DOI: 10.1534/genetics.108.093708

Ancient *Trans*-specific Polymorphism at Pheromone Receptor Genes in Basidiomycetes

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Manuscript received July 8, 2008
Accepted for publication November 5, 2008

ABSTRACT

In the majority of sexual organisms, reproduction occurs almost exclusively through the combination of distinct and alternate forms, called sexes or mating types. In some fungi, there can be dozens to hundreds of alternate alleles that determine compatible mating types. Such extensive polymorphism is expected to be maintained by balancing selection, and in extreme cases may give rise to *trans*-specific polymorphism. Here, we analyzed sequences of two pheromone receptors in the Microbotryum fungal species complex (Basidiomycota), which has only two alternate mating types. Several lines of evidence strongly suggest that the pheromone receptors are two allelic sequences acting to determine the alternate A1 and A2 mating types required for mating in Microbotryum. Phylogenetic trees of pheromone receptors in the Microbotryum species complex indicated a *trans*-specific polymorphism: the Microbotryum sequences from a given mating type were all more similar to the pheromone receptors of distantly related classes of fungi than to the alternate pheromone receptor in the Microbotryum species. A phylogenetic tree built using other known pheromone receptors from basidiomycetes showed that *trans*-specific polymorphism is widespread. The pheromone receptor alleles from Microbotryum appeared as the oldest, being at least 370 million years old. This represents the oldest known *trans*-specific polymorphism known in any organism so far, which may be due to the existence of sex chromosomes, obligate sexuality, mitochondrial inheritance linked to the mating type, and a highly selfing mating system in Microbotryum.

In sexual organisms, reproduction occurs almost exclusively through the combination of distinct and alternate forms, called sexes or mating types. Dimorphic sex chromosomes define mating compatibility in most mammals, while more polymorphic systems, involving dozens to hundreds of alleles, determine mating types in some plants and fungi (Richman 2000). Such extensive polymorphism at mating types is maintained by negative frequency-dependent selection, the most common form of balancing selection (Richman 2000): when a mating-type allele becomes rare, it has a selective advantage because the individuals carrying it can mate with a larger proportion of other individuals. Balanced polymorphism of multiple alleles is therefore frequent at mating-type loci in a wide range of organisms, such as plants, insects, and fungi (May *et al.* 1999; Richman 2000), and it is also pervasive in various somatic self/nonself recognition systems, such as the vertebrate major histocompatibility complex (MHC) (Hughes and Yeager 1998) and the vegetative incompatibility system in fungi (Powell *et al.* 2007). Consistent with rare allele advantage, nonsynonymous mutations have been found to be positively selected in several mating-type and nonself recognition systems (Richman 2000; Cho *et al.* 2006), including in fungi (Wu *et al.* 1998; Karlsson *et al.* 2008).

In addition to potentially maintaining large numbers of alleles, genes under balancing selection also maintain alleles far longer than neutral genetic variation because the selective advantage of an allele when it becomes rare can counteract the effects of loss due to genetic drift. In extreme cases, *trans*-specific polymorphism can be observed, *i.e.*, the maintenance of multiple allelic classes that transcend species boundaries by virtue of being more ancient than the species themselves (Richman 2000). When this occurs, an allele sampled from a particular species can be more related to members of the same functional allelic class in other species than to members of different allelic classes in the same species.

Fungi are highly interesting models for studying the origin and diversity of alleles by balancing and positive selection, in particular at their mating-type genes. In heterothallic fungi, mating-type loci determine sexual compatibility: only gametes differing at these loci can mate, and basidiomycetes often have high numbers of mating types. However, very few studies have been devoted to these aspects in fungi so far (but see May *et al.* 1999; Karlsson *et al.* 2008).

Mating-type loci have very different organizations in the two main groups of fungi. In ascomycetes, the two

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alternate mating types are "idiomorphs," *i.e.*, nonhomologous sequences present at the same locus (Metzenberg and Glass 1990). These idiomorphs are completely dissimilar genes, with no evidence of derivation by common descent and encoding proteins with different functions. In *Cochliobolus heterotrophus* for instance, MAT1-1 encodes an α-domain protein, while MAT1-2 encodes a HMG-domain protein (Stanton and Hull 2007).

In contrast, mating compatibility in basidiomycetes occurs between cells having different alleles at specific genes (*e.g.*, Halsall *et al.* 2000; Kues 2000; Casselton and Kues 2007). In the basidiomycete *Ustilago maydis* for instance, a major fungal pathogen of corn, cell recognition, and fusion are regulated by a pheromone/receptor system that resides at the *a* mating-type locus. All haploid cells have pheromone and receptor genes, and they can fuse only if they have different alleles at these genes. After fusion, maintenance of the dikaryon and the developmental switch to filamentous growth occurs only if the product of mating is heterozygous for the second *b* mating-type locus (Feldbrugge *et al.* 2004). The *b* locus encodes homeodomain proteins that function as transcriptional regulators. The majority of sexual basidiomycete fungi possess a system similar to that in *U. maydis*, called "tetrapolar," where *a* and *b* loci are unlinked and haploids must have different alleles in both loci to mate successfully. Other members of this phylum are "bipolar," either because the *a* and *b* loci are tightly linked (*e.g.*, in *U. hordei*, Bakkeren and Kronstad 1994) or because only one of the loci has a role in determining mating-type specificity (*e.g.*, in *Coprinellus disseminatus*, James *et al.* 2006). The absence of linkage between *a* and *b* loci in a tetrapolar system is likely the ancestral condition (Hibbett and Donoghue 2001) and is often associated with large numbers of alternate alleles (Raper *et al.* 1960). In contrast, bipolar species are often biallelic, although not always (James *et al.* 2006).

In this study, we are interested in investigating whether balancing selection and/or positive selection are acting at mating-type genes in basidiomycete fungi. More specifically, we will study the pheromone receptor gene (Figure 1). In *U. maydis*, each haploid cell contains both a pheromone gene and a pheromone receptor gene at the *a* locus. Only cells having different alleles at these genes can mate (Figure 1A). Only the pheromone from one allele can activate the receptor of the alternative allele. Recombination is suppressed between the pheromone and receptor genes, avoiding the emergence of self-compatible haploids, as the two genes are separated by a region unique to each mating type. Mushroom forming homobasidiomycetes (*e.g.*, *Coprinopsis cinerea*) have evolved multiple versions of their pheromones and receptors (Figure 1B) (Casselton and Kues 2007). The B locus in these fungi (homologous to the *a* locus of Ustilago species) contains several subloci, with different paralogs of the pheromone and of the pheromone receptor genes. For two haploid cells to be compatible, it is required and sufficient that they have different alleles for at least one of the subloci. Pheromones can only activate receptors within the same sublocus class in *C. cinerea* (Casselton and Kues 2007). The genes within each sublocus are maintained as a functional unit by being separated by a unique DNA sequence that is very dissimilar from other allelic and paralogous versions (Casselton and Kues 2007). This lack of DNA homology extends into the flanking regions, which prevents recombination and keeps together sets of genes so that sexual development is impossible in the absence of mating. These highly dissimilar flanking regions in basidiomycetes could be suggestive of the idiomorphs in ascomycetes. However, in contrast to ascomycetes, homologs can be easily recognized in the core mating-type genes themselves, in particular in the pheromone receptor genes (Casselton and Kues 2007) (Figure 1). Alleles can therefore be defined for pheromone receptor genes and this term is commonly used for the mating-type genes in basidiomycetes (*e.g.*, Casselton and Kues 2007). This is true in particular for all basidiomycetes that are basal to the homobasidiomycetes, which have a single copy of the pheromone receptor gene.

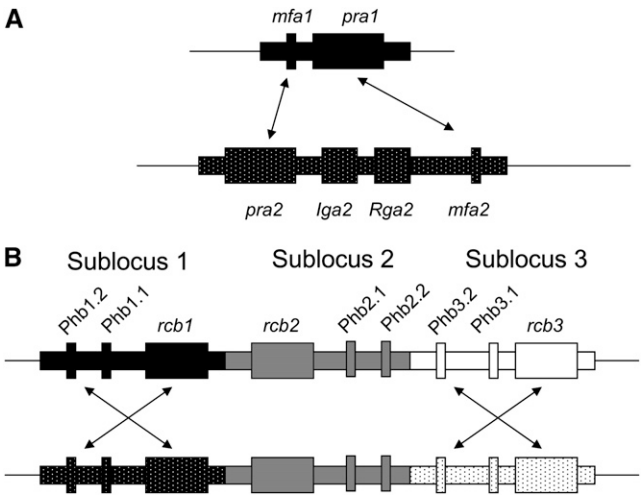

Figure 1.—Organization of the locus encompassing the pheromone (narrow vertical rectangles) and pheromone receptor (wide horizontal rectangles) genes in *Ustilago maydis* (A) and *Coprinopsis cinerea* (B). Arrows indicate receptor and pheromone combinations that are compatible. Different degrees of shading (solid, shaded, open) indicate paralogs and different filled motifs indicate alleles. The *a* locus in *U. maydis* has one a-receptor gene (pra) and one a-pheromone gene (mfra). The products of *Iga2* and *Rga2* (squares) have no obvious role in sexual development but are involved in mitochondrial function (Bortfeld *et al.* 2004). The B locus of *C. cinerea* is composed of three subloci, each containing a receptor gene and two pheromone genes. For two haploid cells to be compatible, it is required and sufficient that they have different alleles for at least one of the sublocus. Pheromones can only activate receptors within the same sublocus class. This figure has been adapted from Casselton and Kues (2007) and Stanton and Hull (2007).

The region with suppressed recombination around the core mating-type genes can extend very far and encompass other genes. Mating-type loci in some fungi are indeed found in chromosomal regions that, in several cases, present clear parallels to sex chromosomes characterized in plant and animal systems (Hood *et al.* 2004; Fraser and Heitman 2005; Menkis *et al.* 2008). An example of incipient sex chromosomes is found in the basidiomycete *Cryptococcus neoformans*, a ubiquitous human fungal pathogen with a bipolar mating-type system: recombination suppression around the core mating-type genes include >20 genes and extends >100 kbp in length (Fraser *et al.* 2004). An even more extreme example is *Microbotryum violaceum*, which is also bipolar and possesses true-size dimorphic sex chromosomes, with suppressed recombination throughout much of their lengths (Hood 2002; Hood *et al.* 2004; Hood and Antonovics 2004).

M. violaceum causes a sexually transmitted disease, anther smut, in plants of the Caryophyllaceae family (Thrall *et al.* 1993). Diploid teliospores of the fungus are produced in the anthers of infected plants, replacing the pollen, and female structures are aborted. Deposited by pollinators on a new host, teliospores germinate, and the fungus then undergoes meiosis. Sexual conjugation between two haploid cells of the two opposite mating types (A1 and A2) is required prior to infection, selfing being predominant (Hood and Antonovics 2000; Giraud 2004; Giraud *et al.* 2005, 2008). Conjugation, and thus cell fusion, is initiated only between sporidia of different mating types (Garber and Ruddat 2002), indicating that pheromones and pheromone receptors are involved in this process, as in Ustilago species (Feldbrugge *et al.* 2004). *M. violaceum* is a complex of sibling species, specialized on different hosts and showing postzygotic isolation, but the taxonomy has not been fully revised yet (Kemler *et al.* 2006; Le Gac *et al.* 2007a,b; Sloan *et al.* 2008). Size-dimorphic sex chromosomes are present in multiple Microbotryum species, exhibiting large-scale recombination suppression and high levels of allelic and structural heterozygosity (Hood 2002; Hood and Antonovics 2004). Two putative STE3-like pheromone receptors have recently been characterized among sequences expressed during mating in the Microbotryum species infecting *Silene latifolia* (Yockteng *et al.* 2007). They were respectively associated with A1 and A2 mating behavior of haploid sporidia, thus probably constituting alternative alleles of the homolog for the pheromone receptor at the *a* locus in *U. maydis*, although their function has not been experimentally demonstrated yet.

Here we analyzed the sequences of the two characterized pheromone receptors, pr-MatA1 and pr-MatA2, in multiple Microbotryum species to investigate their evolution. In particular, our aims were: (1) to obtain more information on the putative allelic status of the two sequences of pheromone receptors characterized in Microbotryum; (2) to look for footprints of positive selection among Microbotryum species, which might occur for instance if the pheromone receptor is involved in species recognition during mating or if there is regular selection for novel mating-type specificities; (3) to look for footprints of balancing selection and *trans*-specific polymorphism at the pheromone receptor genes in Microbotryum; *trans*-specific polymorphism will be detected if alleles of a given species are not monophyletic, as is usually the case, but rather are closer to alleles from other species than to alternate alleles from their own species; and (4) to compare the Microbotryum pheromone receptors with those of other fungi to gain more general insight into the evolution of fungal mating types, in particular to assess whether balancing selection and *trans*-specific polymorphism could be detected more generally in basidiomycetes.

MATERIALS AND METHODS

Fungal isolates, DNA extraction, PCR, and sequencing: For DNA sequencing, we used 38 Microbotryum strains, belonging to at least 13 different fungal species (Le Gac *et al.* 2007a), collected from 18 host species in western Europe and America (Table 1). Species have been recognized on the basis of congruence between multiple phylogenies and await full taxonomic revision. Diploid teliospores were stored and grown as previously described (Le Gac *et al.* 2007a). Single sporidial colonies, representing haploid clonal colonies derived from a single meiotic product, were isolated and cultured for DNA extraction as previously described (Le Gac *et al.* 2007a). For each diploid sample (teliospores in flowers), necessarily heterozygous at the mating-type locus, we thus obtained both colonies of A1 haploid sporidia and colonies of A2 haploid sporidia. The mating type of sporidia was determined by crossing them *in vitro* against stock sporidia of known mating type, as previously described (Thomas *et al.* 2003). DNA was extracted using the Chelex (Bio-Rad) protocol (Giraud 2004).

Most of the length of the two putative pheromone receptors, pr-MatA1 and pr-MatA2 (Figure 2), was amplified and sequenced, using the primers given in Table 2 and Touchdown PCR programs with annealing temperatures from 60° down to 45°. PCR products were purified and sequenced in both directions as previously described (Le Gac *et al.* 2007a). The sequences are available in GenBank (accession nos: FJ200750–FJ200809).

The eight primer pairs in Table 2 were also used for PCR at low annealing temperature to detect other putative pheromone receptors in DNA extracted from the stock sporidia of known mating types for the Microbotryum species MvSl. The eight primer pairs were used with a PCR program having an annealing temperature of 48° and using three replicate DNA extractions from each of A1 and A2 mating-type sporidia. PCR products were then run on agarose gels. For band isolation on agarose gels, the QIAEX2 gel extraction kit from QIAGEN was used.

Positive selection: We wanted to investigate whether positive Darwinian selection had influence in the evolution of each of the A1 and A2 pheromone receptors in the Microbotryum species complex. We analyzed pr-MatA1 and pr-MatA2 separately, testing for positive selection across the sites of each nucleotide (codon) alignment. Positive selection was tested

TABLE 1

Characteristics of the Microbotryum isolates used in this study

Host species	Isolate name	Geographical origin	Date of collection	Obtained sequences		Microbotryum species	
				pr-MatA1	pr-MatA2	Le Gac *et al.* (2007a)	Kemler *et al.* (2006); Denchev (2007)
Dianthus carthusianorum	2002 Site 23	Sestrière, Italy	2002		X	MvDsp1	*M. dianthorum*
D. carthusianorum	DC70.42	The Alps, Switzerland	2001	X	X	MvDsp1	*M. dianthorum*
D. carthusianorum	70.22	The Alps, Switzerland	2001		X	MvDsp1	*M. dianthorum*
D. carthusianorum	433.09	Disentis, Switzerland	2006	X	X	MvDc	*M. dianthorum*
D. neglectus	19	The Alps, France	2004		X	MvDsp2	*M. dianthorum*
D. neglectus	IT03 St. Anna (Site 8)	Saint Anna, Italy	2003	X		MvDsp2	*M. dianthorum*
D. sylvestris	IT02 19-2A	The Alps, Italy	2002	X	X	MvDsp1	*M. dianthorum*
Gypsophila repens	GR4	Grosio, Italy	NC		X	MvDc	*M. dianthorum*
Lychnis flos cuculi	92.04	The Alps, Switzerland	2001	X	X	MvLf	*M. silenes-inflatae*
L. flos cuculi	92.05	The Alps, Switzerland	2001	X		MvLf	*M. silenes-inflatae*
L. flos-cuculi	L.fc fGS12	Sheffield, United Kingdom	NC	X	X	MvLf	*M. silenes-inflatae*
L. flos-jovis	IT03 ValDePesio Site 5 Sporidia E (A2)	Valle de Pesio, Italy	2003		X	MvLfj	
Saponaria ocymoides	02 Site21 Tet1	Sestrière, Italy	2002	X	X	MvSoff	*M. saponariae*
S. ocymoides	IT03 Sta1 Plant 2	Saint Anna, Italy	2003	X	X	MvSoff	*M. saponariae*
S. ocymoides	IT02 19-1	Cesana Torinese, Italy	2002		X		
S. officinalis	441	Sazos, French Pyrénées	2006	X	X	MvSoff	*M. saponariae*
Silene acaulis	FR02 Site 5	Gare de Peyrou d'Amont, France	2002	X		MvSa	*M. violaceum*
S. acaulis	89.01	The Alps, Switzerland	2001	X	X	MvSa	*M. violaceum*
S. acaulis	2002 Site 26 D A1	Sestrière, Italy	2002	X	X	MvSa	*M. violaceum*
S. caroliniana	384	Blue Ridge Parkway, USA	NC		X	MvSspA	*M. violaceum*
S. dioica	01 Site	Charterhouse, United Kingdom	2001	X	X	MvSd	*M. lychnidis-dioicae*
S. latifolia	41.06	Essonne, France	2001	X	X	MvSl	*M. lychnidis-dioicae*
S. latifolia	100.06	The Alps, Switzerland	2001	X	X	MvSl	*M. lychnidis-dioicae*
S. latifolia	UK00 Aldeburgh 14-1	Aldeburgh, United Kingdom	2000	X	X	MvSl	*M. lychnidis-dioicae*
S. latifolia	UK00 5.1H.9	United Kingdom	2000	X	X	MvSl	*M. lychnidis-dioicae*
S. latifolia	IT01 Lamole Site 15Plant 1	Lamole, Italy	2001	X	X	MvSl	*M. lychnidis-dioicae*
S. maritima	UK02 Charter House 1-2 (liz) C (A1)	Charterhouse, United Kingdom	2002	X	X	MvSv1	*M. silenes-inflatae*
S. maritima	365	Grunda, Olafsvik, Iceland	2005		X	MvSv2	*M. silenes-inflatae*
S. nutans	IT03 Guarda 1	Guarda, Switzerland	2003	X	X	MvSn	*M. violaceum*
S. nutans	70.01	The Alps, Switzerland	2001	X		MvSn	*M. violaceum*
S. paradoxa	IT04-2	Lamole, Italy	2004	X			
Atocion rupestris	02 Site 46-	Chambéry, France	2002	X	X	MvSv1	*M. lagerheimii*
S. virginica	387	Charlottesville Reservoir, Virginia, USA	NC		X	MvSspA	*M. violaceum*
S. vulgaris	FR02 Site 44-1	Chambéry, France	2002	X	X	MvSv1	*M. lagerheimii*
S. vulgaris	S.v.Sestri IT03	Sestrière, Italy	2003	X	X	MvSv2	*M. silenes-inflatae*
S. vulgaris	300.28	French Pyrénées, pic du midi de bigorre	2003	X		MvSv1	*M. lagerheimii*
S. vulgaris	84.19	The Alps, Switzerland	2001		X	MvSv2	*M. silenes-inflatae*
S. vulgaris	300.26	French Pyrénées, pic du midi de bigorre	2003		X	MvSv2	*M. silenes-inflatae*

Host species, isolate name, geographical origin, date of collection, obtained sequences (pr-MatA1 and/or pr-MatA2), and name of the Microbotryum species based on previous studies are shown.

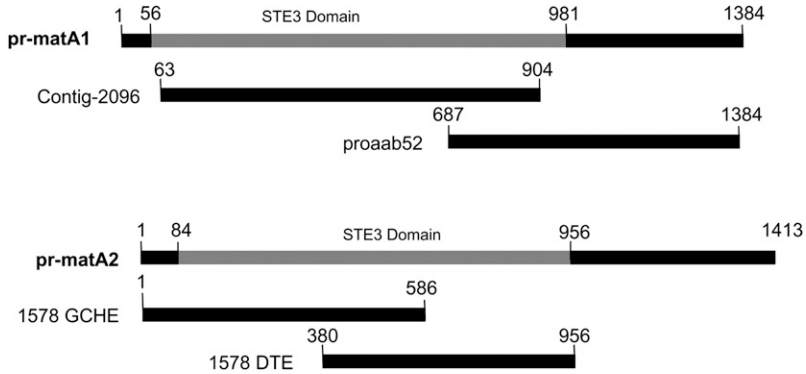

FIGURE 2.—Illustration of the fragments of the pheromone receptors pr-MatA1 and pr-MatA2 that were sequenced in multiple Microbotryum species. Two overlapping fragments were amplified per receptor using the primers indicated in Table 2.

for with the CODEML program of the PAML package (YANG 1997). Selective pressure was measured by using the nonsynonymous (d_N)/synonymous (d_S) substitution rate ratio ω. An $\omega < 1$ suggests purifying selection, $\omega = 1$ is consistent with neutral evolution, and $\omega > 1$ is indicative of positive selection (YANG and BIELAWSKI 2000). Nested codon models implementing the ω ratio can be compared by means of a likelihood ratio test (LRT) (ANISIMOVA *et al.* 2001). We used the null model M1a, which assumes two site classes with $1 > \omega_0 > 0$, and $\omega_1 = 1$, which therefore assumes no sites under positive selection, and compared it with the alternative model M2a, which adds an extra class of sites that allows ω to take values >1. We also compared the null model M7, which assumes a beta distribution of ω across sites, with the alternative model M8, which adds an extra class of sites to M7 where ω can take values >1. Thereby positive selection can be detected if a model allowing for positive selection is significantly more likely (as estimated by the LRT) than a null model without positive selection.

Phylogenetic trees for investigating *trans*-specific polymorphism: As the nucleotides sequences of pr-MatA1 and pr-MatA2 were too divergent to be reliably aligned, phylogenetic trees were built using amino acid sequences. Sequences from other fungi available in GenBank were also included to have the appropriate sampling scope for investigating *trans*-specific polymorphism. All sequences were aligned and assembled using Bioedit v6.0.7 and were manually edited when necessary. We kept only the alignment sections that were unambiguously aligned and without gaps. Phylogenetic trees were reconstructed by neighbor joining (NJ) and maximum likelihood (ML).

We first built a NJ tree including all the Microbotryum sequences (Figure 3) obtained by sequencing 1200 bp of pr-MatA1 and 1000 bp of pr-MatA2 (Figure 2 and Table 1). For the sake of clarity, we added here only the first GenBank hits for both pr-MatA1 and pr-MatA2 (Figure 3). The NJ tree was obtained using the Protdist and Neighbor programs in the Phylip package (FELSENSTEIN 1989). Distances were derived assuming the JTT + G model (JONES *et al.* 1992), a commonly used probabilistic amino acid replacement matrix at the 85% identity level, and allowing for rate heterogeneity among sites. The Seqboot program in the PHYLIP package was used to produce 100 resampled data sets, and bootstrap support proportions were obtained using the Consense program in

TABLE 2

Characteristics of the primers used in this study

Primer name	Sequence	Tm (°)	Receptor	Amplified part
Contig2096F	TCCTTTGTCACGACAAGCAC	52	pr-Mat A1	5′ part
Contig660R	CTGCGTCACGATACCTTTCTT	52	pr-Mat A1	5′ part
Contig2096F2[a]	ATAGTGCACGTCTGCCCAAC	62	pr-Mat A1	5′ part
Contig660R2[a]	GTGTGGCATTGGCGAGTC	58	pr-Mat A1	5′ part
pr0aab52-F	ATGACTCAGGCATCACCATC	60	pr-Mat A1	3′ part
pr0aab52-R	TTGAAGGCGCTCTTCTATTACA	52	pr-Mat A1	3′ part
pr0aab52-F2[a]	TACCAATTTTCGCGCCTACT	58	pr-Mat A1	3′ part
pr0aab52-R2[a]	CCGACTGGGACGCTTCTT	58	pr-Mat A1	3′ part
1578-GCHE-F	TCTTCACTTCGCGATACCAA	58	pr-Mat A2	5′ part
1578-GCHE-R	GCCATATCCCTTCACAATGG	60	pr-Mat A2	5′ part
1578-GCHE-F2[a]	GCGATACCAAGACACGATCA	60	pr-Mat A2	5′ part
1578-GCHE-R2[a]	CACAATGGCAAACCTTGTTG	58	pr-Mat A2	5′ part
1578-DTE-F	GAATAGGTGCCGAGATAGGG	62	pr-Mat A2	3′ part
1578-DTE-R	TCAAGATAACGACCTGTGATGC	52	pr-Mat A2	3′ part
1578-DTE-F2[a]	TTTCTGTGTTGCACGCTTTC	58	pr-Mat A2	3′ part
1578-DTE-R2[a]	TCAGATCGATGATAGTCCCGTA	51	pr-Mat A2	3′ part

Name, sequence, annealing temperature (Tm), corresponding pheromone receptor (pr-MatA1 or pr-MatA2), and amplified part of the pheromone receptor (see also Figure 1) are shown. Two pairs of primers were used per pheromone receptor, amplifying overlapping fragments (respectively, 5′ and 3′ parts).

[a] For these contigs, alternative primer pairs were designed when amplification failed.

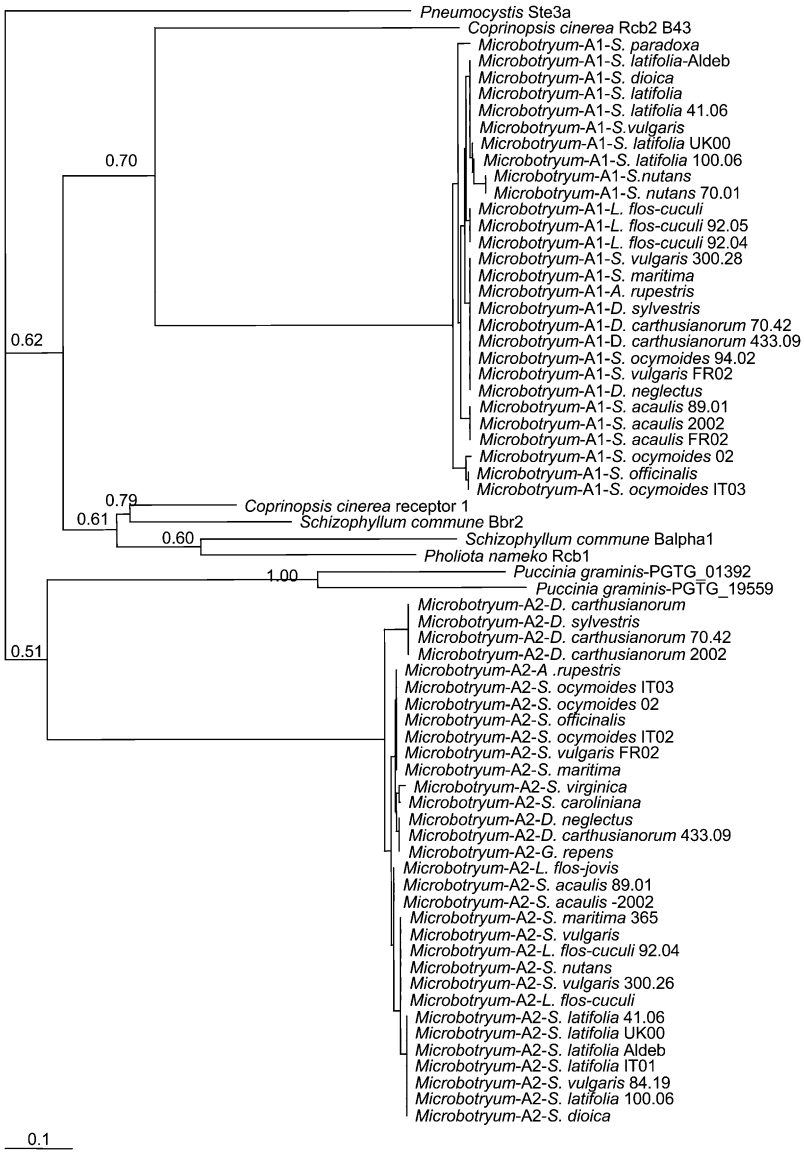

FIGURE 3.—Phylogeny based on the amino acid sequences of parts of the pheromone receptors pr-MatA1 and pr-MatA2 of Microbotryum and other fungi. Nodes not strongly supported are represented as unresolved. Statistical supports indicate NJ bootstraps. Taxa labels for Microbotryum sequences correspond to the mating type (A1 or A2) and to the host plant on which fungal strains were collected.

the PHYLIP package, assuming the majority rule criterion to derive the consensus tree (FELSENSTEIN 1989). We corroborated the NJ tree by constructing an ML tree with the same sequences using TreeFinder version May 2007 (JOBB 2007). The ModelTest v3.5 program (POSADA and CRANDALL 1998) with Akaike information criterion (AIC) was used to select the models that best fit our data. The PMB (probability matrix from Blocks) model of sequence evolution was assumed, which uses the Blocks database of protein alignments along with the additivity of evolutionary distances, to approximate the amino acid substitution probabilities as a function of actual evolutionary distance (VEERASSAMY *et al.* 2003).

We then built a second tree by ML using other characterized complete STE3-like pheromone receptors from basidiomycetes, including the two from Microbotryum (Figure 4). Accession numbers are as follows: *M. violaceum* pr-MatA2 (EF584741) and pr-MatA1 (EF584742); *C. cinerea* RCB1.3 (AAO17255), RCB1.6 (CAA71964), RCB2.6 (CAA71963), RCB2.42 (AAF01419), RCB2.43 (AAQ96345), RCB2.44 (AAQ96344), RCB3.6 (CAA71962), and RCB3.42 (AAF01420); *Schizophyllum commune* BAR1 (Q92275), BAR2 (CAA62595), BAR3 (P56502), BAR8 (AAR99618), BBR1 (P78741), and BBR2 (AAD35087); *Pleurotus djamor* PDSTE3.3 (AAS46748), PDSTE3.2 (AAP57506), and PDSTE3.1 (AAP57502); *C. neoformans* CPRa (AAF71292) and STE3a (AAN75156); *C. gatti* STE3p (AAV28793); *U. maydis* PRA1 (P31302) and PRA2 (P31303); *U. hordei* PRA1 (Q99063) and PRA2 (AAD56044); *Pneumocystis carinii* (AAG38536); *Saccharomyces cerevisiae* (P06783); *Sporisorium reilianum* pheromone receptors a1 (CAI59749), a2 (CAI59755), and a3 (CAI59763); *Puccinia graminis* hypothetical protein (PGTG_195592.2), conserved hypothetical protein (PGTG_00333.2), hypothetical protein similar to B beta mating-type pheromone receptor (PGTG_01392.2); *C. disseminatus* putative pheromone receptors CDSTE3.1 (AAP57492), CDSTE3.2 (AAZ04776), CDSTE3.3 (AAZ14943), and CDSTE3.4 (AAY88882). For *P. graminis*, we used the three proteins that yielded a significant hit when the pheromone receptors of *M. violaceum* were blasted against the complete genome (http://www.broad.mit.edu/annotation/genome/puccinia_graminis), although a single one was annotated as "pheromone receptor." The trees based on the resulting 297-amino-acid alignment were rooted using the sequences from the ascomycetes *P. carinii* and *S. cerevisiae*. To build this phylogenetic tree under a maximum likelihood framework we used the program PHYML (GUINDON and GASCUEL 2003). We assumed the protein model WAG + G + I (WHELAN and GOLDMAN 2001), which unlike the JTT model

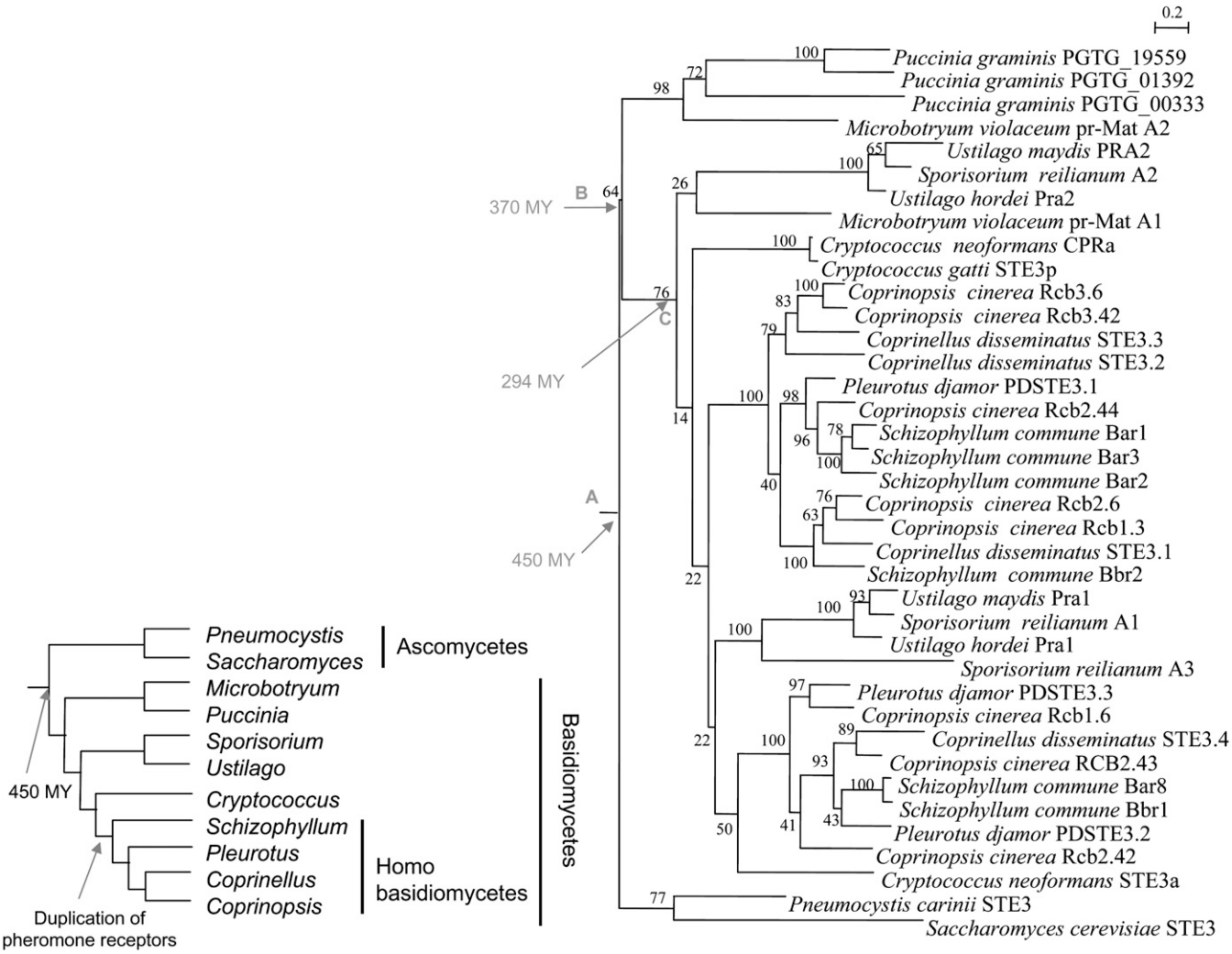

FIGURE 4.—Phylogeny of all known complete STE3-like pheromone receptors from basidiomycete fungi, obtained from the PHYML analysis. Statistical supports indicate ML bootstraps. The calibration point of 450 MY is indicated (node A), as well as the dates for nodes B and C inferred from the molecular clock analysis, whose resulting phylogeny is shown in supplemental Figure S3. The phylogeny of the same genera based on nuclear gene sequences obtained in previous works (ROUX *et al.* 1998; LUTZONI *et al.* 2004; BLACKWELL *et al.* 2006; JAMES *et al.* 2006; GARNICA *et al.* 2007; JAMES 2007; MATHENY *et al.* 2007) is given as an inset. The calibration point of 450 MY, which represents the divergence between ascomycetes and basidiomycetes, is also indicated, as well as the duplication of pheromone receptor genes at the mating-type locus. The time calibration was determined using a molecular clock analysis performed with PAML (supplemental Figure S3) and was placed on the PHYML tree for convenience.

employs an approximate maximum-likelihood method to estimate amino acid replacement matrices on the basis of a large sample of protein families. We allowed for among-site rate variation by using the discrete gamma model with four rate classes, and we estimated and optimized the proportion of invariable sites. The bootstrap analysis was done directly with PHYML, with which we generated 100 data set replicates and estimated a tree for each. The consensus tree was obtained by the majority rule criterion using the program Consense in the PHYLIP package (FELSENSTEIN 1989).

To verify the placement of pr-MatA1 and pr-MatA2 in the ML tree inferred for the STE3-like pheromone receptors in basidiomycetes, we conducted different phylogenetic tests comparing three topologies: (i) the tree previously obtained (Figure 4), (ii) an enforced topology placing the pr-MatA1 as branching on the same lineage as pr-MatA2, either at its base or as a sister leaf (supplemental Figure S1, A and B), and (iii) an enforced topology placing pr-MatA2 as branching on the same lineage as pr-MatA1, either at its base or as a sister leaf (supplemental Figure S2, A and B). Other than the displacement of either pr-MatA1 or pr-MatA2 in the two respective enforced phylogenies, the trees under comparison have the same topology. The topologies were compared using the CONSEL v0.1i software (SHIMODAIRA and HASEGAWA 2001), which implements the AU test (SHIMODAIRA and HASEGAWA 2001), the KH test (KISHINO and HASEGAWA 1989), the SH test (SHIMODAIRA and HASEGAWA 1999), and the RELL bootstrap proportions (SHIMODAIRA and HASEGAWA 1999). These tests compare the *P*-value associated with each tree, which represents the possibility of that tree being the true tree. We could thus rank the competing topologies according to their *P*-values to determine the most likely topology.

Inference of allele age: To infer the age of the divergence between alleles, we used the CODEML program in the PAML package (YANG 1997), which employs maximum likelihood to estimate node dates. Under the molecular clock assumption,

the distance between sequences increases linearly with their time of divergence, and by using an external point of reference (*e.g.*, a fossil record) the clock can be calibrated to estimate sequence distances and then infer the associated dates. CODEML allows the use of multiple calibration points, confidence intervals around these points, as well as the relaxation of the clock assumption. However, given the sparse geologic fossil record available for fungi, we used the only reliable calibration point available for our data set and fixed it to calibrate the clock. We used the Global Clock method assuming the WAG model for node date estimation, instead of the multiple local clock option that would be preferred had we more available fossil estimates for the phylogenetic scope of the present study. A recent study used different fossils as calibration points (Taylor and Berbee 2006) and estimated a minimal age of 330 MY for the divergence between Cryptococcus and Coprinopsis on the one hand, and Ustilago and Puccinia on the other hand. However, the placement of Puccinia in the same clade with Ustilago is not supported in all published phylogenies; instead, within the basidiomycetes Puccinia species are often more basal than Coprinus and Ustilago (Lutzoni *et al.* 2004; James *et al.* 2006; Aguileta *et al.* 2008). We did not use the divergence between Cryptococcus and Coprinopsis as a calibration point because of the presence of paralogs at pheromone receptor genes in these species. We therefore chose to use the divergence between ascomycetes and basidiomycetes, estimated between 450 and 1500 MY (Taylor and Berbee 2006), as a fixed calibration point. The minimal age of node A on Figure 4 was fixed according to the lower estimate, *i.e.*, 450 MY.

RESULTS

Allelic status of the two pheromone receptors identified in Microbotryum: The two pheromone receptor sequences characterized in Microbotryum are strictly associated with the mating behavior (A1 or A2) of sporidia (Yockteng *et al.* 2007). The most straightforward hypothesis is that they constitute alleles at the pheromone receptor gene involved in the determination of the A1 *vs.* A2 mating types. They should then be the sole pheromone receptor sequences expressed during mating. They could alternatively be linked paralogs with somehow different functions in the mating process, although such a case has never been reported in basidiomycetes. In this latter case, alternative alleles of each of the two characterized sequences, pr-MatA1 and pr-MatA2, should exist. The alternative allele of pr-MatA1 should then be found in A2 sporidia, the alternative allele of pr-MatA2 should be found in A1 sporidia, and at least four sequences of pheromone receptors should be expressed during mating.

To look for these hypothetical alternative alleles, we first performed PCRs using four primer pairs designed on each of pr-MatA1 and pr-MatA2 (Table 2) at low annealing temperature (48°), on each of two stock A1 and A2 sporidia. Primer pairs designed on pr-MatA2 never amplified any band in any A1 sporidia. The proaab52 primer pair designed on pr-MatA1 did not amplify any band in any A2 sporidia. The contig-2096 primer pair designed on pr-MatA1 amplified a band in A2 sporidia, at a smaller size than the expected band in A1 sporidia. This smaller band was also present in A1 sporidia, in addition to the band of expected size. We sequenced this PCR product after isolation on gel, but this second band did not correspond to pheromone receptors. We therefore could not amplify in A1 sporidia any sequence similar to pr-MatA2, and vice versa.

To further investigate the existence of possible alternative alleles of pr-MatA2 and pr-MatA1, we looked for other pheromone receptor sequences in the four cDNA libraries built on each of four Microbotryum species during mating, from which 40,000 clones have been sequenced by the Genoscope (Yockteng *et al.* 2007; and our unpublished data). These libraries represent excellent coverage of the expressed genes of Microbotryum, yielding between 5000 and 7000 unisequences each (Yockteng *et al.* 2007); and our unpublished data). The unisequences are all contigs and singlets, the contigs being assembled ESTs and the singlets being ESTs that cannot be assembled with other ESTs. The libraries were built using conjugating sporidia, *i.e.*, the stage at which the function of pheromone receptors is required, and in fact pr-MatA1 and pr-MatA2 were first identified among the ESTs of the library on *S. latifolia* (Yockteng *et al.* 2007). We performed BLASTn and tBLASTx searches using pr-MatA1 and pr-MatA2 as queries on the four cDNA libraries (Table 3). Several ESTs gave significant blast scores against each of pr-MatA1 and pr-MatA2, showing again good coverage of our libraries, especially those on the Microbotryum species from *S. latifolia* and *Dianthus carthusianorum.* BLASTn searches hit only sequences with ~99% similarity with the queries, *i.e.*, only with exactly the same transcript. The tBLASTx searches hit EST sequences with ~99% similarities to the queries but hit also the known alternative pheromone receptor with lower similarity, as expected because the tBLASTx program aligns protein sequences (Table 3). The tBLASTx searches were therefore able to retrieve distant pheromone receptors, but they hit only sequences with ~99% similarity with either pr-MatA1 or pr-MatA2, but not other sequences. This strongly suggests that no other pheromone receptors are expressed in Microbotryum during mating, apart from the previously characterized pr-MatA1 and pr-MatA2 receptors.

Lack of positive selection at Microbotryum pheromone receptors: Comparison among 38 Microbotryum strains revealed sequence variability at 14.72% of nucleotide positions among pr-MatA1 sequences and at 33.07% of sites in sequences of pr-MatA2. The somewhat higher variability among pr-MatA1 was due to the presence of sequences from a Microbotryum species distant from the other ones (from the host plant *S. paradoxa*), which was not sequenced for pr-MatA2.

The A1 sequences on the one hand and the A2 sequences on the other hand were compared among Microbotryum species. No evidence of positive selection was detected at any site in either pheromone receptor,

TABLE 3

Results from Blast searches on the four cDNA libraries of Microbotryum using the pheromone receptors pr-MatA1 and pr-MatA2 as queries

Blast with pr-MatA1		Blast with pr-MatA2	
Blastn	tblastx	Blastn	tblastx
		cDNA library of *Microbotryum* from *Silene latifolia*	
Contig 660 (5 ESTs)	Contig 660 (5 ESTs)	**Contig 330 (2 ESTs)**	**Contig 330 (2 ESTs)**
Contig 2096 (3 ESTs)	Contig 2096 (3 ESTs)	**Singlet 87y06**	**Singlet 87y06**
Contig 326 (6 ESTs)	Contig 326 (6 ESTs)	**Contig 588 (2 ESTs)**	**Contig 588 (2 ESTs)**
Singlet 55yc24	Singlet 55yc24	**Singlet 21ym21**	**Singlet 21ym21**
	Singlet 87y06		Contig 660 (5 ESTs)
	Contig 588 (2 ESTs)		Contig 2096 (3 ESTs)
		cDNA library of *Microbotryum* from *Dianthus cartusianorum*	
Singlet 92yg21	Singlet 92yg21	**Contig 64 (9 ESTs)**	**Contig 64 (9 ESTs)**
Contig 1508 (4 ESTs)	Contig 1508 (4 ESTs)	**Singlet 107ym05**	**Singlet 107ym05**
	Contig 64 (9 ESTs)		Singlet 92yg21
			Contig 1508 (4 ESTs)
		cDNA library of *Microbotryum* from *Silene nutans*	
Contig 297 (4 ESTs)	Contig 297 4 ESTs)	**Contig 1578 (3 ESTs)**	**Contig 1578 (3 ESTs)**
	Contig 1578 (3 ESTs)		
		cDNA library of *Microbotryum* from *Silene vulgaris*	
	Contig 3266 (2 ESTs)	**Contig 3266 (2 ESTs)**	**Contig 3266 (2 ESTs)**
	Singlet 57yk03	**Singlet 57yk03**	**Singlet 57yk03**

Underlined entries correspond to contigs or singlets with ~99% similarity to pr-MatA1 and entries in boldface type correspond to contigs or singlets with ~99% similarity to pr-MatA2. The number of ESTs assembled in each contig is indicated within parentheses. Singlets are made of single EST by definition.

indicating that there has not been detectable selection for changes in amino acids among the analyzed Microbotryum species. For A1 we found an average d_N/d_S ratio of 0.35 across sites, and for A2 we estimated an average d_N/d_S ratio of 0.17 across sites. These values are well below 1, which indicates purifying selection acting to maintain the function of both alleles. Likelihood ratio tests comparing models M1a and M2a, a formal test for positive selection, were not significant with *P*-values of 0.66 and 0.84 in the case of A1 and A2, respectively. Likelihood ratio tests comparing models M7 and M8 were also not significant, with *P*-values of 0.56 and 0.09 in the case of A1 and A2, respectively.

***Trans*-specific polymorphism at Microbotryum pheromone receptors:** The pr-MatA1 and pr-MatA2 sequences could only be aligned to each other when translated into protein but still exhibited variable sites at 71.62% of amino acid positions. The percentage of variable sites was 9.4% among A1 protein sequences and 7.5% among A2 protein sequences within the Microbotryum species complex. The sequences were also aligned with their best hits in GenBank and in fully sequenced fungal genomes, corresponding to pheromone receptors of other fungi. In the phylogenetic tree inferred from these data (Figure 3), all the A1 sequences of the different Microbotryum species were much more similar to each other than to any A2 sequences of their own species, and vice versa. Even more interestingly, Microbotryum sequences from pr-MatA2 appeared closer to the putative pheromone receptors identified here in the genome of *P. graminis* than to pr-MatA1 in Microbotryum species, and the Microbotryum sequences from pr-MatA1 appeared closer to pheromone receptors of *C. cinerea* and *S. commune* than to pr-MatA2 in Microbotryum species. If pr-MatA1 and pr-MatA2 are indeed alleles, the polymorphism in Microbotryum must therefore have been maintained from a time before the divergence between these basidiomycete fungal classes.

***Trans*-specific polymorphism at pheromone receptors in basidiomycetes:** To investigate whether *trans*-specific polymorphism was general in basidiomycetes, we inferred a phylogenetic tree of other STE3-like pheromone receptors from basidiomycete fungi (Figure 4). The pheromone receptor sequences from the bipolar *P. graminis* appeared relatively young, with no evidence of *trans*-specific polymorphism. In contrast, several fungi appeared to have *trans*-specific polymorphism at their pheromone receptor.

Let us consider first the fungi basal to homobasidiomycetes (see inset in Figure 4): because they have a single pheromone receptor gene at their MAT locus (paralogs being only present in homobasidiomycetes; Casselton and Kues 2007), there is no ambiguity in recognizing alleles of the receptor gene. The A1 sequences of *U. maydis*, *U. hordei*, and *S. reilianum* are closer to each other than they are to the A2 sequences from

their own species, and vice versa. The A1 and A2 alleles at the pheromone receptor of these three phylogenetically close species must therefore have been maintained since before their common ancestor. Our calibration analysis indicated that the alleles in these species must be at least 294 million years old (Figure 4). Similarly, the two alleles of the pheromone receptor gene in the bipolar Cryptococcus species look ancient, each allele being closer to sequences from other species than to the alternative sequence of Cryptococcus. Our calibration analysis indicated that the divergence must be at least 269 million years old (Figure 4). The *trans*-specific polymorphism of the Microbotryum pheromone receptor appeared however to be the most ancient. Using the available calibration date (Taylor and Berbee 2006), we estimated the minimal age of divergence for the two alternate Microbotryum pheromone receptors to be 370 million years old (Figure 4). This is a conservative estimate, as we used the more recent fossil date suggested so far for the ascomycetes–basidiomycetes divergence. Furthermore, the AU, KH, SH, and RELL *P*-value estimates (supplemental Table S1), confirmed that the phylogeny we recovered (Figure 4) has the topology most likely to be correct in comparison with the enforced topologies placing the Microbotryum A1 and A2 sequences in the same clade (supplemental Figures S1 and S2).

Regarding the homobasidiomycetes (*S. commune, C. disseminatus, C. cinerea*), the intermingled sequences of their receptors with those of other species may also reflect *trans*-specific polymorphism and/or the presence of ancient paralogous sequences. For *C. cinerea* for instance three pheromone receptors are present at the MAT locus, rcb1, rcb2, and rcb3 (Figure 1). The sequence variants of these rcb1, rcb2, and rcb3 genes are each found intermingled with other species (Figure 4): the variant 43 from rcb2 is for instance closer to the variant 6 from rcb1, and to sequences to other fungal species, than to the variant 44 from rcb2. The same pattern is found for sequence variants at the paralogous receptors Bbr and Bar in *S. commune* (Figure 4). The Bar8 sequence for instance is found closer to the Bbr1 sequence and to sequences from other species, than to the Bar1 variant from *S. commune*. This has been previously interpreted as complex evolution between paralogous sequences by recombination and/or gene shuffling (Riquelme *et al.* 2005; Casselton and Kues 2007), but our data on other basidiomycetes indicate that ancient *trans*-specific polymorphism at each paralog could also help explain the pattern.

DISCUSSION

The allelic status of the two pheromone receptors identified in Microbotryum: Our results yielded several lines of evidence strongly suggesting that pr-MatA1 and pr-MatA2 are two alleles involved in the determination of the A1 *vs.* A2 mating types: no other pheromone receptor sequence could be detected either by PCR at low annealing temperature or by blast searches in four high-covered cDNA libraries specific to mating cells. The tBLASTx searches in the cDNA libraries always hit the two distant pr-MatA1 and pr-MatA2 identified sequences, but no other sequences. The two characterized pheromone receptors pr-MatA1 and pr-MatA2, therefore, most probably constitute the two alternative alleles controlling the cell recognition and fusion in Microbotryum. The alternative hypothesis, that they are paralogs with different functions cannot, however, be entirely ruled out without functional experiments, but seems highly unlikely given the data at hand. Furthermore, the coexistence of different pheromone receptors having different functions in mating has never been described in any basidiomycetes so far. Yet another hypothesis is that pr-MatA1 and pr-MatA2 could be paralogs that have retained the same function and are the sole receptors in the genome, being the result of ancient duplications and differential gene losses. Some basidiomycetes indeed have paralogs at pheromone receptors, but the duplications seem to have arisen in the mushroom lineage, after the divergence from Microbotryum, Puccinia, and Cryptococcus (Figure 4; Casselton and Kues 2007), which makes this last hypothesis also unlikely.

Lack of evidence for positive selection at pheromone receptors in Microbotryum: No evidence of positive selection was detected at any sites, in either the A1 or A2 receptor, which indicates that there has not been any change in amino acids attributable to diversifying selection between the Microbotryum species analyzed here, in either the A1 or the A2 receptor. This was actually not surprising. We might have expected positive selection if the pheromone receptors were involved in species recognition during mating, but no evidence of such a role in interspecific gamete fusion has been detected within the *M. violaceum* complex (Le Gac *et al.* 2007b). We might also have expected positive selection if there had regularly been selection for the appearance of novel mating-type specificity within the *M. violaceum* complex. However, there are only two alternate mating types in all Microbotryum species studied to date, and the fungus has a mating system that is predominantly selfing (Hood and Antonovics 2000; Giraud 2004; Giraud *et al.* 2005, 2008). Any diploid teliospore that arrives and germinates on a plant should be heterozygous at the mating-type locus and thus be able to self. A rare third allele may therefore not have any advantage in a highly selfing basidiomycete like Microbotryum (Giraud *et al.* 2008). Positive selection for regular appearance of new receptor sequences is thus in fact not expected in such a case, in contrast to highly outcrossing species, where any new, rare allele will increase its chance of finding a compatible mate. It

is, however, unlikely that selfing has been the only factor maintaining only two alleles in Microbotryum for so long. Other mechanisms have been proposed that restrain the number of mating types. The most popular hypothesis is the need for the nucleus to reduce opportunities for conflict among organelles, which can be achieved by their uniparental inheritance, one sex, or mating type transmitting mitochondria (Hurst and Hamilton 1992). The number of mating types is then maintained down to two, except in rare cases where a complex hierarchy has been established between several mating types (Hurst and Hamilton 1992). Interestingly, mitochondria are in fact transmitted by one of the mating types, in Microbotryum (Wilch *et al.* 1992; and our unpublished data) and in other fungi (Barr *et al.* 2005). Other hypotheses for why the number of mating types does not increase despite balancing selection include the existence of sheltered load (Uyenoyama 2005). Recombination suppression around mating-type loci indeed leads to the fixation of recessive deleterious alleles in a permanently heterozygous state (Uyenoyama 2005). Such sheltered load can impede invasion by new mating types because the shared deleterious mutations in linkage to the ancestral and the derived mating types can then be revealed in a homozygous state (Uyenoyama 2003). This may be particularly important in species like Microbotryum having sex chromosomes with suppressed recombination (Hood 2002; Hood *et al.* 2004). Deleterious alleles linked to mating types have in fact been detected at high frequencies in Microbotryum (Oudemans *et al.* 1998; Hood and Antonovics 2000; Thomas *et al.* 2003). Mating dynamics has also been invoked as a force capable of maintaining only two mating types, in particular when gametes can wait for a suitable mate (Iwasa and Sasaki 1987). Under this hypothesis, however, there should be a regular turnover of alleles, and they should not be particularly old (Iwasa and Sasaki 1987).

In this study, we did not test for positive selection in lineages predating the divergence of A1 and A2 because the corresponding nucleotide sequences could not be reliably aligned. However, it remains an interesting possibility that positively selected substitutions predated, and were possibly associated with, the divergence of pr-MatA1 and pr-MatA2 in Microbotryum but have been fixed and are under purifying selection in the two alleles.

***Trans*-specific polymorphism at pheromone receptors:** All the pr-MatA1 sequences of the different Microbotryum species were much more similar to each other than to any pr-MatA2 sequences of their own species, and vice versa. Furthermore, pr-MatA1 was more similar to sequences from distant classes of fungi than to pr-MatA2, and vice versa. If pr-MatA1 and pr-MatA2 are indeed alternate allelic forms, as many lines of evidence strongly suggest, the polymorphism at the locus would then be deeply *trans*-specific, and would have been maintained from a time before the radiation of the Microbotryum species complex, and even well before the ancient separation from other classes of fungi. We have estimated the divergence between pr-MatA1 and pr-MatA2 to be at least 370 million years old. Alleles in a given species are usually closer to each other than they are to alleles from other species, due to the action of genetic drift and recombination, except in rare cases where balancing selection is occurring (Richman 2000). Mating types are genes where balancing selection is expected to exert a strong pressure, because mating can only occur between individuals with different alleles. Rare alleles should therefore have a strong advantage, which will limit their loss by genetic drift. In Microbotryum, where the mating type has only two alleles and where sexual reproduction is a prerequisite for infection, the two alleles are in fact indispensable in the life cycle and it seems intuitive that they should be maintained over huge periods of time. Several additional factors, as explained above, may help explain the maintenance of the same two alleles over a huge period of time: a new, third allele is not expected to be selected for in a highly selfing species like *M. violaceum*, the mating types control mitochondria transmission, and the sex chromosomes are not recombining (see above).

The divergence between pr-MatA1 and pr-MatA2 was in fact estimated to be at least 370 million years old, which would represent the oldest known *trans*-specific polymorphisms known in any group of organisms so far. *Trans*-specific polymorphisms reported in other organisms have been estimated to be much younger: 30 MY at a vegetative incompatibility locus in fungi (Wu *et al.* 1998), 14 MY at the sex-determining locus in bees (Cho *et al.* 2006), 80 MY at the major histocompatibility complex-encoded proteasome in amphibians (Nonaka *et al.* 2000), and 50 MY at an immunoglobulin variable region in rabbits (Su and Nei 1999). The polymorphism at mating-type loci had not been characterized with regard to being *trans*-specific so far, although balancing selection had been shown to occur (May *et al.* 1999).

In the phylogenetic tree of the known STE3-like pheromone receptors from basidiomycete fungi, several other fungi appear, however, to have *trans*-specific polymorphism at their pheromone receptors. The receptors of the tetrapolar *U. maydis* and *S. reilianum*, and of the bipolar *U. hordei*, three phylogenetically close species, appear to have been maintained since well before their common ancestor. The A1 sequences of the three species are indeed closer to each other than they are to the A2 sequences from their own species, and vice versa, and we dated their divergence node to be at least 294 million years old. This is in agreement with functional studies that showed that the *U. hordei a* locus (with the A1 pheromone receptors and pheromones), when introduced by transformation are necessary and sufficient to make *U. maydis* intercompatible with *U. hordei* MAT-2 strains but not MAT-1 strains (Bakkeren

and Kronstad 1996). The *trans*-specific polymorphism thus truly indicates here conserved function across speciation events by balancing selection. The phylogenetic tree of the known STE3-like pheromone receptors also indicated that sequences from the two mating types of *C. neoformans* must have been maintained since at least 269 million years ago. The presence of widespread *trans*-specific polymorphism shows that balancing selection is a common and consistent process at pheromone receptors in basidiomycetes.

Regarding the homobasidiomycetes (*S. commune*, *C. disseminatus*, and *C. cinerea*) the intermingled sequences of their receptors with those of other species may also reflect *trans*-specific polymorphism and/or the presence of ancient paralogous sequences. The fact that sequence variants of rcb1, rcb2, and rcb3 genes in *C. cinerea* for instance were found intermingled with sequences of other species from different genera has been previously interpreted as complex evolution between paralogous sequences by recombination and/or gene shuffling. It could also reflect ancient *trans*-specific polymorphism at each paralog. If there had been recombination between the paralogs, one would have expected homogenization of the sequences, so that they would appear clustered, or yield a tree with low bootstrap support. The pattern found could be explained by sequence shuffling among ancient paralogs (Riquelme *et al.* 2005; Casselton and Kues 2007). The activation of pheromone and receptors only within the rcb1, rcb2, and rcb3 classes in *C. cinerea* (Casselton and Kues 2007), however, suggest that the paralogs have maintained their integrity for a long time. This, together with the evidence here for ancient *trans*-specific polymorphism in basidiomycetes basal to the homobasidiomycetes (Microbotryum, Ustilago, Sporisorium, and Cryptococcus), make *trans*-specific polymorphism a more parsimonious explanation. The two hypotheses are, however, not mutually exclusive. Both balancing selection and gene shuffling among paralogs may have acted together to produce the complex pattern observed among pheromone receptor genes in homobasidiomycetes. In fact, some evidence supports the occurrence of gene shuffling: some paralogs were found very similar in sequences (*e.g.*, Rcb2.6 and Rcb1.3 in *C. cinerea* on Figure 4) and crossactivation between different sets of pheromone/pheromone receptor has been reported in *S. commune* (Fowler *et al.* 2004). However, it is important to note that, even under the hypothesis of gene shuffling among paralogs in homobasidiomycetes, strong balancing selection must be invoked to explain how such divergent copies, having the same function, are maintained across many speciation events. Gene shuffling should indeed rapidly erase genetic diversity among the copies unless this diversity is maintained by selection.

It is then interesting to look at the relationship between the number and the age of alleles (Takahata 1990; Vekemans and Slatkin 1994). The existence of multiple alleles at a sex-determining locus may mean that none is indispensable. Even though negative frequency-dependent selection can act to preserve alleles in the face of genetic drift, there can be turnover and the loss of old alleles as new allelic forms arise (Wright 1939). The polymorphism at mating types in fact appears older in *M. violaceum*, a bipolar selfing fungus with two alleles, than in tetrapolar outcrossing species with multiple alleles, such as *S. commune* and *C. cinerea*. The rationale would be that outcrossing selects for multiple mating-type alleles (Giraud *et al.* 2008) that then allows for allelic turnover despite balancing selection. Duplication of pheromone receptors also favors the emergence of a higher number of mating-type alleles, so that outcrossing should also select for gene duplications. In contrast, having a new, rare mating type does not widen the number of potential mates under selfing, as it might under outcrossing, because any diploid individual already has the two mating types needed to self. Rare alleles may thus not be selected for under selfing, favoring the same two alleles being maintained over very long periods of time. Selfing basidiomycetes would therefore be expected to be bipolar, with only two mating-type alleles, having diverged for a very long time. In contrast, outcrossing basidiomycetes would be expected to have multiple alleles, still with *trans*-specific polymorphisms, but younger than in selfing species. Further studies are required to elucidate the mating systems of basidiomycetes in nature and to determine if the number of alleles is linked to the level of outcrossing (Giraud *et al.* 2008). As explained above, however, factors other than selfing can also act to constrain the number of mating types, such as the existence of sheltered load linked to mating types (Uyenoyama 2005), which impedes the invasion of a new mating type because of shared deleterious mutations in linkage to the ancestral and the derived mating types (Uyenoyama 2003), or such as the need for the nucleus to reduce opportunities for conflict among organelles, which can be achieved by their uniparental inheritance, and is most easily achieved with only two mating types (Hurst and Hamilton 1992).

A striking feature of the tree including all the known fungal pheromone receptors is the presence of numerous sequences in the clade containing *M. violaceum* pr-Mat A1, including several species, while only Microbotryum and Puccinia sequences fall within the *M. violaceum* pr-Mat A2 clade. This may stem from an ascertainment bias due to the characterization of pheromone receptors based on similarity to the first characterized sequences. The major basidiomycete group to which Microbotryum and Puccinia belong, the Pucciniomycotina, has been much less well studied than the Ustilaginomycotina or Agaricomycotina groups, and other species with pheromone receptors falling in the same clade as pr-MatA2 may wait to be discovered. This view is actually supported by the recent genome

sequence of *Melampsora larici-populina*, where a putative gene was found to be very similar to pr-MatA2 (S. Duplessis, personal communication), while none was found to be more similar to pr-MatA1.

The fungal pheromone receptors of basidiomycetes appear as excellent models to investigate balancing selection and *trans*-specific polymorphism, although they have not yet been thoroughly studied on these aspects. Interestingly, no *trans*-specific polymorphism has been detected in the homedomain proteins that constitute the second part of the basidiomycete mating type loci among *U. hordei*, *U. maydis*, and *S. reilianum* (Schirawski *et al.* 2005), while they are expected to be under the same balancing selection as pheromone receptors. Analyses of homeodomain proteins of a wider range of basidiomycetes are required to assess whether *trans*-specific polymorphism also exists at the homeodomain protein genes of the mating type. Recent studies however suggest that the homeodomain proteins could have been recruited for the mating-type function later than the pheromone receptors in the evolution of the basidiomycetes (Coelho *et al.* 2008), which could explain that they do not show ancient *trans*-specific polymorphism.

Conclusion: Here we bring new light into the evolution of the mating types in fungi. Indeed, we have shown how *trans*-specific polymorphisms that are particularly ancient appear to be widespread at the genetic loci that determine mating recognition and compatibility of basidiomycetes, which deserves further studies. If the allelic status of the two pheromone receptors of Microbotryum can be confirmed, they would represent the oldest *trans*-specific polymorphism known to date, which may be due to its obligate sexuality and highly selfing mating system. The genomic evolution of bipolar mating systems, such as that of Microbotryum, seems to resemble the sex chromosomes of mammals more than the extensively polymorphic mating types of many other fungi. The first event marking the beginnings of the X–Y differentiation in mammals have for instance been estimated to have occurred ~240–320 million years ago (Lahn and Page 1999), which is close to the divergence between pr-MatA1 and pr-MatA2. The important distinction is that the two mating types are allelic in basidiomycetes, while in mammals the differentiation arose from the presence of specific genes on the Y chromosome that are now absent from the X. The evolution of suppressed recombination in the sex chromosomes of Microbotryum, and whether this has occurred by the addition of successive regions (*i.e.*, "strata"), as in animal and plant sex chromosomes (Lahn and Page 1999; Handley *et al.* 2004; Nicolas *et al.* 2005), as well as in fungi (Menkis *et al.* 2008), will be of particular importance to advancing our understanding of sex chromosomes in general and of the forces that constrain the number and age of alternate alleles in bipolar systems.

We thank S. Billiard, J. A. Shykoff, R. Debuchy, P. Gladieux, D. M. de Vienne, Y. Brygoo, G. Refrégier, M. Le Gac, M. Uyenoyama, and two anonymous referees for comments on the manuscript. We also thank J. Abbate for providing some of the DNAs, S. Duplessis for analyses on the unpublished genome of *Melampsora larici-populina*, and A. Gautier for her help with sequencing. We acknowledge the grants Agence Nationale de la Recherche (ANR) 06-BLAN-0201 and ANR 07-BDIV-003 to T.G. and the award of DEB-0346832 to M.E.H. from the National Science Foundation.

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Communicating editor: M. K. Uyenoyama