A* α and *A* β subloci (Fig. 1B).

The *C. bilanatus* *A* α locus

The ca 5-kb *A* α locus was further analysed using transformation and DNA sequencing. *C. cinereus* monokaryon FA2222 was transformed with pCbAalpha1 (which contained *mip* and about 2.0 kb of the *A* α region) and with pCbAalpha2 carrying 2.8 kb of *A* α DNA on a 7.7-

kb *Hind*III fragment subcloned from cosmid 28D4 into pBluescript KS⁻ (Fig. 1B). However, neither plasmid induced *A*-regulated clamp cells. Attempts to subclone larger fragments of the *A* α region overlapping the *Hind*III site between these two fragments were not successful. The large deletion identified in cosmid 11A2 (Fig. 1A) and a number of similar deletions observed when amplifying other cosmids (12E5, 11F6, 28D4, 45A8, 38F10) through growth of *E. coli* (not shown) suggest that the *C. bilanatus* *A* α sequences are poorly tolerated in this host. Cloning experiments where DNA fragments from *C. bilanatus* were mixed with fragments of similar size from an unrelated source supported this conclusion. Although we regularly identified pBluescript KS⁻ clones containing the control fragment, we did not obtain plasmids with *C. bilanatus* DNA (data not shown).

Sequencing the region between *mip* and that hybridizing to the *C. cinereus* ‘homologous hole’ identified individual *HD1* and *HD2* genes (Fig. 1B). The *C. bilanatus* *HD1* gene, designated *a1-1*, is present in pCbAalpha1 but is truncated at the 5' end. A further 169 bp of *a1-1* was cloned in pCbAalpha2 along with the entire *HD2* gene, designated *a2-1*. These *a1-1* and *a2-1* genes are divergently transcribed (Fig. 1B), as is typical for *HD1* and *HD2* genes belonging to the same gene pair (Kües and Casselton 1993). The presumptive start codons for both genes are separated by a short 205-bp sequence of palindromic structure, with two centrally located, inversely orientated CAAT motifs (Fig. 2). TATA elements were not identified.

The products of the *C. bilanatus* *A* α locus

Database homology searches revealed the predicted a1-1 and a2-1 protein products are most closely related to the

Table 1 Phenotypic expression of *Coprinus bilanatus* *A1* mating type DNA in heterologous monokaryons of the same species and of *C. cinereus*

Cosmid	Host	Trp ⁺ transformants		
		Total examined	Total with clamp cells	Percentage co-expression
28D4	<i>C. bilanatus</i>			
	R8 (<i>A2 B3</i>)	57	1	1.8
	S61 (<i>A3 B1</i>)	29	2	6.9
	38F10			
38F10	R8 (<i>A2 B3</i>)	69	1	1.4
	S61 (<i>A3 B1</i>)	24	1	4.2
28D4	<i>C. cinereus</i>			
	FA2222 (<i>A5 B6</i>)	47	15	32
	AT8 (<i>A43 B43</i>)	87	33	38
	38F10			
38F10	FA2222 (<i>A5 B6</i>)	110	50	45
	AT8 (<i>A43 B43</i>)	92	39	42
45A8	LN118 (<i>A42 B42</i>)	147	12	8.2
	FA2222 (<i>A5 B6</i>)	103	12	12
	AT8 (<i>A43 B43</i>)	42	13	31
	11F6			
11F6	FA2222 (<i>A5 B6</i>)	45	14	31
	AT8 (<i>A43 B43</i>)	23	8	35
11A2	FA2222 (<i>A5 B6</i>)	51	18	33
	AT8 (<i>A43 B43</i>)	9	1	11
12E5	FA2222 (<i>A5 B6</i>)	54	16	30
	AT8 (<i>A43 B43</i>)	8	2	16

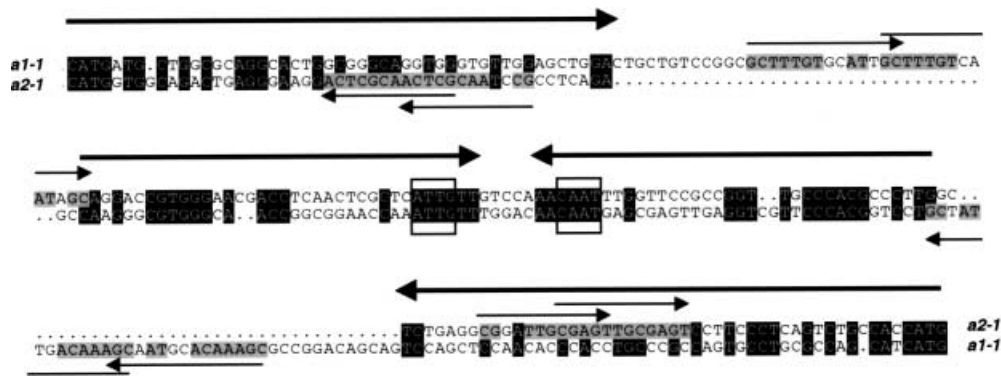


Fig. 2 The 205 bp promoter region between the ATG start codons of *C. bilanatus* genes *a1-1* and *a2-1* has an unusual palindromic structure (indicated by the large arrows). The first three bases of each sequence represent complementing bases of ATG start codons; and the last three bases represent ATG start codons of the respective genes whose positions are given to the left or right of the sequence. Identical bases in the palindromic sequence are highlighted in black. Small arrows and grey-shaded bases mark repetitive elements within the sequence that might be important for gene expression. Two inversely orientated CAAT motifs are boxed

products of the *HD1* and *HD2* genes found in the *C. cinereus* *A* locus (Fig. 3A, B). The most conserved regions in each protein class are the homeodomains (HD1 homeodomains: 57–67% identity with *C. cinereus*; HD2 homeodomains: 73–83% identity). The least conserved regions are the specificity domains (specificity domains of HD1 proteins: 16–20% identity and 39–48% similarity; specificity domains of HD2 proteins: 17–18% identity and 34–44% similarity) The rest of the HD1 proteins exhibited 30–34% identity and 46–50% similarity; and the rest of the HD2 proteins exhibited 39–42% identity and 55–56% similarity.

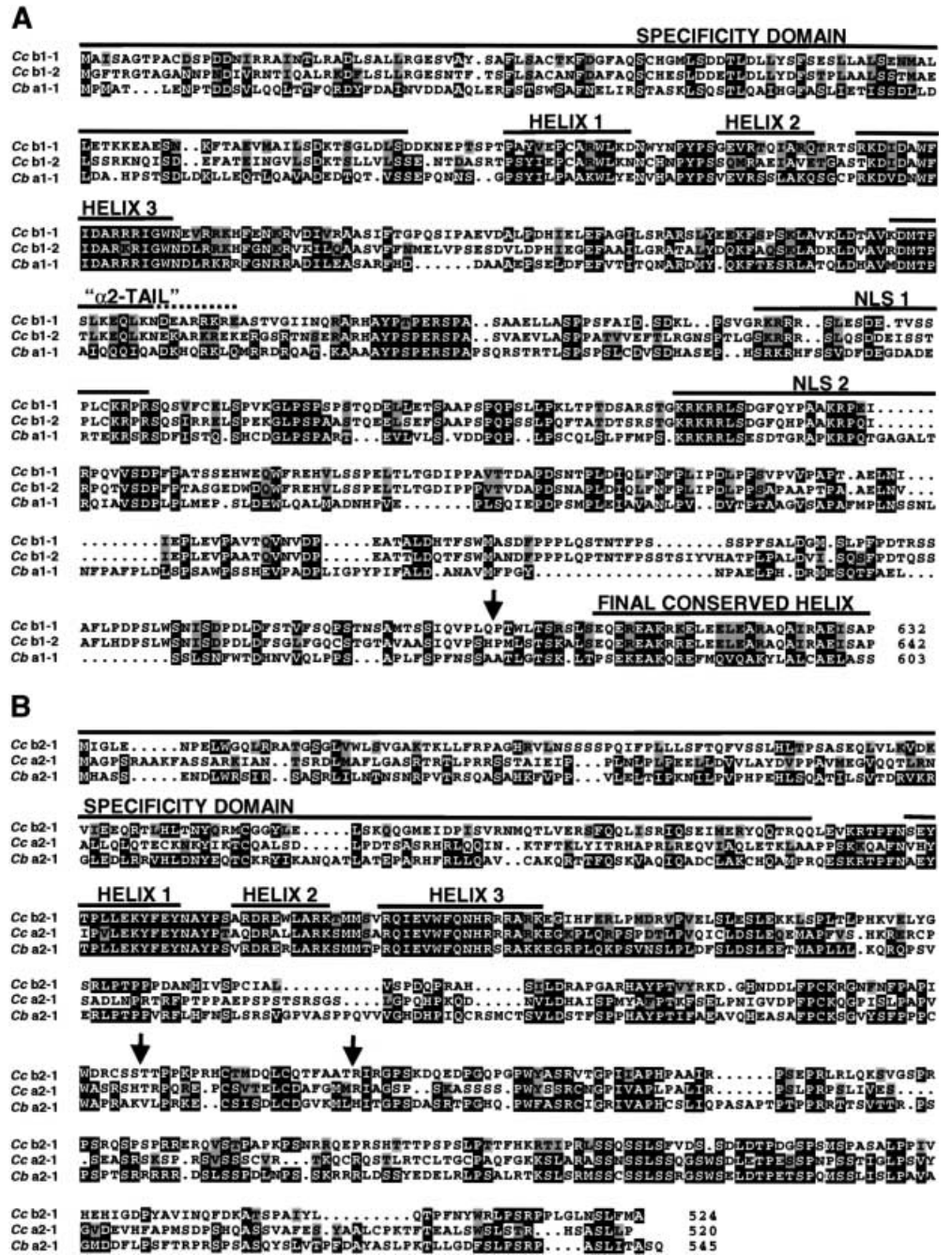
Other HD1 protein motifs were identified in *C. bilanatus* *a1-1* (Fig. 3A). Two nuclear localization signals, NLS1 and NLS2 characterized in *C. cinereus* (Asante-Owusu et al. 1996; Spit et al. 1998), also exist in *C. bilanatus* (Fig. 3A). Upstream of the less conserved NLS1 signal is a short conserved sequence thought to be a helical dimerization motif (Badrane and May 1999). This motif resembles the C-terminal tail of *Saccharomyces cerevisiae* mating type protein $\alpha 2$ (Kües 2000; Kües et al. 1994a; Fig. 3A), known to contact the homeodomain of mating type protein **a1** to stabilize the $\alpha 2$ -**a1** protein complex for DNA-binding (Johnson 1995). At the C-terminus of *C. bilanatus* *a1-1* is a highly conserved helical region found in all *C. cinereus* HD1 proteins (Badrane and May 1999; Fig. 3A). This helix can be deleted in *C. cinereus* without loss of protein function, unlike the directly adjacent amino acid sequences (Tyman et al. 1992) that are possibly required for a transactivation function (Asante-Owusu et al. 1996). Further domains were not identified in the *C. bilanatus* HD2 protein (Fig. 3B). Work in *C. cinereus* has shown that the final third of the HD2 proteins can be deleted, suggesting the absence of essential domains (Kües et al. 1994a; Fig. 3B).

Discussion

The isolation of the chromosomal DNA region of *C. bilanatus* containing the *A1* mating type sequences and flanking genes presented in this paper is only the third *A* mating type locus cloned from a homobasidiomycete; and it is the first from a secondarily homothallic basidiomycete. Similar to the heterothallic species *C. cinereus* and *S. commune* (Casselton and Kües 1994; Raper 1966), the *C. bilanatus* *A* locus is bipartite with *A α* and *A β* subloci (Fig. 1B) and is composed of *HD1* and *HD2* genes encoding two different classes of homeodomain transcription factors (Fig. 3A, B). One pair of divergently transcribed *HD1* and *HD2* genes was identified in the *A α* locus of *C. bilanatus* (Fig. 1B), as in *C. cinereus* (Kües et al. 1994c; Pardo et al. 1996) and *S. commune* (Specht et al. 1994; Stankis et al. 1992). In all three organisms, the *HD1* gene in the *A α* locus is directly flanked by a conserved *mip* gene for a mitochondrial intermediate peptidase (Casselton et al. 1995; Isaya et al. 1995; Kües et al. 1992, 1994c; Stankis et al. 1992; Figs. 1B and 4). This character expedited isolation of the *C. bilanatus* *A* mating type region and may also facilitate cloning of *A* mating type loci from other fungi.

In *S. commune*, an unessential gene of unknown function and conserved sequence, gene *X*, appears at the *A α* sublocus on the opposite side to *mip* and directly next to the *A α* *HD2* gene (Marion et al. 1996; Fig. 4). In *C. cinereus*, transcripts for such a gene were not detected (Kües et al. 1992, 1994c) and no related sequences were found in the region separating the *A α* and *A β* subloci in *C. bilanatus* (T.Y. James, unpublished results). In contrast, the position of the *pab-1* gene in relation to *mip* and the *A α* sublocus is conserved and the distance between *mip* and *pab-1* is very similar: 50 kb in *S. commune* (Giasson et al. 1989), 40 kb in *C. cinereus* (Mutasa et al. 1990; Kües et al. 1992) and 39 kb in *C. bilanatus* (this study). In both *Coprinus* species, the *pab-1* gene is found upstream of the *A α* sublocus, which itself is upstream of *A β* (Day 1960; Lukens et al. 1996; Figs. 1A, 4). In *S. commune* *pab-1* is located in between the two *A* subloci (Raper 1966; Giasson et al. 1989; Fig. 4), suggesting some kind of translocation has taken place in evolution. Also found between the *A α* and *A β*

Fig. 3 Alignment of predicted amino acid sequences for: **A** *C. bilanatus* HD1 protein a1-1 to HD1 proteins from the *C. cinereus b* gene pair and **B** *C. bilanatus* HD2 protein a2-1 to HD2 proteins from *C. cinereus A42* haplotype. *C. cinereus* sequences are from Badrane and May (1999) and Kües et al. (1994a). *C. bilanatus* amino acids indicated in *black* are residues where identical (*boxed in black*) or conserved residues (*boxed in grey*) are found in one or more of the *C. cinereus* proteins. *Ruled lines* indicate specificity domains as defined in *C. cinereus* (Banham et al. 1995; Kües et al. 1994a), the extension of the three helical regions (*helix 1–helix 3*) of the homeodomains (Bürglin 1994), a sequence motif of predicted helical structure in HD1 proteins (Badrane and May 1999) resembling the C-terminal dimerization motif of *S. cerevisiae* mating type protein $\alpha 2$ (Kües 2000; Kües et al. 1994a), two nuclear localization signals *NLS1* and *NLS2* in the HD1 proteins (Badrane and May 1999; Spit et al. 1998) and a conserved but non-essential C-terminal helical region in the HD1 proteins (Tymon et al. 1992). *Black arrows* indicate positions where *C. cinereus* proteins can be truncated without loss of function (Kües et al. 1994a)



subloci in *S. commune* is the adenine synthesis gene *ade-5* (Raper 1966; Giasson et al. 1989; Fig. 4) for aminoimidazole ribonucleotide synthetase (Alic et al. 1990). It is possible that the gene is functionally equivalent to *C. cinereus ade-8*, a gene closely linked to the *Aβ* sublocus (Day 1960; Lukens et al. 1996; Fig. 4). To date, the entire extension of the *Aβ* sublocus in *S. commune* and the number of genes within it are not established (Shen et al. 1996). It is also unknown whether the *Aβ* sublocus is flanked by the gene *β-fg*, which could help establish the type of gene rearrangements that are responsible for structural differences between *Coprinus* and *Schizophyllum A* mating type loci.

It is likely that there are additional *Aβ* genes in *C. bilanatus*. Our transformation studies with cosmid 11A2 indicate the presence of at least one active gene in the 4–5.6 kb *Aβ* mating type DNA not present in cosmid 28D4 (Fig. 1A). The gene(s) responsible for *A* mating type activity of cosmid 28D4 must be present either within the *Aα* sublocus or in the short DNA stretch belonging to the *Aβ* sublocus (Fig. 1A, B). Preliminary sequence data suggest that the entire *Aβ HD1* gene *b1-1* and part of the affiliated *HD2* gene *b2-1* are located within the 7.7-kb *HindIII* fragment cloned in pCcAalpha2 and are separated by about 0.6 kb of non-coding DNA from the *HD2* gene *a2-1* of the *Aα*

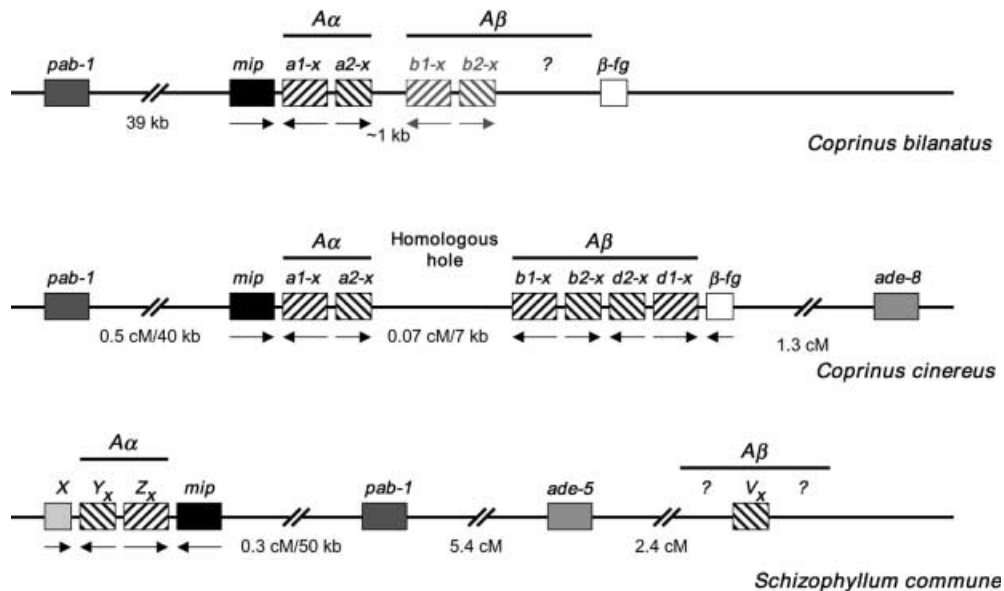


Fig. 4 Comparison of the *C. bilanatus* *A* mating type locus region and associated genes with other homobasidiomycetes, *C. cinereus* and *Schizophyllum commune*. In *C. cinereus*, the number of genes per gene pair and even the number of gene pairs vary between different *A* haplotypes. Therefore, an archetypal model of the *A* mating type locus is given with two gene pairs in the *Aβ* sublocus (*b* pair and *d* pair) that matches most of the analysed *A* haplotypes (although in exceptional cases there are more genes; Casselton and Olesnicky 1998; Hiscock and Kües 1999; Kües 2000). *HD1* genes are boxed with stripes upwards from left to right; and *HD2* genes are boxed with stripes downwards from left to right. Specific names for *HD1* and *HD2* genes are indicated above and *x* refers to different alleles. *C. bilanatus* gene pair *b1-1* and *b2-1* are predicted from sequence data (T.Y. James, unpublished) and are indicated by grey-shaded boxes. Conserved gene sequences flanking the variable *A* mating type DNA are shown as black boxes (*mip*) and white boxes (*β-fg*). Gene *X* and more distantly linked genes are indicated with grey-shaded boxes. Arrows indicate the direction of transcription where known; and question marks indicate positions where further *A* genes are predicted

sublocus (T.Y. James, unpublished results; Fig. 4). Since neither the pCbAα1 or pCbAα2 subclones conferred *A* mating type regulated development in *C. cinereus* monokaryon FA2222 in contrast to the whole cosmid DNA, the active gene of 28D4 must be the *HD1* gene *a1-1* of the *Aα* sublocus that is truncated at the 5' end in pCbAα1. Previous work, using subcloned mating type genes from the *A42* haplotype of *C. cinereus*, indicated that *HD2* proteins of *C. cinereus* interacted with the *HD1* proteins of *C. bilanatus*. Unlike *HD2* genes, *HD1* genes of *C. cinereus* did not induce clamp cell formation in *C. bilanatus* (Challen et al. 1993). From these former results, activity of *C. bilanatus HD2* genes in *C. cinereus* might not be expected. This could explain the inactivity of the *HD2* gene *a2-1* in the heterologous *C. cinereus* monokaryon FA2222 but not the inactivity of the putative *HD1* gene *b1-1*. It would be interesting to determine whether the *C. bilanatus HD1* protein *b1-1* has the same specificity as the *HD1* protein *b1-4* present in *C. cinereus* monokaryon FA2222 (Pardo et al. 1996) and is therefore incompatible with the native *HD2* protein *b2-4*.

From our experiments with both species, it is possible that *C. bilanatus HD2* gene products do not interact properly with *C. cinereus HD1* proteins in formation of protein complexes and binding to promoters. This is unlikely to be due to the homeodomain DNA binding motifs which are very similar in both species (Fig. 3 A, B), especially at the amino acids that are known to contact the DNA in other homeodomain proteins (Sharkey et al. 1997). Work with modified genes in *C. cinereus* and *S. commune* has revealed that the homeodomain motif in the *HD1* proteins is not essential for the function of heterodimeric *HD1-HD2* complexes (Kües et al. 1994b; Luo et al. 1994; Asante-Owusu et al. 1996). It would therefore be interesting to elucidate the nature of the specificity domains and determine how *C. bilanatus HD1-C. cinereus HD2* combinations are distinguished from *C. bilanatus HD2-C. cinereus HD1* combinations. Further work should reveal the complete structure of the *C. bilanatus A1* haplotype and permit analysis of all *A1* genes and their products in homologous and heterologous hosts.

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References

- Alic M, Clark EK, Kornegay JR, Gold ME (1990) Transformation of *Phanerochaete chrysosporium* and *Neurospora crassa* with adenine biosynthetic genes from *Schizophyllum commune*. *Curr Genet* 17: 305–311
- Asante-Owusu RN, Banham AH, Böhnert HU, Mellor EJC, Casselton LA (1996) Heterodimerization between two classes of homeodomain proteins in the mushroom *Coprinus cinereus* brings together potential DNA-binding and activation domains. *Gene* 172: 25–31

- Badrane H, May G (1999) The divergence-homogenization duality in the evolution of the *b1* mating type of *Coprinus cinereus*. *Mol Biol Evol* 16: 975–986
- Banham AH, Asante-Owusu RN, Göttgens B, Thompson SAJ, Kingsnorth CS, Mellor EJC, Casselton LA (1995) An N-terminal dimerization domain permits homeodomain proteins to choose compatible partners and initiate sexual development in the mushroom *Coprinus cinereus*. *Plant Cell* 7: 773–783
- Binninger DM, Skrzynia C, Pukkila PJ, Casselton LA (1987) DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. *EMBO J* 6: 835–840
- Bottoli APF, Kertesz-Chaloupková K, Boulianne RP, Granado JD, Aebi M, Kües U (1999) Rapid isolation of genes from an indexed genomic library of *C. cinereus* in a novel *pab-1*⁺ cosmid. *J Microbiol Methods* 35: 129–141
- Bürglin TR (1994) A comprehensive classification of homeobox genes. In: Duboule D (ed) *Guidebook to the homeobox genes*. Oxford University Press, New York, pp 25–72
- Burrows DM (1991) Transformation studies in the basidiomycete fungi *Coprinus cinereus* and *Coprinus bilanatus*. PhD thesis. Queen Mary and Westfield College, London University, London
- Burrows DM, Elliott TJ, Casselton LA (1990) DNA-mediated transformation of the secondarily homothallic basidiomycete *Coprinus bilanatus*. *Curr Genet* 17: 175–177
- Casselton LA, Kües U (1994) Mating-type genes in heterobasidiomycetes. In: Wessels JGH, Meinhardt F (eds) *The Mycota*, vol I. Growth, differentiation and sexuality. Springer, Berlin Heidelberg New York, pp 307–322
- Casselton LA, Olesnick NS (1998) Molecular genetics of mating recognition in basidiomycete fungi. *Microbiol Mol Biol Rev* 62: 55–70
- Casselton LA, Asante-Owusu RN, Banham AH, Kingsnorth CS, Kües U, O'Shea SF, Pardo EH (1995) Mating type control of sexual development in *Coprinus cinereus*. *Can J Bot* 73: S266–S272
- Challen MP, Elliott TJ, Kües U, Casselton LA (1993) Expression of *A* mating type genes of *Coprinus cinereus* in a heterologous basidiomycete host. *Mol Gen Genet* 241: 474–478
- Challen MP, Bhattiprolu GR, Warner PJ, Elliott TJ (1994) Cloning the *Coprinus bilanatus* *TRP2* gene and its use as a selectable marker in transformation. *Mycol Res* 98: 179–185
- Day PR (1960) The structure of the *A* mating type locus in *Coprinus lagopus*. *Genetics* 45: 641–650
- Elliott TJ, Challen MP (1983) Genetic ratios in secondarily homothallic basidiomycetes. *Exp Mycol* 7: 170–174
- Giasson L, Specht CA, Milgrim C, Novotny CP, Ullrich RC (1989) Cloning and comparison of the *A α* mating-type alleles of the basidiomycete *Schizophyllum commune*. *Mol Gen Genet* 218: 72–77
- Granado JD, Kertesz-Chaloupková K, Aebi M, Kües U (1997) Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. *Mol Gen Genet* 256: 28–36
- Hiscock SJ, Kües U (1999) Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. *Int Rev Cytol* 193: 165–295
- Hopple JS Jr, Vilgalys R (1999) Phylogenetic relationships in the mushroom genus *Coprinus* and dark-spored allies based on sequence data from the nuclear gene coding for the large ribosomal subunit RNA: divergent domains, outgroups, and monophyly. *Mol Phylogenet Evol* 13: 1–19
- Isaya G, Sakati WR, Rollins RA, Shen GP, Hanson LC, Ullrich RC, Novotny CP (1995) Mammalian mitochondrial intermediate peptidase – structure–function analysis of a new homolog from *Schizophyllum commune* and relationship to thimet oligopeptidases. *Genomics* 28: 450–461
- Johnson AD (1995) Molecular mechanisms of cell-type determination in budding yeast. *Curr Opin Genet Dev* 5: 552–558
- Kemp RFO (1974) Bifactorial incompatibility in the two-spored basidiomycetes *Coprinus sassii* and *C. bilanatus*. *Trans Br Mycol Soc* 62: 547–555
- Kües U (2000) Life history and developmental processes in the basidiomycete *Coprinus cinereus*. *Microbiol Mol Biol Rev* 64: 316–353
- Kües U, Casselton LA (1993) The origin of multiple mating types in mushrooms. *J Cell Sci* 104: 227–230
- Kües U, Richardson WVJ, Tymon AM, Mutasa ES, Göttgens B, Gaubatz S, Gregoriades A, Casselton LA (1992) The combination of dissimilar alleles of the *A α* and *A β* gene complex, whose proteins contain homeodomain motifs, determine sexual development in the mushroom *Coprinus cinereus*. *Genes Dev* 6: 568–577
- Kües U, Asante-Owusu RN, Mutasa ES, Tymon AM, Pardo EH, O'Shea SF, Göttgens B, Casselton LA (1994a) Two classes of homeodomain proteins specify the multiple *A* mating types of the mushroom *Coprinus cinereus*. *Plant Cell* 6: 1467–1475
- Kües U, Göttgens B, Stratmann R, Richardson WVJ, O'Shea SF, Casselton LA (1994b) A chimeric homeodomain protein causes self-compatibility and constitutive sexual development in the mushroom *Coprinus cinereus*. *EMBO J* 13: 4054–4059
- Kües U, Tymon AM, Richardson WVJ, May G, Gieser PT, Casselton LA (1994c) *A* mating-type factors of *Coprinus cinereus* have variable numbers of specificity genes encoding two classes of homeodomain proteins. *Mol Gen Genet* 245: 45–52
- Kühner R, Romagnesi H (1978) *Flore analytique des champignons supérieurs*. Masson, Paris
- Little PFR (1987) Choice and use of vectors. In: Glover DM (ed) *DNA cloning*, vol III. A practical approach. (Practical approach series) IRL Press, Oxford, pp 19–42
- Lukens L, Yicun H, May G (1996) Correlation of genetic and physical maps at the *A* mating-type locus of *Coprinus cinereus*. *Genetics* 144: 1471–1477
- Luo YH, Ullrich RC, Novotny CP (1994) Only one of the paired *Schizophyllum commune* *A α* mating-type, putative homeobox genes encodes a homeodomain essential for *A α* -regulated development. *Mol Gen Genet* 244: 318–324
- Marion AL, Bartholomew KA, Wu J, Yang HL, Novotny CP, Ullrich RC (1996) The *A α* mating-type locus of *Schizophyllum commune*: structure and function of gene *X*. *Curr Genet* 29: 143–149
- May G, Le Chevanton L, Pukkila PJ (1991) Molecular analysis of the *Coprinus cinereus* mating type *A* factor demonstrates an unexpectedly complex structure. *Genetics* 128: 529–538
- Mutasa ES, Tymon AM, Göttgens B, Mellon FM, Little PFR, Casselton LA (1990) Molecular organisation of an *A* mating type factor of the basidiomycete fungus *Coprinus cinereus*. *Curr Genet* 18: 223–229
- Pardo EH, O'Shea SF, Casselton LA (1996) Multiple versions of the *A* mating type locus of *Coprinus cinereus* are generated by three paralogous pairs of multiallelic homeobox genes. *Genetics* 144: 87–94
- Raper CA, Raper JR, Miller RE (1972) Genetic analysis of the life-cycle of *Agaricus bisporus*. *Mycologia* 64: 1088–1117
- Raper JR (1966) *Genetics of sexuality in higher fungi*. Ronald Press, New York
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sharkey M, Graba Y, Scott MP (1997) *Hox* genes in evolution: protein surfaces and paralog groups. *Trends Genet* 13: 145–151
- Shen GP, Park DC, Ullrich RC, Novotny CP (1996) Cloning and characterization of a *Schizophyllum* gene with *A β 6* mating-type activity. *Curr Genet* 29: 136–142
- Specht CA, Stankis MM, Novotny CP, Ullrich RC (1994) Mapping the heterogeneous DNA region that determines the nine *A α* mating-type specificities of *Schizophyllum commune*. *Genetics* 137: 709–714
- Spit A, Hyland RG, Mellor EJC, Casselton LA (1998) A role for heterodimerization in nuclear localization of a homeodomain protein. *Proc Natl Acad Sci USA* 95: 6228–6233
- Stankis MM, Specht CA, Yang H, Giasson L, Ullrich RC, Novotny CP (1992) The *A α* mating type locus of *Schizophyllum*

- commune* encodes two dissimilar multiallelic homeodomain proteins. Proc Natl Acad Sci USA 89: 7169–7173
- Tymon AM, Kües U, Richardson WVJ, Casselton LA (1992) A fungal mating type protein that regulates sexual and asexual development contains a POU-related domain. EMBO J 11: 1805–1813
- Zhou B, Chen N, Li Q (1988) Application of partial restriction procedure in both shotgun and non-random strategies for nucleotide sequencing. Gene 70: 405–409
- Zolan ME, Pukkila PJ (1986) Inheritance of DNA methylation in *Coprinus cinereus*. Mol Cell Biol 6: 195–200