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

Michael E. Hood[1]

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

Manuscript received July 25, 2001
Accepted for publication November 9, 2001

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 evolved 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-

[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 micromanipulation, 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 (Bio-Rad, 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× 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 *Eco*RI/*Mse*I AFLP analysis. To increase the number of AFLP products available for analysis, the restriction enzyme combination *Xho*I/*Msp*I was additionally used, with the linkers and primers being the same as for *Eco*RI/*Mse*I 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 as primers in this study were 5′-CTTCGGAATAACGAGAAGGC-3′ and 5′-AGGTATGAGCAGTGGATCGG-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 (*Xho*I and *Msp*I) 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

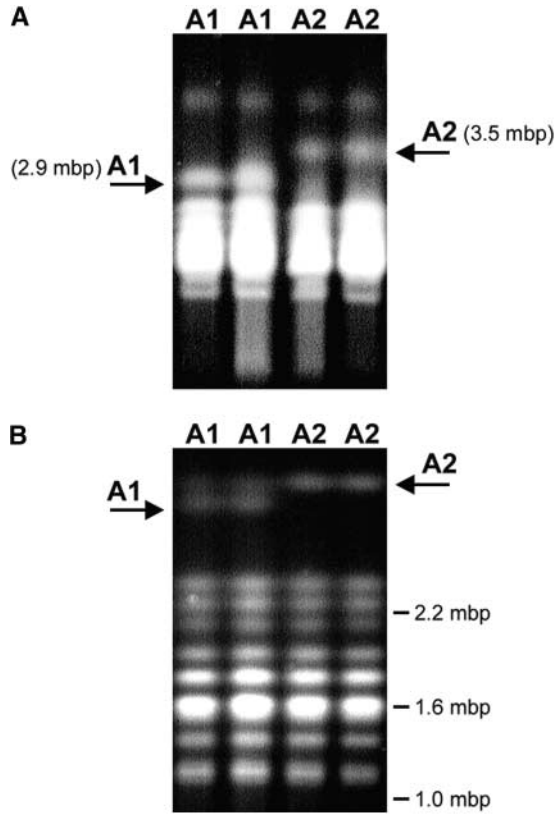

Figure 1.—Electrophoretic karyotypes for a linear tetrad of *Microbotryum violaceum* from *Silene latifolia* showing size dimorphism of A1 and A2 sex chromosomes (arrows). The mating types of the tetrad cells are indicated. This pattern of size dimorphism was consistent for each mating type across 36 tetrads. (A) Run conditions were designed to separate large (>3 Mbp) chromosomes. Note that there is a homozygous autosome larger than the sex chromosomes, but this gel doesn't resolve most autosomes. (B) Separation of smaller (1–3 Mbp) chromosomes. This gel resolves most of the autosomes, showing that there is structural homozygosity among them.

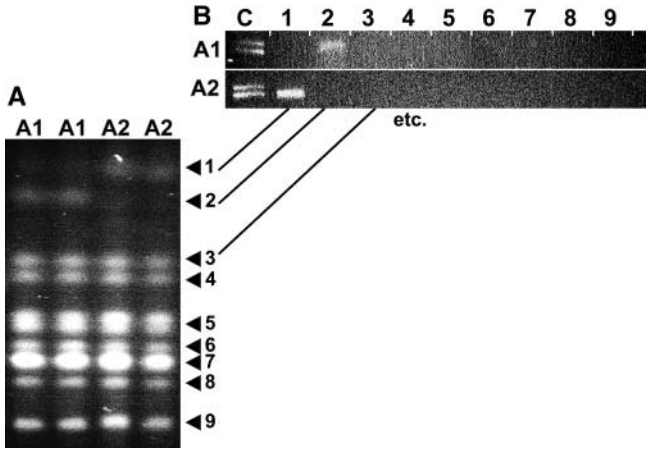

FIGURE 2.—(A) Electrophoretic karyotype of *Microbotryum violaceum* from *Silene latifolia* showing location of gel-isolated chromosomes. Chromosomes were isolated from lanes 1 (A1) and 4 (A2) of the electrophoretic karyotype. (B) PCR products from corresponding gel-isolated chromosomes using mating-type-specific primers. Amplification is limited to the dimorphic chromosomes. "C" indicates a combined ladder of A1- and A2-specific products as a positive control.

tetrads showed that the A2 sex chromosome is larger than the A1. However, the sex chromosomes, ranging from 2.2 to 2.7 Mbp in length, are intermediate in size relative to the rest of the karyotype. The autosomes numbered from 9 to 13 pairs and ranged in size from 1.3 to 3.5 Mbp.

Within each lineage of *M. violaceum* from *S. 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 *Saccharomycodes 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. Even though the predominance of the haploid stage can be quite variable between fungi and bryophytes, the mechanisms involved in sex chromosome divergence may be similar. These mechanisms potentially include the addition of genetic material by translocation of large regions of DNA to one of the sex chromosomes, gradual divergence in size by the amplification of functional genes, and insertion of self-proliferating elements like transposons. The latter mechanism operates frequently in diploid systems, particularly as heterogametic Y chromosomes effectively trap transposons (ERLANDSSON *et al.* 2000; STEINEMANN and STEINEMANN 2000).

In support for this process, or at least for the potential action of transposons in *M. violaceum*, such elements are easily identified among DNA sequences linked to mating type. The suppression of recombination near

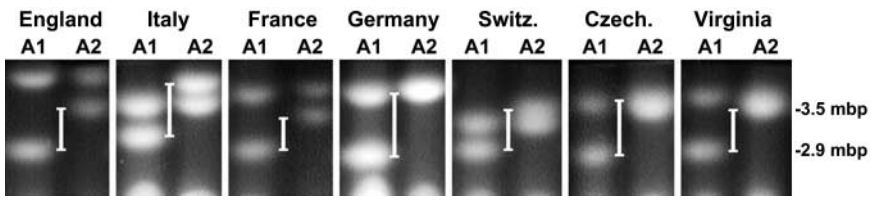

FIGURE 3.—A1 and A2 sex chromosomes of *Microbotryum violaceum* from *Silene latifolia* collected in Europe and North America. Vertical bars mark the size variation between the smaller A1 chromosome and the larger A2 chromosome in each sample. Variation occurs in both the sizes of the sex chromosome and the degree of dimorphism. Note that both lanes from a locality are from a single tetrad. Also note that a large autosome is homozygous in each tetrad. Smaller autosomes are not included.

TABLE 1

Comparison of mating-type-linked AFLP fragments to sequences in protein databases

Sequence ID (Accession no.)	Linkage	Length (bp)	Similarity	*E* value
XC/MG454 (AF398756)	A2	454	Retroelement	5*e*-07
XC/MG430 (AF398757)	A2	430	Retroelement	4*e*-07
XC/MA410 (AF398758)	A2	410	Retroelement	0.017
EC/MAC239 (AF398759)	A1	239	Retroelement	1*e*-04
EGT/MGA572 (AF398760)	A1	572	Helicase	2*e*-25

Similarity identified by BLASTx algorithm at National Center for Biotechnology Information. *E* value indicates the number of similar matches expected by chance. The DNA source was from the Giles County population, strain 135HT.

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.

I thank Janis Antonovics for advice and discussion, Doug Taylor and Miriam Heuhsen for collecting samples, and Hilary Heishman and Cathy Arnette for technical assistance.

LITERATURE CITED

Alexander, H. M., P. H. Thrall, J. Antonovics, A. M. Jarosz and P. V. Oudemans, 1996 Population dynamics and genetics of plant disease: a case study of anther-smut disease. Ecology **77:** 990–996.

Allen, C. E., 1917 A chromosome difference correlated with sex in *Sphaerocarpus.* Science **46:** 466–467.

Antonovics, J., D. Stratton, P. H. Thrall and A. M. Jarroz, 1996 An anther-smut disease (*Ustilago violacea*) of fire-pink (*Silene virginica*): its biology and relationship to the anther-smut disease of white campion (*Silene alba*). Am. Midl. Nat. **135:** 130–143.

Bauer, R., F. Oberwinkler and K. Vanky, 1997 Ultrastructural markers and systematics in smut fungi and allied taxa. Can. J. Bot. **75:** 1273–1314.

Bucheli, E., B. Gautschi and J. A. Shykoff, 2000 Host specific differentiation in the anther smut fungus *Microbotryum violaceum* as revealed by microsatellites. J. Evol. Biol. **13:** 188–198.

Bull, J., 1978 Sex chromosomes in haploid dioecy: a unique contrast to Muller's theory for diploid dioecy. Am. Nat. **112:** 245–250.

Charlesworth, B., 1978 Model for evolution of Y chromosomes and dosage compensation. Proc. Natl. Acad. Sci. USA **75:** 5618–5622.

Charlesworth, B., 1991 The evolution of sex chromosomes. Science **251:** 1030–1033.

Cummins, J. E., and A. W. Day, 1973 Cell cycle regulation of mating type alleles in the smut fungus *Ustilago violacea*. Nature **245:** 259–260.

Day, A., 1976 Communication through fimbriae during conjugation in a fungus. Nature **262:** 583–584.

Erlandsson, R., J. F. Wilson and S. Paeaebo, 2000 Sex chromosome transposable element accumulation and male-driven substitutional evolution in humans. Mol. Biol. Evol. **17:** 804–812.

Filatov, D. A., F. Moneger, I. Negrutiu and D. Charlesworth, 2000 Low variability in a Y-linked plant gene and its implications for Y-chromosome evolution. Nature **404:** 388–390.

Gallegos, A., D. J. Jacobson, N. B. Raju, M. P. Skupski and D. O. Natvig, 2000 Suppressed recombination and a pairing anomaly on the mating-type chromosome of *Neurospora tetrasperma*. Genetics **154:** 623–633.

Gaudet, D. A., J. Gusse and A. Laroche, 1998 Origins and inheritance of chromosome-length polymorphisms in the barley covered smut fungus, *Ustilago hordei*. Curr. Genet. **33:** 216–224.

Hood, M. E., and J. Antonovics, 1998 Two-celled promycelia and mating-type segregation in *Ustilago violacea* (= *Microbotryum violaceum*). Int. J. Plant Sci. **159:** 199–205.

Hood, M. E., and J. Antonovics, 2000 Intratetrad mating, heterozygosity, and the maintenance of deleterious alleles in *Microbotryum violaceum* (=*Ustilago violacea*). Heredity **85:** 231–241.

Hood, M. E., O. J. Rocha and J. Antonovics, 2001 Differences in teliospore germination patterns of *Microbotryum violaceum* on European and North American *Silene* species. Mycol. Res. **105:** 532–536.

Korablev, V. P., V. I. Radashevsky and G. P. Manchenko, 1999 The XX–XY (male heterogametic) sex chromosomes in *Polydora curiousa* (Polychaeta: Spionidae). Ophelia **51:** 193–201.

Lee, B. S., L. Bi, D. J. Garfinkel and A. M. Bailis, 2000 Nucleotide excision repair/TFIIH helicases Rad3 and Ssl2 inhibit short-sequence recombination and Ty1 retrotransposition by similar mechanisms. Mol. Cell. Biol. **20:** 2436–2445.

Lee, N., G. Bakkeren, K. Wong, J. E. Sherwood and J. W. Kronstad, 1999 The mating-type and pathogenicity locus of the fungus *Ustilago hordei* spans a 500-kb region. Proc. Natl. Acad. Sci. USA **96:** 15026–15031.

Lewis, K. R., and B. John, 1963 *Chromosome Marker*. Little Brown, Boston.

McCluskey, K., and D. Mills, 1989 Identification and characterization of chromosome length polymorphisms among strains representing fourteen races of *Ustilago hordei*. Mol. Plant-Microbe Int. **3:** 366–373.

McCluskey, K., B. W. Russell and D. Mills, 1990 Electrophoretic karyotyping without the need for generating protoplasts. Curr. Genet. **18:** 385–386.

Merino, S. T., M. A. Nelson, D. J. Jacobson and D. O. Natvig, 1996 Pseudohomothallism and evolution of the mating-type chromosome in *Neurospora tetrasperma*. Genetics **143:** 789–799.

Metzenberg, R. L., and N. L. Glass, 1990 Mating type and mating strategies in *Neurospora*. BioEssays **12:** 53–59.

Mills, D., and K. McCluskey, 1990 Electrophoretic karyotypes of fungi: the new cytology. Mol. Plant-Microbe Interact. **3:** 351–357.

Muller, H. J., 1914 A gene for the fourth chromosome of *Drosophila*. J. Exp. Zool. **17:** 325–336.

Perlin, M. H., C. Hughes, J. Welch, S. Akkaraju, D. Steinecker *et al.*, 1997 Molecular approaches to differentiate subpopulations or *formae speciales* of the fungal phytopathogen *Microbotryum violaceum*. Int. J. Plant Sci. **158:** 568–574.

Randall, T. A., and R. L. Metzenberg, 1998 The mating type locus of *Neuropsora crassa*: identification of an adjacent gene and characterization of transcripts surrounding the idiomorphs. Mol. Gen. Genet. **259:** 615–621.

Rice, W. R., 1987 Genetic hitchhiking and the evolution of reduced genetic activity of the Y sex chromosome. Genetics **116:** 161–167.

Sato, M., and M. Ikeda, 1992 Chromosomal complements of 2 forms of Neanthes-Japonica (Polychaeta, Nereeididae) with evidence of male-heterogametic sex-chromosomes. Mar. Biol. **112:** 299–307.

Steinemann, M., and S. Steinemann, 2000 Common mechanisms of Y chromosomes evolution. Genetica **109:** 105–111.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. V. Lee *et al.*, 1995 AFLP a new technique for fingerprinting. Nucleic Acids Res. **23:** 4407–4414.

White, M. J. D., 1973 *Animal Cytology and Evolution*, Ed. 3. Cambridge University Press, Cambridge.

Wilch, G., S. Ward and A. Castle, 1992 Transmission of mitochondrial DNA in *Ustilago violacea*. Curr. Genet. **22:** 135–140.

Yamazaki, T., and Y. Oshima, 1996 *Saccharomycodes ludwigii* has seven chromosomes. Yeast **12:** 237–240.

Zolan, M. E., 1995 Chromosome-length polymorphism in fungi. Microbiol. Rev. **59:** 686–698.

Communicating editor: M. E. Zolan