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Using phylogenies of pheromone receptor genes in the *Microbotryum violaceum* species complex to investigate possible speciation by hybridization

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Abstract: Several cases of speciation by hybridization have been reported in fungi, mostly involving recent hybridization between closely related species. In the basidiomycete genus *Microbotryum* by contrast some species were suspected to have arisen by hybridization between moderately distant species. In particular two species, *M. lagerheimii* and *M. silenes-acaulis*, had different placements in phylogenetic trees depending on the genes considered. *Microbotryum* species exhibit bipolar heterothallism, and here we analyzed sequences of the two alternate pheromone receptors to obtain further insights on the occurrence of hybridization. Indeed because mating-type loci are always heterozygous homoploid hybrid speciation should leave a permanent footprint at the mating-type locus by retaining the alternate alleles from their respective parental species. The trees obtained with each of the two pheromone receptors were well resolved, and the species relationships were in agreement with published phylogenies. Fungal pheromone receptor genes of basidiomycetes thus appear useful for phylogenetic studies, although it may not be true for the homobasidiomycetes where duplications of these genes have occurred. Furthermore an incongruence between the phylogenies of the two pheromone receptors was found for one species, *M. lagerheimii*, as previously observed between other nuclear genes. However additional species analyzed here revealed that the incongruence involved the whole clade including both *M. lagerheimii* and the *Microbotryum* species parasitizing *Lychnis flos-cucucli*. The ancestor of these species thus possibly arose via hybridization between distant ancestral lineages, although further studies should address alternative hypotheses, such as chance events during lineage sorting.

Key words: homoploid speciation, incomplete lineage sorting, introgression, mating type, novel host, STE3-like pheromone receptors

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INTRODUCTION

Many fungal species do not exhibit complete intersterility (Le Gac and Giraud 2008), which provides the opportunity for hybridization when they occur in sympatry. In fact several cases of speciation by hybridization have been reported in fungi (see Olson and Stenlid 2002, Schardl and Craven 2003, Giraud et al. 2008a for reviews), which may favor the emergence of adaptations to novel ecological niches, in particular for pathogenic fungi (Arnold 2004, Giraud et al. 2008a). Hybrid speciation is usually classified according to the ploidy level of the resulting individuals; when hybrids have a haploid chromosomal number that equals that of the parental species the process is called allopolyploid speciation, whereas hybrids with ploidy identical to that of the parents are referred to as allodiploids or homoploids. This classification system largely divides hybrids according to the reproductive isolating mechanisms responsible for their emergence as new species; allopolyploidy is thought to result directly in reproductive isolation of the hybrid from the parental species, whereas homoploid hybrids require some other genetic or ecological barrier to prevent backcrossing. Selfing and ecological specialization have been suggested as a factor in facilitating homoploid speciation in plants by isolating hybrids from their parental species (Lowe and Abbott 2004, McCarthy et al. 1995).

Allopolyploid hybrids have been identified in diverse fungal genera, such as *Botrytis allii*, the agent of gray mold neck rot of onion and garlic (Staats et al. 2005), several *Neotyphodium* species, symbiotic endophytes of grasses (Moon et al. 2004) and some *Saccharomyces* species used for brewing (Masneuf et al. 1998). A well described case of homoploid speciation is the rust *Melampsora* × *columbiana* that emerged from hybridization of the closely related *M. medusa* and *M. occidentalis*, parasites of *Populus* species (Newcombe et al. 2000). It is suspected that

some species in the basidiomycete genus *Microbotryum* have origins through hybridization between moderately distant species (Le Gac et al. 2007a). For two *Microbotryum* species, *M. lagerheimii* and *M. silenes-acaulis*, different placements in phylogenetic trees are observed depending on the genes considered. This suggested that these two species might have arisen by hybridization between different lineages and have given rise to novel pathogens on new host species.

Most *Microbotryum* species cause a sexually transmitted disease, anther smut, in plants of the Caryophyllaceae (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 undergo meiosis. *Microbotryum* species exhibit a heterothallic bipolar breeding system, where sexual conjugation between haploid cells of the two opposite mating types (A1 and A2) is required before infection. Automictic self fertilization is predominant (Hood and Antonovics 2000; Giraud 2004; Giraud et al. 2005, 2008b). Previously subsumed under the name *Microbotryum violaceum* anther smut fungi have been shown to be a complex of sibling species, specialized on different hosts (de Vienne et al. 2010) and showing post-zygotic isolation in the form of hybrid inviability and sterility (Le Gac et al. 2007a, b; Sloan et al. 2008; de Vienne et al. 2009). Taxonomy is currently being revised, with several new species described (Lutz et al. 2005, 2008; Kemler et al. 2006; Denchev 2007; Denchev et al. 2009). Of interest, the high rates of selfing (Hood and Antonovics 2000; Giraud 2004; Giraud et al. 2005, 2008b) and ecologically specialization on different host plants (Le Gac et al. 2007a, b; Sloan et al. 2008; de Vienne et al. 2009) are factors that should aid homoploid speciation (McCarthy et al. 1995, Lowe and Abbott 2004).

Two putative STE3-like pheromone receptors were characterized among sequences expressed during mating in the *Microbotryum* species infecting *Silene latifolia* (Yockteng et al. 2007, Devier et al. 2009). Several lines of evidence, such as linkage to the mating-type behavior and expression data, suggested that they probably constitute alternative alleles of the pheromone receptor at the mating-type locus, although their function has not been demonstrated (Yockteng et al. 2007, Devier et al. 2009). These two putative alleles showed ancient transspecific polymorphism (Devier et al. 2009), as is expected for genes under balancing selection (Richman 2000). These mating-type genes are interesting, not only because of what they reveal about the evolution of sex-linked genes but because they also might provide insights into the histories of speciation in cases of hybridization. Specifically, because mating-type loci are always heterozygous in the diploid or dikaryotic stage in heterothallic fungi homoploid hybrid speciation should leave a permanent footprint at the mating-type locus by retaining the alternate alleles from their respective parental species. In contrast a hybridization event giving rise to introgression that involves backcrossing to one of the parental species can restore congruence of phylogenetic histories of the alternate mating-type alleles even while the signals of introgression persist in other parts of the genome.

We therefore constructed phylogenies based on the nucleotide sequences of the two pheromone receptors, pr-MatA1 and pr-MatA2, in multiple *Microbotryum* species. Mushroom-forming homobasidiomycetes (e.g. *Coprinopsis cinerea*) have evolved multiple versions of their pheromones and receptors (Casselton and Kues 2007), which could impair their use as phylogenetic markers because of paralogy. Duplications of pheromone receptors however have not been observed for other basidiomycetes in more basal positions, such as *Microbotryum* (Devier et al. 2009). Sequence similarity analyses (i.e. BLAST) in extensive cDNA libraries and PCR under low annealing temperatures further support the result that pr-MatA1 and pr-MatA2 are likely to be alleles of a single-copy pheromone receptor per haploid genome (Devier et al. 2009).

To gain insights into possible speciation events by hybridization our aim was to compare phylogenies based on the two pheromone receptors and to incorporate studies characterizing other genomic regions (Le Gac et al. 2007a, Refrégier et al. 2008). Incongruent placement of *Microbotryum* species between phylogenies of the two pheromone receptors would be further evidence for an ancient event of speciation by hybridization. We therefore compared the phylogenetic topologies obtained for pr-MatA1 and pr-MatA2, in particular assessing the two *Microbotryum* species showing incongruent placements among trees based on genes unlinked to mating type (i.e. *M. lagerheimii* and *M. silenes-acaulis*). We also added some species that were not included in the previous tree comparison (Le Gac et al. 2007a), in particular the *Microbotryum* species parasitizing *Lychnis flos-jovis*. This species indeed might provide important information because it has been shown to be the sister species of *M. lagerheimii* (Refrégier et al. 2008) that similarly exhibited incongruent placements in different gene phylogenies.

MATERIALS AND METHODS

Sequences.—We used nucleotide sequences alignments obtained by Devier et al. (2009) to construct phylogenetic

trees for the loci pr-Mat 1 and pr-MatA2 (GenBank accession numbers FJ200750–FJ200809) in addition to novel sequences (GenBank accession numbers GU073457–GU073464). These sequences were amplified by PCR, sequenced, assembled, corrected by hand and then aligned with the others sequences following the same methods as described by Devier et al. (2009). Devier et al. (2009) constructed phylogenetic trees based only on amino acids alignments and did not investigate relationships among *Microbotryum* species. A total of 37 *Microbotryum* diploid strains were analyzed, belonging to at least 12 fungal species (Le Gac et al. 2007a) and collected from 19 host species in western Europe and America (TABLE I). Species were recognized based on congruence between multiple gene phylogenies, and taxonomic revision is under way (Kemler et al. 2006, Denchev 2007, Lutz et al. 2008, Denchev et al. 2009). Some species still await formal description. The *Microbotryum* species parasitizing *Lychnis flos-cuculi*, *S. caroliniana* and *S. virginica* have for instance not yet received Latin names. Species therefore will be referred to by the nomenclature of Le Gac et al. (2007a), often using the initials of their host species (e.g. MvSl for *Microbotryum* on *S. latifolia*) (see TABLE I and FIG. 1 for details and correspondence with existing Latin names). The alignment for pr-MatA1 is 1197 bp long and composed of 30 samples with 183 variable sites excluding gaps. The alignment for pr-MatA2 is 905 bp long and composed of 32 samples with 148 variable sites excluding gaps. The majority of length for the two putative pheromone receptors, pr-MatA1 and pr-MatA2 (86% and 64% respectively), were used in this study (see Devier et al. 2009 for details).

Phylogenetic trees.—The pr-MatA1 and pr-MatA2 nucleotide sequences are highly divergent and cannot be aligned within acceptable parameter ranges. Trees based on nucleotide sequences were constructed separately for the two pheromone receptors. DNA sequences generated in this study were added to the alignment with CodonCode 3.0.3 and corrected by hand when necessary. Only the sections that were unambiguously aligned were retained for further analysis. Phylogenetic trees were inferred by maximum likelihood (ML). ML analyses were performed with PhyML (Guindon and Gascuel 2003). Modeltest 3.5 (Posada and Crandall 1998) with Akaike information criterion (AIC) was used to select the models that best fit our data. The Tamura-Nei 93 model with invariable rate among sites (TN + I) (Tamura and Nei 1993) was chosen for the pr-matA1 tree (FIG. 1A), which accounts for unequal base frequencies, transition/transversion bias and different purine and pyrimidine transition rates. The Hasegawa, Kishino and Yano (1985) model with rate-heterogeneity among sites (HKY + G) (Hasegawa et al. 1985) was chosen for the pr-matA2 tree (FIG. 1B), which also accounts for unequal base frequencies and transition/transversion bias, but unlike the TN93 model it does not allow for different rates of pyrimidine and purine transitions. Bootstrap confidence values were calculated from 10 000 pseudoreplicates.

To root phylogenetic trees the *Microbotryum* strains from the North American hosts *S. caroliniana* and *S. virginica* were used because they were shown to branch at the base of all species analyzed in the present study (Kemler et al. 2006). Given the overall high congruence among the present mating-type phylogenies and the previous phylogenies based on house-keeping genes (Le Gac et al. 2007a, Refrégier et al. 2008), there is no reason to doubt that the *Microbotryum* species parasitizing the North American hosts *S. caroliniana* and *S. virginica* should not be suitable outgroups here.

To test whether the difference in the placement of the clade including the species MvSv1 and MvLfj was significantly different between the two trees we used CONSEL 0.1i 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 thus ranked the competing topologies according to their *P* values to determine the most likely topology. We compared the topology of the pr-MatA2 tree with an enforced topology placing the clade with MvSv1 and MvLfj in the pr-MatA2 tree as in the pr-MatA1 tree. Other than the placement of MvSv1 and MvLfj clade the trees under comparison had the same topology.

RESULTS

Nucleotide sequences of the two pheromone receptors were too divergent to be aligned, so we constructed phylogenetic trees of the *Microbotryum* species separately for each receptor. Comparison among *Microbotryum* genotypes revealed sequence variability at 15.82% of nucleotide positions among pr-MatA1 sequences and at 16.76% of sites in sequences of pr-MatA2. The species nodes were well supported in the majority of cases, although node support was stronger in the pr-MatA1 tree (FIG. 1).

Most of the phylogenetic relationships among the *Microbotryum* species were in agreement with species relationships obtained with other nuclear genes (Kemler et al. 2006, Le Gac et al. 2007a, Refrégier et al. 2008) and congruent between the pr-MatA1 and pr-MatA2 trees with two interesting exceptions. The clade including species MvSv1 (i.e. *M. lagerheimii*), parasitizing *S. vulgaris* (see TABLE I for species nomenclature) and the species MvLfj parasitizing *Lychnis flos-jovis* exhibited different phylogenetic positions between trees constructed with the A1 pheromone receptor, pr-MatA1, versus the A2 pheromone receptor, pr-MatA2 (incongruence indicated by the arrow in FIG. 1). Each of the two placements corresponded to a placement supported by at least one gene unlinked to mating type (Le Gac et al. 2007a). The two species, MvSv1 and MvLfj, were resolved as sister species in the two trees, as found

TABLE I. Characteristics of the *Microbotryum* isolates used in this study: host species, isolate name, geographical origin, date of collection, locus sequenced (pr-MatA1 and/or pr-MatA2) and name of the *Microbotryum* species based on previous studies

Host species	Isolate name	Geographic origin	Year of collection[a]	Locus sequenced		*Microbotryum* species[b]	*Microbotryum* species[c]
				pr-MatA1	pr-MatA2		
Dianthus carthusianorum	2002	Sestriere, Italy	2002		X	MvDsp1	*M. shykoffianum*
Dianthus carthusianorum	70.42	The Alps, Switzerland	2001	X	X	MvDsp1	*M. shykoffianum*
Dianthus carthusianorum	70.22	The Alps, Switzerland	2001		X	MvDsp1	*M. shykoffianum*
Dianthus carthusianorum	433.09	Disentis, Switzerland	2006	X	X	MvDc	*M. carthusianorum*
Dianthus neglectus	19	The Alps, France	2004		X	MvDsp2	*M. superbum,*
Dianthus neglectus	IT03	Saint Anna, Italy	2003	X		MvDsp2	*M. superbum*
Dianthus sylvestris	IT02	The Alps, Italy	2002	X	X	MvDsp1	*M. shykoffianum,*
Gypsophila repens	GR4	Grosio, Italy	NC		X		*M. violaceum sens. lat.*
Lychnis flos-cuculi	92.04	The Alps, Switzerland	2001	X	X	MvLf	*M. violaceum sens. lat.*
Lychnis flos-cuculi	92.05	The Alps, Switzerland	2001	X		MvLf	*M. violaceum sens. lat.*
Lychnis flos-cuculi	Tc1	Sheffield, United Kingdom	NC	X	X	MvLf	*M. violaceum sens. lat.*
Lychnis flos-jovis	W410	Sweden	NC	X	X	MvLfj	*M. violaceum sens. lat.*
Saponaria ocymoides	2002	Sestrière, Italy	2002	X	X	MvSoff	*M. saponariae*
Saponaria ocymoides	IT03	Saint Anna, Italy	2003	X	X	MvSoff	*M. saponariae*
Saponaria officinalis	441	Sazos, French Pyrénées	2006	X	X	MvSoff	*M. saponariae*
Silene acaulis	FR02	Gare de Peyrou d'Amont, France	2002	X		MvSa	*M. silenes-acaulis*
Silene acaulis	89.01	The Alps, Switzerland	2001	X	X	MvSa	*M. silenes-acaulis*
Silene acaulis	2002	S Sestriere, Italy	2002	X	X	MvSa	*M. silenes-acaulis*
Silene caroliniana	384	US, Blue Ridge Pkway	NC	X	X	MvSspA	*M. violaceum* sens. lat
Silene dioica	2001	Charterhouse, United Kingdom	2001	X	X	MvSd	*M. silenes-dioicae*
Silene latifolia	41.06	Essonne, France	2001	X	X	MvSl	*M. lychnidis-dioicae*
Silene latifolia	100.06	The Alps, Switzerland	2001	X	X	MvSl	*M. lychnidis-dioicae*
Silene latifolia	Aldeburgh	Aldeburgh United Kingdom	2000	X	X	MvSl	*M. lychnidis-dioicae*
Silene latifolia	UK00	United Kingdom	2000	X	X	MvSl	*M. lychnidis-dioicae*
Silene maritima	UK02	Charterhouse, United Kingdom	2002	X	X	MvSv1	*M. lagerheimii*
Silene maritima	365	Grunda, Olafsvik, Iceland	2005		X	MvSv2	*M. silenes-inflatae*
Silene nutans	IT03	Guarda, Switzerland	2003	X	X	MvSn	*M. violaceum* sens. str.
Silene nutans	70.01	The Alps, Switzerland	2001	X		MvSn	*M. violaceum* sens. str.
Atocion rupestris	2002	Chambéry, France	2002	X	X	MvSv1	*M lagerheimii*
Silene virginica	387	C'ville Res,Virginia, USA	NC	X	X	MvSspA	*M. violaceum* sens. lat.
Silene vulgaris	FR02	Chambéry, France	2002	X	X	MvSv1	*M lagerheimii*
Silene vulgaris	IT03	Sestriere, Italy	2003	X	X	MvSv2	*M. silenes-inflatae*
Silene vulgaris	300.28	French Pyrénées, pic du midi de bigorre	2003	X		MvSv1	*M lagerheimii*
Silene vulgaris	84.19	The Alps, Switzerland	2001		X	MvSv2	*M. silenes-inflatae*
Silene vulgaris	300.26	French Pyrénées, pic du midi de bigorre	2003		X	MvSv2	*M. silenes-inflatae*
Silene ottites	338	The Alps	2003	X	X		*M. violaceum sens. lat.*
Stellaria sp.	325	Denmark	2005	X	X		*M. violaceum sens. lat.*

[a] NC : non communicated.

[b] (Le Gac et al. 2007a).

[c] (Denchev 2007, Denchev et al. 2009, Kemler et al. 2006, Lutz et al. 2008).

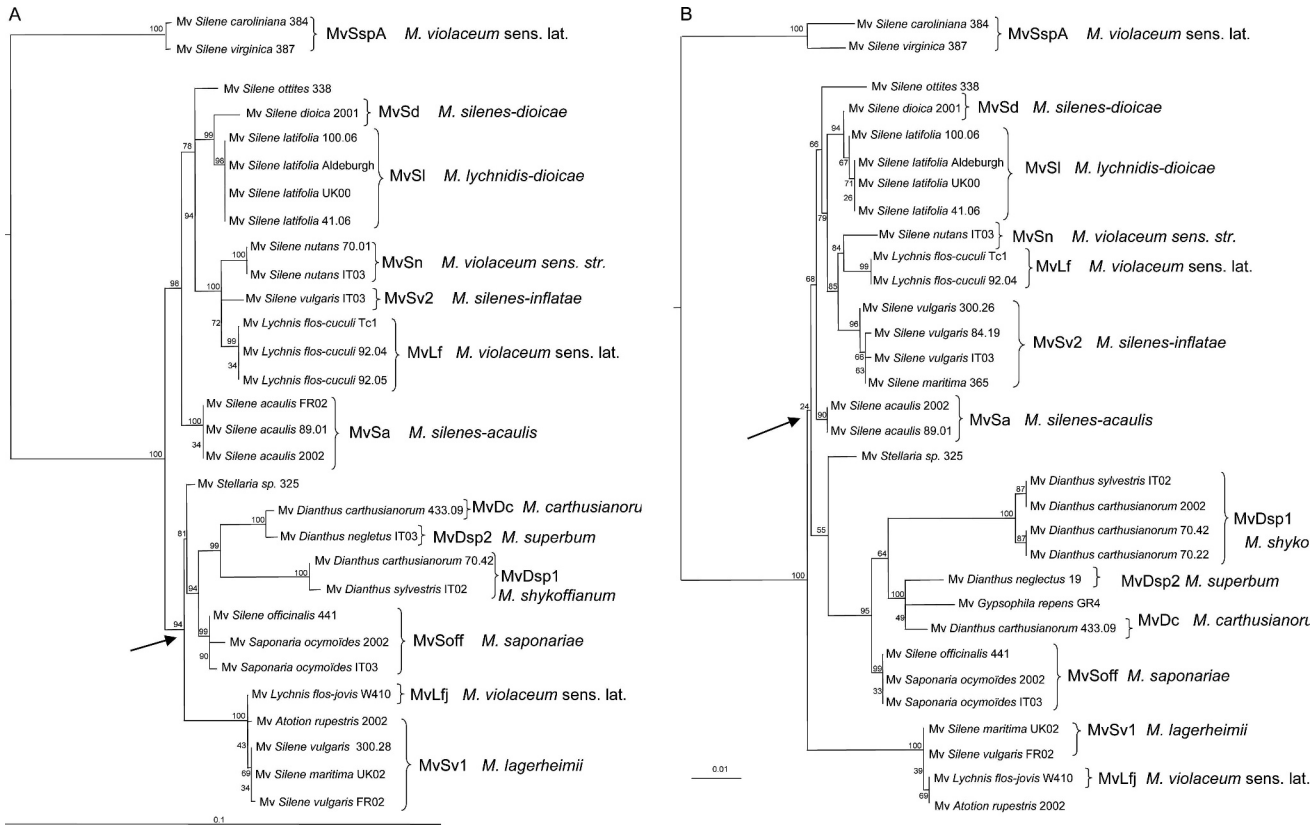

FIG. 1. Phylogenies based on the nucleotide sequences of parts of the pheromone receptors of *Microbotryum* species (A) pr-MatA1, (B) pr-MatA2. Unsupported nodes are represented as polytomies. Statistical support based on ML bootstraping. Taxon labels correspond to the host plant on which fungal strains were collected (Mv stands for *Microbotryum* and is followed by the name of the host plants; see TABLE I for details). Brackets indicate the fungal species name delimited based on multigene phylogenies (Le Gac et al. 2007a) plus the Latin name given by formal descriptions (Kemler et al. 2006, Denchev 2007, Lutz et al. 2008) (see TABLE I for details). The arrow indicates conflicting branches between the two pheromone receptor phylogenies. The trees are rooted based on previous studies (see text).

with other nuclear genes (Refrégier et al. 2008). It was thus the entire clade including species MvSv1 and MvLfj that exhibited different placements in the pr-MatA2 and pr-MatA1 trees. Node support for the placement of this clade however was not high in the pr-MatA2 tree. We therefore ran the AU test to check whether the sequence data of pr-MatA2 could be consistent with a placement of MvSv1 and MvLfj as in the pr-MatA1 tree. The results indicated that the sequence data of pr-MatA2 significantly supported the placement of the clade including MvSv1 and MvLfj in a different position than in the pr-MatA1 tree (TABLE II). The AU test thus indicates that the two trees indeed have significantly different topologies.

The species MvSa (i.e. *M. silenes-acaulis*) exhibited the same placement in both A1 and A2 phylogenies, which was also the same placement as in phylogenies based on two other nuclear genes (β-tubulin and elongation factor 1α) (Le Gac et al. 2007a). However the placement based on the pheromone receptors is markedly different from that obtained with the nuclear γ-tubulin gene (Le Gac et al. 2007a).

DISCUSSION

Analysis of fungal mating-type genes in basidiomycetes have shed new light on the unique evolutionary dynamics of sex-related genetics (Devier et al. 2009). The current study expands the significance of such studies to an understanding of speciation processes via hybridization. The trees obtained with each of the two *Microbotryum* pheromone receptors were well resolved, and the species relationships were in general agreement with the phylogenies of Lutz et al. (2005, 2008), Kemler et al. (2006), Le Gac et al. (2007a) and Refrégier et al. (2008). Therefore the divergence in the DNA sequence of mating-type genes are useful for phylogenetic studies, as already shown for ascomycetes (O'Donnell et al. 2004, Du et al. 2005, Voigt et al. 2005, Rau et al. 2007). Mushroom-forming homobasidiomycetes (e.g. *Coprinopsis cine-*

TABLE II. Results from CONSEL v0.1i comparing the pr-MatA2 tree and an A2 enforced tree with the clade including MvSv1 and MvLfj placed as in the pr-MatA1 tree

Tree	Rank	au	np	bp	pp	kh	sh	wkh	wsh
A2	1	0.919	0.604	0.622	0.602	0.723	0.723	0.723	0.723
Enforced A2	2	0.081	0.396	0.378	0.398	0.277	0.277	0.277	0.277

au, *p*-values for the approximately unbiased (AU) test; np, bootstrap probability of the selection; bp, same asnp, but calculated directly from the replicates; pp, Bayesian posterior probability (pp) calculated by the BIC criterion; kh, *p*-values for the Kishino-Hasegawa (KH) test; sh, *p*-values for the Shimodaira-Hasegawa (SH) test; wkh, *p*-values for the weighted Kishino-Hasegawa (WKH) test; wsh, *p*-values for the weighted Shimodaira-Hasegawa (WKH) test; For more details see (Shimodaira and Hasegawa 2001).

rea) have evolved multiple copies of their pheromones and receptors (Casselton and Kues 2007), suggesting that paralogy might impair the use of pheromone receptors for inferring phylogenies in that group. However duplications have never been reported for basidiomycetes other than the homobasidiomycetes (Devier et al. 2009). The present study thus indicates that pheromone receptors may be good candidate genes for constructing phylogenies in the basidiomycetes that are basal to the mushrooms.

Incongruence among the trees based on genes not linked to mating type, including γ-tubulin, β-tubulin and elongation factor 1α, suggested the occurrence of hybridization events among *Microbotryum* species (Le Gac et al. 2007a). In particular both species MvSv1 (*M. lagerheimii*) and MvSa (*M. silenes-acaulis*) exhibited incongruencies that involved deep positions in the trees and with nodes that were supported strongly. However such patterns of incongruence give little insight as to whether the breakdown of reproductive isolation between species gave rise to a new species via hybridization or subsequent backcrossing that results in introgression (Le Gac et al. 2007a). The current study on the phylogenies of the mating-type genes is aimed at disentangling these two alternative fates for hybridization events.

The two pheromone receptor phylogenies were congruent for the placement of MvSa (*M. silenes-acaulis*), but the nuclear γ-tubulin gene showed a different phylogenetic placement for this species (Le Gac et al. 2007a). This pattern may be explained either by hybridization or by chance events in lineage sorting during rapid speciation. In the case of hybridization the event involving MvSa must have been followed by back-crossing with a parental species that suggests introgression because the phylogenies of the pheromone receptors genes were congruent. In an ever increasing diversity of organisms such transient existence of hybrid genotypes has been shown to be a powerful evolutionary force by providing a bridge for genetic material between species (Mallet 2005, 2007). Further studies however should be conducted to disentangle hybridization and chance events during lineage sorting.

Our analysis of sequences from MvSv1 (*M. lagerheimii*) and MvLfj are more consistent with the scenario of hybrid speciation giving rise to the clade containing these species because the A1 and A2 pheromone receptor phylogenies retrace incongruent species histories; the enforced topology test indicated that the placements of the clade with MvSv1 and MvLfj were significantly different in the A1 and A2 trees. We detected no evidence of polyploidy or aneuploidy at any loci sequences (Le Gac et al. 2007a), including particularly the pheromone receptors analyzed here. The ancestor of MvSv1 and MvLfj species thus might have arisen by homoploid hybrid speciation without subsequent backcrosses with a parental species. Of note, this possible hybrid speciation event is associated with the only known case of such closely related *Microbotryum* species being found naturally on three host genera (i.e. *Silene*, *Lychnis* and *Atocion*; see Refrégier et al. 2008), which might suggest a role for hybridization in favoring the parasitism of novel host species. It remains possible however that backcrossing occurred with genes linked to the mating-type locus being favored for introgression. A more general explanation for phylogenetic incongruence involves chance events during lineage sorting due to rapid or recent speciation (Pollard et al. 2006), which is expected to yield incongruence among close species. This possibility cannot be completely ruled out here, and further studies are needed to positively document cases of homoploid speciation and to exclude alternative scenarios. Novel methods based on coalescence are beginning to be devised to tease out hybridization and incomplete lineage sorting (e.g. Joly and McLenachan 2009), but so far these analyses can be applied only to recent sister species with incomplete monophyly.

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