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ORIGINAL ARTICLE

Variation in mate-recognition pheromones of the fungal genus *Microbotryum*

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Mate recognition is an essential life-cycle stage that exhibits strong conservation in function, whereas diversification of mating signals can contribute directly to the integrity of species boundaries through assortative mating. Fungi are simple models, where compatibility is based on the recognition of pheromone peptides by corresponding receptor proteins, but clear patterns of diversification have not emerged from the species examined, which are few compared with mate signaling studies in plant and animal systems. In this study, candidate loci from *Microbotryum* species were used to characterize putative pheromones that were synthesized and found to be functional across multiple species in triggering a mating response *in vitro*. There is no significant correlation between the strength of a species' response and its genetic distance from the pheromone sequence source genome. Instead, evidence suggests that species may be strong or weak responders, influenced by environmental conditions or developmental differences. Gene sequence comparisons reveals very strong purifying selection on the a_1 pheromone peptide and corresponding receptor, but significantly less purifying selection on the a_2 pheromone peptide that corresponds with more variation across species in the receptor. This represents an exceptional case of a reciprocally interacting mate-recognition system in which the two mating types are under different levels of purifying selection.
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INTRODUCTION

Mating compatibility is a unifying and fundamental process to most eukaryotic species and forms an essential criterion upon which to view reproductive isolation that may lead to evolutionary divergence. In many organisms, structural or chemical traits of one individual must match a corresponding component of the mating partner—thus, compatibility is the result of coevolutionary dynamics within species and is also, in many systems, analogous to lock-and-key diversification that contributes directly to species boundaries. For example, from fruit flies to waterfowl, rapid evolution of male genitalia can be considered an adaptive response to mating compatibility (Acebes *et al.*, 2003; Brennan *et al.*, 2010). Similar lock-and-key organization is found in fungi, though not as morphological compatibilities. Instead, fungi most often use the recognition of mating-pheromone peptides, which have been detected in all fungal lineages (Kothe, 2008).

In fungi, mate recognition occurs during the haploid stage of the life cycle, when cells of different mating types are frequently isogamous (Billiard *et al.*, 2012). With a lack of morphological differentiation in structure, mating recognition occurs at a molecular level between pheromones and pheromone receptors. Cells of both mating types produce pheromones and receptors that interact in two reciprocally matching pairs of mating signals (Bakkeren *et al.*, 2008; Raudaskoski and Kothe, 2010; Lee *et al.*, 2010). Pheromone systems are essential to determining mating compatibility even in fungi that also exhibit anisogamy; the determination of gamete morphology is very rarely linked to molecular mating types (Billiard *et al.*, 2011). There are analogies to the self-incompatibility systems of flowering plants, where molecular rather than morphological mechanisms govern reproductive compatibility (reviewed by Hiscock *et al.*, 1996). Nevertheless, compatible isogamous gametes in fungi do not necessarily exhibit identical roles in cell fusion, and in particular the initiation of conjugation structures can be a behavior specific to only one mating type (Day, 1976).

The pheromone signals are small, nonpolar peptides secreted from a cell in response to appropriate mating conditions. They are produced from longer pheromone precursors that contain an N-terminal extension and a C-terminal short motif that are removed by proteolytic cleavage within the cells (Jones and Bennett, 2011). Despite the hydrophobic nature of these peptides, mating pheromones are capable of diffusing to nearby cells where they trigger a response if the cell has the corresponding transmembrane receptor protein (Bakkeren *et al.*, 2008).

Pheromone and receptor coevolution within species and divergence between species leads to clear expectations of assortative mating. Yet, a limited number of studies in fungi have systematically tested the sequence divergence in pheromone and receptor genes in relation to the strength of prezygotic reproductive isolation (see May *et al.*, 1999; Karlsson *et al.*, 2008; Devier *et al.*, 2009; Kellner *et al.*, 2011). Furthermore, studies generally have not found that diversification rates of pheromones and their matching receptors are correlated in a manner expected from coevolution (Nygren *et al.*, 2012), noting that these genes do not have major roles outside of mating (Billiard *et al.*, 2012). A lack of evidence for coevolutionary patterns may result from the contradicting influence of major selective forces upon mate signaling; in particular, pheromone–receptor recognition is so essential to the life cycle that evolutionary conservation might be expected, but

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the ability to discriminate intra- from inter-species mating could select for divergence of signaling compatibilities (James, 2012). In addition, mating type-specific functions (that is, signaling and initiation of conjugation structures) could mean that selective pressures on pheromones and receptors differ between the reciprocally interacting pairs produced by both mating types that, to our knowledge, has not been investigated previously. The biology of these pheromone-mating type interactions in fungi thus can provide valuable insights into the evolution of mating mechanisms, serving as understudied models for sexual eukaryotes.

Kniep (1919) demonstrated the classification of fungal strains into two opposite haploid mating compatibility types, and among the species he used were anther-smut fungi in the genus *Microbotryum* (formerly *Ustilago violacea*). The *Microbotryum* genus has served as a valuable model for sexual reproduction because mating between haploid cells is a prerequisite for infection of the host plant (Day, 1979), and thus the sexual cycle must occur every generation (that is, every disease transmission). *Microbotryum* is a very tractable model where the haploid cells are easily cultured and crossed *in vitro*. Several species in this genus have been well studied for the evolution of reproductive interactions (see, for example, Day, 1979; Le Gac *et al.*, 2007a; Giraud *et al.*, 2008), and cells are known to respond to the presence of the compatible mating type by halting the cell cycle and induction of conjugation tubes, resulting in fusion of the compatible cells. *Microbotryum* haploids are divided into two mating types, called a_1 and a_2, that exhibit similar developmental responses during mating, though initiation of the conjugation tubes is earlier and to a greater extent from cells of the a_2 mating type (Day, 1976). Direct evidence of assortative mating has been lacking, even when tested for directly (Le Gac *et al.*, 2007a, but see Büker *et al.*, 2013). It is important to note, however, that the mating system of *Microbotryum* tends to be a form of selfing called automixis, with low levels of outcrossing (Giraud *et al.*, 2008), and this mating system may limit the type of selection that results in assortative mating.

In this study, genome sequence data of several *Microbotryum* species were used to identify putative pheromone loci. The corresponding peptides were synthesized to assess whether mating responses could be triggered. Patterns of diversification and selection were quantified for the alternative pheromones and compared with prior work on their corresponding pheromone receptors. In addition, the synthetic pheromones from one species were applied to fungi across the *Microbotryum* genus to test for a correlation between genetic distance and mating response. Particularly for groups like the *Microbotryum* fungi, where studies have not shown assortative mating (Giraud, 2004; Le Gac *et al.*, 2007a), this combination of approaches can illuminate the molecular and phenotypic variation associated with reproductive compatibilities across species boundaries.

MATERIALS AND METHODS

Study system and origin of specimens

Specimens of *Microbotryum* (Basidiomycota, Microbotryomycetes) were collected as diploid teliospores from natural populations in North America and Europe of host plants in the Caryophyllaceae (Table 1). Recent studies have aimed to define species of *Microbotryum*, the names of which are used here, whereas unnamed species are commonly referred to by the epithet *Microbotryum violaceum sensu lato* followed by the host of origin (Kemler *et al.,* 2006; Denchev, 2007; Lutz *et al.,* 2008; Denchev *et al.,* 2009). In general, the anther-smut fungi found on different host species form highly specialized and reproductively isolated *Microbotryum* species, conforming to the Genealogical Concordance Phylogenetic Species Recognition criterion (Le Gac *et al.*, 2007a; Cai *et al.*, 2011). In addition, reproductive isolation between *Microbotryum* species has been observed in the form of postzygotic inviability and sterility (de Vienne *et al.*, 2009). For each species, haploid cultures of opposite mating types and originating from the same diploid parent were obtained by micromanipulation of the postmeiotic yeast-like sporidia. Haploid cultures were grown on potato dextrose agar (BD Difco, Franklin Lakes, NJ, USA) and stored frozen under desiccation (according to protocols in Hood and Antonovics, 2004).

Table 1 List of *Microbotryum* samples used in this study, their origins and genetic distance to *Microbotryum lychnidis-dioicae*

Species names[a]	*Host species*	*Sample origin*	*Genetic distance*[b]
M. lychnidis-dioicae	*Silene latifolia*	Lamole, Italy	0
M. silenes-dioicae	*Silene dioica*	Olivone, Switzerland	9
M. violaceum sensu lato	*Silene notarisii*	Gran Sasso, Italy	34
M. silenes-acaulis	*Silene acaulis*	Valle de Pesio, Italy	36
M. coronariae	*Lychnis flos cuculi*	Great Cumbrae Is., UK	38
M. violaceum sensu stricto	*Silene nutans*	Bois Carre, France	42
M. violaceum sensu lato[c]	*Silene lemmonii*	El Dorado County, California, USA	45
M. saponariae	*Saponaria ocymoides*	Cesana Tor, Italy	65
M. lagerheimii	*Silene vulgaris*	Chambery, France	66
M. lagerheimii	*Atocion rupestre*	Chambery, France	67
M. lagerheimii	*Lychnis flos jovis*	Valle de Pesio, Italy	68
M. lagerheimii	*Silene suecica*	Kirkjubaejarklaustur, Iceland	68
M. violaceum sensu lato	*Silene italica*	St. Steffano di Sessanio, Italy	75
M. violaceum sensu lato[c]	*Silene parryi*	Washington, USA	79
M. dianthorum	*Dianthus neglectus*	Valle de Pesio, Italy	80
M. carthusianorum	*Dianthus carthusianorum*	Sestriere, Italy	81
M. violaceum sensu lato	*Silene virginica*	Charlottesville, Virginia, USA	82
M. shykoffianum	*Dianthus sylvestris*	Cesana Tor, Italy	86
M. violaceum sensu lato	*Silene paradoxa*	Gran Sasso, Italy	87
M. violaceum sensu lato	*Silene caroliniana*	Virginia Beach, Virginia, USA	92

[a]As described elsewhere (Kemler *et al.,* 2006; Denchev, 2007; Lutz *et al.,* 2008; Denchev *et al.,* 2009).
[b]Genetic distance measured as numbers of nucleotide differences from *M. lychnidis-dioicae* based upon 1404 bp sequence concatenation of elongation factor 1α and β-tubulin.
[c]For these samples, only one mating-type haploid was available for testing.

Mating types of haploid cultures were identified by pairing with cultures of known mating types and examining the conjugation response that is elicited (Day, 1979). In addition, PCR primers that discriminate between a_1 and a_2 pheromone receptors (Devier *et al.*, 2009) were used to test for mating types following DNA extraction with the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). According to these methods, alternate alleles at the mating pheromone/pheromone receptor locus define the mating types of the isolated haploid cultures. For fungi in general, both mating types produce a pheromone product and possess a linked receptor for detection of the complementary pheromone (Bakkeren *et al.*, 2008; Lee *et al.,* 2010; Raudaskoski and Kothe, 2010).

Identified pheromone peptides and functional analysis in *M. lychnidis*-dioicae

Mating-pheromone sequences were identified using published pheromone sequences for a_1 and a_2 mating types in the related red yeast, *Rhodosporidium* (also a member of the Microbotryomycetes) (Coelho *et al.*, 2008). The *Rhodosporidium* sequences were used as tBLASTx search queries (ftp://ftp.ncbi.nlm.nih.gov/blast/executables/) against *Microbotryum lychnidis-dioicae* genomic survey sequences, including EST databases (Microbase http://genome.jouy.inra.fr/funybase/FUNYEST/WEB/CGI-BIN/funyest_home.cgi; Yockteng *et al.*, 2007). Candidate loci were further identified by searching for a C-terminus CAAX motif (C indicating cysteine, A an aliphatic amino acid and X various amino acids) characteristic of fungal mating pheromones (Jones and Bennett, 2011).

To assess whether loci coding for the mating pheromones had been correctly identified, the DNA sequences from *M. lychnidis-dioicae* were translated into peptide sequences for commercial synthesis by AnaSpec (Fremont, CA, USA), attempting to match the peptide boundaries and modifications described for other basidiomycete fungi (Kües *et al.*, 2011). The three synthesized peptides were subject to farnesylation, the addition of a methyl group to the C-terminal cysteine residue and high-performance liquid chromatography purification. Peptide sequences and purity were obtained as follows: a_1 pheromone YPKATYTLPQC$_{(Farnesyl)\text{-}Ome}$ (>95% purity), an a_2 pheromone ERNPM SNFWVAVC$_{(Farnesyl)\text{-}Ome}$ (>80% purity) and a second a_2 pheromone ERNPKDNVLGGESC$_{(Farnesyl)\text{-}Ome}$ (>93% purity). Lyophilized peptides were suspended in methanol for use in functional assays, and preliminary tests led to the use of pheromones at 1 mM concentration.

Assays were performed at 22 °C on 1% water agar medium with the addition of α-tocopherol (Phytol) (Sigma-Aldrich, St Louis, MO, USA) according to Hood and Antonovics (2000). Such low nutrients, temperatures not >25 °C and host extracts, in particular α-tocopherol, have been shown to stimulate production of conjugation structures in *Microbotryum* (Day *et al.*, 1981). Preliminary tests also included combinations of 5C, 15C, without the addition of α-tocopherol, and the minimal medium (minus glucose) reported for *Microbotryum* (Day and Jones, 1968), but there were not qualitative differences from the employed assay conditions.

Each of the three synthesized peptides and a methanol-only control was tested with a_1 and a_2 cultures of *Microbotryum* species. For each test, a 1-μl drop of 1 mM pheromone was first spotted onto the agar surface and allowed to stand for 5 min for the methanol to evaporate or disperse. Afterwards, a 1-μl drop of cells was spotted onto the same location as the pheromone. Cell suspensions were diluted in sterilized, deionized water such that a 1-μl drop on the agar surface distributed cells that were not in direct contact with other cells. The cells were observed with an inverted microscope after 24 and 48 h and 1 week.

Interspecific response to the pheromone peptides from *M. lychnidis-dioicae*

Using a range of *Microbotryum* species (Table 1), the proportions of cells producing conjugation tubes (on a scale corresponding to 20% increments) were recorded while blind to the synthetic pheromone, species and mating-type treatments. Responses to the three synthetic mating pheromones were measured at 24 h and 1 week after exposure.

Genetic distances among the *Microbotryum* species were calculated as the numbers of nucleotide differences from *M. lychnidis-dioicae* (the source of the synthetic pheromone peptide sequences) based upon 1404 bp concatenation of Elongation factor 1α and β-tubulin sequences produced by Le Gac *et al.* (2007b). Correlation between genetic distances and pheromone response strengths (that is, the proportions of cells producing conjugation tubes) was tested using Spearman's rho nonparametric test, and differences in response strengths between the two a_2 pheromones were assessed by a paired sample *t*-test in PASW Statistics v18 (SPSS Statistics, Chicago, IL, USA).

Sequence variation at pheromone loci and correspondence with pheromone receptor loci

Mating-pheromone loci from other *Microbotryum* species were obtained using pheromone sequences from *M. lychnidis-dioicae* as BLASTn search queries for genomic survey sequence libraries from several *Microbotryum* species (ENA accession numbers PRJEB6548; http://www.ebi.ac.uk). In addition, PCR primers were designed to regions flanking the mating pheromones using the web-based program Primer3 (http://frodo.wi.mit.edu/primer3/) with the default settings. For a_1 pheromones, the primers were: phero_a_1_F: 5′-AGCC TGTGCACCGGATAG-3′ and phero_a_1_R: 5′-ACACCTCCAGCCTCAATAC TAACATCTC-3′. For the a_2 pheromones, the primers were: phero_a2_F: 5′-AGCCGCCTCGAAGAGC-3′ and phero_a2_R: 5′-AGTTCCGAAGGGC CACA-3′. In order to detect multiple copies of the pheromones per genome, PCR products were cloned using the TOPO TA cloning kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions, and 10 clones per species were sequenced by Sanger methodologies in both directions.

DNA sequences corresponding to the mating-pheromone peptides were aligned separately for the a_1 and a_2 alleles using Clustal X Version 1.6 (Thompson *et al.,* 1997), corrected visually, for both nucleic acid and peptide sequences. Analyses of sequence variation were conducted in MEGA5 (Tamura *et al.,* 2011). The two-tailed *Z*-test for patterns of selection that depart from neutrality (based upon nonsynonymous and synonymous substitutions rates, dN and dS, respectively) was conducted in MEGA5, using a set of species where comparable representation was possible for both a_1 and a_2 sequence alignments (Figure 1) and with variance estimates based upon 500 bootstrap replications. A Kolmogorov–Smirnov test of whether a_1 and a_2 pheromone-coding codons could be differentiated by rates of nonsynonymous to synonymous substitutions was performed in PASW Statistics v18. Assessment of the distribution of conserved versus variable amino-acid positions between the mature a_2 pheromone peptide and the N-terminal extension region was performed by randomization tests, comparing the proportion of 1000 simulated random distributions of conserved amino-acid positions to the observed number in the pheromone-coding region.

Pheromone receptor sequences were obtained from Devier *et al.* (2009, 2010) for a comparable set of species from which pheromone peptide sequences were obtained. Phylogenetic trees were reconstructed based on aligned protein sequences in MEGA5, using the neighbor-joining method (Saitou and Nei, 1987), with evolutionary distances computed by the Kimura 2-parameter method (Kimura, 1980) using the number of substitutions per site. Pairwise deletions were used for the analyses. For each set of aligned pheromone receptor sequences, the average pairwise distance was calculated using the numbers of differences per 100 amino acids, with s.e. determined by the bootstrap method with 100 replications.

RESULTS

Identified pheromone peptides and functional analysis in *M. lychnidis-dioicae*

Searching haploid *M. lychnidis-dioicae* genomic sequences for mating-pheromone peptides yielded multiple loci with nonidentical DNA sequences in both a_1 and a_2 genomes (Figure 1). Mating-pheromone loci in the a_1 genome coded for tandem repeats of the pheromone peptide that were identical in amino-acid sequence within and between loci. The a_2 genome contained loci, each with a single iteration of the mating-pheromone peptide, that did not code for identical pheromone peptides between loci. Where variation in the pheromone-coding region was observed within a_2 genomes, there existed both nonsynonymous nucleotide substitutions and a

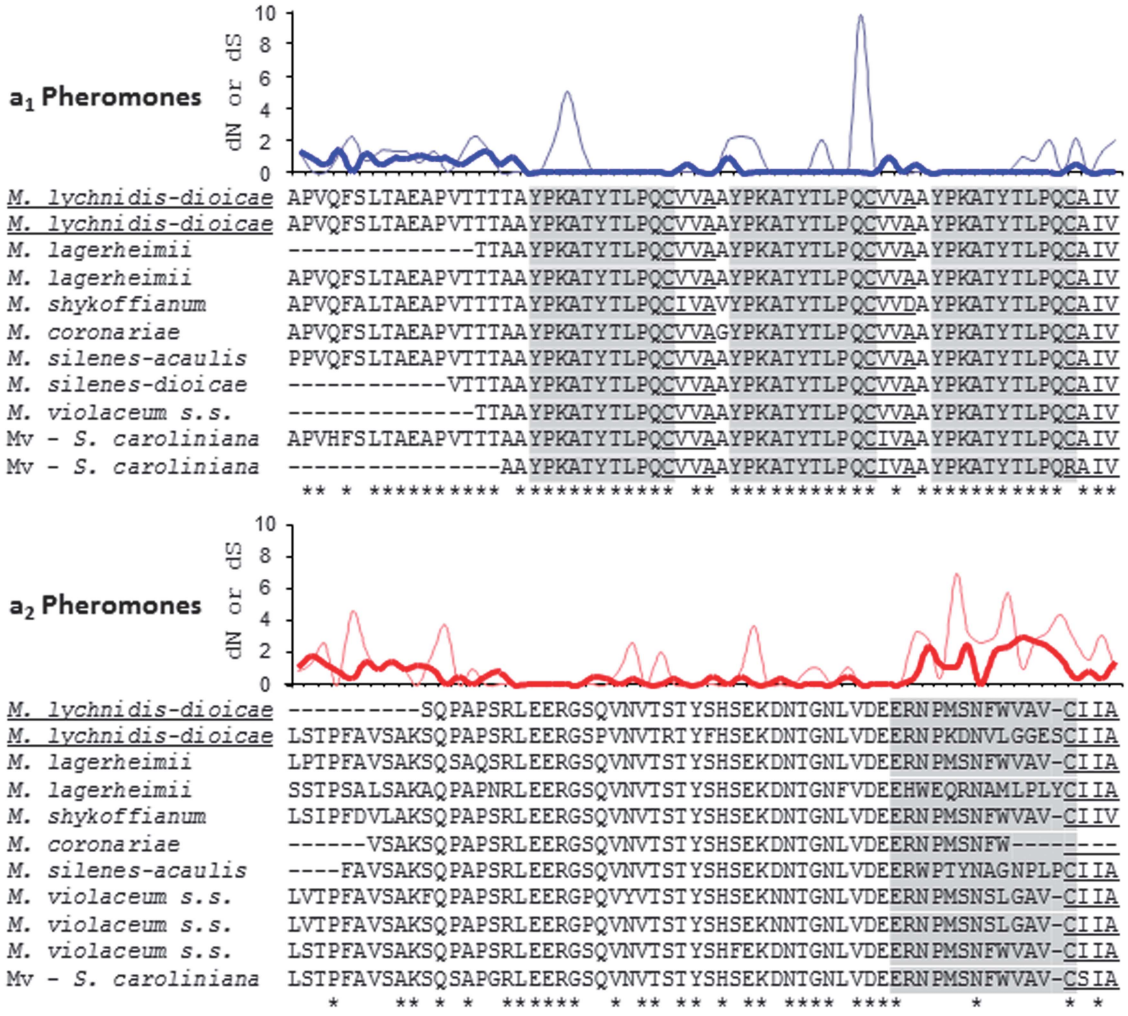

Figure 1 Amino-acid sequence alignments from a_1 and a_2 mating-pheromone loci in the genus *Microbotryum*. Putative pheromone peptides are identified in gray background, and underlined taxon entries indicate the source and sequences of synthesized pheromone peptides. CAAX motifs are indicated by underlined text, and invariable residues are shown with asterisks. Above the alignments are plotted the nonsynonymous DNA sequence substitutions per nonsynonymous site (dN, thick lines) and synonymous substitutions per synonymous site (dS, thin lines).

three-nucleotide insertion/deletion mutation corresponding to an amino-acid length change in the putative pheromone peptide.

Using the three synthesized peptides (one a_1 and two a_2 mating-pheromone variants), cell-cycle arrest and conjugation structures as previously described (Day, 1976) were induced in *M. lychnidis-dioicae* a_1 and a_2 cells following exposure to mating-pheromone peptides derived from their complementary mating-type genomes (Figure 2). Both synthesized variants of a_2 pheromone peptides induced conjugation reactions in a_1 cells. When exposed to pheromone peptides from their own mating-type genome, a_1 and a_2 cells continued to divide and appeared unaffected compared with the methanol-only control. Consistent with expectations for *M. lychnidis-dioicae* (Day, 1976), a_2 cells developed longer conjugations tubes than a_1 cells that produced only short protuberances or pegs that were most visible after 48 h (Figures 2 and 3). At 48 h, some cells of either mating type had begun to revert to mitotic divisions (Figure 3).

Interspecific responses to the pheromone peptides from *M. lychnidis-dioicae*

Species across the genus *Microbotryum* responded to the synthetic a_1 and a_2 pheromone peptides in a manner similar to *M. lychnidis-dioicae*, with arrest of cell division and production of conjugation tubes. There was no significant correlation between the strength of response (that is, proportions of cells producing conjugation tubes) to the mating-pheromone peptides and the genetic distance of the species to *M. lychnidis-dioicae* from which the synthesized peptide sequences were obtained. For the invariant synthesized a_1 pheromone and two a_2 pheromones at 22 °C and scored after 24 h or 1 week, the correlation coefficients between response strength and genetic distance ranged from $r = 0.29$ to -0.16, and the smallest two-tailed P-value was 0.21 (Supplementary Figure S2).

There were significant patterns of the response strengths that revealed differences among the *Microbotryum* species. In particular, the strength of a species' response to the a_1 pheromone was positively correlated with the strengths of response to each of the two a_2 pheromones, noting that these responses were measured with different cell cultures of alternate a_2 and a_1 mating types, respectively. The 24-h measurements produced responses to the a_1 pheromone that were weakly, but not significantly, positively correlated with responses to the two a_2 pheromones (peptides ERNPMSNFWVAVC and ERNPKDNVLGGESC, respectively) per species, with $r = 0.41$, $P = 0.07$, and $r = 0.37$, $P = 0.11$. The 1-week measurements produced responses

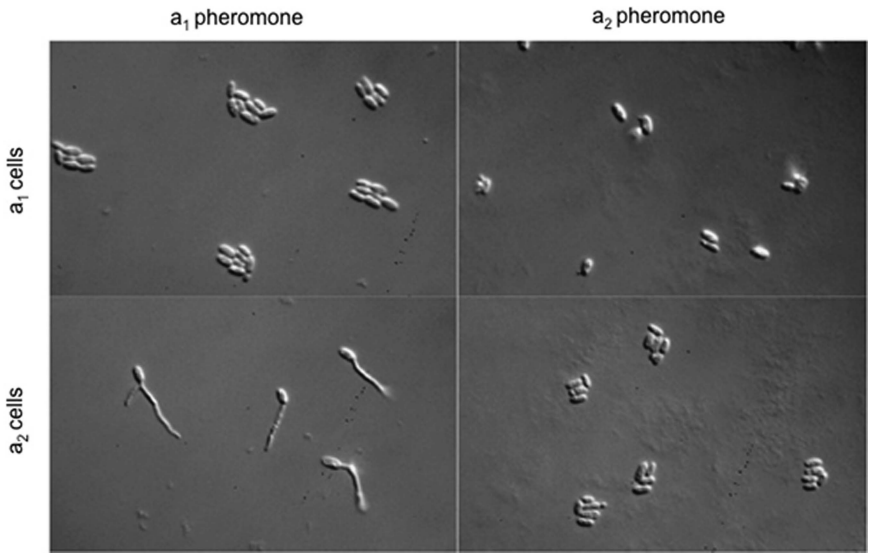

Figure 2 *In vitro* responses by haploid *Microbotryum lychnidis-dioicae* cells cultures to synthetic a_1 and a_2 mating-pheromone peptides at 24 h after exposure. Differential interference contrast micrographs show arrest of cell division and production of conjugation structures following treatment of cells with peptides derived from pheromone sequences in opposite alternate mating-type genomes. Cells exposed to peptides derived from pheromone sequences in their own genomes continue cell division. Long conjugation tubes are produced by a_2 cells, but not by a_1 cells.

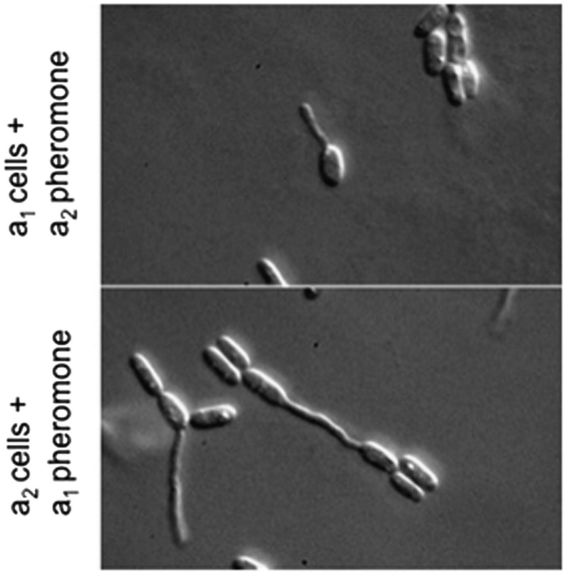

Figure 3 *In vitro* responses by haploid *Microbotryum lychnidis-dioicae* cells cultures to synthetic a_1 and a_2 mating-pheromone peptides at 48 h after exposure. Differential interference contrast micrographs show extension of conjugation peg structures in a_1 cells and reversion to cell division in a_2 cells.

to the a_1 pheromone that were strongly positively correlated with responses to the two a_2 pheromones per species, with $r=0.65$, $P<0.01$, and $r=0.567$, $P=0.01$. Between the two forms of a_2 pheromones identified from *M. lychnidis-dioicae*, the peptide ERNPMSNFWVAVC, which is the a_2 peptide sequence found in several other *Microbotryum* species (Figure 1), induced stronger responses in a_1 cells across species than the ERNPKDNVLGGESC peptide, which is unique to *M. lychnidis-dioicae* among the species sampled; paired samples *t*-test, $t=-2.99$, $P=0.01$.

Sequence variation at pheromone loci and comparison with pheromone receptors

Greater variation in the a_2 pheromones compared with the uniform a_1 pheromone resulted from the relaxation of purifying selection rather than diversifying selection. In particular, both a_1 and a_2 mating-pheromone loci showed statistically significant purifying selection across species when considering the pheromone peptide and the N-terminal extension peptide sequence, but with greater deviation from neutrality observed for a_1 than the a_2 pheromone loci (*Z*-test statistic $=-3.87$, $P<0.01$ for a_1, and *Z*-test statistic $=-2.39$, $P=0.02$ for a_2). Average rates of synonymous substitutions were 2.8 and 1.8 times greater than nonsynonymous substitutions for the a_1 and a_2 loci, respectively. Specifically for the pheromone peptide-coding region, again both mating types showed purifying selection (*Z*-test statistic $=-3.92$, $P<0.01$ for a_1, and *Z*-test statistic $=-2.91$, $P<0.01$ for a_2). Two codons for the a_2 mating-pheromone peptide had dN values greater than dS (Figure 1), but neither showed statistical significance of the individual codon dN/dS ratio (smallest *P*-value $=0.31$). Nevertheless, the dN/dS rates for pheromone-coding section of the more variable a_2 pheromone were significantly less purifying (that is, selection relaxed to a greater extent) than for the uniform a_1 pheromone (Kolmogorov–Smirnov test, $P=0.02$). Interestingly however, the relaxation of selection was not uniform across the pheromone locus, with the a_2 pheromone peptide sequence itself containing significantly fewer conserved amino-acid positions than the N-terminal extension peptide sequence (randomization of conserved residues test, $N=1000$, $P=0.02$).

This greater variation in the a_2 pheromone was potentially because of one locus being conserved while a paralogous locus being under relaxed selection within the *Microbotryum* a_2 genomes. Across all *Microbotryum* species yielding a_2 pheromone sequences, with the exception of *M. silenes-acaulis*, the pheromone peptide ERNPMSNFWVAVC was found, whereas in several species variant a_2 pheromone forms existed additionally. Among just the variant forms of the a_2 pheromone, the test for deviation from selection

neutrality was not significant (Z-test statistic $= -0.82$, $P < 0.42$; Supplementary Figure S1). However, even among these variant forms of the a_2 pheromone, the pheromone peptide sequence itself contained significantly fewer conserved amino-acid positions than the N-terminal extension peptide sequence (randomization of conserved residues test, $N = 1000$, $P < 0.001$), indicating variation in selection among different parts of the a_2 pheromone precursor.

Comparable sets of species from which mating-pheromone sequences were obtained and where pheromone receptor sequences are available from the literature (Devier *et al.*, 2009, 2010) revealed a corresponding pattern of diversity (Figure 4). The topologies were not identical, with the placement of *M. silenes-doicae* that may reflect poor resolution because of small number of differences; this species is altered in its placement here from the a_2 nucleotide sequence tree of Devier *et al.* (2010) by a single amino-acid difference from *M. lychnidis-dioicae*. The average pairwise distance, using numbers of differences per 100 amino acids, for a_1 pheromone receptor sequences (4.53, s.e. 0.62) was several times greater than for the a_2 sequences (1.79, s.e. 0.50). Thus, the very highly conserved a_1 mating pheromone interacts with a_2 pheromone receptors that also show comparatively lower protein sequence diversification, and this is contrasted by the variable a_2 mating pheromone that interacts with the more diverse a_1 pheromone receptor.

DISCUSSION

Mating pheromones provide exceptional insights into diversification, where the phenotype under selection is determined by peptides of a very short length and nucleic acid sequences are available for analysis from numerous fungal genomes. As one of the longest-studied models for sexual reproduction in fungi, *Microbotryum* is revealed to have significantly purifying but nonsymmetrical patterns of variation between alternative mating types. These results broaden our knowledge of such an essential life-cycle process particularly when viewed in the context of prior work on breeding system ecology and comparisons with related basidiomycete fungi.

The *in vitro* effects of synthetic pheromone peptides, identified in the *M. lychnidis-dioicae* genome by the characteristic C-terminus CAAX motif, were consistent with observations of mixing-mating-type cultures, including the arrest of cell cycle and production of conjugation tubes (Day, 1976). As Day (1976) reported of mating cells, a_2 cells exhibited conjugation tube growth more rapidly and to a greater extent than a_1 cells, which produced only short pegs at a later stage in mating. The genetic characteristics were also similar to mating-pheromone loci of other fungi, including the presence of multiple loci in each haploid genome and tandem iterations of the pheromone within precursor molecules, in particular for the a_1 pheromone (Kües *et al.*, 2011). Although there remains the possibility that a complete inventory of *Microbotryum* pheromones was not achieved, these results indicate that the loci examined play a central role in the mate recognition process.

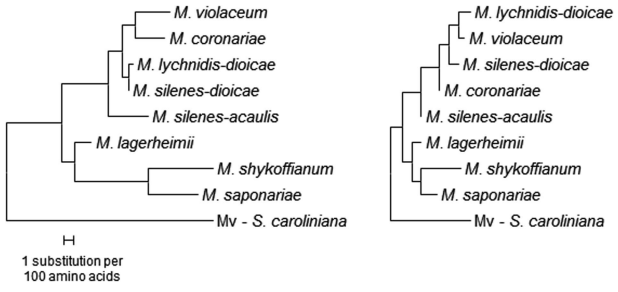

Figure 4 Phylogenetic reconstructions of mating-pheromone receptor protein sequences from a_1 and a_2 mating types across species of *Microbotryum*. Sequences were obtained from Devier *et al.* (2009, 2010). The two phylogenetic trees contain comparable species and are displayed on a common distance scale.

Throughout the diversification of *Microbotryum* species, the mating-type signals have remained functionally conserved, with synthesized pheromones from *M. lychnidis-dioicae* able to induce conjugation structures across the genus. Conservation of mating signal is consistent with reports of both experimental and natural interspecific hybridization (Le Gac *et al.*, 2007a; Gladieux *et al.*, 2011; Büker *et al.*, 2013). The pheromone recognition system in *Microbotryum* is ancient, and the alternate a_1 and a_2 receptor alleles have been maintained as a trans-specific polymorphism predating the genus and estimated as 370 million years old (Devier *et al.*, 2009). Although the age of the genus is not well established, early work by Kniep (1926) demonstrated some limits of the *Microbotryum* pheromone to induce mating structures in other fungi. At that time, the anther-smut fungi were grouped in the genus *Ustilago* with many cereal smuts, and Kniep (1926) found that conjugations would occur readily between smut fungi from diverse dicot host families, but not between dicot- and monocot-infecting smuts, reflecting the divide now recognized between the Microbotryales and Ustilaginales fungi, respectively (Piepenbring and Oberwinkler, 2003).

The selfing mating system of *Microbotryum* likely contributes to the lack of diversification in the mating-pheromone signals. The anther-smut fungi tend to mate by automixis, where haploid products of the same meiosis conjugate to complete the sexual cycle (Giraud *et al.*, 2008; Gibson *et al.*, 2012). Substantial evidence supports this mode of reproduction, from direct observation to the predicted genomic consequences upon heterozygosity (Hood and Antonovics, 2000; Thomas *et al.*, 2003), although some low level of outcrossing and even interspecific hybridization has been observed (Gladieux *et al.*, 2011). With the requirement of mating per generation (that is, disease transmission) and selective pressure being rare to discriminate intra- versus inter-species mating, diversification by positive selection may not be expected at the mating pheromone and receptor, and indeed the patterns observed here were strongly purifying. Prior work has not found evidence for reinforcement or reproductive character displacement (Refrégier *et al.*, 2010), also consistent with the general lack of selection driving assortative mating (Le Gac *et al.*, 2007a; but see also Büker *et al.*, 2013).

Complete identity of peptide sequences across the *Microbotryum* genus, particularly for the a_1 mating-type pheromone, underlies the cross-species pheromone recognition and displays an exceptional level of genetic conservation. Even for the a_2 pheromone there was significant purifying selection, although significantly less strong than for the a_1 pheromone; in addition, there appears to be a common a_2 variant of the peptide in the most distant *Microbotryum* species studied here (that is, *M. lychnidis-dioicae* and *M. violaceum sensu lato* from *Silene caroliniana*) that may help explain the lack of assortative mating. These strong patterns of conservation may not be typical of other fungi. For example, Martin *et al.* (2011) found that mating pheromones in ascomycetes experience a range of purifying to neutral selection, with the Eurotiomycetes alpha pheromone even displaying positive diversification. Recent work in the basidiomycete Sporidiobolales (Coelho *et al.*, 2008, 2011), also in the Microbotryomycetes, indicates that pheromone evolution may occur more rapidly in those related fungi than in *Microbotryum*. Coelho *et al.* (2011) showed that 5

of 11 amino acids differ between the a_1 mating-type pheromones of *Sporidiobolus* and *Sporobolomyces* (accessions FJ183441, AF444509 and HG008766), even though these fungi represent a genetic distance comparable to that among *Microbotryum* species; Jukes–Cantor ITS distance of 0.126 versus 0.121, respectively (see Supplementary Table S1). Between two species of *Sporidiobolus* (*S. salmonicolor* and *S. johnsonii*), the pheromone sequences were the same but the genetic distance of 0.042 was much smaller than among the *Microbotryum* species used here. In another comparison between *Microbotryum* and the related Sporidiobolales, the anther-smut fungi pheromone precursors had fewer repeat iterations of the a_1 pheromone peptide (three compared with four or five repeats, respectively) and a_2 pheromone peptide (one compared with two, respectively). It may provide useful insights to further study pheromone-related behaviors across the Microbotryomycetes because most of the species mate as yeast-like cells that are easy to observe *in vitro*, and there is a growing wealth of genomic, mating system and life-history data (Giraud *et al.*, 2008, Coelho *et al.*, 2013; Fontanillas *et al.*, 2015).

With the level of pheromone sequence conservation, it is not surprising that this study found no significant evidence of assortative mating. The invariant a_1 pheromone, which induces the conjugation structures in a_2 cells, does not provide the opportunity for discrimination among species. However, variation in a_2 pheromones might have led to some species differentiation. The prior study by Le Gac *et al.* (2007a) shows strong differences in mating rates among hybrid pairs of *Microbotryum* species, but the variation was not correlated with genetic distance as would indicate assortative mating. In the current study, there appeared to be a very strong source of variation that was independent of mating-pheromone sequence; specifically, the induction of conjugation structures was significantly correlated between alternate mating types of the same species. Under the test conditions, some species were strong responders, both a_1 and a_2, whereas others were weak, probably indicating that responses to nutrient levels, temperature or other factors overwhelm any ability to detect assortative mating based on variation in the a_2 pheromone signal, if it exists. In addition, it was also suggested that different species of *Microbotryum* vary in their responses to the presence of host extracts as external cues (Day *et al.*, 1981; Castle and Day, 1984). Therefore, it might be expected that mating responses will differ among species based on the testing medium and conditions. Indeed, other fungi have been found to change their mating behavior in response to extrinsic signals such as light (Idnurm and Heitman, 2005) or to rely upon reproductive kinetics (that is, developmental timing) (Leu and Murray, 2006). Therefore, further studies are needed to control for such nonpheromone responses to the environment before assortative mating based on the a_2 pheromone in *Microbotryum* is ruled out completely, as suggested by Büker *et al.* (2013).

Even though the haploid cells of *Microbotryum* are morphologically indistinguishable before mating (that is, isogamous), this study revealed that genetic patterns at the pheromone loci differed between mating types in ways that may correspond with their contrasting roles in the conjugation process. Day (1976) described the initial stage of conjugation as transmission of the pheromone ('inducer molecules') from the a_1 to the a_2 cells. We observe that this conserved a_1 signal is present in a greater number of repeated pheromone sequences within the precursor peptide, which are liberated through post-translational processing (Jones and Bennett, 2011), and may suggest greater diffusion of the a_1 signal to communicate with cells at a distance of tens of microns (Day, 1976). However, similar genetic copy-number patterns are known in other fungi, including ascomycete (Martin *et al.*, 2011) and basidiomycetes (Fuchs *et al.*, 2006), where growth of the conjugation structure is not as strikingly asymmetrical as in *Microbotryum*. It is possible that the role of the a_1 pheromone in initiating mating, while the a_2 pheromone plays a responsive role over shorter distances, places a greater constraint on the evolution of the a_1 signal that was found to have stronger purifying selection that the a_2 pheromone.

Overall, both a_1 and a_2 pheromones, identified here using functional analysis with synthetic peptides, are significantly conserved in the evolution of *Microbotryum* species. At the same time, differences in the strength of conservation between the mating-type pheromones corresponded with diversification of their matching pheromone receptors, suggesting distinct coevolutionary patterns between the two pheromone–receptor pairs that has not been shown previously. The uniform a_1 pheromone is recognized by the a_2 receptor that is less diverse than the a_1 receptor, which then recognizes the more diverse a_2 pheromone. These results represent an intriguing comparison within the fungal genus and should be investigated more broadly, using other fungi where the mating types have distinct roles in the conjugation process.

DATA ARCHIVING

Sequence data are provided at Genbank under accessions KP635064–KP635085.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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