Extensive divergence between mating type chromosomes of the anther-smut fungus

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Running title: Divergence between mating type chromosomes

Key Words: sex chromosomes, recombination suppression, mating types, Microbotryum

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Abstract

Genomic regions that determine mating compatibility are subject to distinct evolutionary forces that can lead to a cessation of meiotic recombination and the accumulation of structural changes between members of the homologous chromosome pair. The relatively recent discovery of dimorphic mating type chromosomes in fungi can aid the understanding of sex chromosome evolution that is common to dioecious plants and animals. For the anther-smut fungus, Microbotryum lychnidis-dioicae (= M.
violaceum isolated from *Silene latifolia*), the extent of recombination cessation on the
dimorphic mating type chromosomes has been conflictingly reported. Comparison of
restriction digest optical maps for the two mating type chromosomes shows that
divergence extends over 90% of the chromosome lengths, flanked at either end by two
pseudoautosomal regions. Evidence to support the expansion of recombination cessation
in stages from the mating type locus toward the pseudoautosomal regions was not found,
but evidence of such expansion could be obscured by ongoing processes that affect
genome structure. This study encourages the comparison of forces that may drive large-
scale recombination suppression in fungi and other eukaryotes characterized by
dimorphic chromosome pairs associated with sexual life cycles.
Introduction

The cessation of meiotic recombination is a hallmark of sex chromosome evolution, and recent studies on mating type chromosomes in fungi have broadened our understanding of this phenomenon (Fraser et al. 2004; Fraser and Heitman 2004; Hood et al. 2004; Whittle and Johannesson 2011). As in plants and animals, the chromosomes determining reproductive compatibility in fungi are derived from an autosome pair. Through processes of recombination suppression and a suite of associated evolutionary forces (e.g. sheltering genetic load, reduced effective population size, and the accumulation of repetitive DNA), a pair of sex or mating type chromosomes becomes differentiated from the autosomes and from each other (Bergero and Charlesworth 2009).

While still relatively poorly understood compared to plant and animal systems, further studies of mating type chromosomes in fungi can help to illuminate the evolution of dimorphic chromosome pairs, identified as "allosomes" sec. Montgomery (1911) (also "heterosomes"), as being a fundamental genomic feature of many sexual eukaryotes.

The first size dimorphic fungal mating type chromosomes were described in the anther-smut fungus, Microbotryum lynchidis-dioicae (= M. violaceum isolated from Silene latifolia) (Hood 2002), but there have been recent conflicting reports regarding the extent of recombination suppression between complementary mating types (referred to as a₁ and a₂). Data provided by Hood and Antonovics (2004) suggested that regions of allelic heterozygosity in linkage to mating type is sufficiently great to comprise the majority of the mating type chromosome lengths. In brief, 11-12% of random genomic markers was heterozygous and linked to mating type, while the mating type chromosome themselves represent only ~13% of the total genome size. More recently, a study
provided contradictory evidence based on allelic divergence and segregation analysis using artificial crosses, suggesting that the mating type-specific region was small and roughly one quarter the length of the mating type chromosomes (Votintseva and Filatov 2009). This latter study added evidence that the mating type locus is flanked by two pseudoautosomal regions, and the authors suggested that there was a gradient of divergence between the $a_1$ and $a_2$ chromosomes that was greatest at the mating type locus and decreased toward these pseudoautosomal regions.

Across fungal species, the size and degree of dimorphism for mating type chromosomes can be evolutionarily dynamic traits. In some fungal species of the genera *Neurospora*, *Ustilago* and *Cryptococcus*, regions of recombination suppression linked to mating type have been shown to extend from a few thousand base pairs to over 75% of the chromosome length (Fraser et al. 2004; Menkis et al. 2008; Bakkeren et al. 2008; Ellison et al. 2011; Whittle et al. 2011). Similar to mammalian X and Y chromosomes, mating type chromosomes can exhibit overall length polymorphism and have frequent localized rearrangements, possibly mediated by the build up of transposable elements in non-recombining regions (Fraser et al. 2004; Whittle et al. 2011).

Evolutionary explanations for the origin of dimorphic mating type chromosomes remain elusive, as do the connections to the evolution of dimorphic sex chromosomes in plants and animals (Abbate and Hood 2010). A very satisfying conceptual model, involving the repeated recruitment of genes determining sexually antagonistic traits, has gained acceptance for the formation of age-structured "evolutionary strata" of recombination cessation in sex chromosomes (Rice 1987; Lahn and Page 1999; Bergero and Charlesworth 2009). However, in the haploid determination of mating compatibility
of fungi, the absence of sexes (i.e. the lack of an association between anisogamy and 
mating types, see Billiard et al. 2010) and the absence of mating type-specific ecological 
traits aside from processes involved in syngamy, suggests that forces aside from sexual 
antagonism have given rise to convergence between fungi and other sexual eukaryotes 
(Fraser et al. 2004; Abbate and Hood 2010). There has also been the recent suggestion 
that the importance of sexually antagonistic traits in the evolution of sex chromosomes in 
plants and animals has been overstated relative to the available data (Ironside 2010). 
Then, for fungi the reporting of evolutionary strata in Cryptococcus neoformans, 
Neurospora tetrasperma, and Microbotryum lychnidis-dioicae (Metin et al. 2010; Menkis 
et al. 2008; Votintseva and Filatov 2009, respectively), adds strength to this call for 
reevaluating the theories and empirical support on sex chromosome evolution. 
In particular, a stronger empirical understanding of recombination suppression in 
fungi is needed so that generalities can be established and compared to the predominating 
concepts in the sex chromosome literature. Toward this goal, we describe the non-
recombining region of mating type chromosomes in the anther-smut fungus M. lychnidis-
dioicae using full-length assemblies of restriction digest optical maps for integration with 
prior studies. Microbotryum lychnidis-dioicae is a basidiomycete fungal pathogen in the 
Pucciniomycotina sub-phylum; this fungus has been previously reported under the epithet 
M. violaceum when isolated from the host Silene latifolia. It is an exceptionally well-
studied model for the evolutionary ecology of infectious disease (Bernasconi et al. 2009), 
fungal speciation, and genetics (Giraud et al. 2008). It is among the fungi first used to 
demonstrate bipolar segregation of mating compatibility factors (Goldschmidt 1928). 
Sexual reproduction is an obligate component of the life cycle, with meiosis and syngamy
occurring prior to infection of each new host plant. The size-dimorphic mating type chromosomes in *M. lycnidis-dioicae* are known to be rich in transposable elements and poor in other genic content relative to the autosomes (Hood et al. 2004). By establishing the extensive divergence between the alternate mating type chromosomes and support for the presence of two pseudoautosomal regions, this study better resolves our understanding of fungi and the characteristics of genome evolution across sexual eukaryotes.

**Materials and Methods**

The genotype of *Microbotryum lycnidis-dioicae* used in this study was isolated from *S. latifolia* in Lamole, Italy, and haploid products of a meiosis were isolated by micromanipulation and cultured on growth medium (as in Hood and Antonovics 2004). This is the same strain used in the majority of previous studies by Hood et al. cited herein, but not in the study by Votintseva and Filatov (2009). The mating types of haploid cultures were identified by pairing with cultures of known mating types and examining the conjugation response that is elicited by the alternate mating pheromone (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 type following DNA extraction with the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA).

Chromosomes were separated by pulsed-field electrophoresis (as in Hood 2002) using low melt agarose. Regions of the gels containing bands representing the a$_1$ and a$_2$ mating type chromosomes identified by segregating size dimorphism (Hood 2002) were excised and supplied to OpGen, Inc. (Gaithersburg, Maryland, USA) for production of
optical maps. High molecular weight DNA was isolated from the agarose, and loaded into microfluidic channels for stretching and immobilization. The restriction endonucleases NheI and AflII were each used as replicate treatments to separately digest immobilized DNA molecules, followed by image analysis of cut site distributions. Overlapping fragment patterns of separate DNA molecules were assembled into high-resolution consensus maps of the full-length chromosomes. Maps of the $a_1$ and $a_2$ mating type chromosomes were compared to each other with the MapSolver software (OpGen, Inc.) to calculate alignment scores based on a cumulative scoring function that rewards matching cut sites and penalizes mismatch or missing sites. The software's default settings were used to calculate alignment scores. As described in Latreille et al. (2007) (see also Tang and Waterman 2001), alignment in MapSolver are generated by a searching algorithm that compares two optical maps in forward and inverse orientations, and alignment scores are positively scaled to the log of an alignment's length and the number of matching restriction cut sites within them, but missing or mismatched cut sites penalize the score value.

For the enzyme AflII, which produced fewer cut sites overall, the map alignments were also analyzed by relaxing two parameters that contribute to the stringency of the score. Specifically, for this secondary AflII analysis, the number of standard deviation in sizing error allowed in an alignment between fragments was doubled from the default of 2 to 4, and the parameter determining the degree to which mismatched cut sites contribute to the score was adjusted to apply a lesser penalty by doubling the value from the default of 0.2 to 0.4.
Scores of alignments between a₁ and a₂ chromosomes were analyzed separately for the NheI and AlfII restriction site optical maps for the presence of extreme values based on mean and standard error calculations among observed alignments; this was used to assess confidence in describing the pseudoautosomal regions.

Position data for alignments were used to assess whether more or stronger matches between regions the a₁ and a₂ chromosomes were distributed closer to the pseudoautosomal regions as suggested by the results of Votintseva and Filatov (2009); note that distribution alignments nearer to the pseudoautosomal regions would be expected from the evolutionary strata hypothesis, which assumes additions to an ancestral area of suppressed recombination extending up to the pseudoautosomal boundary where recombination continues (e.g. Lahn and Page 1999). The relationship of proximity to the pseudoautosomal regions and strengths of alignment scores was tested using Pearson bivariate correlation analysis in PSAW Statistics v18 (SPSS Inc., Chicago, USA). The distributional proximity of alignments was tested against random expectations by simulating random positions for a₁ and a₂ sites involved in alignments for an equal number of alignments as in the observed data, with 1,000 replicated randomizations for comparison to the observed mean distances to the pseudoautosomal regions.

Results

Optical maps of restriction site distributions show that alignments of the a₁ and a₂ mating type chromosomes in the anther-smut pathogen, M. lychnitis-dioicae, were poor over ca. 90% their lengths, indicating the greatest extent of non-recombination yet shown in a fungal species (Fig. 1). The replicate restriction optical maps generated with the
enzyme NheI and AflII gave the same result in this regard (Fig. S1). Regions located at
each end of the chromosome arms showed the highest alignment scores and should
therefore correspond to pseudoautosomal regions, each being ca. 4-6% of the
chromosome length and extending to each end of the chromosomes. For the optical map
generated from NheI restriction sites, only the pseudoautosomal regions had alignment
scores greater than 1.96 standard errors from the mean of observed alignment scores (i.e.
exceeding the 95% confidence interval) (Table S1). For the optical map generated from
AflII restriction sites, only the alignments of two pseudoautosomal regions were obtained
under the alignment's default parameter settings (Table S1). Under the relaxed parameter
setting described above, necessitated by the lower density of cut sites overall with this
enzyme, again only the pseudoautosomal regions had alignment scores greater than 1.96
standard errors from the mean of observed alignment scores. Also, only the
pseudoautosomal regions represented alignments in common to both NheI and AflII
restriction optical maps. In agreement with pulsed-field electrophoretic gels published
previously (Hood 2002), the a1 chromosome is shorter, estimated from the optical maps
to be 3.3 Mbp. Thus the non-recombinating region of the a1 chromosome represents a
slightly smaller proportion than for the larger 4.0 Mbp a2 chromosome; non-recombinating
regions being 89% versus 91% of the a1 and a2 chromosomes, respectively.

Alignments scores for sections in the non-recombinating regions of the mating type
chromosome were weaker and covered shorter lengths than for the pseudoautosomal
regions (Fig. 1). Using position data based upon the distance in kilo-basepairs to
pseudoautosomal regions, alignments between the a1 and a2 chromosomes along the
length of the non-recombinating regions were not nearer to pseudoautosomal regions than
expected by chance alone. In particular, the mean distance of alignments to the pseudoautosomal regions was not closer than 95% (n = 1,000 replications) of simulated randomized alignment positions (Fig. 2). These results were similar for both mating type chromosomes, but with the a1 chromosome approaching significance with the mean distance to pseudoautosomal regions being closer than 91% (n = 1,000 replications) of simulated randomized alignment positions. Moreover, there was no apparent positive relationship between the strength of the alignment scores and proximity to the pseudoautosomal regions as would also have been expected by more recent cessation of recombination in those regions (a1 correlation coefficient -0.171, p = 0.4; a2 correlation coefficient 0.060, p = 0.8).

This distribution of the alignments in the non-recombining region could potentially be interpreted to suggest a large scale inversion involving the majority of the mating type chromosomes, followed by further localized rearrangements that obscure the inverse co-linearity of alignment order that is expected to result from such an inversion event. Alternatively, the distribution of alignments may suggest "hot-spots" (see Richard et al. 2008) of segmental duplications where sites in the non-recombining region of one chromosome match multiple sites on the complementary chromosome.

Discussion

The region of the genome containing the mating type locus in *M. lychnitis-dioicae* has likely been shaped by long-term evolutionary processes, in contrast to fungi suggested to serve as models for the early evolution of sex chromosomes of plants and animals (Fraser and Heitman 2004; Menkis et al. 2008). Alternate alleles at the mating
pheromone receptor locus in *Microbotryum* actually exhibit the most ancient pattern of
balancing selection and maintenance across speciation events for a locus in any known
group of organisms (Devier et al. 2009). The observation that optical maps could not be
aligned over most of the mating type chromosome lengths indicates that the
recombination suppression is sufficiently ancient to allow the extensive accumulation of
differentiating mutations, noting that non-recombining regions may be subject to higher
substitution rates than recombining regions (Charlesworth 1994).

There are two technical issues that likely contribute to disagreement between the
prior study suggesting the non-recombining region of the mating type chromosomes in *M.
lychnidis-dioicae* was small (Votintseva and Filatov 2009) and the two studies indicating
extensive divergence of the chromosome pair (i.e. these optical maps and the previous
measure of allelic heterozygosity in linkage to mating type by Hood and Antonovics
2004). The smaller size estimate of the non-recombining region was based upon a set of
shotgun DNA fragments that were originally isolated from the mating-type chromosome
bands in electrophoretic karyotypes by Hood et al. (2004). Allelic identity versus
divergence at these loci between haploid *a*₁ and *a*₂ cultures was used to assign these loci
to the pseudoautosomal versus non-recombining regions of the mating type chromosomes,
respectively. The first issue relates to background autosomal contamination that may be
present in the gel-isolated electrophoretic bands from which the shotgun fragments
originated. Autosomal contamination would not create a false positive in assessing
differences between the mating type chromosomes and autosomes in Hood et al. (2004),
but mistakenly assigning homozygous autosomal loci to mating type chromosomes would
inflate the estimated size of the recombining pseudoautosomal regions in Votintseva and
Filatov (2009). Quantification of such autosomal contamination may be achieved when a well-assembled genome sequence is produced. The second issue is related to the nature of the 61 loci found by Votintseva and Filatov (2009) that have allelic identity between sequenced PCR products from haploid \(a_1\) and \(a_2\) cultures and were thus assigned to pseudoautosomal regions. Among these sequences, ca. 20% were identified as probable transposable elements in Hood et al. (2004). As repetitive elements, they are expected to be present as sequences in multiple genomic locations, at non-syntenic positions on both mating type chromosomes as well as on autosomes. Therefore, including transposable elements sequences with the 0% divergence between PCR products from \(a_1\) and \(a_2\) cultures for would cause the proportion of loci assigned to the pseudoautosomal regions to be substantially overestimated in Votintseva and Filatov (2009).

The conclusions from the optical maps are in strong agreement with the estimate of heterozygosity in the majority of the mating type chromosomes from the genome-wide survey of marker variation and linkage to mating type (Hood and Antonovics 2004). In general, recent studies have cited the smaller size estimate of the non-recombining region in *M. lychnidis-dioicae* in Votintseva and Filatov (2009), but they have not used that estimate in additional quantitative analyses that would necessitate reassessment (e.g. Ellison et al. 2011; Kües et al. 2011). Further studies are needed to ascertain whether there is sufficient variation among strains of *M. lychnidis-dioicae* that might contribute to the contradictory results, particularly as Votintseva and Filatov (2009) used different strains for the segregation analysis versus the source of loci that they characterized. However, at least the presence and direction of the mating type chromosome size
dimorphism has been consistent among multiple field-collected specimens of this fungal species (Hood 2002).

In order to advance the analogies between mating type chromosomes and sex chromosomes, greater consideration is needed regarding the nature of the non-recombining regions. Relative to dioecious plants and animals, few traits have the potential to be harmful when recombined between the mating types in fungi. The exception might be traits involved directly in the process of syngamy (Billiard et al. 2011), but these are not normally considered under the concept of sexual antagonism in plants and animals. Consequently, the most accepted adaptive model for expansion of recombination suppression in sex chromosome evolution does not likely apply to fungi (Bergero and Charlesworth 2009). Moreover, even among plants and animals, broad empirical support for sexually antagonistic traits as the driving force for recombination suppression has been suggested to be lacking (Ironside 2010); non-adaptive processes that fix recombination-blocking mutations (e.g. inversions) in linkage to regions of permanent heterozygosity by drift or under the fitness effects of genetic load was proposed as alternative models (Ironside 2010) which may just as well be applicable to fungi.

Linkage to mating types in fungi has been the subject of considerable study, and reports of large regions of recombination suppression in diverse species prompts the need for mechanistic explanations (e.g. Metin et al. 2010; Menkis et al. 2008; Votintseva and Filatov 2009). Particular to basidiomycete fungi, recombination suppression has been suggested to allow linkage between the two loci determining pre- and post-syngamy viability (Bakkeren and Kronstad 1994). Also, linkage of mating type with the
centromere is frequently associated with the mating system of automixis in fungi, as in
some insects (i.e. intra-tetrad selfing) (Mogie 1986; Zakharov 2005; Lewis and John
1963). Under these scenarios, further expansion of a non-recombining region is not
necessarily expected because a single step of recombination cessation is sufficient, as
might have achieved centromere linkage of mating type in *N. tetrasperma* (Ellison et al.
2011). However, theoretical studies regarding the automictic mating system, in particular,
have described the spread of modifiers that suppress recombination with mating type, and
the accumulation of load loci (i.e. deleterious recessive mutations) or overdominant loci
(i.e. heterozygote advantage mutations) can accelerate this evolution (Antonovics and
Abrams 2004; Johnson et al. 2005). Lending strength to this theoretical scenario, both *M.
lychnidis-dioicae* and *N. tetrasperma*, two fungi with cessation of recombination across
the majority of the mating type chromosomes, are in fact strongly automictic (Giraud et al.
2008; Ellison et al. 2011).

The test of whether alignments of restriction digest optical maps supported the
presence of evolutionary strata in *M. lychnidis-dioicae* provided a negative but non-
conclusive result. Divergence of mating type chromosomes by evolutionary strata would
predict that the most recent cessation of recombination, and thus the least divergence
between the *a*₁ and *a*₂ mating type chromosomes would be nearest the pseudoautosomal
regions. However, neither the strengths nor the distributions of alignments in the non-
recombining region were greater nearer to the pseudoautosomal regions. Importantly,
these results do not prove that a history involving evolutionary strata is absent in this
species, but the results indicate that perhaps other types of data, including physical
mapping of DNA sequences, should be pursued to help resolve the issue. At present,
genome sequencing of the a strain of *M. lychnidis-dioicae* by the Broad Institute (www.broadinstitute.org) has not provided long enough sequence assemblies for the placement on the optical maps, perhaps challenged by elevated densities of transposable elements on the mating type chromosomes of *M. lychnidis-dioicae* (Hood et al. 2004).

The confirmation and characterization of two pseudoautosomal regions in *M. lychnidis-dioicae*, representing just ~10% of the mating type chromosomes, draw additional similarity between sex-related allosomes in fungi and other eukaryotes. Like the evolution of non-recombining regions of sex chromosomes, the pseudoautosomal regions have received recent attention (reviewed by Otto et al. 2011). Pseudoautosomal regions at either side of the sex-determining locus are found in the majority of dioecious plants and about half of animals. The role of pseudoautosomal regions is often assumed to be to facilitate the proper alignment and segregation of sex chromosomes during meiosis in the heterogametic sex, although other selective forces that favor recombination may be important (Otto et al. 2011). In fungi, however, the haploid determination of mating compatibility creates the distinction that pseudoautosomal regions would be under more frequent selection to maintain this homologue-pairing role during every meiosis, rather than in just half of meioses, because there is no homogametic diploid. Similarly, the role of partial sex linkage in plants and animals as an influence on the evolution of pseudoautosomal regions (Otto et al. 2011) would be contrasted by the absence of sexes in fungi. Thus, the distribution and structure of fungal pseudoautosomal regions may contribute substantially to the understanding of their occurrence in dioecious plants and animals by the contrasting expectation about the relevant evolutionary forces.
Recent work has shown that the evolution of linkage to mating type occurs independently in various species of *Microbotryum* (Abbate and Hood 2010; Petit et al. 2012). The results presented here reflect the mating type chromosome characteristics of the best-studied species in this genus, including the \( a_1 \) genotype that is the subject of a current genome sequencing project (Broad Institute). However, variation in the overall structure of mating type chromosomes is known to occur in both distantly and very closely related fungi. With indications that variation in reproductive mode is a major influence on these genomic structures (e.g. Whittle et al. 2011), fungi provide opportunities to address the role of sexual life cycles for the origins of allosomes in a broad phylogenetic context and for making illuminating comparisons across eukaryotic kingdoms.

**Acknowledgements**

We are grateful to reviewers for valuable comments on the manuscript and to Eric Fontanillas for technical help. MEH acknowledges support from the NSF grant DEB-0747222, and TG acknowledges support from the grant FungiSex ANR-09-0064-01.

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Figure Legend

Fig 1. Alignment of restriction digest optical maps for the a₁ and a₂ mating type chromosomes of *Microbotryum lychnids-dioicae* (=*M. violaceum* isolated from *Silene latifolia*). Horizontal lines within each chromosome represent the distribution of sites cut by the restriction endonucleases Nhel and AflIII; separate restriction enzyme maps are showing in Fig S1. Blue shading indicates the two pseudoautosomal regions, connected by bold lines. Yellow shading indicates areas of weaker alignment, connected by lines, in the non-recombining region. The alignments for the AflIII optical map are included for the less stringent alignment parameters, as described in the text, where the default parameters produced alignments only for the pseudoautosomal regions. The a₁ and a₂ chromosome are estimated to be ca. 3.3 and 4.0 Mbp, respectively.

Fig 2.
Relationship between alignment scores for regions of the NheI and AflIII optical maps and alignment positions on the mating type chromosomes of *Microbotryum lychnidis-dioicae.*

Positions of aligned regions for the $a_1$ (A) and $a_2$ (B) chromosomes were plotted relative to the distance in kilo-basepairs from the closest pseudoautosomal region; thus, the x-axis for each chromosome measures roughly half the chromosome length.
A

Distance from $a_1$ Pseudoautosomal Regions (kbp)

Segment Alignment Score

B

Distance from $a_2$ Pseudoautosomal Regions (kbp)

Segment Alignment Score