Mating Within the Meiotic Tetrad and the Maintenance of Genomic Heterozygosity

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ABSTRACT

Mating among the products of a single meiosis (automixis or meiotic parthenogenesis) is found in diverse groups of plant, animal, and fungal taxa. Restoration of the diploid stage is often strictly controlled and brings together products separated at the first meiotic division. Despite apparent similarities to diploid selfing, the theoretical prediction is that heterozygosity should be maintained on all chromosomes when it is linked to the centromeres and thus also segregates at the first meiotic division. Using the fungus Microbotryum, we directly test this prediction by linear tetrad analysis. The patterns of meiotic segregation for chromosome size variation (electrophoretic karyotypes) and PCR products (AFLP procedures) were determined for Microbotryum lineages native to North America and Europe. Our data reveal a surprisingly dynamic genome that is rich in heterozygosity and where size-dimorphic autosomes are common. The genetic variation agrees with the prediction of centromere-linked heterozygosity. This was observed to the greatest extent in the lineage of Microbotryum native to North America where there was consistent first-division segregation and independent assortment of multiple linkage groups. The data also show properties that distinguish the fungal sex chromosomes from the autosomes in both lineages of Microbotryum. We describe a scenario where the mating system of automixis with first-division restitution is the result of feedback mechanisms to control exposure of genetic load.

ALONG the continuum from selfing to outcrossing, genetic structure is expected to shift from more uniform to more variable, and the patterns are reflected in both the heterozygosity within the genome and the average relatedness within populations. These correlations are the basis for many studies of population genetics. However, a large variety of organisms complete the sexual life cycle through automixis (*sensu* Mogie 1986; *i.e.*, meiotic parthenogenesis), a form of selfing where mating occurs among the products of a single meiosis. Here, the consequences for genetic structure can be quite orthogonal to classic expectations. Automixis is frequent among fungi, particularly the "secondary homothallic" ascomycetes, the yeasts, the smuts, and the bisporic mushrooms (Kirby 1984; Zakharov 1986). It is also common in insects, nematodes, various other metazoans, and some protists (Mogie 1986; van der Beek *et al.* 1998; Normark 2003). In plants, it is frequent in mosses and ferns, but also occurs in flowering plants (Walker 1979; Antonius and Nybom 1995; Cruden and Lloyd 1995). With the products of only a single diploid cell contributing to the next generation, some have questioned the importance of the meiotic cycle and have likened automixis to self-fertilization or even asexuality (reviewed by Mogie 1986, but serious confusion persists). Here we show that mating within the tetrad can produce a surprisingly dynamic genome that is rich in heterozygosity.

When mating occurs randomly among the products of a single meiosis, a tendency toward homozygosity is indeed the expected result (as in diploid selfing, but at a somewhat slower rate; Kirby 1984). However, many automictic organisms regulate the process either cytologically or through the segregation of gamete compatibility factors. This regulation can preserve a high degree of heterozygosity throughout much of the genome in the face of apparent selfing (Lewis and John 1963). As found in many automictic taxa (Ochman *et al.* 1980; Hoffmann 1983; Zakharov 1986; Beukeboom and Pijnacker 2000; Belshaw and Quicke 2003), mating strictly among the products of one meiosis that brings together chromosomes separated at the first meiotic division (often called "central fusion" or "first-division restitution") will restore heterozygosity to all loci that also segregate at the first meiotic division. This specifically means that heterozygosity would be maintained in tight linkage with centromeres. A heterozygous locus segregating at the second meiotic division (*i.e.*, where there is a crossover event between it and the centromere) would then have a one in two chance of being driven to homozygosity in each generation. When mating strictly brings together sister nuclei of meiosis II, the effects are reversed, and heterozygosity is lost more rapidly near centromeres but preserved in distal regions. This is a less likely way of maintaining heterozy-

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gosity because it specifically requires the presence of one and only one chiasma proximal to the centromere rather then generalized suppression of crossing over near the centromere. As the heterozygosity tends toward fixation in particular genomic regions, the automictic progeny increasingly resemble their parents. Thus, genetic diversity within the population is expected to be reduced in a way that is disconnected from heterozygosity at the level of the individual.

These effects on genetic structure have long been recognized in theory (Lewis and John 1963; White 1973), but empirically they remain almost completely unexplored. This is because many organisms do not present tractable models for directly observing meiosis and for genetic analysis of the resulting haploids. However, several population-level studies and genomic surveys have attributed peculiar frequencies of heterozygosity to automixis (*e.g.*, Hoffmann 1983; Summerbell *et al.* 1989; Marescalchi *et al.* 1993; Merino *et al.* 1996; Hood and Antonovics 2000). Here, we directly test the prediction that regularized mating within the meiotic tetrad that brings together chromosomes separated at meiosis I will determine the genomic distribution of heterozygosity in the automictic fungus Microbotryum. The dispersal stage of this fungus consists of diploid spores that undergo meiosis upon germination. In nature, meiosis is followed rapidly by mating among the products, but haploids can be isolated prior to mating and analyzed for the segregation of heterozygous markers.

MATERIALS AND METHODS

Study system: *Microbotryum violaceum* (formerly *Ustilago violacea*) is a basidiomycete that causes anther smut disease in members of the Caryophyllaceae. It is well studied as a pathogen in natural plant populations and as a genetic model for fungi with bipolar mating compatibility (see Antonovics *et al.* 2002; Garber and Ruddat 2002, and references therein). Diploid spores of the fungus are primarily transmitted between hosts by insect pollinators. Upon germination, the fungus undergoes meiosis followed rapidly by mating between haploid cells of opposite mating types (called A1 and A2). Mating is a prerequisite for infection of the new host, and studies have suggested that more rapid mating is favored (Hood and Antonovics 2000; Van Putten *et al.* 2003). As a result, mating would tend to involve the cells first available within the tetrad, either among cells of the septate basidium (the promycelium) or among the initially produced yeast-like cells (sporidia), which are essentially basidiospores and their haploid descendants. As in most basidiomycetes, the nuclear condition after mating and during vegetative growth is heterokaryotic (the two haploid nuclei remain distinct within the cells) and karyogamy occurs at spore formation.

We previously provided evidence that the mating system of *M. violaceum* tends toward automixis, which is regulated by centromere linkage of the mating-type locus. Thus, mating occurs after both meiotic divisions and is between products whose chromosomes were separated at meiosis I (Hood and Antonovics 1998, 2000). It is the first fungus in which the mating-type locus was found to reside on a size-dimorphic pair of chromosomes, and these chromosomes have similarities to sex chromosomes of plants and animals in the distributions of transposable elements and functional genes (Hood 2002; M. E. Hood, J. Antonovics and B. Koskella, unpublished results). Typically 10–12 autosomes are in the haploid genome. As with many fungi, the chromosomes are too small to observe their characteristics using light microscopy, but they can be quantified by electrophoretic techniques as described below. The taxonomy of anther smut fungi on different hosts within the Caryophyllaceae is currently unresolved, and it appears that multiple species are subsumed under the name *M. violaceum* (Perlin *et al.* 1997; Vanky 1998; Hood *et al.* 2001; Freeman *et al.* 2002). For simplicity we refer to the fungus as Microbotryum, and the lineages are informally identified as varieties by using the host's specific name.

In this study, we examined a lineage of Microbotryum native to Europe from the host *Silene latifolia* (*Microbotryum* var. *latifolia*), and a lineage native to North America from *S. caroliniana* (*Microbotryum* var. *caroliniana*). Populations were sampled widely within the natural ranges of the two host plants, and *Microbotryum* var. *latifolia* was also sampled from North America where *S. latifolia* and its Microbotryum lineage have been introduced (Table 1). Collections of diploid spores were kept under desiccation prior to the isolation of linear tetrads by micromanipulation. Meiosis produces a tetrad that is arranged such that the first two cells are separated from the second two cells by a septation immediately after the first meiotic division. Therefore, when the yeast-like sporidia are produced and isolated, their position within the linear tetrad can be recorded (as illustrated in Hood and Antonovics 2000). The haploid sporidia were cultured on potato dextrose agar and used for DNA extraction. Replicate cultures were placed in long-term storage (frozen under desiccation) and are available over the web at http://www.people.virginia.edu/~meh2s/tetradcollection.htm.

The spore content of a single diseased flower in natural populations is presumed to represent a single diploid genotype (Hood 2003) and is referred to as such here. In several cases, multiple tetrads were isolated from the same diploid genotype. These replicate tetrads were used to determine the patterns of segregation and independent assortment across meioses. Mating types of haploid cells were determined by mixing them with cultures of known mating types at 15° on water agar for 24 hr and then examining for mating cells.

Genetic analysis: All diploid genotypes of Microbotryum listed in Table 1 were subjected to karyotype analysis by pulsed-field gel electrophoresis using the CHEF-DR II system and size standards available from Bio-Rad (Hercules, CA). Haploid cell cultures were suspended into agarose without generating protoplasts (McCluskey *et al.* 1990). Electrophoresis conditions consisted of 0.8% chromosomal grade agarose, 1× TBE, and a run time of 96 hr at 14° and 2.7 V/cm with a 200-sec initial switch time and an 1100-sec final switch time. Electrophoretic karyotypes for eight tetrads of *Microbotryum* var. *caroliniana* (Anderson, KY, sample ID MvScGCKY1) were analyzed digitally to determine segregation of chromosomes that were variable in size. Band intensity profiles (electropherograms) were created for the karyotype lanes using Scion Image software (Scion, Frederick, MD; Figure 1).

Markers produced by amplified fragment length polymorphism (AFLP) techniques were used to determine the segregation of allelic variation. Because the AFLP analysis is carried out on meiotic products that are haploid, problems of dominance are not an issue as they are when this technique is applied to diploids. Instead, the segregation of AFLP markers for presence and absence within the linear tetrad indicates heterozygosity (or possibly hemizygosity) for restriction sites within the diploid genotype (Figure 2). The same *Eco*RI/*Mse*I

and *Xho*I/*Msp*I procedures for AFLP analysis were used as described previously (Hood 2002).

To estimate the heterozygous and homozygous proportions of the genome, AFLP products between 100 and 400 bp were analyzed digitally using dye-labeled PCR primers and the GeneScan program on an ABI 377 automated DNA sequencer. Two diploid genotypes of *Microbotryum* var. *latifolia* (Hungerford, UK, sample ID MvSl.80-1H; and Giles County, VA, sample ID MvSl.135HT) and two of *Microbotryum* var. *caroliniana* (Franklin County, NC, sample ID MvSc.CP-A91-26; and Anderson County, KY, sample ID MvScGCKY1) were analyzed for the segregation of AFLP products in two replicate tetrads for each diploid genotype.

In addition, seven of the eight replicate tetrads of *Microbotryum* var. *caroliniana* (Anderson County, KY, sample ID MvScGCKY1) were analyzed for the segregation of heterozygous *Eco*RI/*Mse*I AFLP products by agarose gel electrophoresis. This allowed the collection of data on a much larger number of markers and over a broader range of sizes and was used to characterize cosegregation and independent assortment of heterozygous markers. Analysis of seven linear tetrads for markers segregating at the first division is sufficient in 99% of cases to detect independent assortment of at least 9 of the putative 11 linkage groups if they each present one or more markers.

RESULTS

Segregation of karyotypic variation: Application of linear tetrad analysis to the electrophoretic karyotypes revealed a tremendous amount of chromosome size variation segregating during meiosis. This was true not only for the previously described dimorphism for the fungal sex chromosomes (Hood 2002) but also for many of the autosomes in *Microbotryum* var. *caroliniana* and to a lesser extent in *Microbotryum* var. *latifolia*.

Table 1 shows the distribution of size-dimorphic autosomes that segregated at the first meiotic division. They were a consistent but infrequent feature throughout the range of *Microbotryum* var. *latifolia*. Segregation of an autosome at the second meiotic division was rarely observed in *Microbotryum* var. *latifolia* (in only 2 of the 33 tetrads from 28 diploid genotypes). Although it is difficult to assign homology to particular chromosomes across diploid genotypes, dimorphic autosomes were found throughout the size range of the chromosomes (data not shown), suggesting that the phenomenon is not restricted to a particular autosome pair.

In contrast, nearly half of the 10–12 chromosomes from *Microbotryum* var. *caroliniana* exhibited first-division segregation for size (Table 1). Hence, the patterns of size variation were much more complex, requiring analysis of multiple tetrads from a given diploid genotype. Using this approach, homology of chromosome pairs within the genome could be evaluated by the consistency or independence of their assortment.

In the diploid genotype of *Microbotryum* var. *caroliniana* from Kentucky, where eight linear tetrads were analyzed, five autosome pairs segregated for size at the first meiotic division in each meiosis (Figure 1 and Table 2). The pair of sex chromosomes also had consistent first-division segregation. Three autosome pairs exhibited segregation for size at the second meiotic division, and remarkably, they did so in each meiosis. One chromosome pair consistently exhibited both a major size difference segregating at the first division and a smaller difference segregating at the second division. Another pair showed a single case of second-division segregation (*i.e.*, the smallest autosome pair in tetrad 3 of Table 2). The run conditions for electrophoretic karyotypes were

TABLE 1

Collections of Microbotryum and segregation of size-dimorphic autosomes

	Diploid genotypes	No. of tetrads	First division segregating autosomes
Microbotryum var. *latifolia*			
Virginia			
Giles County	1	4	1
Giles County	2	2	0
Rockingham County	1–8	8	0
Shenandoah County	1,2	2	0
Albemarle County	1	1	0
United Kingdom			
Aldeburgh	1	1	1
Aldeburgh	2	1	0
Hungerford	1	2	1
Hungerford	2	1	0
Ipswich	1	1	0
Italy			
Lamole	1	1	1
Lamole	2,3	2	0
San Gimignano	1,2	2	0
Antrodoco	1	1	0
France			
Orsay	1	1	0
Germany			
Darmstadt	1	1	2
Switzerland			
Oetweil	1	1	0
Czech Republic			
Olomouc	1	1	1
Microbotryum var. *caroliniana*			
Virginia			
Amherst County	1,2	2	5
Princess Anne County	1	1	2
Kentucky			
Anderson County	1	8	5
Jessamine County	1	1	5
North Carolina			
Franklin County	1	4	4

Diploid genotypes from a locality are combined in a single row where they show identical patterns. Second-division segregation was observed for one autosome of *Microbotryum* var. *latifolia* from Darmstadt and from Orsay. Analysis of such variation in *Microbotryum* var. *caroliniana* is presented in Table 2. The range of samples from *Microbotryum* var. *caroliniana* includes two subspecies of this host: Virginia and North Carolina, ssp. *Pensylvanica*; Kentucky, ssp. *wherryi*.

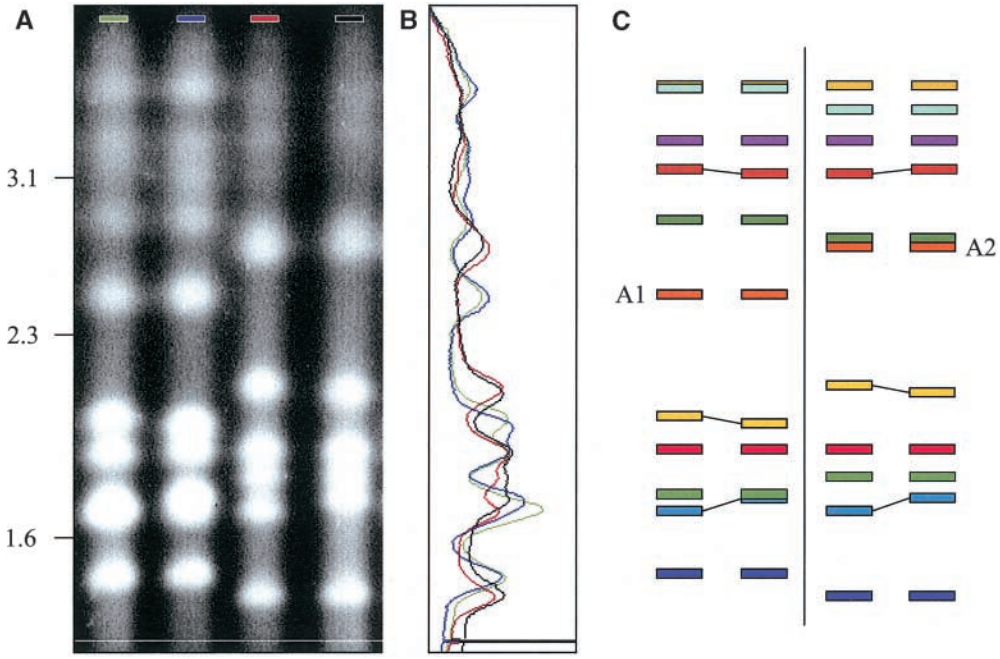

FIGURE 1.—Tetrad analysis of *Microbotryum* var. *caroliniana* for the segregation of chromosome size in electrophoretic karyotypes. (A) Electrophoretic karyotype of a linear tetrad (tetrad 1 from Table 2). Cells within tetrads are ordered from left to right as they originated from different postmeiotic nuclei: as in HOOD and ANTONOVICS (1998), these are the teliospore cell, and the proximal, the middle, and the distal cells of the promycelium, respectively. Chromosome size is given in megabase pairs. (B) Band intensity profiles calculated by Scion Image software. Profiles are matched by color to the respective lanes of the karyotype. (C) Tentative karyotype diagram and assignment of homologous chromosome pairs based upon segregation and independent assortment across multiple meioses (Table 2). The dimorphic sex chromosomes are identified as A1 and A2 on the basis of cosegregation with mating type. The vertical line delineates variation separated at the first meiotic division.

optimized for chromosomes from 1 to 3 Mbp, and there was great confidence in determining size variation for all but the largest four autosome pairs.

The patterns of independent assortment across the eight replicate tetrads were used to assign homology to chromosome pairs. While the level of replication did not allow for detection of ordered segregation in multipair complexes (*i.e.*, hexavalents or higher), two particular chromosome pairs could not be distinguished by independent assortment (Table 2). However, with five autosome pairs and the sex chromosomes segregating at the first meiotic division across seven meioses, the probability of finding consistent assortment between two chromosome pairs was not significant at the $P < 0.1$ level. Tentative assignment of homology within these cosegregating chromosome pairs was nonetheless possible on the basis of size similarity and the fact that one of the pairs also exhibited second-division segregation for size.

Segregation of allelic variation: A high proportion of AFLP markers was heterozygous in the linear tetrads of Microbotryum from both host species (Figure 2, Table 3). However, the host from which the fungus was collected had a major effect on the distribution of the markers within the genomes. This was generally consistent with the differences in patterns of karyotypic segregation.

A total of 212 AFLP products were obtained in the 100- to 400-bp size range in the four tetrads of *Microbotryum* var. *latifolia* (two tetrads from each of two diploid genotypes; Table 3). The two diploid genotypes were very similar in both the number of AFLP products they had in common (97%) and the percentage of AFLP markers that were heterozygous (11 and 12%). All of the heterozygous markers segregated at the first meiotic division in each tetrad. There was no evidence of independent assortment among the markers, and furthermore they all cosegregated with the mating-type locus across the four meioses. Twenty-one of the heterozygous markers were present in the A1 and absent in the A2 cells, and three were present in A2 and absent in A1 cells.

In contrast, AFLP products that were heterozygous in *Microbotryum* var. *caroliniana* had patterns of independent assortment that could be used to infer their presence on multiple chromosomes. In the digital analysis of 100- to 400-bp AFLP products, the two diploid genotypes of *Microbotryum* var. *caroliniana* were much less similar to each other (Table 3), sharing only 46% of bands and differing in the percentage of AFLP markers that were heterozygous (12 and 19%). All but one heterozygous marker exhibited first-division segregation; one marker (unique to the diploid genotype from Anderson County, KY) segregated at the second meiotic

TABLE 2

Segregation of chromosome size variation and AFLP products across replicate tetrads of *Microbotryum* var. *caroliniana*

	Tetrads							
	1	2	3	4	5	6	7	8
Mating types:	1122	1122	2211	1122	1122	2211	2211	1122
Chromosomes								
Dark yellow	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Teal	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Purple	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Dark red	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Dark green	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Orange[a]	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Light yellow	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Light red	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Light green	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Light blue	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -	- - - -
Dark blue	- - - -	- - - -	- - - -[b]	- - - -	- - - -	- - - -	- - - -	- - - -
AFLP Products								
CA/T 620	- -	- -	- -	- -	- -	- -	- -	
T/TG 1000	- -	- -	- -	- -	- -	- -	- -	
GT/GA 460	- -	- -	- -	- -	- -	- -	- -	
GT/GA 495	- -	- -	- -	- -	- -	- -	- -	
GT/GC 250	- -	- -	- -	- -	- -	- -	- -	
GT/AC 770	- -	- -	- -	- -	- -	- -	- -	
GT/GG 60	- -	- -	- -	- -	- -	- -	- -	
T/GT 1200	- -	- -	- -	- -	- -[b]	- -	- -	
T/TG 800	- -	- -	- -	- -	- -	- -	- -	
GT/GT 700	- -	- -	- -	- -	- -	- -	- -	
GT/GC 200	- -	- -	- -	- -	- -	- -	- -	
CA/GC 230	- -	- -	- -	- -	- -	- -	- -	
C/GA 110	- -	- -	- -	- -	- -	- -	- -	
C/GA 100	- -	- -	- -	- -	- -	- -[b]	- -	
GT/AC 750	- -	- -	- -	- -	- -	- -	- -	
GT/AC 230	- -	- -	- -	- -	- -	- -	- -	
C/GA 300	- -	- -	- -	- -	- -	- -	- -	
C/GA 400	- -	- -	- -	- -	- -	- -	- -	
GT/GT 1050	- -	- -	- -	- -	- -	- -	- -	
C/GA 270	- -	- -	- -	- -	- -	- -	- -	
GT/GG 600	- -	- -	- -	- -	- -	- -	- -	
GT/AC 600	- -	- -[b]	- -[b]	- -	- -[b]	- -	- -[b]	

Cells within tetrads are ordered from left to right as they originated from different postmeiotic nuclei: as in Hood and Antonovics (1998), these are the teliospore cell and the proximal, the middle, and the distal cells of the promycelium, respectively. A1 and A2 mating types are abbreviated as 1 and 2. Chromosomes correspond to colors used in Figure 1 and are in the same order according to size (size differences are not to scale). AFLP products are identified by the *Eco*RI/*Mse*I selective primers used in the PCR reactions and the approximate size of the products in base pairs. Markers distinguished by independent assortment are grouped together. A dash indicates that the particular product was obtained with DNA from that haploid culture.

[a] The chromosome pair identified by orange color represents the size-dimorphic sex chromosomes.

[b] Exceptional to the overall patterns of segregation.

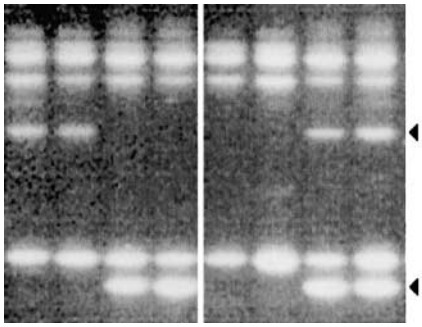

FIGURE 2.—Section of an agarose AFLP gel for *Microbotryum* var. *caroliniana* showing an example of independent assortment of AFLP products across two replicate tetrads. The two heterozygous markers (arrowheads) segregated at the first meiotic division in these linear tetrads, but assortment is alternate in the first tetrad and parallel in the second. Homozygous AFLP products are present in all lanes of the gel.

TABLE 3

Tetrad analysis of AFLP products as a measure of heterozygosity in Microbotryum

	AFLP products	
	Homozygous	Heterozygous (%)
Microbotryum var. *latifolia*		
Hungerford, UK	188	23 (12)
Giles County, VA	185	21 (11)
Products in common	185	20
Microbotryum var. *caroliniana*		
Anderson County, KY	151	35 (19)
Franklin County, NC	149	20 (12)
Products in common	107	8

division in the two replicate tetrads. Independent assortment of heterozygous markers was evident across the tetrads, but the assessment of linkage groups was limited by the level of replication, and this issue was instead determined by analysis of AFLP markers by agarose gel electrophoresis.

Segregation of heterozygous AFLP products across the seven replicate tetrads of one diploid genotype of *Microbotryum* var. *caroliniana* is presented in Table 2. The data show that heterozygous and centromere-linked AFLP markers can be tentatively assigned to at least seven different linkage groups by the patterns of independent assortment. One of these linkage groups, with the greatest number of heterozygous markers (10 of 22), cosegregated with mating-type and the size-dimorphic sex chromosomes. Only one marker showed a high degree of second-division segregation, but this was not in a pattern that would be consistent with the second-division size variation seen in the electrophoretic karyotypes. Two other instances of second-division segregation were observed, but these were in markers where six of the seven tetrads showed first-division segregation. Some AFLP markers did cosegregate with size-dimorphic autosomes (Table 2). However, it should be noted that some degree of cosegregation is expected by chance alone and therefore a formal quantitative assignment of markers to chromosomes was not carried out.

DISCUSSION

The theoretical consequences of mating within the meiotic tetrad that brings together chromosomes separated at meiosis I are confirmed by the high frequency of centromere-linked variation in Microbotryum. The effect was much more pronounced in diploid genotypes of *Microbotryum* var. *caroliniana*, but was also evident in *Microbotryum* var. *latifolia*. Although there was no independent assortment of heterozygous AFLP markers in this latter lineage, autosomes segregating for size at the first meiotic division were a consistent and widespread feature of the genome. Previously we also described deleterious alleles in linkage with autosomal centromeres in a natural population of Microbotryum on *S. latifolia* (HOOD and ANTONOVICS 2000). More recently, THOMAS *et al.* (2003) reported several such centromere-linked deleterious alleles in samples of Microbotryum from *S. latifolia* and other hosts.

Microbotryum var. *caroliniana* was characterized by levels of heterozygosity much higher than those of *Microbotryum* var. *latifolia*. In the former, there was a large degree of allelic heterozygosity, and many autosome pairs were dimorphic in size by as much as hundreds of kilobase pairs (perhaps 10% of their length). Considering this difference between the two lineages of Microbotryum, it may be surprising that they are often subsumed under a single species name, but recent studies suggest that their divergence is ancient (PERLIN *et al.* 1997; HOOD *et al.* 2001; FREEMAN *et al.* 2002). We can add that the lineages have differentiated not only in their karyotype profiles, as in many closely related fungi (ZOLAN 1995), but also in the dynamic nature of their genomes. This probably reflects different rates of mating within *vs.* outside the tetrad. *Microbotryum* var. *latifolia* likely has a mixture of automixis with rare outcrossing (HOOD and ANTONOVICS 2000), whereas in *Microbotryum* var. *caroliniana* automixis may well be obligatory (as discussed below).

The evolution of this type of automixis across fungi, insects, and plants suggests that there are advantages to maintaining heterozygosity (ZAKHAROV 1986). Moreover, this mating system favors recombination suppressors (ANTONOVICS and ABRAMS 2004) that in turn should preserve even larger regions of heterozygosity around the centromeres. However, this type of automixis could itself be reinforced by the very genetic load that it causes to accumulate. In Microbotryum, it is found that some haploid cell lines are associated with deleterious alleles often linked to mating type or the

centromeres of other chromosomes (Kaltz and Shykoff 1997; Oudemans *et al.* 1998; Hood and Antonovics 2000; Thomas *et al.* 2003). However, as such deleterious alleles are allowed to accumulate at the centromeres of autosomes, there would be an increasing fitness cost of mating between tetrads or to closely related diploid genotypes because exposing the alleles to homozygosity would occur at a very high rate. Thus, a feedback process may quickly develop, with mating within the tetrad becoming more frequent, the extensive accumulation of deleterious recessive mutations, and the system being driven ever further from the ability to produce viable progeny by mating outside the tetrad. Following, or in tandem with this cascade toward obligate automixis, the spread of recombination suppressors and the large degree of centromeric suppression of crossing over in Microbotryum (Garber *et al.* 1987) could also help control exposure of genetic load, as has been previously suggested by studies of other automictic fungi (Summerbell *et al.* 1989; Kerrigan *et al.* 1993; Xu 1995).

The conditions that favor such a scenario remain to be explored theoretically. However, we do see greater evidence for deleterious recessive mutations in *Microbotryum* var. *caroliniana* than in *Microbotryum* var. *latifolia* in the form of higher variation in the growth rate of haploid cultures (our unpublished data). Across the diversity of Microbotryum lineages, there are also those for which culturing of haploid cells is difficult and for which all four cells of linear tetrads are inviable (our unpublished data). These latter types of sample would often go underreported and be attributed to spore inviability. To empirically address this issue, the actual fitness costs of mating within *vs.* between tetrads needs to be determined by performing crosses within lineages of Microbotryum that are at different stages in the pathway toward obligatory automixis. By performing the crosses within or between tetrads of the same diploid fungal genotype, the effects of exposing genetic load can be separated from the issues of protoplasmic compatibility sometimes observed to result from outcrossing in fungi (*i.e.*, as with the *het* locus of the automictic fungus *Neurospora tetrasperma*; Jacobson 1995; Powell *et al.* 2001; Saenz *et al.* 2001).

Recent reviews have described high levels of chromosome size variation within and between fungal populations (Mills and McCluskey 1990; Zolan 1995). It has been suggested that such variation should be negatively correlated with the frequency of sexual reproduction (Kistler and Miao 1992) because the difficulties of pairing size-dimorphic chromosomes during metaphase I are expected to limit karyotype changes in sexual species but permit greater plasticity in asexual species. The same reasoning has been applied to karyotypic variation in insects and has been used to support the classification of species that show a rapidly changing karyotype as asexual (specifically as apomictic or ameiotic; Normark 1999). As an obligately sexual fungus, *Microbotryum* var. *caroliniana* clearly contradicts this model, to the extent that nearly half of the autosomes are substantially dimorphic in size. In *Microbotryum* var. *latifolia*, there is also substantial variation of karyotypes even within populations (Table 1; Hood *et al.* 2003). The high level of karyotype variation in Microbotryum is associated with the substantial centromeric suppression of recombination and the maintenance of fixed heterozygosity (Garber *et al.* 1987). Such regions are expected to accumulate repetitive DNA elements such as retrotransposons (Charlesworth and Langley 1989). In fact, we have recently shown that a large proportion of the genome of *Microbotryum* var. *latifolia* consists of such elements, predominantly in the fungal sex chromosomes (15%) but also in autosomes (6%; M. E. Hood, J. Antonovics and B. Koskella, unpublished results). An even higher density of retrotransposons might be expected in *Microbotryum* var. *caroliniana* where heterozygosity is maintained to a greater extent. Repetitive DNA in turn may promote crossing over between similar sequences at nonhomologous regions (ectopic recombination), which results in changes in chromosome size and structure (reviewed in Lönnig and Saedler 2002). A high degree of chromosome size variation is also reported for some automictic insects (*e.g.*, Marescalchi and Scali 1997), and thus the correlations between sexual cycles, repetitive DNA content, and karyotypic plasticity could be tested across very diverse taxa.

The most unexpected result from this study was the presence of variation in chromosome size that segregated at the second meiotic division; the pattern could be seen consistently across all eight of the replicate tetrads of *Microbotryum* var. *caroliniana*. It is well recognized that at least one chiasma will promote proper segregation of chromosomes during meiosis (Roeder 1997). However, our data are consistent with the formation of only one crossover event, and similar examples are very rare (*e.g.*, Gallegos *et al.* 2000). That size variation segregating at the second division was relatively small and that we found no corresponding AFLP markers consistently segregating at the second division suggest that the crossover events were near the ends of the chromosome arms.

The problem still remains as to why second-division size variation in chromosome size was maintained at all because mating within the tetrad should result in a rapid loss of such distal heterozygosity. Apart from the possibility that these were recent chromosome mutations, there is an alternative hypothesis, which depends upon a peculiarity of tetrad development in Microbotryum. We previously reported that mating under natural conditions is often so rapid that a cell containing sister nuclei of the second meiotic division mates before it can complete cytokinesis (Hood and Antonovics 1998). As a result, a trinucleate zygote is produced, containing a haploid nucleus of one mating type and two haploid nuclei of the other mating type. This development ap-

pears to produce viable infection hyphae, but it is unknown at what stage the superfluous chromatin might be lost. We previously puzzled over the significance of such a development, but it does provide a mechanism whereby postmeiotic nuclei can be sorted during vegetative growth into karyotypic combinations that are "balanced" as opposed to "unbalanced" for chromosomal variation (Lewis and John 1963).

While this selective explanation may work for one pair of chromosomes, independent assortment (as can be seen in the example of Figure 1) often means that no combination of A1 and A2 cells from within the tetrad would restore heterozygosity to all chromosome pairs segregating at the second meiotic division. Thus, we may expect karyotypic evolution from this particular diploid genotype of Microbotryum to be extremely rapid and to be detectable over a small number of generations. Of course, the rate of change is also dependent upon the frequency of chromosomal rearrangements, which may be quite high (as discussed above). The karyotype variation is contradicted by the low levels of DNA sequence variation observed within the natural population of Microbotryum (Delmotte *et al.* 1999). Using an experimental approach, future studies could explore the dynamics of genetic structure within genomes and within populations that are founded by single or controlled numbers of diploid genotypes.

The distribution of heterozygous markers in this study supports the previous conclusion that the sex chromosomes in Microbotryum have a genetic structure different from the autosomes (Hood 2002). The patterns can be seen in both lineages of Microbotryum even though the sex chromosomes in *Microbotryum* var. *latifolia* are the largest chromosomes in the genome (~3–4 Mbp) and those in *Microbotryum* var. *caroliniana* are much smaller (~2.2–2.7 Mbp). The proportion of heterozygous markers in *Microbotryum* var. *latifolia* indicated that 11 or 12% of the genome is heterozygous, and most of this variation was associated with the sex chromosomes (although not all cosegregating markers in a small number of tetrads are necessarily linked). This level of heterozygosity is only slightly less than the total proportion of the genome represented by the sex chromosomes on the basis of their estimated sizes in electrophoretic karyotypes (~13%). Therefore, the sex chromosomes in *Microbotryum* var. *latifolia* are probably highly differentiated and heterozygous throughout the vast majority of their lengths. The data for *Microbotryum* var. *caroliniana* similarly suggest greater variation between the pair of sex chromosomes than for any pair of autosomes. In this lineage, a somewhat larger proportion of the genome is heterozygous relative to the size of sex chromosomes (12–19% heterozygous *vs.* ~10% in size, respectively). These data are consistent with some heterozygosity found at the centromeres of autosomes in addition to the sex chromosomes. However, the largest number of the markers cosegregated with the sex chromosomes even though they are intermediate in size relative to the autosomes.

Curiously, in both lineages of Microbotryum, more heterozygous markers linked to mating type were present in the A1 cells than in the A2 cells even though the A2 sex chromosome is larger in both cases and would be expected to yield more markers. The reason for this discrepancy requires further study, but efforts should focus on the extent to which duplicated or repetitive DNA is responsible for the size dimorphism of sex chromosomes and how this may influence the skewed distribution of AFLP products or functional genes.

In summary, our results show that, despite what superficially appears to be an extreme form of selfing, the genome of Microbotryum is extremely rich in heterozygosity and has a surprisingly dynamic karyotype that may be influenced to a large extent by the accumulation of repetitive DNA elements. An important corollary is that the variation we observe is not influenced by the relatedness between individuals in the manner assumed by most measures of population genetics. Therefore, great caution is needed before interpreting within- and among-population heterozygosity in automictic species, particularly when linkage relationships to the centromeres are unknown. Additionally, there seem to be features peculiar to the genome, including evidence of strict control of chiasma formation and consistent second-division segregation of chromosome size. These complexities may interact with development during meiosis to permit nuclear sorting and selection for balanced genomes. Because this mating system can maintain high frequencies of recessive deleterious alleles, the phenomenon of "mating-type bias" appears to be common in Microbotryum. Such deleterious alleles may also further restrict organisms to automixis and reproductive isolation through costly exposure when mating occurs outside the tetrad. Finally, our results show that if mating compatibility is genetically determined in the gametic phase, evolutionary forces for the divergence of haploid sex chromosomes are still effective in highly automictic species.

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