Title
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We dedicate this article to the memory of David Perkins

*Present address: Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111*
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ABSTRACT

A large region of suppressed recombination surrounds the sex-determining locus of the self-fertile fungus *Neurospora tetrasperma*. This region encompasses nearly one-fifth of the *N. tetrasperma* genome and suppression of recombination is necessary for self-fertility. The similarity of the *N. tetrasperma* mating chromosome to plant and animal sex chromosomes and its recent origin (<5 MYA), combined with a long history of genetic and cytological research, makes this fungus an ideal model for studying the evolutionary consequences of suppressed recombination. Here we compare genome sequences from two *N. tetrasperma* strains of opposite mating-type to determine if structural rearrangements are associated with the non-recombining region and to examine the effect of suppressed recombination for the evolution of the genes within it. We find a series of three inversions encompassing the majority of the region of suppressed recombination and provide evidence for two different types of rearrangement mechanisms: the recently proposed mechanism of inversion via staggered single strand breaks as well as ectopic recombination between transposable elements. In addition, we show that the *N. tetrasperma mat a* mating-type region appears to be accumulating deleterious substitutions at a faster rate than the other mating-type (*mat A*) and thus may be in the early stages of degeneration.
ARTICLE SUMMARY

The evolutionary benefit of sex is usually attributed to the action of genetic recombination. Recombination breaks up the associations between gene variants from the same chromosome and shuffles them into a myriad of different combinations. One way to understand the evolutionary importance of recombination is to study regions of the genome where it is absent, such as in the sex chromosomes of many organisms. In mammalian sex chromosomes, the X once shared identity with the Y across its entire length (~160 MYA), however, the Y degenerated after recombination stopped and identity between X and Y can now only be found in a small region. The rest of the Y has been obliterated, making it difficult to interpret the early evolutionary history of these two chromosomes. Here we examined a large, non-recombining, genomic region in the fungus *Neurospora tetrasperma* where recombination was suppressed far more recently (~4.5 MYA). We can clearly identify the changes in chromosome structure that are suppressing recombination, we have developed a model for the order and molecular mechanism by which these changes occurred, and we find subtle evidence of asymmetrical degeneration of the genes within this region between the *N. tetrasperma* mating type chromosomes.
INTRODUCTION

The elimination of recombination can have a dramatic effect on the evolutionary trajectory of a genomic region. Without recombination, selection acts on linked genetic complexes rather than independent genetic elements. Theory predicts the accumulation of deleterious mutations and selfish genetic elements in the absence of recombinational purging and a reduced ability to fix adaptive mutations (CHARLESWORTH and CHARLESWORTH 2000; CHARLESWORTH et al. 2005).

The genetic consequences of suppressed recombination have been best studied in the sex chromosomes of outcrossing eukaryotes, e.g., plants, insects and mammals, because the initial step in the formation of sex chromosomes is posited to be a cessation of recombination across a genomic region that includes the sex-determining locus (CHARLESWORTH et al. 2005). The suppression of recombination across such a region will be selected for if it creates linkage between the sex-determining locus and other genes that are sexually antagonistic in that their functions are beneficial to only one of the sexes. The non-recombining region can be formed from the spread of recombinational suppressors or structural changes to the chromosome that prevent synopsis. In either case, studies in mammals, birds, and plants have shown evidence that present-day non-recombining regions are composed of multiple discrete blockage events that occurred at different time-points in the evolutionary history of the taxon in question (termed “evolutionary strata” by Lahn & Page (CHARLESWORTH et al. 2005; LAHN and PAGE 1999).

Because large, non-recombining, sex-linked regions appear to have evolved independently across a diverse array of taxonomic groups, comparing the evolution of such regions across disparate
taxa will make it possible to understand the evolutionary events associated with their formation as well as the genomic consequences of suppressed recombination.

In several species of fungi, the properties of the chromosomal region surrounding the mating-type locus have been studied extensively because of their similarities to the sex chromosomes of other organisms (Fraser and Heitman 2004; Fraser and Heitman 2005). The mating-type locus of Cryptococcus neoformans occurs within a ~100 kb region where recombination is suppressed due to multiple chromosomal rearrangements (Fraser et al. 2007; Lengeler et al. 2002). The evolutionary history of this region includes the accumulation of transposable elements as well as gene conversion, gene loss and pseudogenization (Fraser et al. 2004; Metin et al. 2010). The mating-type region of Ustilago hordei resides within a 500 kb region that is characterized by suppressed recombination and chromosomal rearrangements (Lee et al. 1999) and the mating-type chromosomes of the fungus Microbotryum violaceum are heteromorphic and contain a region of suppressed recombination that has been roughly estimated to be 1,000 kb in size (Votintseva and Filatov 2009). DNA polymorphism within M. violaceum populations has been compared between the recombinating and non-recombining portions of the genome, however, nucleotide variation within the non-recombinating region did not stand out from the rest of the genome due to overall low levels of polymorphism and high linkage-disequilibrium (Votintseva and Filatov 2010), consistent with previous results showing that M. violaceum is predominantly selfing (Giraud et al. 2005).

The sex-determining locus of Neurospora tetrasperma (termed mat) is surrounded by a region of suppressed recombination that is approximately seven-fold larger than that of M. violaceum and
seventy-fold larger than that of *C. neoformans* (Menkis et al. 2008). From the work presented here, we now know that it includes ca. 2000 genes and spans a distance of ca. 7.8 Mb, which represents 80% of the mating-type chromosome and approximately one-fifth of the *N. tetrasperma* genome. The formation of the region of suppressed recombination was part of a series of evolutionary events that allowed this species to become self-fertile and it is relatively young compared to most vertebrate sex chromosomes (less than ~4.5 MYA compared to ~160 MYA for the XY system in marsupial and placental mammals (Veyrunes et al. 2008) and the ZW system in snakes (O’Meally et al. 2010) and >120 MYA for the ZW system in birds (Mank and Ellegren 2007)). Due to the large size and recent origin of the non-recombining region, *N. tetrasperma* has emerged as an important model for the study of early sex chromosome evolution (Gallegos et al. 2000; Jacobson 2005; Menkis et al. 2008; Menkis et al. 2010; Merino et al. 1996) alongside other organisms with relatively young sex chromosomes such as medaka (Kondo et al. 2006), papaya (Liu et al. 2004), and several species of *Drosophila* (Charlesworth et al. 2005), (reviewed in (Fraser and Heitman 2005)).

Sex in *N. tetrasperma* is controlled by two mating type idiomorphs, *mat a* and *mat A*. The majority of isolates collected from nature are heterokaryotic: haploid nuclei of opposite mating type can be found in a single fungal individual, allowing the individual to be self-fertile (Raju 1992). Self-fertility is maintained between generations by the packaging of two nuclei of opposite mating type into a single sexual spore, however, if recombination occurs in the region between the mating-type locus and the centromere during meiosis, two nuclei of the same, rather than opposite, mating-type will be packaged into the spore (Gallegos et al. 2000; Merino et al. 1996; Raju and Perkins 1994). In this case, the resulting progeny will not be self-fertile. The
region of suppressed recombination is therefore thought to have evolved to ensure the correct packaging of nuclei of opposite mating-type into the sexual spore, thus maintaining the heterokaryotic, self-fertile condition (GALLEGOS et al. 2000; MERINO et al. 1996). Nevertheless, the heterokaryon does occasionally break down via the production of vegetative or sexual spores containing nuclei with only one of the two mating types (RAJU 1992). The haploid, homokaryotic individual that grows from such a spore can mate with another homokaryotic individual of opposite mating type to restore the heterokaryotic condition (RAJU 1992). Thus, the reproductive strategy of N. tetrasperma is likely to include repeated rounds of selfing with an occasional outcrossing event (POWELL et al. 2001). Repeated selfing within a heterokaryon is also supported by previous work showing that allelic differences between mat a and mat A nuclei were confined to the non-recombining region of the mating chromosome (MERINO et al. 1996).

A considerable body of work has built upon the initial observations of Howe and Haysman (HOWE and HAYSMAN 1966) that recombination was reduced on the N. tetrasperma mating-type chromosome. Raju (RAJU 1992) and Raju and Perkins (RAJU and PERKINS 1994) determined the steps during ascus development that are required for correct packaging of mat a and mat A nuclei into a single ascospore. Merino et al. (MERINO et al. 1996) and Gallegos et al. (GALLEGOS et al. 2000) confirmed that recombination is suppressed across the majority of the mating type chromosome and Gallegos et al. visualized the non-recombining region as an anomalous unpaired region visible during pachytene. Jacobson (JACOBSON 2005) reciprocally introgressed mating type chromosomes between N. tetrasperma and N. crassa. His results suggested that the N. tetrasperma mat a chromosome may be collinear with the N. crassa mat A chromosome while the N. tetrasperma mat A chromosome may be structurally rearranged. However, he also found
evidence for the existence of genetic, rather than structural, modifiers of recombination and was unable to determine the relative contributions of these two phenomena with respect to the suppression of recombination in this region. More recently, Menkis et al. (MENKIS et al. 2008) sequenced 35 genes spanning the mating type chromosome from each of the two N. tetrasperma mat a and mat A strains used in this study. Based on sequence divergence between these genes, the authors predicted the existence of two evolutionary strata: one large pericentric region encompassing most of the chromosome, and another much smaller region distal to the mat locus. In two other studies, Menkis et al. (MENKIS et al. 2009) showed that N. tetrasperma is actually a species complex composed of nine genetically isolated lineages and found evidence of differences in the size of the non-recombining region among these lineages (MENKIS et al. 2010). Together, these results suggest that there may be important differences in the region of suppressed recombination around the mating-type locus among the different N. tetrasperma lineages (MENKIS et al. 2009; MENKIS et al. 2008; MENKIS et al. 2010). Whittle et al. (WHITTLE and JOHANNesson 2011; WHITTLE et al. 2011b) have investigated patterns of codon usage and nonsynonymous substitution within the region of suppressed recombination in another N. tetrasperma lineage (strain P4492; lineage #1), one different from the strain that was the main subject of (MENKIS et al. 2008), (JACOBson 2005), and the work presented here (strain P581; lineage #6). They found evidence for relaxed purifying selection within this region in the form of substitutions from preferred to nonpreferred codons and, in a branch-specific analysis using PAML, a higher dN/dS ratio along the N. tetrasperma branch for the genes in the non-recombining region compared to their N. crassa and N. discreta orthologs. However, these analyses assume that the location and number of non-recombining strata in their lineage of interest (lineage #1; see (MENKIS et al. 2009)) is the same as those reported for lineage #6.
(MENKIS et al. 2008), despite the previous evidence suggesting that these regions may have evolved independently (MENKIS et al. 2009; MENKIS et al. 2008; MENKIS et al. 2010). While several other recent studies have investigated the molecular evolution of the strain we study here (NYGREN et al. 2011; WHITTLE et al. 2011c), reviewed in (WHITTLE et al. 2011a), none of these have examined the evolution and structure of the region of suppressed recombination in detail.

More than forty years of genetic and cytological analyses involving N. tetrasperma combined with the open question as to the degree in which changes in chromosome structure are involved in the suppression of recombination, made this organism an ideal candidate for a genome sequencing project. This sequencing project was undertaken by the Joint Genome Institute and involved the sequencing and assembly of two genomes: the haploid mat A and mat a strains derived from a single heterokaryotic N. tetrasperma isolate. These are the same strains studied in (MENKIS et al. 2008) and (JACOBSON 2005) and here we use their genome assemblies to investigate the mechanisms of recombination suppression as well as the evolutionary consequences for the genes residing within the non-recombining region. We find that the majority of the region of suppressed recombination is covered by several large chromosomal rearrangements. Additionally, we show that the young evolutionary stratum identified by Menkis et al. (MENKIS et al. 2008) is located in a region where the two mating chromosomes are collinear and identify an additional stratum, created by an inversion, on the opposite end of the chromosome. We propose a model for the sequence of events and mechanisms of rearrangement that produced the current orientation and show that the region of suppressed recombination within the mat a strain appears to be in the early stages of degeneration.
RESULTS

Chromosomal Rearrangements: To determine if chromosomal rearrangements could be responsible for the suppression of recombination, we created a whole genome alignment between the mat a and mat A strains of *N. tetrasperma* as well as between each *N. tetrasperma* strain and the outgroup species *N. crassa*. We visualized large-scale synteny between these pairs using dotplots (Fig. 1). Synteny is strongly conserved across all genome pairs, with the exception of those involving the *N. tetrasperma* mat A mating type chromosome. Knowing that the *N. tetrasperma* mat a mating type chromosome is collinear with that of *N. crassa*, we concluded that a series of chromosomal rearrangements had occurred on the *N. tetrasperma* mat A chromosome.

Focusing on the mating type chromosome and the comparison between the two *N. tetrasperma* strains, we found that there have been two large inversions (approximately 5.3 and 1.2 Mb), a smaller inversion (68 kb), and an apparent translocation of a ~143 kb segment from one end of the chromosome to the other (Table 1; Fig. 2). Consistent with previous cytological data, the mating-type chromosome ends are collinear and the rearrangements encompass contiguous regions within the central portion of the chromosome, however, none of the rearrangement events include the mat locus itself. We propose that the evolution of the mat A chromosome can be explained by two overlapping inversions which, together, resulted in the movement of the 143 kb segment from one end of the chromosome to the other (Fig. 2). We therefore will hereafter refer to this segment as the “relocated” region. The alternative explanation for this movement, that a 143 kb genomic segment was excised from one end of the mat chromosome and reinserted into the other, appears less likely for reasons explained below.
Mechanisms of rearrangement: Ectopic recombination between transposable elements is generally believed to be a common mechanism for the generation of chromosomal inversions (Casals and Navarro 2007) and its role in creating chromosomal rearrangements has been experimentally verified in yeast (Argueso et al. 2008). However, a study by Ranz et al. (Ranz et al. 2007) found no evidence for ectopic recombination in the majority of fixed inversions between species within the Drosophila melanogaster group. Instead, the authors found short duplications of non-repetitive sequence at the breakpoints of most of the inversions they identified and proposed a novel mechanism for the generation of inversions involving staggered single-strand breaks followed by nonhomologous end joining.

We compared the two breakpoints associated with each inversion shown in our model to determine if there was evidence for either ectopic recombination via transposable elements or the staggered break model of (Ranz et al. 2007). Interestingly, we find evidence for both types of rearrangement mechanisms, adding credence to the novel mechanism proposed by Ranz et al. while also supporting the notion that ectopic recombination is a common mechanism underlying chromosomal rearrangements.

At the breakpoints of the 1.2 Mb inversion, we find in mat A, a short duplication of a 50 basepair segment that is unique in the N. tetrasperma mat a genome. The orientation of this duplication is consistent with those found in Drosophila (Ranz et al. 2007) and with our model of the rearrangement events. In N. tetrasperma, after the 1.2 Mb inversion, the duplicated segments would have been in an inverted orientation as in Drosophila (Ranz et al. 2007). The subsequent
5.3 Mb inversion, which contained the left duplication, would have then returned the leftmost duplication to the same orientation as the right duplication (Fig. 2).

The 5.3 Mb inversion is flanked by inverted Mariner transposable elements. The amino acid sequence of the transposase ORFs of the two elements are ~28% identical and ~59% similar to that of the Pogo family Mariner-3_AN from Aspergillus nidulans (KAPITONOVA 2003). Both transposase ORFs have multiple stop codons suggesting that they are no longer active. These sequence features also support the inclusion of the relocated region within both inversions, providing additional support for our model of how this region moved from one end of the chromosome to the other (Fig. 2). We found no such sequence features supporting the alternative model, in which the inversions occurred independently from the relocation.

The small 68 kb inversion could also have resulted from ectopic recombination. Although the sequences located at the breakpoints of this inversion have no homology to any known transposable elements, both flanks contain several microsatellite regions and regions of low sequence complexity, creating several short blocks of micro-homology that could facilitate ectopic recombination.

**Relative ages of rearrangement events:** We used the whole genome alignment between the two N. tetrasperma strains to locate 192,225 nucleotide differences between them, more than 99% (190,728) of which are located within the boundaries of the non-recombining region on the mating-type chromosome. These two strains are homokaryons that were derived from a single
heterokaryotic strain. The lack of nucleotide differences across most of the genome is most likely due to repeated inbreeding of the self-fertile heterokaryotic strain (MERINO et al. 1996).

If nucleotide differences have accumulated on the mating-type chromosome because of recombination suppression, the number of neutral differences should be proportional to the amount of time since the formation of the non-recombining region. If the rearrangement events that we observed occurred at different evolutionary timepoints, they should have different levels of neutral nucleotide divergence with the oldest event being the most divergent.

As a measure of neutral divergence between the two *N. tetrasperma* strains, we calculated the number of synonymous substitutions per site (Ks) for each gene within each rearrangement event (Fig. 3). The large 5.3 Mb inversion was the rearrangement event that allowed *N. tetrasperma* to become self-fertile by suppressing recombination along the majority of the chromosomal region between the *mat* locus and the centromere. We have therefore compared the distribution of Ks values for genes within the large inversion to those distributions for each of the other rearrangement events.

We found no significant difference between Ks values for the 5.3 Mb inversion and the 1.2 Mb inversion, nor is there a significant difference between Ks values for the 5.3 Mb inversion and the *mat* proximal block (the 218 kb chromosomal region between the *mat* locus and the leftmost rearrangement event; see Fig. 3), suggesting that the suppression of recombination in the 5.3 Mb and 1.2 Mb inversions occurred at the same evolutionary time, and that these inversions also
suppressed recombination in the \textit{mat} proximal block (Bonferroni corrected MWU tests; $P = 0.6$ and $P = 1$, respectively; Fig. 3).

We found two chromosomal regions whose Ks values were much smaller compared to those from the 5.3 Mb inversion: the \textit{mat} distal block (the 850 kb chromosomal region between the recombining left chromosome arm and the \textit{mat} locus; see Fig. 3) and the 68 kb inversion (Bonferroni corrected MWU tests; $P = 1.1\times10^{-15}$ and $P = 4.4\times10^{-5}$, respectively; Fig. 3). The suppression of recombination in these regions most likely occurred after the rearrangement event that created the large inversion.

Surprisingly and counter to our model of the order of rearrangement events, we found that the Ks values for genes within the relocated region were much larger than those for genes within the 5.3 Mb inversion (Bonferroni corrected MWU test; $P = 4.33\times10^{-6}$; Fig. 3). One interpretation of this result is that recombination was suppressed in this region much earlier than that of the 5.3 Mb inversion. The median Ks values between the \textit{N. tetrasperma mat} \textit{A} and \textit{mat} \textit{a} strains for the genes within the relocated region are approximately 2.7 times greater than those for the next most divergent regions. If the substitution rate was the same between these chromosomal regions, the suppression of recombination within the relocated region would have had to occur at an evolutionary timepoint that was more than 2-fold earlier than the other rearrangement events. However, the divergence between \textit{N. tetrasperma mat} \textit{A} and its close relative \textit{N. crassa} is only \~1.3 fold greater than the divergence between the two \textit{N. tetrasperma} strains (median Ks between \textit{N. tetrasperma} and \textit{N. crassa} for genes within the 5.3 and 1.2 Mb inversions: 0.070, median Ks for the same genes between the \textit{N. tetrasperma} strains: 0.054). This sequence of
events would place the timepoint of the relocation well before the divergence of the species. Given that only one of the species now exhibits the relocation, this scenario is very unlikely.

**Investigating the large sequence divergence of the relocated region:** The alternative interpretation for the large sequence divergence of the relocated region is that it has been experiencing an elevated substitution rate relative to the rest of the non-recombining region. However, because we used only synonymous codon positions to calculate divergence, the elevated rate of substitution would have to pertain to nucleotide substitutions that did not change the amino acid, rather than an elevated rate of protein evolution. One possibility is that the elevated synonymous substitution rate is due to relaxed selection for codon usage. Another is that some of the annotated genes within this region are actually pseudogenes and thus, because they are completely unconstrained by purifying selection, are accumulating nucleotide substitutions at a faster rate than synonymous positions. To address the possibility that the divergence measures for this region are biased by pseudogenes, we repeated the analysis on a subset of genes from the region for which we had evidence of expression from the *mat A* expressed sequence tags (ESTs). The increased divergence was also present in the subset of genes showing evidence of expression, suggesting that the potential inclusion of pseudogenes in our analysis was not responsible for the increased divergence we observed. Obviously, such a filter is not infallible because a minority of pseudogenes have been shown to be transcribed based on profiling of mRNA/ESTs from a variety of organisms (between 2% and 15% of pseudogenes studied in rice (ZOU et al. 2009), *Arabidopsis* (ZOU et al. 2009), humans (ZHENG et al. 2007), and yeast (LAFONTAINE and DUJON 2010)). Assuming Neurospora also follows this
general pattern, such a filter, though incomplete, would nevertheless eliminate the majority of pseudogenes from this region.

To test the hypothesis that relaxed selection for codon usage is occurring in the relocated region, we calculated the codon usage in the 100 most highly expressed genes in *N. crassa*. We used these values to calculate the Codon Adaptation Index (CAI) for each one-to-one ortholog between *N. crassa* and the two *N. tetrasperma* strains. CAI values range from 0-1 with higher values indicating more codon usage bias (SHARP and LI 1987). To control for any bias resulting from using *N. crassa* highly expressed genes to identify favored codons, we repeated this analysis with codon usage information gleaned from the 100 most highly expressed genes in *N. tetrasperma mat A* (as determined by EST coverage) and obtained similar results (Fig. S1).

We compared the CAI values for genes from each *N. tetrasperma* strain to their ortholog in *N. crassa*. We found that the genes within the chromosomal region that was relocated in *N. tetrasperma mat A* have a median CAI that is lower than that of their *N. crassa* orthologs (one-sided permutation test: *N. tetrasperma mat A* $P=0.0016$; using only the subset of genes with ESTs: $P=0.0023$)(Fig. 4). This situation is not seen for the other rearrangement events; to the contrary, the genes within the 68 kb inversion appear to be evolving higher codon usage bias in both *N. tetrasperma* strains (one-sided permutation test: $P=0.026$ [mat A], $P=0.003$ [mat a]; Fig. 4).

Given that most amino acid changing mutations are likely to be deleterious, it is reasonable to expect that relaxed selection across a genomic region would result in an increase in the number
of nonsynonymous substitutions per site (Ka). To minimize inflation of Ka due to the inclusion of pseudogenes or misannotated genes in the analysis, we only used genes that had evidence of expression in the form of ESTs.

We found that the genes located within the relocated region of the \textit{N. tetrasperma mat A} strain as well as their orthologs in \textit{N. tetrasperma mat a}, both have a median Ka that is greater than that of the non-recombining region as a whole (one-sided permutation test: \textit{N. tetrasperma mat A} $P=0.005$; \textit{N. tetrasperma mat a} $P=0.013$; Fig. 5). Together, the elevated Ka and the reduced CAI of the genes in the relocated region suggest that it has experienced reduced purifying selection in the \textit{N. tetrasperma} lineage since its divergence from \textit{N. crassa}.

**Evidence for asymmetrical degeneration within the non-recombining region:** The origin of the \textit{N. tetrasperma} region of suppressed recombination is approximately 37x younger than that of the XY sex chromosomes of marsupial and placental mammals (~4.5 MYA versus ~166 MYA) (Veyrunes et al. 2008). Unlike mammals, it is possible for a functionally diploid (heterokaryotic) \textit{N. tetrasperma} individual to become haploid (homokaryotic). These haploid individuals grow and reproduce via mitotic spores in the laboratory and there is evidence of outcrossing between them in the wild (Menkis et al. 2009; Powell et al. 2001). These observations suggest that there should be selection to maintain the function of both copies of the sex-linked genes and one would therefore expect the non-recombining regions to be maintained intact. However, the two strains that we examine here are almost identical across their genomes except for the non-recombining region, implying a long history of inbreeding, and previous studies have observed high instances of sexual dysfunction when \textit{N. tetrasperma} strains are
outcrossed in the laboratory (JACOBSON 1995; SAENZ et al. 2001). These results suggest that the degree of outcrossing in nature may be limited, in which case selection against degeneration within the non-recombining regions of both mating-types may be reduced.

One signal of degeneration that has been found previously in *N. tetrasperma* (WHITTLE and JOHANNESSON 2011; WHITTLE et al. 2011b), as well as in regions of reduced recombination in many other organisms (BACHTROG 2003; BETANCOURT and PRESGRAVES 2002; BETANCOURT et al. 2009; HADDRILL et al. 2007; KLIMAN and HEY 1993; LIU et al. 2004; MARAIS et al. 2008; NICOLAS et al. 2005; PEICHEL et al. 2004; PRESGRAVES 2005; ZHOU et al. 2008), is the accumulation of deleterious alleles. The elimination of recombination across a genomic region will, in essence, lower the effective population size of that region, making fixation more likely for slightly deleterious mutations and less likely for slightly beneficial ones. Given that most amino-acid changing substitutions are deleterious, suppression of recombination should result in an increased proportion of nonsynonymous substitutions compared to synonymous substitutions (Ka/Ks) across the non-recombining region (CHARLESWORTH and CHARLESWORTH 2000).

The previous results from a different lineage within the *N. tetrasperma* species complex found a higher Ka/Ks ratio for a set of *N. tetrasperma* genes located within the non-recombining region but not for a set of genes from outside of this region, consistent with the genes within the non-recombining region being under relaxed purifying selection (WHITTLE and JOHANNESSON 2011; WHITTLE et al. 2011b). To determine if there is evidence supporting this prediction for the genes within the non-recombining region of this *N. tetrasperma* lineage, we calculated Ka and Ks for
all ortholog pairs between each *N. tetrasperma* strain and *N. crassa* and compared the Ka/Ks ratios for the genes within the non-recombining region to those for the genes outside it.

Consistent with the prediction of (CHARLESWORTH and CHARLESWORTH 2000) and the previous results from another *N. tetrasperma* lineage (WHITTLE and JOHANNESSON 2011; WHITTLE et al. 2011b), we found that the genes within the non-recombining region of the mat *a* strain have a significantly higher median Ka/Ks ratio than the genes outside of the non-recombining region, but this pattern did not hold for the genes from the mat *A* strain (MWU test: $P=0.001$ and $P=0.6$, respectively; Fig. 6). One explanation for this is that the mat *a* region may be accumulating deleterious alleles at a faster rate than the mat *A* region. To further explore this possibility, we compared the accumulation of deleterious mutations within the non-recombining region in the form of pseudogenes, codon usage, and nonsynonymous substitutions between the two *N. tetrasperma* mating types. For the pseudogene analysis, we identified candidate pseudogenes from the set of *N. crassa* predicted proteins for which we were unable to find an ortholog in the *N. tetrasperma* set of predicted peptides. Confidently identifying pseudogenes can be difficult because misannotations in the *N. crassa* genome that incorrectly identify start sites or incorrectly predict ORFs that are not actually transcribed, can make the homologous region in *N. tetrasperma* appear to be a pseudogene. For these reasons, we required a candidate pseudogene in *N. tetrasperma* to have either a frameshift or nonsense mutation, full-length homology to functional genes in both *N. crassa* and *N. discreta*, and evidence of expression in *N. crassa*. Applying these conservative criteria, we found a total of ten candidate pseudogenes: two with nonsense and/or frameshift mutations in both *N. tetrasperma* genomes, five with such mutations only in the *N. tetrasperma* mat *a* genome, and three appearing only in the *N. tetrasperma* mat *A*
genome (Table S1). While it is notable that we observed more pseudogenes on the mat a chromosome compared to mat A, the sample size (ten pseudogenes in total) is not large enough to confidently conclude that this represents evidence of asymmetrical degeneration.

As an additional approach to assess evidence of asymmetrical degeneration in one of the two N. tetrasperma sex-linked regions, we used N. crassa as an outgroup to assign the nucleotide substitutions that we identified within the non-recombining region to one of the two N. tetrasperma lineages. After normalizing by the total number of nucleotide substitutions, we compared the frequencies of each nonsynonymous substitution (at the codon level) between the two N. tetrasperma strains and found that nonsynonymous substitutions have occurred at higher frequencies on the N. tetrasperma mat a lineage (one-sided, paired MWU test: $P=2.749\text{e-15}$; Fig. 7A), suggesting that the N. tetrasperma mat a strain may be accumulating deleterious substitutions at a higher rate than the mat A strain.

We also used the codon usage table mentioned previously to identify synonymous changes involving the substitution of a more preferred codon to a less preferred codon and vice versa. After normalizing by the total number of synonymous substitutions within each lineage, we found a tendency for substitutions in N. tetrasperma mat a that involve a change to an unpreferred codon to have occurred at higher frequencies, although this difference is not significant at an $\alpha$ of 0.05 (one-sided, paired MWU test: $P=0.072$; Fig. 7B). We also found that substitutions involving a change to a preferred codon have occurred at lower frequencies compared to N. tetrasperma mat A (one-sided, paired MWU test: $P=0.039$; Fig. 7B). Together, these results suggest that the N. tetrasperma mat a non-recombining region may be in the early
stages of degeneration and are consistent with observations that, within a heterokaryotic *N. tetrasperma* individual, *mat A* nuclei outnumber *mat a* nuclei during growth and early sexual development (Johannesson, *pers com*).

Suppression of recombination in other systems has often been accompanied by the accumulation of transposable elements. We identified *de novo* repetitive elements as well as those with homology to known fungal elements in both *N. tetrasperma* strains and used a permutation test to determine if the region within the non-recombining region is enriched for transposons relative to the rest of the genome. Interestingly, the *mat A* strain has significantly more transposons in the genomic region where recombination is suppressed compared to the rest of the genome (*P*=0.0004), but the *mat a* strain does not (*P*=0.30)(Fig. 8).

**DISCUSSION**

In this study we have used two high quality genome assemblies, representing the nuclei of opposite mating type derived from a single heterokaryotic strain, to discover a series of three inversions within the *N. tetrasperma* region of suppressed recombination. The location of these rearrangements and the collinearity of the chromosome ends are consistent with the cytology, genetic map data, and sequence divergence data from previous studies that have investigated the region of suppressed recombination. The identification of these rearrangements answers the question raised by Jacobson (JACOBSON 2005) as to the relative influence of structural versus genetic modifiers in maintaining this non-recombining region: while we show that structural rearrangements encompass most of the non-recombining region, the *mat a* and *mat A*
chromosomes are collinear in the chromosomal region surrounding the *mat* locus (Fig. 3). This region includes the more recent evolutionary stratum identified in (Menkis et al. 2008) (the ~850 kb *mat* distal block) as well as the *mat* proximal block (the ~218 kb chromosomal segment between the mating type locus and the first chromosomal rearrangement). Genetic evidence assures that this segment is within the non-recombining region, and the presence of sequence divergence within this region (Fig. 3) implies that it has long remained so, but it is unclear why recombination is suppressed. However, the *mat a* and *mat A* loci do not share sequence homology and in addition, we found that within each strain, the mat locus is flanked by regions that have been subject to Repeat Induced Point mutations (RIP (Galagan and Selker 2004)). It is possible that the RIPed regions, together with the idiomorphic mat loci, create enough sequence divergence to disrupt synapsis in this region and thus cause the suppression of recombination to extend past the distal portion of the *mat* locus.

We were able to confirm the finding in (Menkis et al. 2008) that the *mat* distal block has accumulated significantly less sequence divergence than the rest of the non-recombining region (Fig. 3). Although there is no structural difference between the two chromosomes in this region, it is possible that genetic factors are suppressing recombination (as hypothesized by Jacobson (Jacobson 2005) and similar to the *rec* genes of *N. crassa* (Catcheside 1975)), or there may have been fluctuations in the exact location of the boundary of the non-recombining region over evolutionary time.

By combining our analysis of the relative timing of the rearrangement events with the analysis of sequence features associated with the boundaries of these events, we have formulated a cohesive
model for the structural evolution of the *N. tetrasperma* mating-type chromosome (Fig. 2). Based on sequence divergence, the last rearrangement event (a 68 kb inversion) occurred more recently than the first two and therefore represents a second evolutionary stratum. These results, along with those from other fungal phyla (Fraser et al. 2004; Idnurm et al. 2008), suggest that inversion-mediated suppression of recombination on mating chromosomes and the expansion of such non-recombining regions as discrete strata have occurred independently in many fungal lineages and may be a common property of the evolution of sex in fungi, similar to the sex chromosomes of other taxa (Charlesworth et al. 2005; Fraser and Heitman 2005).

An additional similarity between the *N. tetrasperma* non-recombining region and the sex chromosomes from other systems is that it may be under reduced purifying selection. The non-recombining region in the *N. tetrasperma mat A* strain has accumulated an excess of transposons compared to the rest of genome and there is evidence that the *N. tetrasperma mat a* strain may be in the early stages of degeneration (Figures 6-8).

It is unclear why we observe an accumulation of transposons only on the *mat A* chromosome. One possible explanation is that we identified fewer transposons in the *mat a* strain because its genome assembly has a higher proportion of sequence bases in gaps compared to the *mat A* strain (Table S3). Alternatively, we speculate that the additional transposable elements in the *N. tetrasperma mat A* strain could be the result of “genome shock” (McClintock 1984): a single burst of activity specific to the *mat A* nucleus due to the release of transposon suppression during the reorganization of the mating-type chromosome. Of course, it is equally plausible that the relative timing of the chromosome reorganization and the transpositions was reversed, such that
increased transposon activity enabled the 5.3 Mb inversion that led to self-fertility in *N. tetrasperma*, thereby linking the additional transposon copies to the new chromosome orientation.

Our finding that the *mat a* chromosome is accumulating deleterious alleles at a faster rate than the *mat A* chromosome is also somewhat unexpected and stands in contrast to previous theory (Bull 1978). *N. tetrasperma* individuals can grow as haploid homokaryons which are capable of outcrossing and acting as a maternal or paternal parent. Recessive deleterious alleles would not be sheltered under these conditions and, if *N. tetrasperma* exists often as a homokaryon in nature, there should be selection to maintain both copies of the sex-linked genes. However, there is evidence that outcrossing may be limited in nature (Jacobson 1995), in which case degeneration would be reasonable because most individuals would not leave their heterokaryotic, sheltered state. Additionally, asymmetric evolution between sex-determining chromosomes, at least with respect to differences in chromosome size, has been observed in other haploid organisms such as the liverwort *Marchantia polymorpha* (Yamato et al. 2007) and the fungus *M. violaceum* (Hood 2002). In neither of these systems, unfortunately, have the chromosomes of both mating-types been sequenced, making it impossible to determine if the size difference is due to asymmetric gain or loss (i.e. degeneration) of genetic elements.

While Whittle et al. (Whittle et al. 2011b) report an asymmetry between the *mat a* and *mat A* chromosomes in terms of the frequency of substitutions to preferred codons in lineage #1 of the *N. tetrasperma* species complex, a different lineage than studied here, they do not report whether the deviation is statistically significant and a reanalysis of their results shows that it is not
(Fisher’s exact test: \( P = 0.285 \); Table S6). Additionally, there does not appear to be a similar asymmetry with respect to nonsynonymous substitutions in lineage #1 (Whittle and Johannesson 2011). That we are able to observe such an asymmetry in both nonsynonymous substitutions and codon usage between the sex-linked regions in this \textit{N. tetrasperma} lineage may be due to the larger sample of genes we use here (~1300 genes from within the non-recombining region compared to 168 in (Whittle and Johannesson 2011) and 228 in (Whittle et al. 2011b)) or to differences between the lineage studied in (Whittle and Johannesson 2011; Whittle et al. 2011b) (lineage #1) and that studied here (lineage #6).

Future work comparing the structural rearrangements identified here to the other \textit{N. tetrasperma} lineages would address the hypothesis of independent origins of this region of suppressed recombination and lead to further insight into its evolutionary history. The examination of sequence data from \textit{N. tetrasperma} populations within these lineages would be useful for assessing the effect of reduced recombination on nucleotide diversity. Additionally, allele-specific gene expression experiments would be an ideal approach to determine whether there is evidence that the genes within the \textit{mat A} non-recombining region are evolving increased expression to compensate for the increase in deleterious substitutions that are occurring within the \textit{mat a} non-recombining region.

**MATERIALS AND METHODS**

**Genome sequencing and assembly:** Both \textit{N. tetrasperma} strains (FGSC 2508 \textit{mat A} and FGSC 2509 \textit{mat a}) were sequenced using a hybrid approach on Roche 454 pyrosequencing and Sanger
platforms (Tables S3 and S4) and assembled with Newbler. The *N. tetrasperma mat A* assembly was post-processed to close gaps in-silico using JGI gapResolution software for the entire genome and targeted finishing for the mating-type chromosome. Statistics of both assemblies are summarized in Table S4.

**Genome Annotation:** Both *N. tetrasperma* assemblies were annotated using the JGI annotation pipeline with results deposited to the integrated fungal resource MycoCosm (http://jgi.doe.gov/fungi) for further analysis. Genome assembly scaffolds were masked using RepeatMasker (SMIT *et al.* 1996-2010) and tRNAs were predicted using tRNAscan-SE (LOWE and EDDY 1997). Several gene predictors were used on the repeat-masked assembly: (i) ab initio FGENESH (SALAMOV and SOLOVYEV 2000) and GeneMark (ISONO *et al.* 1994), (ii) homology-based FGENESH+ and GeneWise (BIRNEY and DURBIN 2000) seeded by BLASTx alignments against GenBank’s database of non-redundant proteins, and (iii) direct mapping of EST-derived full-length genes to genome assembly. GeneWise models were extended where possible using scaffold data to find start and stop codons. EST BLAT alignments (KENT 2002) were used to extend, verify, and complete the predicted gene models. From the resulting set of models, a non-redundant representative set of best models was selected (Table S5).

All predicted gene models were functionally annotated using SignalP (NIELSEN *et al.* 1997), TMHMM (MELEN *et al.* 2003), InterProScan (ZDOBNOV and APWEILER 2001), BLASTp (ALTSCHUL *et al.* 1990) against nr, and hardware-accelerated double-affine Smith-Waterman alignments (deCypherSW; http://www.timelogic.com/decypher_sw.html) against SwissProt (BOECKMANN *et al.* 2003), KEGG (KANEHISA *et al.* 2008), and KOG (KOONIN *et al.* 2004).
KEGG hits were used to assign EC numbers (GASTEIGER et al. 2003), and Interpro and SwissProt hits were used to map GO terms (ASHBURNER et al. 2000). Multigene families were predicted with the Markov clustering algorithm (MCL)(ENRIGHT et al. 2002) to cluster the proteins, using BLASTp alignment scores between proteins as a similarity metric.

**ESTs:** N. tetrasperma FGSC 2508 was grown in Vogel’s liquid media (VOGEL 1956) and total RNA was extracted by bead-beating in TRizol (Invitrogen Life Science Technologies) with zirconia/silica beads (0.2 g, 0.5-mm diameter; Biospec Products). ESTs were sequenced using 454 pyrosequencing. The sequencing library protocol and sequence processing procedure are described in (SWARBECK et al. 2011).

**Genome synteny:** Whole genome synteny was assessed between the two N. tetrasperma strains as well as between each N. tetrasperma strain and the outgroup Neurospora crassa. Dotplots were created using the program MUMMER (KURTZ et al. 2004) to visualize synteny while more precise identification of rearrangement breakpoints was achieved using a combination of whole-genome orthology map construction with Mercator and whole-genome alignment with MAVID (DEWEY 2007).

**Identification of orthologs:** Orthologs between the two N. tetrasperma strains as well as those between each N. tetrasperma strain and N. crassa were initially identified based on best-reciprocal-BLAST hits and further verified using synteny information from Mercator. This procedure resulted in the identification of a total of 7,693 single-copy orthologs between the three species.
Calculation of nonsynonymous and synonymous substitutions per site (Ka and Ks): Ka/Ks ratios were calculated for each pair of orthologs between the two *N. tetrasperma* strains as well as between each *N. tetrasperma* strain and the outgroup *Neurospora crassa* using the modified Yang-Nielsen method in the program KaKs_Calculator (ZHANG *et al.* 2006). It has previously been shown that the way in which the total number of synonymous sites are counted can bias the calculation of Ks (BIERNE and EYRE-WALKER 2003). For this reason, we also calculated the Ka and Ks values for all pairwise orthologs as in (BIERNE and EYRE-WALKER 2003), using only four-fold degenerate sites for Ks and the physical site definition for both Ka and Ks and found similar results (Figure S2). For consistency, all Ks results reported here are from the modified Yang-Nielsen method in the program KaKs_Calculator.

Identification of pseudogenes: We used the tfasty program within the FASTA sequence comparison package (PEARSON *et al.* 1997) to perform translated searches (allowing for frameshifts and premature stop codons) against the *N. tetrasperma* genome sequence. As queries, we used the subset of *N. crassa* proteins from within the genomic region of suppressed recombination for which we were unable to find *N. tetrasperma* orthologs. We used a custom Perl script to parse the results to identify matches that showed frameshifts and/or nonsense mutations. We additionally extracted the genomic sequence for candidate pseudogenes and used the program Exonerate (SLATER and BIRNEY 2005) to create genomic DNA/protein sequence alignments to confirm the presence of nonsense mutations or frameshifts.

Codon Adaptation Index (CAI): We used the *N. tetrasperma mat A* ESTs and *N. crassa* Illumina RNA-Seq data (ELLISON *et al.* 2011) to identify the top 100 most highly expressed
genes in each species. We used these genes to compute codon usage tables for each species using the *cusp* application in the EMBOSS package (RICE et al. 2000). We then used the EMBOSS application *cai* to calculate the codon adaptation index for each *N. tetrasperma* and *N. crassa* gene. All CAI analyses were performed twice, once with the *N. tetrasperma* codon usage table and once with the *N. crassa* codon usage table. The results were equivalent in all cases and the reported p-values are those from the *N. crassa* codon usage table. We defined substitutions to preferred codons as a change to a synonymous codon whose frequency of usage in the top 100 most highly expressed genes was at least 10-fold greater than that of the ancestral codon. Similarly, we defined substitutions to unpreferred codons as a change to a synonymous codon whose frequency of usage in the top 100 most highly expressed genes was at least 10-fold less than that of the ancestral codon.

**Repetitive elements:** Repetitive elements were identified *de novo* using the program RepeatModeler (SMIT and HUBLEY 2008-2010). The results of RepeatModeler were added to a fungal-specific repeat library downloaded from RepBase (JURKA 2000) and repetitive elements were identified based on sequence homology using this library and the program RepeatMasker (SMIT et al. 1996-2010). Centromeric regions in Neurospora can be easily identified because they are composed almost entirely of transposable element remnants. The number of repetitive elements per kilobase for the chromosomal segment within the *N. tetrasperma* non-recombining region and the homologous region in *N. crassa* were calculated after excluding centromeric regions so that these measures would not be confounded by differences between assemblies in the number of gaps in these repeat-dense regions.
Permutation tests

CAI and Ka: This test was performed on both *N. tetrasperma* strains separately. Twenty-six genes (the number of genes within the relocated region) were drawn randomly from the set of all genes within the non-recombining region. Each gene’s CAI was subtracted from that of its ortholog in *N. crassa* and the median of the differences was calculated for the random sample and compared to that of the real data (*i.e.* the genes within the relocated region). This procedure was repeated 10,000 times and the p-value was calculated as the proportion of random samples (out of the 10,000 permutations) that had a value greater than that of the real data.

Ka permutation tests were performed similarly except the median Ka of randomly sampled *N. tetrasperma mat A* and *mat a* ortholog pairs was compared to that of the genes within the relocated region.

Transposon enrichment: We compared each *N. tetrasperma* strain separately to *N. crassa*. We took the total number of transposons within each genome and shuffled their locations. We then counted the number of shuffled transposons that landed within the boundaries of the non-recombining region in *N. tetrasperma* and within the homologous region in *N. crassa*. We subtracted the *N. crassa* sum from the *N. tetrasperma* sum and compared this difference to the true difference. The p-value was calculated as the proportion of permutations (out of 10,000 total) where the permuted difference was larger than the true difference.

All permutation tests were performed using custom Perl scripts.
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REFERENCES

ALTSCHUL, S. F., W. GISH, W. MILLER, E. W. MYERS and D. J. LIPMAN, 1990 Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

ARGUESO, J. L., J. WESTMORELAND, P. A. MIECZKOWSKI, M. GAWEL, T. D. PETES et al., 2008 Double-strand breaks associated with repetitive DNA can reshape the genome. Proceedings of the National Academy of Sciences of the United States of America 105: 11845-11850.

ASHBURNER, M., C. A. BALL, J. A. BLAKE, D. BOTSTEIN, H. Butler et al., 2000 Gene Ontology: tool for the unification of biology. Nature Genetics 25: 25-29.

BACHTROG, D., 2003 Protein evolution and codon usage bias on the neo-sex chromosomes of Drosophila miranda. Genetics 165: 1221-1232.

BETANCOURT, A. J., and D. C. PRESGRAVES, 2002 Linkage limits the power of natural selection in Drosophila. Proceedings of the National Academy of Sciences of the United States of America 99: 13616-13620.

BETANCOURT, A. J., J. J. WELCH and B. CHARLESWORTH, 2009 Reduced effectiveness of selection caused by a lack of recombination. Current Biology 19: 655-660.
BIERNE, N., and A. EYRE-WALKER, 2003 The problem of counting sites in the estimation of the synonymous and nonsynonymous substitution rates: implications for the correlation between the synonymous substitution rate and codon usage bias. Genetics 165: 1587-1597.

BIRNEY, E., and R. DURBIN, 2000 Using GeneWise in the Drosophila annotation experiment. Genome Research 10: 547-548.

BOECKMANN, B., A. BAIROCH, R. APWEILER, M. C. BLATTER, A. ESTREICHER et al., 2003 The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. Nucleic Acids Research 31: 365-370.

BULL, J. J., 1978 Sex chromosomes in haploid dioecy: unique contrast to Muller's theory for diploid dioecy. American Naturalist 112: 245-250.

CASALS, F., and A. NAVARRO, 2007 Inversions: The chicken or the egg? Heredity 99: 479-480.

CATCHESIDE, D. G., 1975 Occurrence in wild strains of Neurospora crassa of genes controlling genetic recombination. Australian Journal of Biological Sciences 28: 213-225.

CHARLESWORTH, B., and D. CHARLESWORTH, 2000 The degeneration of Y chromosomes. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 355: 1563-1572.

CHARLESWORTH, D., B. CHARLESWORTH and G. MARAIS, 2005 Steps in the evolution of heteromorphic sex chromosomes. Heredity 95: 118-128.

DEWEY, C. N., 2007 Aligning multiple whole genomes with Mercator and MAVID. Methods in Molecular Biology 395: 221-236.
ELLISON, C. E., C. HALL, D. KOWBEL, J. WELCH, R. B. BREM et al., 2011 Population genomics and local adaptation in wild isolates of a model microbial eukaryote. Proceedings of the National Academy of Sciences of the United States of America 108: 2831-2836.

ENRIGHT, A. J., S. VAN DONGEN and C. A. OUZOUNIS, 2002 An efficient algorithm for large-scale detection of protein families. Nucleic Acids Research 30: 1575-1584.

FRASER, J. A., S. DIEZMANN, R. L. SUBARAN, A. ALLEN, K. B. LENGELER et al., 2004 Convergent evolution of chromosomal sex-determining regions in the animal and fungal kingdoms. PLoS Biology 2: 2243-2255.

FRASER, J. A., and J. HEITMAN, 2004 Evolution of fungal sex chromosomes. Molecular Microbiology 51: 299-306.

FRASER, J. A., and J. HEITMAN, 2005 Chromosomal sex-determining regions in animals, plants and fungi. Current Opinion in Genetics & Development 15: 645-651.

FRASER, J. A., Y.-P. HSUEH, K. M. FINDLEY and J. HEITMAN, 2007 Evolution of the mating-type locus: The basidiomycetes, pp. 19-34 in Sex in Fungi: Molecular Determination and Evolutionary Implications-Print, edited by J. HEITMAN, J. W. KRONSTAD, J. W. TAYLOR and L. A. CASSELTON. ASM Press, Washington, D.C.

GALAGAN, J. E., and E. U. SELKER, 2004 RIP: the evolutionary cost of genome defense. Trends in Genetics 20: 417-423.

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.
GASTEIGER, E., A. GATTIKER, C. HOOGLAND, I. IVANYI, R. D. APPEL et al., 2003 ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Research 31: 3784-3788.

GIRAUD, T., O. JONOT and J. A. SHYKOFF, 2005 Selfing propensity under choice conditions in a parasitic fungus, Microbotryum violaceum, and parameters influencing infection success in artificial inoculations. International Journal of Plant Sciences 166: 649-657.

HADDRILL, P. R., D. L. HALLIGAN, D. TOMARAS and B. CHARLESWORTH, 2007 Reduced efficacy of selection in regions of the Drosophila genome that lack crossing over. Genome Biology 8: R18.

HOOD, M. E., 2002 Dimorphic mating-type chromosomes in the fungus Microbotryum violaceum. Genetics 160: 457-461.

HOWE, H. B., and P. HAYSMAN, 1966 Linkage group establishment in Neurospora tetrasperma by interspecific hybridization with N. crassa. Genetics 54: 293-302.

IDNURM, A., F. J. WALTON, A. FLOYD and J. HEITMAN, 2008 Identification of the sex genes in an early diverged fungus. Nature 451: 193-196.

ISONO, K., J. D. McININCH and M. BORODOVSKY, 1994 Characteristic features of the nucleotide sequences of yeast mitochondrial ribosomal protein genes as analyzed by computer program GeneMark. DNA Research 1: 263-269.

JACOBSON, D. J., 1995 Sexual dysfunction associated with outcrossing in Neurospora tetrasperma, a pseudohomothallic Ascomycete. Mycologia 87: 604-617.

JACOBSON, D. J., 2005 Blocked recombination along the mating-type chromosomes of Neurospora tetrasperma involves both structural heterozygosity and autosomal genes. Genetics 171: 839-843.
JURKA, J., 2000 Repbase update: a database and an electronic journal of repetitive elements. Trends in Genetics 16: 418-420.

KANEHISA, M., M. ARAKI, S. GOTO, M. HATTORI, M. HIRAKAWA et al., 2008 KEGG for linking genomes to life and the environment. Nucleic Acids Research 36: D480-484.

KAPITONOV, V. V. a. J., J., 2003 Mariner-3_AN, a family of DNA transposons in the Aspergillus nidulans genome. Repbase Reports 3.

KENT, W. J., 2002 BLAT--the BLAST-like alignment tool. Genome Research 12: 656-664.

KLIMAN, R. M., and J. HEY, 1993 Reduced natural selection associated with low recombination in Drosophila melanogaster. Molecular Biology and Evolution 10: 1239-1258.

KONDO, M., U. HORNUNG, I. NANDA, S. IMAI, T. SASAKI et al., 2006 Genomic organization of the sex-determining and adjacent regions of the sex chromosomes of medaka. Genome Research 16: 815-826.

KOONIN, E. V., N. D. FEDOROVA, J. D. JACKSON, A. R. JACOBS, D. M. KRYLOV et al., 2004 A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes. Genome Biology 5: R7.

KURTZ, S., A. PHILLIPPY, A. L. DELCHER, M. SMOOT, M. SHUMWAY et al., 2004 Versatile and open software for comparing large genomes. Genome Biology 5: R12.

LAFONTAINE, I., and B. DUIJON, 2010 Origin and fate of pseudogenes in Hemiascomycetes: a comparative analysis. BMC Genomics 11: 260.

LAHN, B. T., and D. C. PAGE, 1999 Four evolutionary strata on the human X chromosome. Science 286: 964-967.

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.
LENGELER, K. B., D. S. FOX, J. A. FRASER, A. ALLEN, K. FORRESTER et al., 2002 Mating-type locus of *Cryptococcus neoformans*: A step in the evolution of sex chromosomes. Eukaryotic Cell 1: 704-718.

LIU, Z., P. H. MOORE, H. MA, C. M. ACKERMAN, M. RAGIBA et al., 2004 A primitive Y chromosome in papaya marks incipient sex chromosome evolution. Nature 427: 348-352.

LOWE, T. M., and S. R. EDDY, 1997 TRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Research 25: 955-964.

MANK, J. E., and H. ELLEGREN, 2007 Parallel divergence and degradation of the avian W sex chromosome. Trends in Ecology & Evolution 22: 389-391.

MARAIS, G. A., M. NICOLAS, R. BERGERO, P. CHAMBRIER, E. KEJNOVSKY et al., 2008 Evidence for degeneration of the Y chromosome in the dioecious plant *Silene latifolia*. Current Biology 18: 545-549.

McCLINTOCK, B., 1984 The significance of responses of the genome to challenge. Science 226: 792-801.

MELEN, K., A. KROGH and G. VON HEIJNE, 2003 Reliability measures for membrane protein topology prediction algorithms. Journal of Molecular Biology 327: 735-744.

MENKIS, A., E. BASTIAANS, D. J. JACOBSON and H. JOHANNESSON, 2009 Phylogenetic and biological species diversity within the *Neurospora tetrasperma* complex. Journal of Evolutionary Biology 22: 1923-1936.
MENKIS, A., D. J. JACOBSON, T. GUSTAFSSON and H. JOHANNESSON, 2008 The mating-type chromosome in the filamentous ascomycete Neurospora tetrasperma represents a model for early evolution of sex chromosomes. PLoS Genetics 4: e1000030.

MENKIS, A., C. A. WHITTLE and H. JOHANNESSON, 2010 Gene genealogies indicates abundant gene conversions and independent evolutionary histories of the mating-type chromosomes in the evolutionary history of Neurospora tetrasperma. BMC Evolutionary Biology 10: 234.

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.

METIN, B., K. FINDLEY and J. HEITMAN, 2010 The mating type locus (MAT) and sexual reproduction of Cryptococcus heveanensis: Insights into the evolution of sex and sex-determining chromosomal regions in fungi. PLoS Genetics 6: e1000961.

NICOLAS, M., G. MARAIS, V. HYKELOVA, B. JANOUSEK, V. LAPORTE et al., 2005 A gradual process of recombination restriction in the evolutionary history of the sex chromosomes in dioecious plants. PLoS Biology 3: e4.

NIELSEN, H., J. ENGELBRECHT, S. BRUNAK and G. VON HEIJNE, 1997 Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering 10: 1-6.

NYGREN, K., R. STRANDBERG, A. WALLBERG, B. NABHOLZ, T. GUSTAFSSON et al., 2011 A comprehensive phylogeny of Neurospora reveals a link between reproductive mode and molecular evolution in fungi. Molecular Phylogenetics and Evolution advance online publication, 23 March 2011 DOI 10.1016/j.ympev.2011.03.023.
O’MEALLY, D., H. R. PATEL, R. STIGLEC, S. D. SARRE, A. GEORGES et al., 2010 Non-homologous sex chromosomes of birds and snakes share repetitive sequences. Chromosome Research 18: 787-800.

PEARSON, W. R., T. WOOD, Z. ZHANG and W. MILLER, 1997 Comparison of DNA sequences with protein sequences. Genomics 46: 24-36.

PEICHEL, C. L., J. A. ROSS, C. K. MATSON, M. DICKSON, J. GRIMWOOD et al., 2004 The master sex-determination locus in threespine sticklebacks is on a nascent Y chromosome. Current Biology 14: 1416-1424.

POWELL, A. J., D. J. JACOBSON and D. O. NATVIG, 2001 Allelic diversity at the het-c locus in Neurospora tetrasperma confirms outcrossing in nature and reveals an evolutionary dilemma for pseudohomothallic ascomycetes. Journal of Molecular Evolution 52: 94-102.

PRESGRAVES, D. C., 2005 Recombination enhances protein adaptation in Drosophila melanogaster. Current Biology 15: 1651-1656.

RAJU, N. B., 1992 Functional heterothallism resulting from homokaryotic conidia and ascospores in Neurospora tetrasperma. Mycological Research 96: 103-116.

RAJU, N. B., and D. D. PERKINS, 1994 Diverse Programs of Ascus Development in Pseudohomothallic Species of Neurospora, Gelasinospora, and Podospora. Developmental Genetics 15: 104-118.

RANZ, J. M., D. MAURIN, Y. S. CHAN, M. VON GROTTHUSS, L. W. HILLIER et al., 2007 Principles of genome evolution in the Drosophila melanogaster species group. PLoS Biology 5: 1366-1381.
RICE, P., I. LONGDEN and A. BLEASBY, 2000 EMBOSS: the European Molecular Biology Open Software Suite. Trends in Genetics 16: 276-277.

SAENZ, G. S., J. G. STAM, D. J. JACOBSON and D. O. NATVIG, 2001 Heteroallelism at the het-c locus contributes to sexual dysfunction in outcrossed strains of Neurospora tetrasperma. Fungal Genetics and Biology 34: 123-129.

SALAMOV, A. A., and V. V. SOLOVYEV, 2000 Ab initio gene finding in Drosophila genomic DNA. Genome Research 10: 516-522.

SHARP, P. M., and W. H. Li, 1987 The Codon Adaptation Index - a measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Research 15: 1281-1295.

SLATER, G. S., and E. BIRNEY, 2005 Automated generation of heuristics for biological sequence comparison. BMC Bioinformatics 6: 31.

SMIT, A., and R. HUBLEY, 2008-2010 RepeatModeler Open-1.0. <http://www.repeatmasker.org>.

SMIT, A., R. HUBLEY and P. GREEN, 1996-2010 RepeatMasker Open-3.0. <http://www.repeatmasker.org>.

SWARBRECK, S. M., E. A. LINDQUIST, D. D. ACKERLY and G. L. ANDERSEN, 2011 Analysis of leaf and root transcriptomes of soil-grown Avena barbata plants. Plant and Cell Physiology 52: 317-332.

VEYRUNES, F., P. D. WATERS, P. MIETHKE, W. RENS, D. MCMILLAN et al., 2008 Bird-like sex chromosomes of platypus imply recent origin of mammal sex chromosomes. Genome Research 18: 965-973.

VOGEL, H. J., 1956 A convenient growth medium for Neurospora (Medium N). Microbial Genetics Bulletin 13: 42–43.
Votintseva, A. A., and D. A. Filatov, 2009 Evolutionary strata in a small mating-type-specific region of the smut fungus *Microbotryum violaceum*. Genetics 182: 1391-1396.

Votintseva, A. A., and D. A. Filatov, 2010 DNA polymorphism in recombining and non-recombining mating-type-specific loci of the smut fungus *Microbotryum*. Heredity advance online publication, 17 Nov 2010 DOI 10.1038/hdy.2010.140.

Whittle, C. A., and H. Johannessson, 2011 Evidence of the accumulation of allele-specific non-synonymous substitutions in the young region of recombination suppression within the mating-type chromosomes of *Neurospora tetrasperma*. Heredity advance online publication, 9 March 2011 DOI 10.1038/hdy.2011.11.

Whittle, C. A., K. Nygren and H. Johannessson, 2011a Consequences of reproductive mode on genome evolution in fungi. Fungal Genetics and Biology advance online publication, 27 February 2011 DOI 10.1016/j.fgb.2011.02.005.

Whittle, C. A., Y. Sun and H. Johannessson, 2011b Degeneration in codon usage within the region of suppressed recombination in the mating type chromosomes of *Neurospora tetrasperma*. Eukaryotic Cell 10: 594-603.

Whittle, C. A., Y. Sun and H. Johannessson, 2011c Evolution of synonymous codon usage in *Neurospora tetrasperma* and *Neurospora discreta*. Genome Biology and Evolution advance online publication, 14 March 2011 DOI 10.1093/gbe/evr018.

Yamato, K. T., K. Ishizaki, M. Fujisawa, S. Okada, S. Nakayama et al., 2007 Gene organization of the liverwort Y chromosome reveals distinct sex chromosome evolution in a haploid system. Proceedings of the National Academy of Sciences of the United States of America 104: 6472-6477.
ZDOBNOV, E. M., and R. APWEILER, 2001 InterProScan--an integration platform for the signature-recognition methods in InterPro. Bioinformatics **17**: 847-848.

ZHANG, Z., J. LI, X. Q. ZHAO, J. WANG, G. K. WONG et al., 2006 KaKs_Calculator: calculating Ka and Ks through model selection and model averaging. Genomics Proteomics & Bioinformatics **4**: 259-263.

ZHENG, D., A. FRANKISH, R. BAERTSCH, P. KAPRANOVA, A. REYMOND et al., 2007 Pseudogenes in the ENCODE regions: consensus annotation, analysis of transcription, and evolution. Genome Research **17**: 839-851.

ZHOU, Q., J. WANG, L. HUANG, W. NIE, Y. LIU et al., 2008 Neo-sex chromosomes in the black muntjac recapitulate incipient evolution of mammalian sex chromosomes. Genome Biology **9**: R98.

ZOU, C., M. D. LEHTI-SHIU, F. THIBAUD-NISSEN, T. PRAKASH, C. R. BUELL et al., 2009 Evolutionary and expression signatures of pseudogenes in *Arabidopsis* and rice. Plant Physiology **151**: 3-15.
FIGURE 1.—Whole genome synteny between *N. tetrasperma* strains and *N. crassa*. (A) *N. tetrasperma* mat A compared to *N. tetrasperma* mat a, (B) *N. tetrasperma* mat A compared to *N. crassa*, (C) *N. tetrasperma* mat a compared to *N. crassa*. The mating type chromosome (chromosome I) is rearranged in the *N. tetrasperma* mat A strain compared to both *N. tetrasperma* mat a and *N. crassa*. Alignments occurring between positive strands are colored in red while those occurring between opposite strands are colored in blue and indicate inversions.
FIGURE 2.—Model of the evolution of the *N. tetrasperma mat A* mating-type chromosome. The order of rearrangement events is shown in (A) and begins with the ancestral *mat A* chromosome (1) which was collinear with *mat a* and the mating-type chromosome of *N. crassa*. The 1.2 Mb inversion occurred first and produced the orientation in (2). This event was followed relatively quickly by the 5.3 Mb inversion (3). The 68 kb inversion, shown as the line at the far right of (B), occurred much later to produce the current arrangement of the *mat A* chromosome (B). The 1.2 Mb inversion (breakpoints shown in red) is flanked by unique 50 bp duplications (D) that would have been in an inverted orientation before the occurrence of the large inversion, consistent with rearrangement via staggered single-strand breaks. The 5.3 Mb inversion (breakpoints shown in blue) is flanked by Mariner transposable elements (M), consistent with
rearrangement via ectopic recombination. Mariner remnants were not present in either of the homologous regions in the mat a chromosome. The overlapping nature of these two inversions explains the relocated genomic region. The 68 kb inversion is flanked by microsatellite containing, low-complexity sequence and may have occurred due to ectopic recombination between blocks of micro-homology. MAT denotes the location of the mating-type locus while CEN shows the location of the centromere.
FIGURE 3.—Relative ages of rearrangement events. The bottom panel shows the two *N. tetrasperma* mating-type chromosomes with lines connecting pairs of orthologous genes. The top panel shows the distribution of synonymous substitutions per site (Ks) between ortholog pairs for chromosomal regions that have been rearranged and for two other chromosomal segments that are collinear between the two *N. tetrasperma* strains but have sequence divergence: an 850 kb segment distal to the *mat* locus and a 218 kb segment proximal to the *mat* locus. The amount of synonymous sequence divergence between orthologs within a given rearrangement event will be proportional to the amount of time since the event occurred. Using the 5.3 Mb inversion as a point of reference, the genes within the relocated region have a distribution of Ks values that is significantly larger, while the genes within the *mat* distal unknown block and small 68 kb inversion both have Ks distributions that are significantly smaller (Bonferroni-corrected MWU test: $P = 4.33 \times 10^{-6}$, $P = 1.1 \times 10^{-15}$, and $P = 4.4 \times 10^{-5}$, respectively). The letters A-H denote the chromosomal regions that are referred to in the main text: A: recombining left arm, B: *mat* distal block, C: *mat* proximal block, D: 143 kb relocated region, E: 5.3 Mb inversion, F: 1.2 Mb inversion, G: 68 kb inversion, H: recombining right arm.
FIGURE 4.—Relaxed selection for codon usage in the *N. tetrasperma* mat A genes within the relocated region. The CAI is a measure of codon usage bias and the difference between the *N. crassa* CAI and those from each *N. tetrasperma* strain was calculated for every set of three-way orthologs. The genes within the *N. tetrasperma* mat A relocated region appear to be evolving reduced codon usage bias (one-sided permutation test: \( P=0.0016 \)) while the genes within the small inversion appear to be evolving increased codon usage bias (one-sided permutation test: \( P=0.026 \)[mat A], \( P=0.003 \)[mat a]). Outliers are not shown.
FIGURE 5.—Increased nonsynonymous divergence in the *N. tetrasperma* *mat A* and *mat a* genes within the relocated region. The number of nonsynonymous substitutions per site (Ka) between *N. tetrasperma* *mat A* and *mat a* orthologs within the relocated region are significantly larger than those values for the entire non-recombining region (one-sided permutation test: *P* = 0.005 [*mat A*], *P* = 0.013 [*mat a*]). Although Ka is larger for these genes, no gene has a Ka/Ks ratio significantly greater than one implying that the increased Ka is due to the accumulation of slightly deleterious amino acid substitutions rather than adaptive evolution. Outliers are not shown.

FIGURE 6.—Reduced efficiency of selection in the *mat a* non-recombining region. The ratio of nonsynonymous substitutions per site (Ka) to synonymous substitutions per site (Ks) was calculated for every pair of orthologs between each *N. tetrasperma* strain and *N. crassa*. The distribution of Ka/Ks ratios was compared for genes inside of and outside of the non-recombining region. Consistent with the evidence in Fig. 7, genes within the region of suppressed recombination in the *mat a* strain, but not the *mat A* strain, have significantly larger Ka/Ks ratios than those outside of the non-recombining region (MWU test: *P* = 0.001 and *P* = 0.6, respectively). Outliers are not shown.
FIGURE 7.—Evidence that the *N. tetrasperma mat a* mating-type chromosome may be in the early stages of degeneration. Nucleotide substitutions occurring within the non-recombining region were assigned to either the *N. tetrasperma mat A* or *mat a* lineage depending upon which allele was present in *N. crassa*. Examination of this set of polarized substitutions showed that nonsynonymous substitutions have occurred at higher frequencies in the *N. tetrasperma mat a* lineage compared to *mat A* (one-sided, paired MWU test: $P=2.749 \times 10^{-15}$). In addition, examination of the set of polarized synonymous substitutions showed that, in the *N. tetrasperma mat a* lineage, preferred substitutions have occurred at significantly lower frequencies and there is a trend toward significance with respect to unpreferred substitutions having occurred at higher frequencies, compared to the *mat A* strain (one-sided, paired MWU test: preferred: $P=0.039$; unpreferred: $P=0.072$). Preferred substitutions were defined as a change to a synonymous codon whose frequency of usage in the top 100 most highly expressed genes in *N. crassa* is at least 10-fold larger than that of the ancestral codon while unpreferred substitutions were defined as the opposite (the usage frequency of the derived codon is at least 10-fold smaller than that of the ancestral codon).
FIGURE 8.—The *N. tetrasperma* mat A region of suppressed recombination is enriched for repetitive elements. Repetitive elements were identified *de novo* and based on homology to known elements. Significance was assessed using a permutation test (*P*=0.0004). Using the same test, the homologous region from *N. tetrasperma* mat a did not have significantly more repetitive elements than *N. crassa* (*P*=0.30).
## TABLE 1

| Chromosomal region       | Size (kb) | Single-copy orthologs |
|--------------------------|-----------|-----------------------|
| Recombining left arm     | 930       | 198                   |
| *mat* distal block       | 850       | 166                   |
| *mat* proximal block     | 218       | 29                    |
| Relocated region         | 143       | 26                    |
| 5.3 Mb inversion         | 5300      | 972                   |
| 1.2 Mb inversion         | 1200      | 245                   |
| 68 kb inversion          | 68        | 11                    |
| Recombining right arm    | 728       | 128                   |

The approximate size of each chromosomal region and number of single-copy orthologs are listed for the eight segments of the *N. tetrasperma* mating chromosome that are discussed in this study.
**Figure S1.** Re-analysis of Codon Adaptation Index (CAI) differences between *N. crassa* and *N. tetrasperma*. The analysis shown in Figure 4 was based on codon usage in *N. crassa*. To verify that the results shown in Figure 4 do not depend on the species used to obtain codon usage information, we repeated the analysis with codon usage information from *N. tetrasperma mat A* and obtained similar results. The genes within the *N. tetrasperma mat A* relocated region appear to be evolving reduced codon usage bias (one-sided permutation test: $P=0.0015$) while the genes within the small inversion appear to be evolving increased codon usage bias (one-sided permutation test: $P=0.0006$ [mat A], $P=0.0003$ [mat a]). Outliers are not shown.

**Figure S2.** Re-analysis of the ratio of nonsynonymous (Ka) and synonymous (Ks) substitutions per site between recombining and non-recombining regions of the genome. The results presented in Figure 6 were reanalyzed using only four-fold degenerate sites. Genes within the region of suppressed recombination in the *mat a* strain, but not the *mat A* strain, have significantly larger Ka/Ks ratios than those outside of the non-recombining region (MWU test: $P=0.04$ and $P=0.4$, respectively).
### TABLE S1

**Pseudogenes present within the *N. tetrasperma* non-recombining region**

| Gene ID    | Function                          | Strain | Mutation |
|------------|-----------------------------------|--------|----------|
| NCU03134   | class I alpha-mannosidase 1A      | mat A  | FS       |
| NCU01896   | Unknown                           | mat A  | FS       |
| NCU02817   | Unknown                           | mat A  | PS       |
| NCU00765   | amino acid permease 2             | mat a  | PS       |
| NCU02882   | Unknown                           | mat a  | PS       |
| NCU03288   | Unknown                           | mat a  | PS       |
| NCU10125   | Unknown                           | mat a  | PS       |
| NCU08351   | Unknown                           | mat a  | PS       |
| NCU11480   | Major Facilitator Superfamily     | Both   | mat A: PS; mat a: FS |
| NCU03275   | Unknown                           | Both   | mat A: PS; mat a: PS and FS |

Pseudogenes were identified as genes in one or both of the *N. tetrasperma* strains where mutations or insertions/deletions resulted in premature stop codons (PS) and/or frameshifts (FS) relative to their orthologs in *N. discreta* and *N. crassa*.

### TABLE S2

**Assembly statistics**

|                        | *Neurospora tetrasperma* | *Neurospora tetrasperma* |
|------------------------|--------------------------|--------------------------|
|                        | FGSC 2508 mat A v2.0      | FGSC 2509 mat a v1.0      |
| Sequecing platform     | Hybrid 454/Sanger        | Hybrid 454/Sanger        |
| Scaffold count         | 81                       | 307                      |
| All Contig count       | 551                      | 861                      |
| Scaffold sequence bases total | 39.1 Mb               | 39.1 Mb                |
| Scaffolded (Large) Contig sequence bases total | 38.5 Mb | 38.1 Mb |
| Estimated % sequence bases in gaps | 1.7% | 2.5% |
| Scaffold N50 / L50     | 3 / 5.7 Mb               | 3 / 5.7 Mb               |
| Contig N50 / L50       | 89 / 134.9 Kb            | 117 / 99.8 Kb            |
| Number of scaffolds > 50.0 Kb | 7                      | 7                       |
| % in scaffolds > 50.0 Kb | 99.2%                  | 98.2%                   |
### Table S3

Sequencing libraries for *Neurospora tetrasperma* FGSC 2508 mat A

| Library | Type | Raw Reads | Raw Bases | Trimmed Bases | Assem Reads | Assem Bases | Coverage | Insert Std Dev |
|---------|------|-----------|-----------|---------------|-------------|-------------|----------|----------------|
| FHCI    | SANG | 232,238   | 242,846,510 | 180,826,637   | 228,004     | 176,589,447 | 4.58x    | 2822           | 705          |
| FHNC    | SANG | 209,117   | 181,677,551 | 156,903,722   | 205,454     | 154,099,062 | 3.99x    | 8558           | 2139         |
| FHCO    | SANG | 52,883    | 44,594,073  | 34,316,772    | 49,195      | 32,285,178  | 0.84x    | 37937          | 9484         |
| GBNH    | 454  | 701,361   | 269,672,184 | 268,587,339   | 679,800     | 261,469,578 | 6.78x    |                |              |
| GCSS    | 454PE| 1,329,018 | 329,435,293  | 287,196,298   | 1,229,007   | 277,086,984 | 7.18x    | 17894          | 4473         |
| GSHS    | 454PE| 1,718,929 | 358,804,691  | 317,600,455   | 1,565,911   | 305,662,912 | 7.92x    | 3550           | 887          |
| **Total** |      | 4,243,546 | 1,427,030,302 | 1,245,431,223 | 3,957,371   | 1,207,193,161 | 31.29x  |                |              |

### Table S4

Sequencing libraries for *Neurospora tetrasperma* FGSC 2509 mat a

| Library | Type | Raw Reads | Raw Bases | Trimmed Bases | Assem Reads | Assem Bases | Coverage | Insert Std Dev |
|---------|------|-----------|-----------|---------------|-------------|-------------|----------|----------------|
| FHFBI   | SANG | 39,341    | 29,329,148 | 23,656,065    | 36,529      | 21,613,054  | 0.57x    | 39775          | 9943         |
| GCXS    | 454  | 2,224,123 | 824,396,125 | 821,323,297   | 2,083,410   | 770,085,673 | 20.18x   |                |              |
| GGUB    | 454PE| 306,099   | 90,121,826  | 67,446,884    | 260,379     | 59,046,315  | 1.55x    | 20556          | 5139         |
| GGUI    | 454  | 1,849,195 | 787,934,733  | 783,260,954   | 1,761,107   | 748,048,169 | 19.60x   |                |              |
| GHTG    | 454PE| 968,920   | 257,042,191  | 230,156,414   | 874,202     | 215,706,069 | 5.65x    | 13704          | 3426         |
| **Total** |      | 5,387,678 | 1,988,824,023 | 1,927,843,614 | 5,015,627   | 1,814,499,280 | 47.55x  |                |              |
### TABLE S5

**Gene model statistics**

| Nuclear Genome Annotation | \textit{Neurospora tetrasperma} FGSC 2508 mat A v2.0 | \textit{Neurospora tetrasperma} FGSC 2509 mat a v2.0 |
|---------------------------|---------------------------------------------------|---------------------------------------------------|
| # gene models             | 10,380                                            | 11,192                                            |
| Gene density              | 265                                               | 286                                               |
| Avg. gene length          | 1836                                              | 1800                                              |
| Avg. protein length       | 468                                               | 443                                               |
| Avg. exon frequency       | 2.72 exons/gene                                   | 2.7 exons/gene                                    |
| Avg. exon length          | 579                                               | 548                                               |
| Avg. intron length        | 154                                               | 152                                               |
| % complete gene models    | 94%                                               | 92%                                               |
| (with start and stop codons) |                                                 |                                                   |
| % genes with homology support |                                             | 87%                                               |
| % genes with Pfam domains | 50%                                               | 46%                                               |

### TABLE S6

**Assessment of results from Whittle et al 2011b**

| Substitution type          | mat a | mat A |
|---------------------------|-------|-------|
| Non-preferred to preferred| 134   | 128   |
| Preferred to non-preferred | 232   | 263   |

The numbers reported in Whittle et al 2011b were compared to determine if there is evidence for a statistically significant asymmetry in synonymous codon substitutions between the lineage #1 \textit{N. tetrasperma} mat a and mat A non-recombining regions. There is not a statistically significant difference between these two regions in the proportion of non-preferred to preferred substitutions relative to preferred to non-preferred (Fisher’s exact test: \(P=0.285\)).