Dimorphic Mating-Type Chromosomes in the Fungus *Microbotryum violaceum*

Michael E. Hood

Department of Biology, University of Virginia, Charlottesville, Virginia 22903

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ABSTRACT

Fungi often mate as haploids, and sex chromosomes (i.e., mating-type chromosomes) that are dimorphic for their size or overall DNA content have never been reported in this kingdom. Using electrophoretic techniques for karyotype analysis, a highly dimorphic chromosome pair that determines mating compatibility is shown to occur in populations of the fungus *Microbotryum violaceum*. This substantiates the evolution of such dimorphism as a general feature associated with haploid determination of mating compatibility, which previously had been known only in haplodioecious plants (mosses and liverworts). Size-dimorphic sex chromosomes are present in a lineage of *M. violaceum* native to Europe, as well as a lineage native to North America. However, they are very different in size between these lineages, indicating either independent evolution of the dimorphism or a large degree of divergence since their isolation. Several DNA sequences that show sequence similarity to transposons were isolated from these sex chromosomes.

SEX chromosomes can be defined as those that determine mating compatibility and that often have patterns of inheritance and cytological features distinct from autosomes. While cogent evolutionary theories can explain the origin of highly dimorphic sex chromosomes in diploid-mating organisms (Charlesworth 1991), these theories cannot be applied to the range of organisms where mating compatibility is determined in the haploid stage. With diploid sex, as in most plants and animals, only the heterogametic chromosome (usually Y) is permanently heterozygous, and therefore the sheltering of deleterious mutations is asymmetrical. According to Muller (1914), this allows for degeneration of the heterogametic but not the homogametic sex chromosome. Other theories that explain the evolution of dimorphism as the result of Muller’s ratchet (Charlesworth 1978) or genetic hitchhiking (Rice 1987) also depend upon the “unique permanent heterozygosity” of the Y chromosome to distinguish its fate from that of the homogametic sex chromosome.

When mating compatibility is determined in the haploid stage, the sex chromosomes are equally heterozygous and therefore equally sheltered in the diploid stage. If degeneration occurs it should be symmetrical between them (Bull 1978). Moreover, degeneration should be limited because only genes that are nonessential for haploid growth can accumulate recessive deleterious mutations, other alleles being exposed to selection in the haploid stage.

In spite of these potential limitations on the evolution of sex chromosomes, such dimorphism occurs in many mosses and liverworts, where mating compatibility is expressed in the predominant haploid stage of the life cycle. In fact, the first report of sex chromosomes in plants was in the liverwort Sphaerocarpus (Allen 1917). Similarly, fungi often have haploid determination of mating compatibility, but size-dimorphic sex chromosomes (conventionally referred to as “mating-type chromosomes”) have not been reported. This is perhaps because their chromosomes are often barely visible under the light microscope, and their study has required electrophoretic techniques for karyotype analysis that have become available only recently (Mills and McCluskey 1990). These techniques were applied to the fungus *Microbotryum violaceum*, the cause of anther-smut disease, to investigate whether fungi also can exhibit a high degree of size dimorphism in the chromosome pair determining mating compatibility.

MATERIALS AND METHODS

**Study system and sampling:** *M. violaceum* is an obligately sexual basidiomycete fungus that causes anther-smut disease in many plant species of the Caryophyllaceae, particularly of the genus Silene (Alexander et al. 1996). Mating is controlled by a haploid self-incompatibility system consisting of two mating-type alleles (a1 and a2), which exhibit complete centromere linkage (Hood and Antonovics 1998). This fungus undergoes meiosis upon germination of diploid teliospores, and it is possible to isolate linear tetrads by micromanipulation (Hood and Antonovics 2000).

The current state of species identification in the genus *Microbotryum* is incomplete, but studies indicate that some collections from different hosts represent cryptic species (Antonovics et al. 1996; Bauer et al. 1997; Perlin et al. 1997; Bucheli et al. 2000; Hood et al. 2001).

Populations of *M. violaceum* were sampled from infected Silene *latifolia* at the following localities (the number of tetrads isolated per population is in parentheses): United Kingdom, Aldeburgh (2), Ipswich (1), Hungerford (2); Italy, Lamole (8), San Gimignano (2), Antrodoco (1); France, Orsay (1); Germany, Darmstadt (1); Switzerland, Oetwil (1); Czech Re-

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1 Author e-mail: michael.hood@virginia.edu
public, Olomouc (1); and the United States, Virginia: Giles County (5), Albemarle County (1), Rockingham County (8), Shenandoah County (2). Native North American populations of Microbotryum on \( S. \) caroliniana were sampled in Virginia, Princess Anne County (1), Amherst County (2); Kentucky, Jessamine County (1), Anderson County (6); and North Carolina, Franklin County (4). Samples were kept under desiccation prior to the isolation of tetrads. Once isolated by micro-manipulation, the haploid meiotic products were placed in long-term storage (frozen under desiccation) and are available over the web at http://www.people.virginia.edu/~meh2s/tetradcollection.htm.

Electrophoretic karyotype analysis: A CHEF-DR II system (BioRad, Hercules, CA) was used to generate karyotypes by pulsed-field gel electrophoresis. Haploid cell cultures were suspended into agarose plus without generating protoplasts (McCluskey et al. 1990). Electrophoresis conditions consisted of 0.8% chromosomal grade agarose, \( 1 \times \) TBE, and a run time of 96 hr. The separation of chromosomes ranging in size from 1 to 3 mbp was done at 14° and 2.7 V/cm with 200 sec initial switch time and 1100 sec final switch time. Larger chromosomes were separated with an initial gel temperature of 37° for 3 hr followed by 14° and 2.1 V/cm and with 1100 sec initial switch time and 1700 sec final switch time. Gels were stained in ethidium bromide and viewed under UV light.

Mating-type-specific DNA sequences: Linear tetrads of samples from the Hungerford and Giles County populations were subjected to amplified fragment length polymorphism (AFLP) analysis, and AFLP products were identified that consistently cosegregated with mating type across four meioses. The protocol and primers were similar to those described in Vos et al. (1995) for standard EcoRI/MseI AFLP analysis. To increase the number of AFLP products available for analysis, the restriction enzyme combination XhoI/MspI was additionally used, with the linkers and primers being the same as for EcoRI/MseI AFLP analysis except at the ends corresponding to the restriction sites, which were modified appropriately.

Because the source of DNA for each reaction is a haploid product of meiosis, AFLPs served as codominant markers. AFLP products of interest were isolated from agarose gels and sequenced using the AFLP primers. The sequences were then compared to the databases at the National Center for Biotechnology Information using the BLASTx algorithm (http://www.ncbi.nlm.nih.gov).

The sequences were also used to generate mating-type-specific primers that were internal to the AFLP-derived sequences. Such oligo sequences used in this study were 5'-CTTCGGGATAACGAGAAAGCC-3' and 5'-AGGTATG AGCAGTGAGATCGG-3', a single primer pair producing unequal-sized fragments in each mating type due to indels in the nonrecombining region. Across samples from all countries, there was no evidence of recombination between this size-specific PCR marker and the mating-type locus. Individual chromosome bands from the electrophoretic karyotypes were excised and treated with restriction enzymes (XhoI and MspI) prior to DNA extraction from the agarose gel (QIAGEN, Valencia, CA). These were the same restriction enzymes used for genomic digestion in the AFLP analysis, thus facilitating DNA extraction but avoiding restriction sites in the target sequence. The mating-type-specific primers were then used to determine which karyotype bands contained the target DNA sequences.

RESULTS

Sex chromosomes in \( M. \) violaceum were identified by the cosegregation of mating type and chromosome dimorphism and by mating-type-specific markers. In \( M. \) violaceum from European and introduced North American populations of \( S. \) latifolia, one pair of dimorphic chromosomes exhibited consistent cosegregation with mating type across 36 tetrads (Figure 1). The identity of the sex chromosomes was confirmed using mating-type-specific PCR primers. These primers amplified only target DNA in the isolated dimorphic pair of chromosomes and showed no amplification in any of the other isolated chromosomes (Figure 2). DNA content of the \( A1 \) chromosome of \( M. \) violaceum from \( S. \) latifolia ranges from 2.8 to 3.1 Mbp in length, while the \( A2 \) chromosome is substantially larger, ranging from 3.4 to 4.2 Mbp. The sex chromosomes are among the largest in the genome with only one autosome in the same size range. Other autosomes range from 1.0 to 2.6 Mbp, and the number of chromosomes in the karyotypes varies from 11 to 13 depending on the population.

The sex chromosomes were similarly identified in the North American lineage of \( M. \) violaceum on the native host species \( S. \) caroliniana. Again, consistent cosegregation of size dimorphism and mating type across 14
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them (Randall and Metzenberg 1998; Lee et al. 1999), and they are consequently often referred to as idiomorphs (“distinct types”; Metzenberg and Glass 1990). Genetic studies have shown differences at the molecular level (Merino et al. 1996; Gallegos et al. 2000), but this study is the first demonstration of major quantitative differences in the overall DNA content of the chromosomes determining mating compatibility. Karyotype analyses of fungi using the light microscope have been notoriously difficult, and therefore only recently have fungal chromosomes been studied using electrophoretic separation (Mills and McCluskey 1990). In many fungal species, especially those that are primarily asexual, there is substantial chromosome size variation within and between populations (Zolan 1995). However, only two other fungi have been analyzed for meiotic segregation of karyotypic patterns using tetrad analysis: Ustilago hordei (McCluskey and Mills 1989; Gaudet et al. 1998) and Saccharomyces ludwigii (Yamazaki and Oshima 1996). Both showed segregation of chromosome size within tetrads, but no attempt was made to identify whether these size differences occurred in the sex chromosomes.

Bull (1978) presented the most serious theoretical consideration of sex chromosome evolution when mating compatibility is determined in the haploid stage. He argued in relation to bryophytes that, due to the lack of sheltered degeneration to produce a smaller chromosome as in diploids, it is more likely that dimorphism in haploid sex chromosomes arises by the addition of genetic material to produce a larger sex chromosome. Within each lineage of Microbotryum violaceum from Silene latifolia, distributed across the host’s natural range in Europe and from introduced populations of S. latifolia in North America, overall karyotype patterns are conserved but the size and the degree of dimorphism between the sex chromosomes varies at the regional scale (Figure 3).

The DNA sequences of 20 mating-type-linked AFLP products were obtained, 9 on the A2 chromosome and 11 on the A1. Of these, 4 were sequences similar to known retroelements in other organisms and 1 was similar to helicase, which is known to be involved in Ty1 transposition in yeasts (Lee et al. 2000; Table 1).

DISCUSSION

Alleles at the mating-type loci of fungi often differ to the extent that there is no sequence similarity between them (Randall and Metzenberg 1998; Lee et al. 1999), and they are consequently often referred to as idiomorphs (“distinct types”; Metzenberg and Glass 1990). Genetic studies have shown differences at the molecular level (Merino et al. 1996; Gallegos et al. 2000), but this study is the first demonstration of major quantitative differences in the overall DNA content of the chromosomes determining mating compatibility. Karyotype analyses of fungi using the light microscope have been notoriously difficult, and therefore only recently have fungal chromosomes been studied using electrophoretic separation (Mills and McCluskey 1990). In many fungal species, especially those that are primarily asexual, there is substantial chromosome size variation within and between populations (Zolan 1995). However, only two other fungi have been analyzed for meiotic segregation of karyotypic patterns using tetrad analysis: Ustilago hordei (McCluskey and Mills 1989; Gaudet et al. 1998) and Saccharomyces ludwigii (Yamazaki and Oshima 1996). Both showed segregation of chromosome size within tetrads, but no attempt was made to identify whether these size differences occurred in the sex chromosomes.

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In support for this process, or at least for the potential action of transposons in Microbotryum violaceum, such elements are easily identified among DNA sequences linked to mating type. The suppression of recombination near
the mating-type loci of fungi (Gallegos et al. 2000) may favor the persistence of transposons or other degenerative processes such as Muller’s ratchet, but this per se is insufficient to explain the origin of the dimorphism because there is no reason to expect the process to be unbalanced between the two chromosomes. It is possible, however, that a chance increase in transposon frequency in one mating type generates a feedback process favoring their further insertions into nonfunctional regions. Alternatively, there may be unequal functions associated with the sex chromosomes during reproduction, thus disfavoring structural changes in one relative to the other. This idea has not been studied empirically in a haplodioecious system, but there is evidence that mating types in fungi may differ substantially in their roles during the sexual process. The mating types in M. violaceum differ in determination of uniparental inheritance of mitochondria (Wilch et al. 1992), their dominance in laboratory-derived polyploids (Cummins and Day 1973), and their role in initiating conjugation structures during mating (Day 1976). Further research is needed to address whether the direction of mating-type-specific chromosome dimorphism in fungi is influenced by these reproductive functions.

Molecular studies of mating factors in fungi reveal similarities to hypothesized proto-X and proto-Y chromosomes (i.e., suppressed recombination and sequence dissimilarity; Lee et al. 1999; Gallegos et al. 2000). However, the asymmetrical sheltering that is central to explanations of sex chromosome dimorphism in “higher” diploids cannot occur in organisms with haploid sexes or mating types. This study showing the presence of major size dimorphism in fungal sex chromosomes therefore brings into question the current ideas on sex chromosomes in diploid plants and animals by suggesting that asymmetrical sheltering may not be a prerequisite for the evolution of dimorphism. It may be relevant that in some diploids, such as S. latifolia, the heterogametic Y chromosome is larger than the X. Here, the mechanism proposed for the dimorphism is proliferation of repetitive elements (Filatov et al. 2000). This may be the same mechanism that occurs in haplodioecious organisms. The heterogametic Y chromosome also appears larger than the X in Drosophila melanogaster (Lewis and John 1963) as well as some Polychaete worms (Sato and Ikeda 1992; Korablev et al. 1999) and Anuran frogs (White 1973). Clearly there is much theoretical common ground, but despite the enormous number of taxa where mating compatibility is determined in the haploid stage, no empirically tractable system has yet been employed to address the evolutionary consequences for genomic organization. The ability to rapidly isolate and quantitatively assess DNA content of individual chromosomes in Microbotryum will greatly facilitate research in the origin and maintenance of sex chromosomes in haplodioecious organisms.

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