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Degeneration in Codon Usage within the Region of Suppressed Recombination in the Mating-Type Chromosomes of *Neurospora tetrasperma*[▽]†

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The origin and early evolution of sex chromosomes are currently poorly understood. The *Neurospora tetrasperma* mating-type (*mat*) chromosomes have recently emerged as a model system for the study of early sex chromosome evolution, since they contain a young (<6 million years ago [Mya]), large (>6.6-Mb) region of suppressed recombination. Here we examined preferred-codon usage in 290 genes (121,831 codon positions) in order to test for early signs of genomic degeneration in *N. tetrasperma mat* chromosomes. We report several key findings about codon usage in the region of recombination suppression, including the following: (i) this region has been subjected to marked and largely independent degeneration among gene alleles; (ii) the level of degeneration is magnified over longer periods of recombination suppression; and (iii) both *mat a* and *mat A* chromosomes have been subjected to deterioration. The frequency of shifts from preferred codons to nonpreferred codons is greater for shorter genes than for longer genes, suggesting that short genes play an especially significant role in early sex chromosome evolution. Furthermore, we show that these degenerative changes in codon usage are best explained by altered selection efficiency in the recombinationally suppressed region. These findings demonstrate that the fungus *N. tetrasperma* provides an effective system for the study of degenerative genomic changes in young regions of recombination suppression in sex-regulating chromosomes.

At present, little is known about the origin and early evolution of sex chromosomes. This is because most ancient sex chromosome systems are so highly deteriorated (e.g., Y in X/Y systems) that they retain few traces of the historical events driving their evolution (8, 9, 11, 12, 54). The limited data available to date about early stages of sex chromosome evolution have been derived from young sex chromosomes from certain plants (e.g., *Silene* [41]) and/or from neo-X/Y systems of *Drosophila* (4, 5). Thus, model systems for young sex chromosomes are needed (12). The *Neurospora tetrasperma* mating-type (*mat*) chromosomes have recently emerged as a model system for the study of early stages of sex chromosome evolution (42).

The filamentous ascomycete *N. tetrasperma* is a self-fertile (pseudohomothallic) organism presumed to have evolved from a self-incompatible (heterothallic) ancestor (15, 50). As with most filamentous ascomycetes, the *N. tetrasperma mat* chromosomes contain the *mat* locus, which comprises two dissimilar alleles (*mat a* and *mat A* idiomorphs) that regulate mating and sexual reproduction (10, 53). Pseudohomothallism in *N. tetrasperma* is associated with a specialized meiotic pathway. Specifically, the *mat* chromosomes contain a young (<6 million years ago [Mya]), large (>6.6-Mbp) segment of suppressed recombination, including the segment between the *mat* locus and the centromere, which ensures first division segregation of mating-type idiomorphs. Spindles align in parallel for the second meiotic cell division, ensuring that nuclei of opposite mating types are encased in each ascospore (50). Consequently, the two *mat* chromosomes coexist in separate haploid nuclei in both reproductive and vegetative tissue, consistent with a predominant heterokaryotic and self-fertile state (50). This life history trait differs from that of the closely related heterothallic haploid taxon *Neurospora crassa*, which undergoes normal recombination during meiosis, contains nuclei with a single mating type in each ascospore, and requires two partners containing nuclei of opposite types for mating and sexual reproduction (53).

The *N. tetrasperma mat* chromosomes share many of the key features associated with the dimorphic sex chromosomes of animals and plants. In particular, as in dimorphic sex chromosomes, the segment of recombination suppression is localized to the central region of the *mat* chromosomes (42, 44), is flanked by two normally recombining, pseudoautosomal (PA) regions (21, 22, 32, 42), and contains genes with markedly divergent alleles (32, 35, 42, 44). Furthermore, the region of suppressed recombination comprises at least two distinct evolutionary strata, i.e., regions of suppressed recombination that have arisen successively over time, including an older (stratum 1) and a younger (stratum 2) stratum (42). Such strata have been reported for a wide range of plant and animal sex chromosomes and for mating-type chromosomes from *Microbotryum violaceum* (11, 22, 27, 35, 52, 59). Altogether, it is evident that the *N. tetrasperma mat* chromosomes provide a framework with which to examine genomic alterations within the recombinationally suppressed regions of sex-regulating chromosomes.

Given that the recombinationally suppressed region in *N. tetrasperma mat* chromosomes is young, divergence levels

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among alleles are not saturated (42), and it has not undergone extensive gene loss or other typical signs of massive degeneration (9, 42, 54). Thus, degenerative genomic changes, if present at this early stage, are likely subtle. One genomic trait likely to serve as a signal of early-stage degeneration is synonymous codon usage. Synonymous codons are not used randomly, and in many organisms, natural selection favors the use of a subset of preferred codons (PR). Preferred codon levels (or bias in codon usage) within the genome have been associated with more efficient and accurate translation (16, 17, 57) and represent adaptation at the molecular level in protein-coding DNA (1, 2). In addition, it has been found that shorter genes have a greater bias in codon usage in certain animals and plants (for examples, see references 3 and 17), suggesting that these traits may have coevolved to further promote highly efficient/accurate translation. In total, one may conclude that marked shifts from preferred codons to nonpreferred codons (NPR) provide a means to test for early signs of genomic deterioration in the *N. tetrasperma mat* chromosomes (1, 5).

Given that each of the *mat* chromosomes is recombinationally suppressed in *N. tetrasperma* (42), degenerative codon changes may be expected to occur on both chromosomes. This feature contrasts with those of dimorphic sex chromosome systems, in which only one chromosome is nonrecombining (4, 12). Another factor that could influence the level and patterns of genomic deterioration among differentiating *mat* chromosomes in *N. tetrasperma* is exposure to haploid selection. In dimorphic sex chromosome systems of diploid plants and animals, the marked degeneration of the heterozygous nonrecombining chromosome may be facilitated by the sheltering of mutations by the homologous chromosome and by the restriction of haploidy to the gamete stage (12, 45). In contrast, sex chromosomes in diploid organisms with haploid sex determination systems (e.g., Bryophytes and certain Fungi) are expected to have similar, and low, degeneration levels in the sex chromosomes. This pattern is expected because sex chromosomes should be equally sheltered at the diploid stage, and only those genes not associated with haploid growth may accumulate deleterious recessive mutations (7, 30). The pseudohomothallic system of *N. tetrasperma* has features of both systems. In particular, the heterokaryotic nature of *N. tetrasperma* may allow the sheltering of recessive mutations on the *mat* chromosomes, paralleling that observed in diploid organisms (49). In turn, the fact that each nucleus is haploid in *N. tetrasperma* might also allow for haploid selection. Specifically, homokaryotic sectors may arise within the heterothallic mycelium, as has been observed in certain heterokaryotic basidiomycetes (56). In addition, findings that as many as 20% of the asexual and sexual spores produced in the laboratory for *N. tetrasperma* are homokaryotic (44, 48) suggest that a segment of the natural population is haploid. Thus, it is possible that the *mat* chromosomes may evolve in a manner similar to that of dimorphic sex chromosomes and/or similar to that of a haploid system. Further data are needed to ascertain whether the young recombinationally suppressed region in *N. tetrasperma* is subjected to degeneration and which of these factors drive such processes.

The objective of the present study was to evaluate degenerative changes in preferred-codon usage within the young region of suppressed recombination in the *N. tetrasperma mat* chromosomes. For this purpose, we compared preferred-codon usage between *mat a* and *mat A* linked alleles among genes from various regions of the *mat* chromosomes (two pseudoautosomal regions and the younger [stratum 2] and older [stratum 1] regions of suppressed recombination), examined the role of gene length, and identified the possible mechanisms driving the degeneration.

MATERIALS AND METHODS

For our analysis, we generated DNA sequence data (coding sequences [CDS]) for *mat a* and *mat A* linked alleles for each of 290 genes located on the *N. tetrasperma* strain P4492 *mat* chromosomes by using Solexa technology. The *mat a* and *mat A* linked alleles were isolated and sequenced using haploid, single-mating-type component strains of the *N. tetrasperma* heterokaryotic strain P4492 (referred to here as P4492a and P4492A, respectively). The gene order within *N. tetrasperma* chromosomes was based on the order known for *N. crassa*, since these two genomes have been shown to be highly correlated (32). The gene order is available from the National Center for Biotechnology Information (NCBI) genome browser MapViewer (http://www.ncbi.nlm.nih.gov/mapview/). Following earlier conventions, we assumed that normally recombining gene alleles are identical or nearly identical in normally recombining genomic regions in a highly inbred taxon such as *N. tetrasperma* and that genes located in recombinationally suppressed regions show greater synonymous divergence (K_s) among their alleles over time (27, 35, 42, 43, 52).

Fungal strains. We examined genomic sequence data derived from the two haploid, single-mating-type component strains (*mat a* and *mat A*) of the *N. tetrasperma* heterokaryotic strain P4492 (P4492a and P4492A, respectively) and from the *N. crassa* strain FGSC 2489. P4492 originates from the Perkins collection of *Neurospora* strains from nature, curated by the Fungal Genomics Stock Center (FGSC), University of Missouri. The FGSC identification numbers (IDs) for the *mat a* and *mat A* component strains are FGSC 9034 and FGSC 9033, respectively. *N. tetrasperma* strain P4492 has previously been shown to belong to phylogenetic lineage 1 of the *N. tetrasperma* species complex (43).

DNA sequencing. The DNA sequences for the single-mating-type component strains P4492a and P4492A were generated by Geneservice, Source BioScience plc, using Solexa technology. The 55-bp paired-end DNA library was constructed with an insert size of ~170 bp. The sequencing process followed the protocol for Genome Analyzer II (GA II), and the images from GA II were processed by the GA II software pipeline for the purpose of base calling. All 55-bp paired-end reads for P4492a and P4492A were mapped against the reference genome of *N. tetrasperma* FGSC 2508 (available from the DOE Joint Genome Institute [http://www.jgi.doe.gov]) using Maq (http://maq.sourceforge.net/) (36). The maximum number of mismatches allowed was set to 3 in the mapping process. The consensus sequences were filtered and called with a minimum read depth of 3, a minimum mapping quality of 40, and a minimum neighboring quality of 20. This mapping protocol is conservative, limiting false-positive mapping. Coding DNA, intergenic regions, and introns were identified from the consensus sequences of linkage group I (the *mat* chromosome) of P4492a and P4492A. For *N. crassa*, the DNA sequences and NCU IDs were obtained from the *Neurospora crassa* Database (annotation version 3; FGSC 2489) (http://www.broad.mit.edu/annotation/genome/neurospora/).

Identification of gene sequences. A gene set of 290 genes was identified and utilized for analysis in our investigation (3 sequences per gene—1 each for P4492a, P4492A, and *N. crassa*—for a total of 870 sequences). For this purpose, we first identified a total of 2,309 *N. crassa* genes located on the *mat* chromosomes that have been defined by an NCU number at the *Neurospora crassa* Database (NCU IDs have been assigned to well-defined genes and to those encoding hypothetical and predicted proteins [http://broad.mit.edu/annotation/fungi/neurospora/]). The genes located on the *mat* chromosomes of P4492a and P4492A were subsequently identified by comparison to this *N. crassa* sequence database: the consensus sequences of linkage group I for P4492a and for P4492A were each defined as a database and compared to the *N. crassa* gene set (i.e., the CDS regions for each gene) using BLASTN (http://www.ncbi.nlm.nih.gov/). The *N. crassa* gene with the lowest E value (with a cutoff of $\leq 10^{-6}$) for a particular region of *N. tetrasperma* P4492a and P4492A linkage group I was considered a match. The genomic region contained within and surrounding each match for P4492a and for P4492A was isolated and aligned to the matching *N. crassa* CDS region using ClustalW (58). Introns and/or indel sequences identified within the P4492a and P4492A sequences from the alignment were removed. Genomic regions from P4492a and P4492A that matched an entire mRNA from *N. crassa*,

including the start codon and the stop codon, and that did not contain unknown or ambiguous nucleotides were identified for our analysis. Each P4492a and P4492A gene was translated to ensure the accurate identification of reading frames. Synonymous substitution rates (K_s) between alleles of each of the 290 genes described above were determined using default settings in DnaSP (37). The final aligned gene sequences, with gaps removed, used for P4492a and for P4492A are provided in File S1 in the supplemental material (sequence designations ending with P4492_a and P4492A are from the *mat a* and *mat A* chromosomes, respectively).

Identification and analysis of preferred codons. The preferred codons for our analysis were determined using the complete genomewide codon usage data available for *N. crassa* at the Codon Usage Database (http://www.kazusa.or.jp/codon/). In particular, we identified the most frequent codons per amino acid; one preferred codon was identified for amino acids encoded by two, three, or four codons, and two preferred codons were identified for amino acids encoded by six codons. The final preferred-codon list contains 21 codons (see Table SA1 in the supplemental material), which are highly similar to those in the list of optimal codons (i.e., codons present in the most highly expressed genes) reported for other fungi; the latter was based on examination of only a subset of the genomic sequences (38). All codons not listed in Table SA1 in the supplemental material were defined as nonpreferred codons. In order to further verify the preferred-codon list, we determined the relative synonymous codon usage (RSCU) for each codon for the pseudoautosomal regions in *N. tetrasperma* by using CodonW (J. Peden) (http://codonw.sourceforge.net). RSCU values measure the usage of each codon relative to all synonymous codons for a particular amino acid; higher RSCU values denote greater usage of a specific codon relative to its synonymous codons. These RSCU values for the normally recombining region of *N. tetrasperma* (see Table SA2 in the supplemental material) are in agreement with the preferred-codon list estimated from *N. crassa* shown in Table SA1.

Using the preferred-codon list discussed above, we conducted a comparative analysis of preferred-codon frequency among genes, among alleles per gene, among genes of different lengths, and among the recombinationally suppressed and pseudoautosomal regions of *N. tetrasperma*. Codon usage counts were determined using CAIcal (47).

Assessment of the allele-specific switches from preferred to nonpreferred codons. In order to identify specific switches from preferred codons (PR) to nonpreferred codons (NPR) within the recombinationally suppressed and pseudoautosomal regions of the *N. tetrasperma mat* chromosomes, we examined the codons found in *N. tetrasperma* P4492a, *N. tetrasperma* P4492A, and *N. crassa* for every codon position in our 290 genes. For this purpose, the DNA sequences for all 290 genes were concatenated in the sequential order in which they occur on the *mat* chromosomes of *N. crassa*. Using these data, we identified cases where *N. crassa* and one of the *N. tetrasperma mat* chromosomes contained the same preferred codon (i.e., the ancestral codon) while the other *mat* chromosome contained a different, nonpreferred codon. This is consistent with an allele-specific degenerative switch on one chromosome (from a preferred to a nonpreferred codon [PR → NPR]). Based on the same approach, we also identified allele-specific switches from a nonpreferred to a preferred codon (NPR → PR) associated with either the *N. tetrasperma mat a* or the *N. tetrasperma mat A* chromosome. Using these two variables, we measured the net excess of allele-specific switches to nonpreferred codons (PR → NPR) over switches to preferred codons (NPR → PR) (1) for each genomic region within each of the *mat* chromosomes. In addition, we standardized the number of excess switches from preferred to nonpreferred codons relative to the total number of codons examined per chromosomal segment (frequency $[Fr]_{Excess\ NPR}$ = excess PR → NPR switches per 1,000 codon positions) in order to directly compare values among genomic regions. A total of 25,502 codon positions from the pseudoautosomal regions, 18,419 codon positions for stratum 2, and 77,910 codon positions for stratum 1 were examined (a total of 121,831 codon positions).

Identification and analysis of introns. In order to ascertain the role of mutational pressure in changes in preferred-codon usage in *N. tetrasperma*, we compared the GC contents of introns (GCI) to the GC contents of third codon positions (GC3) of the genes under study. For this purpose, we first identified introns in *N. crassa* based on the alignments between genomic DNA and the CDS regions (data are available from the *Neurospora crassa* Database). Alignments were conducted for each of the 290 genes included in this study by using ClustalW. Gaps identified within the genomic DNA that exceeded 30 nucleotides were identified as introns. For *N. tetrasperma* P4492a and P4492A, introns were identified by alignment of the consensus sequence (i.e., the genomic region previously defined for each gene) with the *N. crassa* CDS region; gaps in the genomic DNA were identified as introns. In some cases, the *N. tetrasperma* introns contained internal gaps relative to the *N. crassa* intron, which could result from small insertions (in *N. crassa*) or deletions (in *N. tetrasperma*) and/or from gaps in the Solexa sequence assembly. Introns from *N. crassa*, P4492a, and P4492A were aligned. and gaps were removed. For genes with more than one intron, the introns were concatenated prior to analysis.

TABLE 1. Genes identified for the *Neurospora tetrasperma* single-mating-type component strains used in the present analysis[a]

Genomic region	No. of genes identified	Alleles located on the *mat a* vs *mat A* chromosome	
		Mean K_s	Fraction (%) of genes with a K_s of >0
Pseudoautosomal region 1	32	1.8×10^{-4}	1/32 (3.1)
Region of suppressed recombination			
Stratum 2	38	0.0038	13/38 (34.2)
Stratum 1	190	0.0180	149/190 (78.4)
Pseudoautosomal region 2	30	1.7×10^{-4}	1/30 (3.3)

[a] Strains P4492a and P4492A.

RESULTS

Demarcations within the *mat* chromosomes. As an initial step in our analysis, we identified the location of each of our 290 genes on the *N. tetrasperma mat* chromosomes. The various genomic regions on the *mat* chromosomes have previously been shown to occur in the following sequential order: pseudoautosomal region 1 (PA1), the younger region of suppressed recombination (stratum 2), the older region of suppressed recombination (stratum 1, containing the centromere), and pseudoautosomal region 2 (PA2). Based on the *a priori* assumption that normally recombining gene alleles are identical or nearly identical in *N. tetrasperma* (42, 43), as well as the demarcations on the *mat* chromosomes previously reported by Menkis et al. (42), we found that 228 of the 290 genes examined here are located in the region of suppressed recombination and 62 are located in the flanking pseudoautosomal regions. This region of suppressed recombination contains a high percentage of genes with a K_s of >0 in the comparisons of *N. tetrasperma* P4492a and P4492A alleles (>78% of genes in this region have diverged). We infer that 190 of the genes in the region of suppressed recombination are located within the older stratum 1, while 38 are in the younger, less divergent stratum 2 (Table 1; see also Table SA3 in the supplemental material). Of the 62 genes located in the PA regions, 32 are contained within PA1, the flanking region adjacent to stratum 2, and 30 are located in PA2, the flanking region adjacent to stratum 1 (Table 1; see also Table SA3). The two pseudoautosomal regions had a preponderance of genes with a K_s of zero (i.e., <3.3% of genes show a K_s of >0 [Table 1]).

The divergence values among gene alleles suggest that recombination suppression might not be perfectly enforced within the central region of the *mat* chromosomes. For example, although a K_s of >0 was observed among alleles for many genes in stratum 1 and stratum 2 (Table 1), a substantial fraction of genes in these regions, particularly in the younger stratum 2, have a K_s of zero (like the pseudoautosomal regions) among the *mat a* and *mat A* linked alleles (stratum 2 has 65.8% of genes with a K_s of zero, while stratum 1 has 21.6%

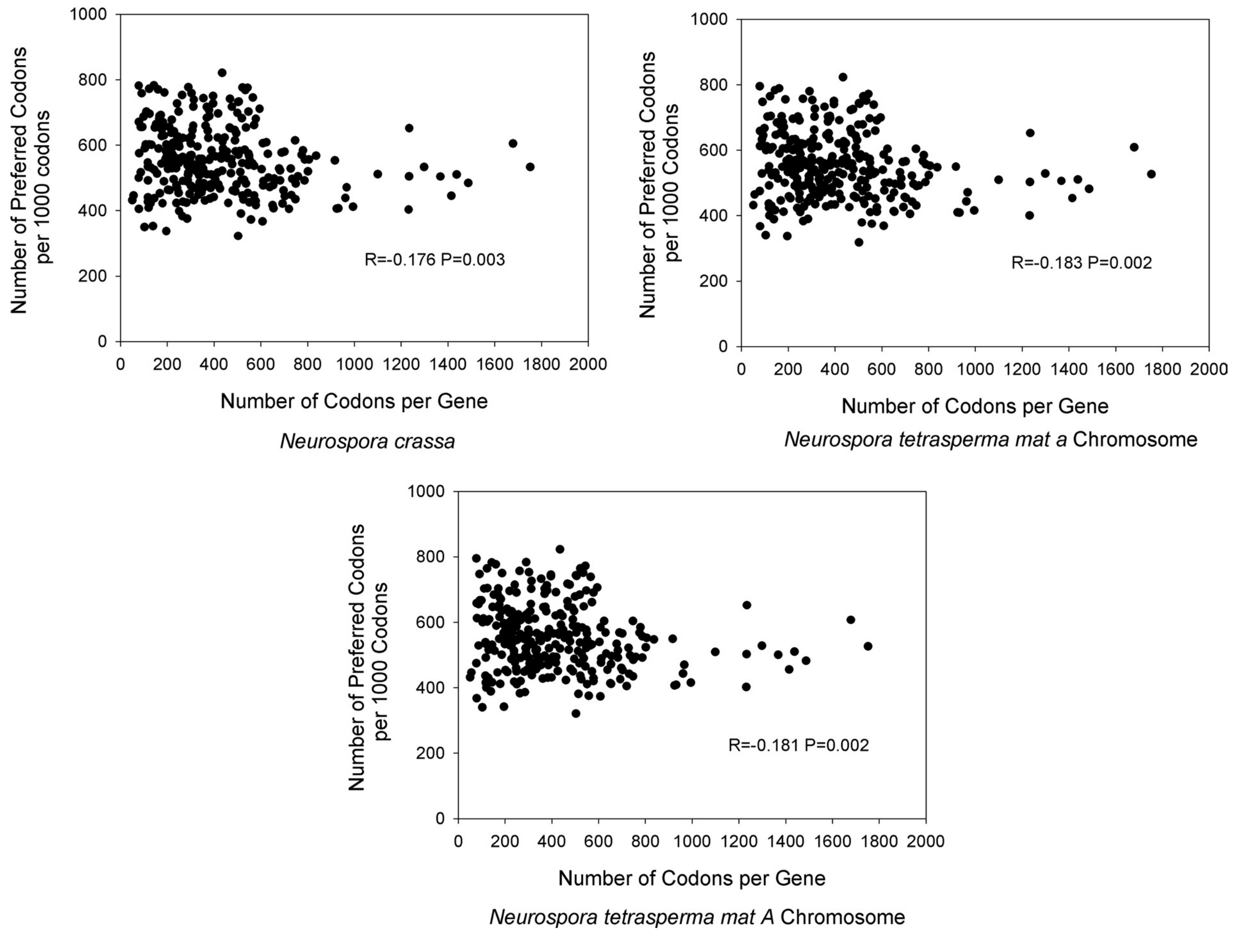

FIG. 1. Relationship between gene length (number of codons) and the number of preferred codons per 1,000 codons for the 290 genes examined in this study. Pearson correlation coefficients and *P* values are shown.

[Table 1]). It is possible that some level of gene conversion, the nonreciprocal transfer of DNA during meiosis, which homogenizes gene alleles (29), and/or crossover events occur during the early stages of recombination suppression in the *mat* chromosomes. It is also possible that recombination suppression has existed for too short a time to affect all genes in a similar manner, and that thus, there is inherent stochasticity in divergence among genes.

Preferred codons and gene length. In order to assess the relationship between gene length and codon usage bias in *Neurospora* using the 290 genes examined here, we standardized the frequency of preferred codons for each gene under study as follows: number of preferred codons per 1,000 codons = number of preferred codons/total number of codons × 1,000. Our data show that the number of preferred codons per 1,000 codons is inversely correlated with gene length (i.e., the number of codons per gene) for *N. crassa* and for each of the *N. tetrasperma* strains (P4492a and P4492A) ($P < 0.05$) (Fig. 1; see also Table SA4 in the supplemental material). Nonetheless, we noted that the correlation coefficients are relatively low (R, ≤0.183 [Fig. 1]) and that the correlation is not universally detected across all gene length categories (see Table SA4). For example, the short (<300 codons) and medium-length (≥300 and <500 codons) genes have similar frequencies of preferred codons (mean, between 562.9 and 570.5 preferred codons per 1,000), while the longest genes (>500 codons) have statistically significantly ($P < 0.05$) lower numbers of preferred codons (between 526.2 and 529.2 preferred codons per 1,000 [see Table SA4]). Thus, the *Neurospora* genes show an inverse relationship between gene length and codon usage bias, similar to that reported for other organisms (e.g., 3, 17), with the caveat that short and medium-length genes (with as many as 500 codons) each have high levels of preferred-codon usage.

Variation in preferred-codon levels among gene alleles. Marked divergence in the number of preferred codons was detected among the *mat a* and *mat A* linked alleles for genes located in the recombinationally suppressed region, but not in the pseudoautosomal regions, of the *N. tetrasperma mat* chromosomes. Specifically, the numbers of preferred codons were identical for *mat a* and *mat A* linked alleles in the vast majority of genes located in the pseudoautosomal regions, with less than 3.3% of genes showing differences (Fig. 2). In contrast, we found that 63.1% of genes in the older stratum (stratum 1) and 28.9% of genes in the younger stratum (stratum 2) of the recombinationally suppressed region had different levels of preferred codons among alleles. The magnitude of the differences among gene alleles differed markedly among the segments of the *mat* chromosomes, with much greater differentials

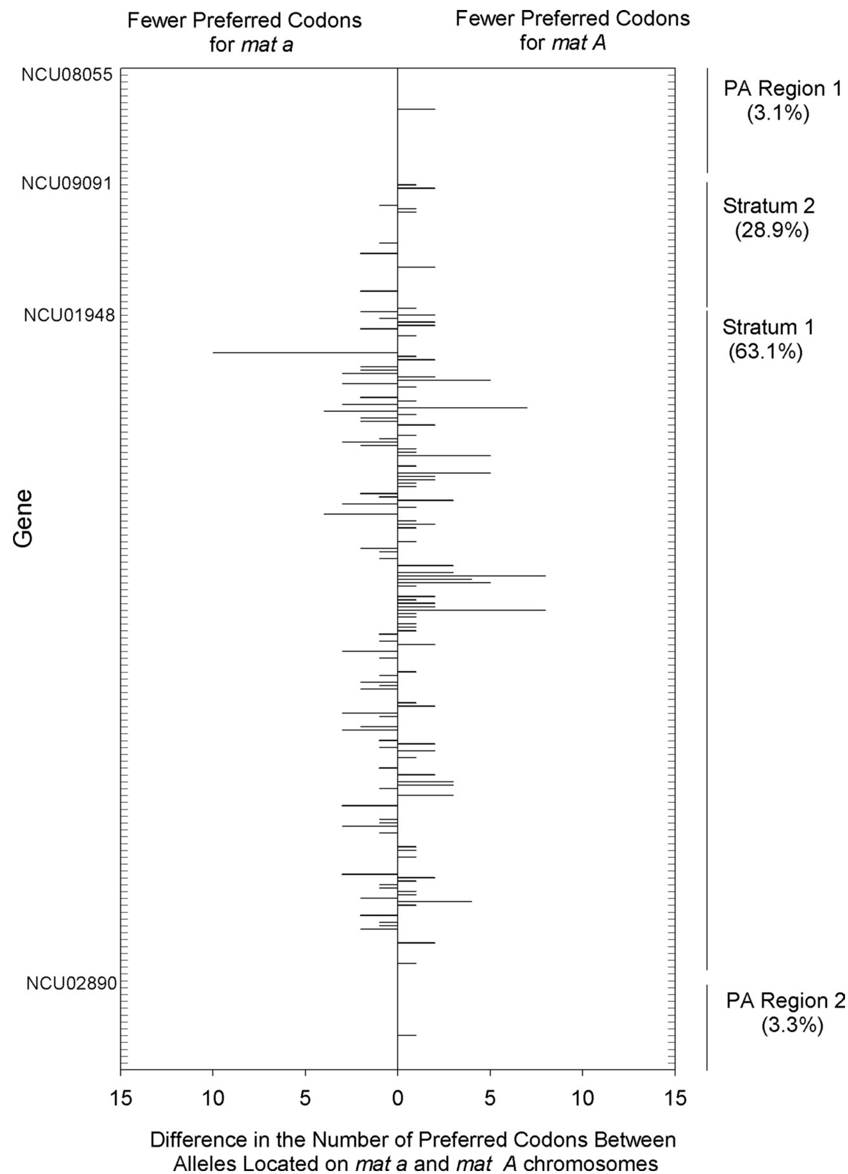

FIG. 2. Differences in the number of preferred codons between alleles for the 290 genes located on the *mat a* and *mat A* chromosomes in *Neurospora tetrasperma* strain P4492. The NCU ID for the first gene in each chromosomal segment is shown. The percentage of genes that have differences in the number of preferred codons among alleles is shown for each chromosomal region. Two or more genes are represented within each grid point on the vertical scale. The genes are listed in the order in which they occur on the *mat* chromosomes.

observed for genes located in stratum 1 than in stratum 2, where one allele had an excess of as many as 10 preferred codons over the other. The data also reveal that the specific allele with more, or fewer, preferred codons could occur on either the *mat a* or the *mat A* chromosome and that this parameter was largely gene specific.

The data also show that the level of divergence in the frequency of preferred codons among homologous gene alleles in the recombinationally suppressed region is associated with gene length. For example, for each of the 228 genes in the centrally located region of suppressed recombination, we found that the absolute value of the difference in the number of preferred codons (per 1,000 codons) between the *mat a* and *mat A* linked alleles is statistically significantly inversely correlated with gene length ($R = -0.427$; $P = 4.5 \times 10^{-7}$ [Fig. 3]). Thus, these findings demonstrate that there is substantially greater divergence in the levels of preferred codons among homologous alleles for shorter genes than for longer genes; this is consistent with greater rates of genomic deterioration in shorter genes within the recombinationally suppressed region.

Excess of allele-specific switches from preferred to nonpreferred codons. We determined the net excess of synonymous allele-specific switches to nonpreferred codons based on comparisons of PR → NPR and NPR → PR switches (Table 2) at codon positions where amino acids were conserved among the *mat* chromosomes. Marked differences were found in the level of synonymous allele-specific codon switches among the various segments of the *N. tetrasperma mat* chromosomes (Table

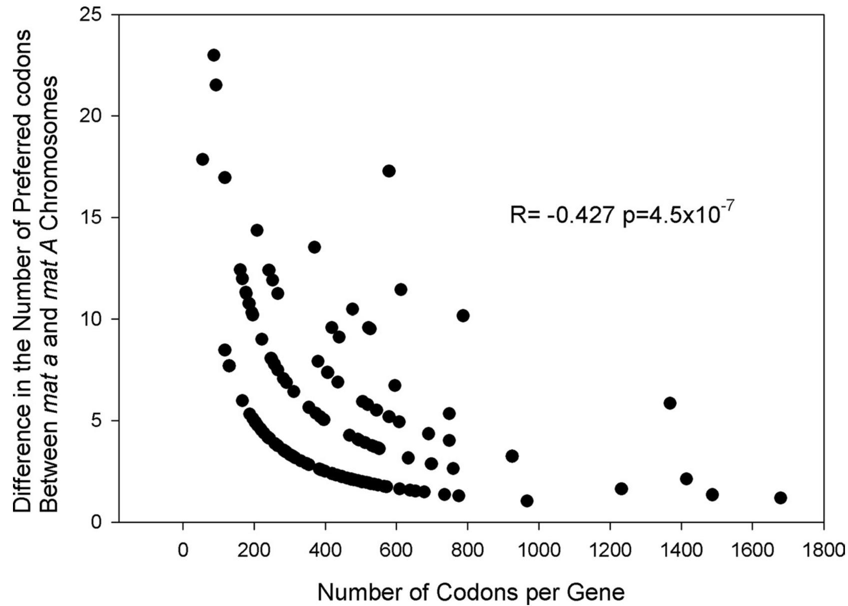

FIG. 3. Absolute value of the difference in the number of preferred codons (per 1,000 codons) between the alleles of genes located in the region of suppressed recombination of the *N. tetrasperma mat a* versus *mat A* chromosomes, plotted against the number of codons per gene. Genes with no differences have been excluded.

2). For the pseudoautosomal regions, we found exceptionally low numbers of allele-specific switches within the *N. tetrasperma mat a* and *mat A* chromosomes. In these regions, we observed only two allele-specific switches from a preferred to a nonpreferred codon (PR → NPR; on the *N. tetrasperma mat A* chromosome) and one switch from a nonpreferred to a preferred codon (NPR → PR; on the *mat a* chromosome) (Table 2). Thus, these data are consistent with normal recombination in the pseudoautosomal regions. In the recombinationally suppressed stratum 2, however, we found elevated levels of allele-

TABLE 2. Numbers of synonymous allele-specific switches from preferred to nonpreferred codons and from nonpreferred to preferred codons that have occurred on the *Neurospora tetrasperma mat a* and *mat A* chromosomes (relative to *Neurospora crassa*)

N tetrasperma chromosome[a]	Type of allele-specific switch[b]	Type of codon located in:			No. of switches[c]	Excess of switches from PR to NPR[d]	$Fr_{Excess\ NPR}$[e]
		N. crassa	*N. tetrasperma mat a*	*N. tetrasperma mat A*			
Pseudoautosomal regions							
mat a	PR to NPR	PR	NPR	PR	0	−1	−0.04
	NPR to PR	NPR	PR	NPR	1		
mat A	PR to NPR	PR	PR	NPR	2	+2	0.08
	NPR to PR	NPR	NPR	PR	0		
Stratum 2							
mat a	PR to NPR	PR	NPR	PR	9	+5	0.27
	NPR to PR	NPR	PR	NPR	4		
mat A	PR to NPR	PR	PR	NPR	7	+4	0.22
	NPR to PR	NPR	NPR	PR	3		
Stratum 1[f]							
mat a	PR to NPR	PR	NPR	PR	232 A	+98	1.26
	NPR to PR	NPR	PR	NPR	134 B		
mat A	PR to NPR	PR	PR	NPR	263 A	+135	1.73
	NPR to PR	NPR	NPR	PR	128 B		

[a] The total numbers of codon positions examined for the pseudoautosomal region, stratum 2, and stratum 1 are 25,502, 18,419, and 77,910, respectively.
[b] PR, preferred codons; NPR, nonpreferred codons.
[c] Within each stratum, values followed by different letters indicate a statistically significant difference ($P < 0.05$) between the number of switches from PR to NPR and the number of switches from NPR to PR.
[d] Data shown are based on total numbers of switches from PR to NPR and from NPR to PR where the amino acid is conserved. When all possible switches, regardless of amino acid status, are included, the net excess remains the same for the pseudoautosomal region; the values for stratum 2 are +5 for both the *mat a* and *mat A* chromosomes; and the values for stratum 1 are +100 for *mat a* and +138 for *mat A*.
[e] Frequency of excess PR-to-NPR switches per 1,000 codon positions, calculated as (number of PR-to-NPR switches − number of NPR-to-PR switches)/total codon positions examined × 1,000.
[f] Three codon positions in stratum 1 had a codon switch on both the *mat a* and *mat A* chromosomes and were excluded from these results.

specific changes in preferred-codon usage. Specifically, we detected nine allele-specific switches from a preferred to a nonpreferred codon on the *mat a* chromosome. Only four cases were found where a nonpreferred codon had switched to a preferred codon; thus, we conclude that there is a net excess of five nonpreferred codons in stratum 2 on the *mat a* chromosome. Similar observations were made for alleles located on the *mat A* chromosome, where we found a net gain of four allele-specific nonpreferred codons in stratum 2 (Table 2). The greatest level of allele-specific switches was detected in stratum 1. Specifically, we found that a total of 232 allele-specific synonymous switches had occurred from a preferred to a nonpreferred codon on the *mat a* chromosome, while only134 were detected in the opposite direction (nonpreferred to preferred) (this difference is statistically significant [P, <0.05] by Fisher's exact test) (Table 2), resulting in an excess of 98 nonpreferred codons among the gene alleles examined on this chromosome. Similarly, 263 allele-specific switches from preferred to nonpreferred codons were detected for the *mat A* chromosome, while only 128 switches from nonpreferred to preferred codons were found (P, <0.05 by Fisher's exact test), yielding an excess of 135 nonpreferred codons. Standardization of the number of excess switches from preferred to nonpreferred codons relative to the total number of codons examined per chromosomal segment ($Fr_{Excess\ NPR}$) further verified the differences between the various genomic regions. In particular, the frequency of excess switches from preferred to nonpreferred codons was found to be 4.7-fold higher in stratum 1 than in stratum 2 for the *mat a* chromosome (calculated as $Fr_{Excess\ NPR(stratum1)}/Fr_{Excess\ NPR(stratum2)}$, or 1.26/0.27) and 7.9-fold higher for the *mat A* chromosome (1.73/0.22) (Table 2), consistent with greater degeneration with extended periods of recombination suppression. Taken together, these findings show that there is a net excess of allele-specific switches from preferred to nonpreferred codons within the recombinationally suppressed regions in *N. tetrasperma mat* chromosomes (but not in the pseudoautosomal regions) and that the level of degeneration increases with longer periods of recombination suppression. Furthermore, these data also reveal that there is a marked excess of preferred-to-nonpreferred codon switches on both the *mat a* and the *mat A* chromosomes and thus that there is not a single "degenerative" chromosome in *N. tetrasperma*.

The net excess of synonymous allele-specific switches to nonpreferred codons described above was determined using PR → NPR and NPR → PR switches for synonymous codon positions where the amino acid was conserved (Table 2). Examination of the total excess of allele-specific switches among preferred and nonpreferred codons, i.e., independent of amino acid status and including both synonymous and nonsynonymous changes, yielded highly similar results. We found a net excess of 100 allele-specific switches from preferred to nonpreferred codons on the *mat a* chromosome and 138 on the *mat A* chromosome for stratum 1 (Table 2, footnote *d*; see also Table SA5 in the supplemental material). The similarity of the findings is consistent with the fact that the vast majority of allele-specific codon switches were synonymous (95.8% [*mat a*] and 93.5% [*mat A*] of allele-specific codon switches in stratum 1 were synonymous).

In addition to allele-specific switches from preferred to nonpreferred codons (and vice versa), we also determined the frequency of other types of preferred codon changes for each of the 121,831 codon positions examined in this study (see Table SA5 in the supplemental material). For example, we found a relatively high number of codon positions in which *N. crassa* contained a preferred codon and *N. tetrasperma* contained nonpreferred codons that were identical for P4492a and P4492A (and vice versa) in stratum 1 and stratum 2. This is indicative of a codon switch that occurred either in the *N. crassa* or the *N. tetrasperma* lineages (prior to recombination suppression). Table SA5 in the supplemental material provides a summary of the frequencies of all types of preferred codon changes among the *mat* chromosomes.

Mutation versus selection. In order to assess whether mutational or selective forces drive the evolution of preferred *Neurospora* codons, we compared the GC contents of third codon positions (GC3) and the GC contents of introns (GCI) (55, 60) among the 290 genes examined here. If mutational pressures drive preferred-codon usage in *Neurospora*, a direct association between GC3 and GCI would be expected. The results show no evidence of a correlation between GC3 and GCI for *N. crassa* genes or for genes located on the *N. tetrasperma* P4492 *mat a* and *mat A* chromosomes (se Fig. SA1 in the supplemental material). Given that preferred *Neurospora* codons end in G or C nucleotides (see Table SA1 in the supplemental material), this is consistent with a lack of a role for mutational bias. Thus, the data suggest that selective pressure variation is driving changes in preferred-codon usage in the *mat* chromosomes.

The finding that codon usage in *N. tetrasperma mat* chromosomes is driven largely/primarily by selection (and not mutational bias) suggests that the divergence in preferred-codon usage in the segment of suppressed recombination in the *mat* chromosomes is attributable to altered selective constraints. Nonetheless, it is conceivable that small-scale/localized mutational biases could also play a role (39). In particular, gene-specific mutational biases associated with the region of suppressed recombination could drive the observed divergence (e.g., allele-specific mutational biases, specific to each gene). In order to assess this possibility, we compared the difference in the frequency of preferred codons to the difference in the frequency of GC nucleotides in introns for *mat a* versus *mat A* linked alleles from among genes located in the region of suppressed recombination (using the genes showing differences in preferred codons and in GCI per allele). The data show that the direction of the differences in preferred-codon frequency among alleles per gene has no association with the difference in GCI frequency (see Fig. SA2 in the supplemental material). For example, among the genes where the *mat a* linked allele has a higher number of preferred codons than the *mat A* linked allele, fewer than half (44.1%) have a parallel trend in GC frequency (i.e., higher GCI frequency in the *mat a* than in the *mat A* linked allele). In fact, 55.9% of genes have the opposite GCI trend (lower GCI frequency in the *mat a* linked allele). Genes with a greater frequency of preferred codons in *mat A* than in *mat a* linked alleles also show a lack of association between preferred-codon frequency and GCI frequency (see Fig. SA2 in the supplemental material). Thus, these data indicate that mutational bias has no substantive relationship at the intragene level with the divergence of preferred-codon usage

in the region of suppressed recombination within the *mat* chromosomes.

DISCUSSION

The net excess of allele-specific codon switches from preferred to nonpreferred codons within the recombinationally suppressed regions of the *N. tetrasperma mat* chromosomes (Table 2; Fig. 2) is consistent with genomic degeneration. This effect is time dependent, as indicated by higher levels of allele-specific changes in the older stratum 1 than in stratum 2 (Table 2). The accumulation of nonpreferred codons is likely the result of reduced selective efficiency in regions of suppressed recombination. The nonindependence of gene alleles in chromosomal regions with suppressed recombination is believed to reduce the effective population size, giving rise to decreased selective efficiency and genetic hitchhiking (26, 28). For example, the fixation of accumulated deleterious mutations, such as switches from preferred to nonpreferred codons, in nonrecombining regions may be promoted by processes such as background selection (which promotes the fixation of mildly deleterious mutations [11]) and/or by Muller's ratchet (with the stochastic loss of chromosome classes with the lowest levels of mutations [46]). Positive selection might also contribute to degeneration in regions with suppressed recombination; for instance, positive-selection events may drag deleterious mutations at linked sites to fixation via selective sweeps (6, 11, 51). In this regard, the net decline in preferred-codon usage in the recombinationally suppressed region of the *N. tetrasperma mat* chromosomes is likely driven by relaxed purifying selection and/or by positive-selection events.

The lack of an association between preferred-codon usage and GCI suggests that mutational pressure does not play a significant role in the degeneration of the *N. tetrasperma mat* chromosomes (see Fig. SA1 and SA2 in the supplemental material). Biased gene conversion toward GC-ending codons has been proposed to be a potential force driving GC content in coding regions and, thus, preferred-codon evolution (19, 23, 24, 25, 33). This is an unlikely factor in our findings, because such events have been associated primarily with regions of very high recombination in outbreeding species (19), whereas we found preferred-codon degeneration within a region of suppressed recombination within the inbreeding species *N. tetrasperma*. In addition, biased gene conversions have been predicted to have similar impacts on coding and intron regions (20) and to preferentially convert AT to GC (40), neither of which was found in our data (see Fig. SA2 in the supplemental material; we found elevated numbers of switches from GC-ending to AT-ending codons). Accordingly, the totality of our data point toward the conclusion that degeneration in preferred-codon usage in the *mat* chromosomes results from altered selective pressure in the young regions of suppressed recombination.

Codon changes and gene length. The elevated bias in codon usage in shorter genes (CDS regions) found here (Fig. 1) has been reported for certain animals and plants (e.g., *Drosophila*, *Arabidopsis*. *Caenorhabditis*, and *Populus* [3, 14, 17]). This might result from enhanced gene expression (3, 18) and/or from higher recombination rates in shorter genes (13), each of which likely leads to enhanced selection pressure for preferred-codon usage (3, 13). The findings of markedly higher levels of degenerative changes in preferred-codon usage for shorter genes (Fig. 3) suggest that they are highly susceptible to genomic alterations at the onset of recombination suppression. It may be speculated that a history of highly efficient selection for preferred-codon usage in shorter genes, possibly resulting from elevated gene expression and/or recombination rates (3, 13), makes these genes most vulnerable to reduced/altered selection pressures after the onset of recombination suppression (11). Given that the accumulation of deleterious mutations is a key factor shaping the degeneration of Y chromosomes (11, 34), our data suggest that short genes might play a relatively greater role than longer genes in the earliest stages of degeneration. Further studies will be needed to ascertain the precise reasons why elevated levels of degenerative codon changes are inherent to shorter genes.

Patterns of degeneration. Our present data reveal that the degeneration in preferred-codon usage occurs in the recombinationally suppressed region for each of the two *mat* chromosomes of *N. tetrasperma* (Table 2). This finding corresponds with the fact that each of the *mat* chromosomes is recombinationally suppressed, and it is consistent with trends expected for systems with haploid sex determination, i.e., degeneration on both chromosomes (7). Although both of the *mat* chromosomes of *N. tetrasperma* show degeneration, their degeneration levels differ; for instance, in stratum 1, the excess of allele-specific switches from preferred to nonpreferred codons in *mat A* versus *mat a* is 37 (calculated as $135 - 98$), which is >27% more in *mat A* (Table 2). This suggests a deviation from the expectation of similar levels of degeneration for organisms with extensive haploid selection. Furthermore, in contrast to theoretical predictions for organisms with extended haploid stages of development, our data do not suggest that there has been a low level of degeneration in the region of suppressed recombination. For example, in stratum 1, the excesses of allele-specific switches from preferred to nonpreferred codons were 1.26 and 1.73 per 1,000 codons for *mat a* and *mat A*, respectively, values more than 21-fold higher than those for the pseudoautosomal regions (≤0.08 per 1,000 codons) (Table 2). Moreover, we found that the net differences in the preferred-codon levels among *mat*-linked alleles are also substantial (the preferred codon differential for some genes is >10 [Fig. 2]). Thus, it is possible that the recombinationally suppressed region in *N. tetrasperma mat* chromosomes evolves in a hybrid manner, with degeneration on both chromosomes as expected under haploid systems (Table 2) and with substantial degeneration and unequal levels of degeneration along the *mat* linked alleles, as predicted for dimorphic sex chromosomes (Table 2; Fig. 2).

Haploid selection is believed to be a possible contributing factor in the lack of degeneration reported for recombinationally suppressed segments of the large mating-type locus (>100 kb) of *Cryptococcus neoformans*. In this system, no losses in gene functionality or changes in chromosome size have been found (23). However, it is not known whether *C. neoformans* shows more-subtle degenerative genomic changes, such as a decline in preferred-codon usage, as reported here. Notably, *C. neoformans* can exist as a haploid or a diploid and often occurs as haploid cells in the environment (23); thus, there may be marked opportunity for haploid selection to limit degeneration

in this taxon. The opportunity for haploid selection may be lower in *N. tetrasperma.* Haploid selection in *N. tetrasperma* might arise due to homokaryotic sectors in the heterokaryon or due to the presence of occasional haploid individuals within the population (48, 56). However, the predominance of a heterokaryotic state in *N. tetrasperma* may permit the sheltering of mutations among haploid nuclei during the life cycle (49), a feature that might facilitate greater degeneration in this species than in organisms that exist primarily or solely as haploid individuals.

The complex pattern of degeneration in the region of suppressed recombination in *N. tetrasperm*a could result from the combined effects of a predominant heterokaryotic state (31) that allows both haploid selection and mutational sheltering. It is also possible that other factors, such as chromosome-specific structural rearrangements on the *mat A* chromosome (32) and/or rare outcrossing or interspecific hybridization events (31, 43), may contribute to the pattern of degeneration.

Conclusions. Based on the present data, several key conclusions can be drawn regarding genomic changes associated with the young regions of recombination suppression in *N. tetrasperma mat* chromosomes. First, the early stages of recombination suppression are characterized by marked and largely independent degeneration in preferred-codon usage among gene alleles. Second, the level of degeneration is magnified over longer periods of recombination suppression, as evidenced by substantially higher levels of degeneration in stratum 1 than in stratum 2. Third, both *mat a* and *mat A* chromosomes are subjected to degeneration, i.e., there is no single "degenerative" chromosome, in contrast to the pattern in ancient dimorphic (X/Y) systems, although a greater level of degeneration was found on the *mat A* than on the *mat a* chromosome. Further studies from other recently emerging model systems of young sex chromosomes (e.g., *Silene* [41]) will be needed to ascertain whether this pattern of degeneration is a universal feature of early stages of recombination suppression or is specific to the *N. tetrasperma mat* chromosomes. Fourth, our data indicate that the rate of divergence in preferred-codon usage is greater for short genes, which have evolved for highly efficient translation. In the future, as more genomic data for *N. tetrasperma* become available, studies should focus on the transposition frequency and the onset of gene-inactivating mutations within the region of restricted recombination in order to reveal whether these other degenerative traits are also inherent to early sex chromosome evolution.

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