ORIGINAL ARTICLE

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*

CA Whittle and H Johannesson

Department of Evolutionary Biology, Uppsala University, Uppsala, Sweden

Currently, little is known about the origin and early evolution of sex chromosomes. This is largely due to the fact that ancient non-recombining sex chromosomes are highly degenerated, and thus provide little information about the early genomic events in their evolution. The *Neurospora tetrasperma* mating-type (*mat*) chromosomes contain a young (<6 Mya) and large region (>6.6 Mb) of suppressed recombination, thereby providing a model system to study early stages of sex chromosome evolution. Here, we examined alleles of 207 genes located on the *N. tetrasperma mat a* and *mat A* chromosomes to test for signs of genomic alterations at the protein level in the young region of recombination suppression. We report that the *N. tetrasperma mat a* and *mat A* chromosomes have each independently accumulated allele-specific non-synonymous codon substitutions in a time-

dependent, and gene-specific manner in the recombinationally suppressed region. In addition, examination of the ratio (ω) of non-synonymous substitutions (*dN*) to synonymous substitutions (*dS*) using maximum likelihood analyses, indicates that such changes are associated with relaxed purifying selection, a finding consistent with genomic degeneration. We also reveal that sex specific biases in mutation rates or selection pressures are not necessary for genomic alterations in sex chromosomes, and that recombination suppression in itself is sufficient to explain these results. The present findings extend our current understanding of genomic events associated within the young region of recombination suppression in these fungal sex-regulating chromosomes.

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Introduction

The early stages of sex chromosome evolution are currently poorly understood. The reason for this is that most ancient sex chromosomes are highly deteriorated and retain little information about the early stages of genomic differentiation and degeneration (Charlesworth and Charlesworth, 2000). Thus, model systems of young sex chromosomes, that is, systems with newly established regions of recombination suppression, are necessary to study early steps in sex chromosome evolution (Charlesworth *et al.*, 2005). One emerging model system is the *Neurospora tetrasperma* mating type (*mat*) chromosomes.

N. tetrasperma is a self-fertile (pseudohomothallic) filamentous ascomycete assumed to have evolved from a self-incompatible (heterothallic) ancestor (Raju and Perkins, 1994; Dettman *et al.*, 2003). As in most filamentous ascomycetes, the *N. tetrasperma mat* chromosomes contain the *mat* locus, which is comprised of two highly dissimilar alleles (*mat a* and *mat A* idiomorphs)

that regulate reproduction and sexual compatibility (Shiu and Glass, 2000; Casselton, 2008). The two *mat* chromosomes of *N. tetrasperma* are found in separate haploid nuclei, of which both are present in the sexual ascospores and in vegetative cells, consistent with a predominant heterokaryotic and self-fertile state (Shear and Dodge, 1927; Jacobson, 1995). This feature of *N. tetrasperma* differs from closely related heterothallic *Neurospora* species, such as *N. crassa*, which is haploid and requires two distinct partners containing nuclei of opposite type for mating and sexual reproduction (Shiu and Glass, 2000). The pathway of meiosis and spore development leading to the packaging of nuclei carrying the two opposite mating types into single ascospores of *N. tetrasperma* is highly novel (Raju and Perkins, 1994). In association with the evolution of a pseudohomothallic mating system, the *N. tetrasperma mat* chromosomes have evolved the unusual feature that they contain a young and large region of suppressed recombination surrounding the *mat* locus (<6 Mya and >6.6 Mbp, respectively) (Menkis *et al.*, 2008), which share marked parallels with dimorphic plant and animal sex chromosomes. Specifically, the region of suppressed recombination is located in the central region of the *mat* chromosomes and surrounds the *mat* locus (Merino *et al.*, 1996; Menkis *et al.*, 2008). This region is flanked by two normally recombining pseudoautosomal regions, contain genes

Correspondence: Professor H Johannesson, Department of Evolutionary Biology, Uppsala University, Uppsala 752 36, Sweden.

E-mail: Hanna.Johannesson@ebc.uu.se

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with markedly diverged alleles, and is comprised of at least two distinct evolutionary strata, that is, recombinationally suppressed regions that have developed successively over time (Fraser and Heitman, 2004, 2005; Menkis *et al.*, 2008). Thus, the *N. tetrasperma* *mat* chromosomes provide a model system for the study of genomic changes inherent to young regions of recombination suppression in sex-regulating chromosomes (Menkis *et al.*, 2008).

In ancient sex chromosomes, massive genomic degeneration has occurred on the permanently heterozygous chromosome. This degeneration is believed to be because of a reduced efficiency of natural selection; non-independence of linked loci is expected to reduce the effective population size of the non-recombining chromosome (Hill-Robertson Effect; Hill and Robertson, 1966; Gordo and Charlesworth, 2001), thus decreasing the efficiency of both positive and negative selection, and enhancing the role of genetic drift (Charlesworth and Charlesworth, 2000). The fixation of accumulated mutations on non-recombining regions may be enhanced by background selection (a net effect of accelerating the fixation of mildly deleterious mutations and the loss of positive mutations (Charlesworth and Charlesworth, 2000)) and/or by Muller's Ratchet (wherein mutations accumulate in an asexual manner, with the stochastic losses of chromosome classes carrying the fewest deleterious mutations (Muller, 1964)). It is also plausible that positive selection contributes to degeneration in the recombinationally suppressed regions by dragging deleterious mutations at linked alleles/sites to fixation through selective sweeps (Rice, 1987; Charlesworth and Charlesworth, 2000; Bachtrog, 2004). To date, only limited data has been available regarding evolution of young sex chromosomes, primarily from *Silene* (Marais *et al.*, 2008) and from the neo-X/Y chromosomes of *Drosophila* (Bachtrog, 2003, 2004). Each of these studies has shown evidence consistent with genomic degeneration. Further data regarding the onset of genomic changes in young sex regulating chromosomes may be revealed from the study of the recently established region of recombination suppression within the *N. tetrasperma* *mat* chromosomes.

In protein coding genes, selective constraint can be measured using the ratio of non-synonymous to synonymous substitution rates over evolutionary time (dN/dS , or ω) (Yang, 2007). Specifically, values of $\omega < 1$, $\omega = 1$ and $\omega > 1$ indicates purifying selection, neutral evolution and positive selection, respectively (Yang and Nielsen, 2002). Methods have been developed based on maximum likelihood analyses that allow the assessment of ω among individual lineages in a phylogeny (Branch Model analyses, Yang, 1998; Yang, 2007). This approach may be particularly useful for the identification of variation in selective pressures inherent to the young region of recombination suppression in the *N. tetrasperma* *mat* chromosomes, that is, constraints that have changed after the divergence from a heterothallic ancestor with normal recombination rates.

In this study, we investigated DNA sequence divergence in the young recombinationally suppressed region of the *N. tetrasperma* *mat* chromosomes. We examined 207 genes located on the *mat* chromosomes of *N. tetrasperma* and its close normally recombining relatives *N. crassa* and *N. discreta*. The main objective was to reveal the

frequency of allele-specific non-synonymous and synonymous changes in the recombinationally suppressed region as compared with the normally recombining pseudoautosomal regions. In addition, we conducted branch-model analysis of ω to reveal the variation in evolutionary constraints within and among *N. tetrasperma* *mat* chromosomes.

Materials and methods

Several traits of *N. tetrasperma* *mat* chromosomes have been previously established, and thus, were assumed in our present analysis. Most importantly, restricted recombination in the central segment of the *N. tetrasperma* *mat* chromosomes has been repeatedly demonstrated in scientific analyses, and was assumed for our study. Specifically, recombination suppression has been demonstrated by cytological inspection of the *mat* chromosomes during meiosis (Gallegos *et al.*, 2000), by analysis of genetic marker segregation in *N. tetrasperma* wild crosses (Gallegos *et al.*, 2000), and by *mat* chromosome introgressions between *N. crassa* and *N. tetrasperma* in the laboratory (Howe and Haysman, 1966; Jacobson, 2005). Furthermore, population level analyses have revealed that gene alleles located in the recombinationally suppressed region are linked to each other and to the *mat a* or the *mat A* chromosome in *N. tetrasperma* (Merino *et al.*, 1996; Gallegos *et al.*, 2000; Jacobson, 2005). Finally, a key indicator of recombination suppression is that alleles in this region have undergone substantial sequence divergence (Lahn and Page, 1999; Handley *et al.*, 2004; Sandstedt and Tucker, 2004), which is not observed in the normally recombining flanking pseudoautosomal regions, where alleles are nearly completely homogenous (Merino *et al.*, 1996; Menkis *et al.*, 2008, 2009).

We also assumed a conserved gene order between the studied species. It has been reported that the gene order in *N. tetrasperma* and *N. crassa* is highly conserved and collinear, including the *mat* chromosomes (Howe and Haysman, 1966; Perkins, 1985; Jacobson, 2005). Thus, the gene order for *N. tetrasperma* *mat* chromosomes examined herein was based on the known order for *N. crassa*, as has proven effective in previous studies (for example, Gallegos *et al.*, 2000). The gene order for *N. crassa* is available from MapView (National Center for Biotechnology Information, NCBI, <http://www.ncbi.nlm.nih.gov/mapview/>).

For our investigation, we obtained sequences for *N. crassa* and *N. discreta* available from the *N. crassa* database (FGSC 2489, Annotation Version 3 <http://www.broad.mit.edu/annotation/genome/neurospora/>); the mRNA was also accessed using the RefSeq database at NCBI, <http://www.ncbi.nlm.nih.gov/RefSeq/>) and from the Joint Genome Initiative (<http://www.jgi.doe.gov/>), respectively. The data for *N. tetrasperma* was generated using Solexa technology and were assembled as described below.

Solexa data assembly of *N. tetrasperma* strain P4492

For our study, we gathered genomic sequence data from each of the two separate homokaryotic single mating-type component strains (that is, *mat a* and *mat A*) of the *N. tetrasperma* heterokaryon P4492. The strain P4492 originates from the Perkins collection of *Neurospora* from nature, curated by the Fungal Genomics Stock Center

(FGSC), University of Missouri (FGSC ID for the *mat a* component strain is FGSC 9034, and for *mat A* is FGSC 9033) and has previously been shown to be contained within phylogenetic lineage 1 of the *N. tetrasperma* species complex (Menkis *et al.*, 2009). The *N. tetrasperma mat a* and *mat A* strains/chromosomes are referred to in the present study as NTA and NTA, respectively. Genomic sequences were obtained separately for the NTA and the NTA strain by Solexa technology by Geneservice, Source BioScience plc group (<http://www.geneservice.co.uk/home/>). The 55 bp paired-end DNA library was constructed with insert size ~170 bp. The sequencing process followed the protocol instruction for Genome Analyzer II, and the image from the Genome Analyzer II were processed by GA pipeline software in the purpose of base calling. The 55 bp paired-end sequence reads associated with the *mat* chromosomes were identified and assembled through sequence similarity searches relative to the Linkage Group I (*mat* chromosomes) for *N. tetrasperma* FGSC 2508 (available from the Joint Genome Institute, <http://www.jgi.doe.gov>) using the MAQ (Mapping and Assembly with Qualities) software (<http://maq.sourceforge.net/maq-man.shtml>). We set the maximum number of mismatches to three in the process of mapping raw reads to the reference *N. tetrasperma* genome. Consensus sequences were extracted with the minimum read depth of three. The average depth (coverage) across all non-gap regions calculated by MAQ mapcheck module were 12.54 (NTa) and 11.73 (NTa), which represents standard levels for a genomic survey analysis. The average base error probability was lower than 0.24% (NTa) and 0.15% (NTa), thus demonstrating very high quality sequencing and base calling (calculated based on the Illumina quality). The Solexa DNA sequence covered more than 69 and 70% of the ~10 Mbp *mat* chromosomes for NTA and NTA, respectively; the fraction not covered ($\leq 31\%$) may reflect bias in library construction or sequence amplification. Nonetheless, the fraction of sequences obtained was highly accurate ($\geq 99.85\%$ accuracy). The sequences include genomic regions identified as coding DNA, intergeneric regions and introns.

Identification of genes

N. crassa has historically served as a highly studied model system for fungi, and genes for this species have been annotated (FGSC 2489 <http://www.broad.mit.edu/annotation/genome/neurospora/>). Accordingly, the gene sequences and annotation for this species were used as a reference for gene identification in *N. tetrasperma* and *N. discreta*, whose genomes have not previously been fully annotated. For gene identification, we compared genes (mRNA) from the *N. crassa* sequence database to the DNA sequences for linkage group I of *N. tetrasperma* and to the putative mRNA dataset of *N. discreta*.

For *N. tetrasperma*, the available sequence for NTA and NTA linkage group I was compared with the mRNA dataset from *N. crassa*. We first identified 2309 *N. crassa* mRNA associated with the *mat* chromosomes, which have been defined by an NCU number at the *Neurospora crassa* database. An NCU ID has been assigned for well defined genes, and those encoding hypothetical and predicated proteins (FGSC 2489 <http://www.broad.mit.edu/annotation/genome/neurospora/>).

Subsequently, the available sequence from linkage group I sequence for NTA and for NTA were each defined as a database and compared with the *N. crassa* mRNA gene set using BLASTN (<http://www.ncbi.nlm.nih.gov/>). Note that X's were assigned for regions not covered by the Solexa sequencing. The *N. crassa* gene with the lowest e-value (with a cutoff of $e \leq 10^{-6}$) for a particular region of NTA and for NTA linkage group I was considered a match. The genomic region contained within and surrounding each match for NTA and for NTA was isolated and manually aligned to the matching *N. crassa* mRNA using CLUSTALW (Thompson *et al.*, 1994). Introns and/or indel sequences identified within the NTA and NTA sequence from the alignment were removed. Genomic regions from NTA and NTA, which matched an entire mRNA from *N. crassa*, including the start codon, the stop codon and which did not contain unknown or ambiguous nucleotides were identified for our analysis. Each NTA and NTA gene was translated to ensure the accurate identification of reading frames. For *N. discreta*, the putative open reading frames from JGI (generated from assembled ESTs and genomic data) were compared against the 2309 genes from *N. crassa* using TBLASTX (<http://www.ncbi.nlm.nih.gov/>), and the gene with the lowest e-value was used for identification (with a cutoff of $e \leq 10^{-6}$).

Alignments were conducted between the gene sequences for NTA, NTA, *N. crassa* and *N. discreta*, with *N. crassa* used as the template for the identification of all open reading frames. Only genomic regions in NTA, NTA, *N. crassa* and *N. discreta* fully corresponding to regions in *N. crassa* were included in analysis. Thus, the genes identified herein are not always the complete mRNA regions (for example, there may be an insertion in NTA, NTA or *N. discreta* that is not inherent to *N. crassa*), and do not include premature stop codons, but nonetheless we refer to them using the term 'genes' herein. Altogether, we identified 207 genes in NTA, NTA and *N. discreta* that fully match the complete mRNA sequence from *N. crassa*, which were used in our investigation. The DNA sequences for the *N. tetrasperma mat a* and *mat A* linked alleles for each of the 207 genes identified herein are available in FASTA format at Dryad (<http://dx.doi.org/10.5061/dryad.8429>; note that the sequence names that end in P4492_a and P4492A in this file are from the *mat a* and *mat A* chromosomes, respectively). The reads data are available from the Sequence Reads Archive at NCBI (<http://www.ncbi.nlm.nih.gov/sra/>) under the accession numbers SRR099222 (P4492_a) and SRR099270 (P4492A).

The location of each of the 207 genes on the *N. tetrasperma mat* chromosomes was determined using the gene order for *N. crassa*. Genes were subsequently categorized as being located in stratum 1, stratum 2, pseudoautosomal region 1 or pseudoautosomal region 2. These categorizations were based on demarcation points within the *mat* chromosomes previously reported by Menkis *et al.* (2008) (see Supplementary Table S1). These previous findings, dividing the regions of the chromosome into stratum 1, stratum 2 and the PA regions, were based on the fact that gene alleles are identical or nearly identical in normally recombining genomic regions in a highly inbred taxon such as *N. tetrasperma* (Menkis *et al.*, 2008) and that that genes located in the recombination-

ally suppressed regions have greater synonymous divergence among their alleles over time (Lahn and Page, 1999; Handley *et al.*, 2004; Sandstedt and Tucker, 2004; Menkis *et al.*, 2008).

Synonymous and non-synonymous nucleotide substitution rates

For each of the 207 genes identified above, we determined the synonymous (*dS* or *Ks*) and non-synonymous (*dN* or *Ka*) substitution rates among the *mat a* and *mat A* chromosomes in *N. tetrasperma*. The values were determined for genes located in stratum 1, stratum 2, and for each of the two pseudoautosomal regions. Substitution rates were determined using DNASP (Librado and Rozas, 2009).

Identification of allele-specific codon substitutions

To more fully understand the dynamics of the *mat* chromosome divergence, we examined each of the 82 793 codon positions spanning all 207 genes to identify allele-specific codon substitutions located on the *N. tetrasperma mat a* and *mat A* chromosomes. Allele-specific codon substitutions were utilized to reveal whether there has been independent accumulation of genomic changes on the *mat a* and *mat A* chromosomes. For this analysis, we identified each codon position wherein *N. crassa* and one *N. tetrasperma mat* linked allele contained the same codon (ancestral codon), and the other *N. tetrasperma mat* linked allele contained a different codon (note that *N. crassa* is more closely related to *N. tetrasperma* than *N. discreta* (Dettman *et al.*, 2003), therefore *N. crassa* was used for this purpose). Allele-specific codon substitutions that were associated with the same amino acid on both *N. tetrasperma mat* chromosomes were identified as allele-specific synonymous codon substitutions. Allele-specific codon substitutions that were associated with a change in the amino acid on one *N. tetrasperma mat* chromosome and not the other were identified as allele-specific non-synonymous codon substitutions. For comparisons among genes and genomic regions, the number of allele-specific codon substitutions was standardized per 1000 sites as follows: number of allele-specific codon substitutions/total number of codons examined $\times$ 1000.

In addition to the analysis of allele-specific codon substitutions, we also assessed all possible categories of codon substitutions among the *N. tetrasperma mat* chromosomes. These include codon positions showing no evidence of substitutions, those with substitutions that were identical among chromosomes, and those positions having different codons on both *mat* chromosomes.

Branch model analyses

Differences in ω between the species over evolutionary time were investigated by using the codeml program of phylogenetic analysis by maximum likelihood (PAML; Yang, 2007). For this, the branch-model mode (model = 2, NS = 0) was utilized to test for differences in ω in the NTA and NTA branches (as a group) relative to the *N. crassa* and *N. discreta* branches, the latter two of which exhibit free recombination along the *mat* chromosomes. The tree files used for this comparison were ((NTa no. 1, NTA no. 1), *N. crassa* no. 1, *N. discreta* no. 1) versus ((NTa no. 1, NTA no. 1), *N. crassa* no. 2, *N. discreta* no. 2), wherein different numbers within a single tree file represent lineages permitted to have different ω values. Likelihood-ratio tests were conducted among the two models (*df* = 1). An elevated ω for the branches for NTA and NTA was tested both for concatenated data of the region of suppressed recombination and for the pseudoautosomal regions; the latter case was used to reveal whether the pseudohomothallic *N. tetrasperma* mating system/life history in itself is associated with an elevated ω as compared with the heterothallic species *N. crassa* and *N. discreta*, or whether differences in ω are only associated with the *N. tetrasperma* recombinationally suppressed regions.

Branch-model analysis was also used to reveal differences in ω among *N. tetrasperma mat a* and *mat A* linked alleles for each of the individual 207 genes examined in this study. For this, two models were compared; one where two distinct ω were permitted for the NTA and NTA branches, and another with a single ω for the NTA and NTA branches. The tree files utilized in phylogenetic analysis by maximum likelihood for these analyses were ((NTa no. 1, NTA no. 1), *N. crassa*, *N. discreta*) versus ((NTa no. 1, NTA no. 2), *N. crassa*, *N. discreta*).

Results

Identification and characterization of chromosomal regions

A total of 207 genes were identified for our study. On the basis of the demarcations within the *N. tetrasperma mat* chromosomes reported by Menkis *et al.* (2008), we found that 168 genes are located in the recombinationally suppressed region of the *N. tetrasperma mat* chromosomes. Among these, 27 genes are located in the younger stratum 2 (11 631 codon positions) and 141 genes in the older stratum 1 (54 506 codon positions). A total of 39 genes are located in the pseudoautosomal regions, with

Table 1 The number of genes located on the *mat* chromosomes of *Neurospora crassa*, *N. discreta* and *N. tetrasperma* that were identified and examined in the present study

<i>Mat</i> chromosomal region	Number of genes identified	Total number of codon positions	Mean <i>dS</i> NTA vs NTA per gene (std error)	Mean <i>dN</i> NTA vs NTA per gene (std error)
PA1	18	5618	0.0003 (0.0003)	0 (0)
Stratum 2	27	11 631	0.0046 (0.0018)	0.0009 (0.0004)
Stratum 1	141	54 506	0.0190 (0.0015)	0.0019 (0.0002)
PA2	21	11 038	0 (0)	0 (0)
Total	207	82 793		

Abbreviations: Nta, *N. tetrasperma mat a*; NTA, *N. tetrasperma mat A*; PA1, pseudoautosomal region 1; PA2, pseudoautosomal region 2. The *dN* and *dS* values among the *N. tetrasperma mat a* and *mat A*-linked alleles are provided. A total of 207 genes were examined.

18 genes in pseudoautosomal region 1 (5618 codons) and 21 genes in pseudoautosomal region 2 (11038 codons) (Table 1 and Supplementary Table S1). We found that the mean *dS* values among the *N. tetrasperma* *mat a* and *mat A*-linked alleles were markedly higher for genes located in stratum 1 (mean (std error (s.e.))_{Stratum 1} = 0.0190 (0.0015)) than for genes located in stratum 2 (mean (s.e.)_{Stratum 2} = 0.0046 (0.0018)), and was lowest in the pseudoautosomal regions (equal or near equal to zero (mean (s.e.)_{PA1} = 0.0003 (0.0003), mean (s.e.)_{PA2} = 0 (0)) (Table 1); this pattern is consistent with previous findings by Menkis *et al.* (2008) of a greater allelic sequence divergence with extended time periods of recombination suppression. The genes from the pseudoautosomal regions, and from the recombinationally suppressed stratum 1 and stratum 2 regions are listed in sequential order that they occur on the *mat* chromosomes in Supplementary Table S1.

The mean *dN* value among the *N. tetrasperma* *mat a* and *mat A*-linked gene alleles was markedly lower than *dS* values for genes in stratum 1 (mean (s.e.)_{Stratum 1} = 0.0019 (0.0002), representing about 10% of *dS*). The effect is magnified in stratum 2, where *dN* was only 2% of *dS*; in addition, the mean values of *dN* are markedly lower (mean (s.e.)_{Stratum 2} = 0.0009 (0.0004)) and the variation is much higher in stratum 2 than stratum 1 (s.e. is 44% of the mean in stratum 2). No non-synonymous substitutions were detected in the normally recombining pseudoautosomal regions, consistent with normal recombination and selection pressures. In totality, these data suggest that marked time-dependent protein sequence divergence has occurred among alleles of genes located in stratum 1 and stratum 2 (that is, higher divergence in stratum 1 than stratum 2), while no such differences are inherent to the pseudoautosomal regions (Table 1).

Mat allele-specific protein evolution

To further reveal the dynamics of the *mat* chromosome divergence (Table 1), we examined each of the 82793 codon positions spanning all 207 genes to identify codon substitutions located on the *N. tetrasperma* *mat a* and *mat A* chromosomes (relative to *N. crassa*). The data show that the vast majority of codon positions in stratum 1, stratum 2 and the pseudoautosomal regions show no evidence of codon changes (>932 codon positions per 1000; see category 1 in Table 2). This is not surprising given that these species are closely related, with <6 Mya as divergence from a common ancestor (believed to approximately correspond to the formation of stratum 1, Menkis *et al.*, 2008). We also identified numerous codon substitutions that were shared among the *N. tetrasperma* *mat* chromosomes relative to *N. crassa* (Categories 2 and 3 in Table 2). These types of homoallelic changes could result from a codon switch in either the *N. tetrasperma* or the *N. crassa* lineages. We noted that the level of homoallelic synonymous and non-synonymous substitutions each decreased in the following manner: PA > stratum 2 > stratum 1 (Table 2). This trend is coincident with an uninterrupted history of normal recombination in the pseudoautosomal regions and a longer history of normal recombination in stratum 2 than in stratum 1 of the *N. tetrasperma* *mat* chromosomes. Thus, many of these homoallelic substitutions in stratum

Table 2 The different categories (Cat) of synonymous and non-synonymous codon substitutions among the *N. tetrasperma* *mat a* and *mat A* chromosomes

Cat	mat	Allele-specific substitution	Type of change	NC	NTa	NTA	Pseudoautosomal region 1		Stratum 2		Stratum 1		Pseudoautosomal region 2	
							No. codons	No. per 1000 codons	No. codons	No. per 1000 codons	No. codons	No. per 1000 codons	No. codons	No. per 1000 codons
1	No	None		Codon1 (AA1)	Codon1 (AA1)	Codon1 (AA1)	5238	932.36	10990	944.80	51513	945.08	10422	944.19
2	No	Shared Synonymous		Codon1 (AA1)	Codon2 (AA1)	Codon2 (AA1)	333	59.27	522	44.88	1712	31.41	515	46.66
3	No	Shared Non-synonymous		Codon1 (AA1)	Codon2 (AA2)	Codon2 (AA2)	45	8.01	81	6.96	363	6.66	101	9.15
4	Yes, <i>mat a</i>	Non-synonymous		Codon1 (AA1)	Codon2 (AA2)	Codon1 (AA1)	0	0	6	0.52	116	2.13	0	0
5	Yes, <i>mat A</i>	Non-synonymous		Codon1 (AA1)	Codon1 (AA1)	Codon2 (AA2)	0	0	13	1.12	117	2.15	0	0
6	Yes, <i>mat a</i>	Synonymous		Codon1 (AA1)	Codon2 (AA1)	Codon1 (AA1)	0	0	11	0.95	320	5.87	0	0
7	Yes, <i>mat A</i>	Synonymous		Codon1 (AA1)	Codon1 (AA1)	Codon2 (AA2)	2	0.35	8	0.69	350	6.42	0	0
8	Yes (excluded) ^a	Non-synonymous Both		Codon1 (AA1)	Codon2 (AA2)	Codon3 (AA3)	0	0	0	0	5	0.09	0	0
9	Yes (excluded) ^a	Synonymous Both		Codon1 (AA1)	Codon2 (AA2)	Codon3 (AA3)	0	0	0	0	10	0.18	0	0
Total number of codons							5618		11631		54506		11038	

Abbreviations: AA, Amino acid; NTA, *N. tetrasperma* *mat a*; NTa, *N. tetrasperma* *mat A*.

The type of substitution was determined based on comparison of codons and amino acids (AA) to the closely related and normally recombining *mat* chromosomes from *N. crassa* (NC). Data is based on concatenated sequences for each genomic region. Allele-specific substitutions are in bold. Codons and amino acids followed by different numbers within a single category are indicative of a substitution.

^aCodon positions that have switched to different codons on both the *mat a* and *mat A* chromosomes were not included in allele-specific counts and were categorized separately.

1 and in stratum 2 likely originated in the *N. tetrasperma* lineage and were fixed in the time period before recombination suppression. Accordingly, these data are consistent with a greater frequency of homoallelic substitutions on the *N. tetrasperma* *mat* chromosomes with extended periods of normal recombination.

The primary focus of our examination of concatenated sequences across all 82793 codon positions was to identify allele-specific codon substitutions on the *N. tetrasperma* *mat a* and *mat A* chromosomes. These include codon positions wherein *N. crassa* and one *N. tetrasperma* *mat*-linked allele contained the same codon (ancestral codon), and the other *N. tetrasperma* *mat*-linked allele contained a different codon (Categories 4, 5, 6 and 7 in Table 2). Our results reveal evidence of the independent accumulation of protein level changes in the recombinationally suppressed region of the *mat a* and *mat A* chromosomes. For example, we found ~2.13 allele-specific non-synonymous substitutions per 1000 codons in stratum 1 of the *mat a* chromosome (Table 2). A similar value of 2.15 was detected within the *mat A* chromosome (Table 2); these findings were attributable to different (allele-specific) substitutions on each chromosome. Similar findings were detected for stratum 2, wherein 0.52 and 1.12 allele-specific non-synonymous codon substitutions were identified per 1000 codons on the *mat a* and *mat A* chromosomes, respectively. This is consistent with independent accumulation of genomic changes in this chromosomal region. The level of allele-specific non-synonymous and allele-specific synonymous codon substitutions were each found to be markedly higher in stratum 1 than in stratum 2 (Table 2 Supplementary Figure S1), consistent with a longer time period of recombination suppression in stratum 1. Overall, these

data further support the conclusion that the *mat* chromosomes have independently accumulated substitutions at the protein level in a time-dependent manner within the region of suppressed recombination.

As a further analysis, we determined the frequency of allele-specific non-synonymous codon substitutions on the *N. tetrasperma* *mat* chromosomes for each of the 207 genes examined herein. These results reveal that 37% of genes in stratum 2 have at least one allele-specific non-synonymous codon substitution, similar to the fraction of genes with synonymous codon substitutions (not necessarily the same genes, Figure 1). In contrast, in stratum 1, 66.2% of genes contain at least one allele-specific non-synonymous codon substitution, which is marginally lower than for synonymous substitution (79%). No allele-specific non-synonymous substitutions have occurred in genes located in the pseudoautosomal regions (Figure 1). In sum, these data show that there has been time-dependent and independent accumulation of allele-specific non-synonymous codon substitutions on the *mat a* and on the *mat A*-linked alleles for the majority of genes located in the recombinationally suppressed region (Figure 1).

Correlation coefficients among allele-specific substitutions per gene in stratum 2 revealed a strong-positive correlation between non-synonymous and synonymous changes on the *mat A* chromosome ($R=0.71$, $P=2.2 \times 10^{-5}$), suggesting that many codon sites may be evolving neutrally in this region. No such correlation was found for the *mat a* chromosome in stratum 2 ($R=-0.007$ $P=0.972$), indicating that there may be marked differences in how the two *mat* chromosomes evolve in this genomic segment. Weaker, but statistically significant correlations were detected in stratum 1 (*mat a*

Figure 1 The number of allele-specific codon substitutions per gene (per 1000 codons) on the *N. tetrasperma* *mat a* chromosome and on the *mat A* chromosome (based on comparison to *N. crassa*). (a) Number of allele-specific non-synonymous codon substitutions. (b) Number of allele-specific synonymous codon substitutions. The percentage of genes in each genomic region having at least one allele-specific substitution (on either of the *mat* chromosomes) is provided. Genes are listed on the Y axis in the order they occur on the *mat* chromosomes. The various segments of the *mat* chromosomes, including pseudoautosomal region 1 (PA1), stratum2, stratum 1 and pseudoautosomal region 2 (PA2) are indicated on the Y axis.

$R = 0.236$ $P = 0.004$; *mat A* $R = 0.174$ $P = 0.040$), suggesting that there is a less direct and more complex relationship between synonymous and non-synonymous allele-specific codon substitutions in this genomic region, possibly reflecting variation in selection pressures.

Branch-model analysis

Phylogenetic analysis by maximum likelihood (PAML) branch-model analysis was utilized to assess whether the switch in mating-system and/or the onset of recombination suppression in the *N. tetrasperma* *mat* chromosomes (NTa and NTA as a group) are associated with changes in ω as compared with its close relatives *N. crassa* and *N. discreta*. The results show no differences in ω among *N. tetrasperma* (*mat a* and *mat A* lineages) and *N. crassa* and *N. discreta* for the concatenated gene sequences from the two normally recombining pseudoautosomal regions (Table 3). One would expect differences in the branch-model analysis in the pseudoautosomal region if mating system in itself (pseudohomothallism) alters ω in *N. tetrasperma*; thus, this finding suggests that mating system does not substantially alter ω . In contrast, however, we found that the concatenated gene sequences of the *N. tetrasperma* *mat* chromosomes have a statistically significantly higher ω value in the recombinationally suppressed regions, stratum 1 and stratum 2, than the homologous genes in the heterothallic and normally recombining species *N. crassa* and *N. discreta* (Table 3). Thus, the totality of these findings reveals that there is an elevated ω in the recombinationally suppressed regions of *N. tetrasperma* *mat* chromosomes as compared with the homologous normally recombining regions in *N. crassa* and *N. discreta*, and that this result is directly associated with recombination suppression in *N. tetrasperma* and not the pseudohomothallic mating system.

We subsequently used branch-model analysis to assess differences in ω between *N. tetrasperma* *mat a* and *mat A*-linked alleles for all genes examined in this study. For the analysis, we focused on the older region of suppressed recombination, stratum 1 ($N = 141$ genes), as the younger stratum 2 is insufficiently diverged among *mat* chromosomes to give reliable estimates among these chromosomes (Table 1; Yang, 2007). The results from the branch-model analysis of concatenated *N. tetrasperma* sequences for the *mat a* and *mat A* chromosomes for stratum 1 show that ω does not differ among the *mat* chromosomes (constrained versus unconstrained models $P = 0.3482$, Table 4; note that only the *N. tetrasperma* branch lengths, which are being tested, are shown). This finding, of similar ω for both *mat* lineages does not suggest that there are not major differences among the *mat a* and *mat*

A chromosomes, but rather suggests that ω has shifted independently and to similar levels on both chromosomes (for *mat a* and *mat A*, $\omega = 0.1321$ and 0.1132 , respectively). That is, this can be inferred given that allele-specific non-synonymous substitutions are prevalent on both the *mat a* and on the *mat A* chromosomes in stratum 1 (Tables 1 and 2, Figure 1, Supplementary Figure S1). We also found that the ω values are markedly < 1 in stratum 1 for each of the *mat* chromosomes, which is consistent with a history of purifying selection. Nonetheless, given that the values of ω in stratum 1 are markedly higher ($\omega > 0.1121$) than for the pseudoautosomal regions (wherein dN and thus $\omega = 0$, Tables 1 and 4), the results suggest that there has been relaxed selection and/or positive selection in the recombinationally suppressed region.

Examination of each of the 141 individual genes in stratum 1 reveals that ω is substantially higher on one of the *mat*-linked alleles than the other (that is, *mat a* versus *mat A*) for 16 genes ($P < 0.1$ Table 4, Supplementary Table S2). We used P -values of < 0.1 for discerning genes with substantial differences in ω because lower values are overly conservative for our purposes to detect lineage-level differences in ω (as ω represents a global value per branch). These 16 genes span a range of functions, including genes encoding hypothetical proteins (predicted from genomic data, but not supported by expression analyses), predicted proteins (evident from genomic and expression data) and genes involved in mitochondrial processes (NCU02502, NCU07362) and RNA splicing and/or synthesis (NCU02633, NCU02675) (<http://www.broadinstitute.org/annotation>). We found that the allele with the higher values of ω occurred on the *mat a* chromosome for some of the genes and on the *mat A* chromosome for others, and this trait was gene-specific (Table 4). We also found that ω values are < 1 for *mat a* and *mat A* lineages for most of these genes, suggesting that both gene alleles have evolved under purifying selection (Table 4). The findings of higher ω on one allele in each of these genes strongly suggest that there has been relaxed purifying selection, which gives rise to the enhanced fixation of slightly deleterious mutations on one of the copies. Another potential factor that could also contribute to different ω values among gene alleles is that one allele has been subjected to positive selection (Gerrard and Filatov, 2005).

Discussion

Previous reports have shown that alleles of ancient non-recombining sex chromosomes have elevated non-synonymous substitution rates as compared with the gametologs found on their homologous recombining chromosome (that is, Y- versus X-linked alleles in humans and W- versus Z-linked alleles in birds (Fridolfsson and Ellegren, 2000; Wyckoff *et al.*, 2002)). The few available studies in young or neo-sex chromosomes have also revealed signs of genomic degeneration in regions of suppressed recombination, including accelerated protein evolution (Bachtrog, 2003; Marais *et al.*, 2008). A key indicator of reduced molecular level adaptation in recombinationally suppressed genes is elevated non-synonymous substitutions (Bachtrog, 2003; Marais *et al.*, 2008). Accordingly, we conclude that our findings showing the accumulation of allele-specific

Table 3 Branch model analysis of ω for *N. tetrasperma* *mat a* and *mat A* (NTa, NTA) versus *N. crassa* and *N. discreta* (NC, ND)

Chromosomal region	Direction of difference in ω	P-value
PA1	NA	0.354
Stratum 1	(NTa, NTA) > (NC, ND)	$< 10^{-4}$
Stratum 2	(NTa, NTA) > (NC, ND)	$< 10^{-4}$
PA2	NA	1

Abbreviations: NA, not applicable; Nta, *N. tetrasperma* *mat a*; NTA, *N. tetrasperma* *mat A*; PA1, pseudoautosomal region 1; PA2, pseudoautosomal region 2.

P -values of likelihood ratio tests (LRTs) are provided.

Bold values are statistically significant.

Table 4 Genes in stratum 1 with markedly different ($P < 0.1$) dN/dS (ω) ratios among the *Neurospora tetrasperma* *mat a* and *mat A*-linked alleles based on the branch model analysis in PAML

Gene	Gene description ^a	$\omega_{mat\ a}$ ^b	$\omega_{mat\ A}$ ^b	Direction of difference	LRT P-value
Concatenated sequences of all stratum 1 genes		0.1321	0.1142	$\omega_{mat\ a} > \omega_{mat\ A}$	0.3482
<i>Specific genes with differences</i>					
NCU02502	Hypothetical protein similar to S-adenosyl-methionine-sterol-C	>0.0001	0.0001	$\omega_{mat\ a} > \omega_{mat\ A}$	0.0514
NCU02510	Clathrin heavy chain	0.0001	0.3233	$\omega_{mat\ A} > \omega_{mat\ a}$	0.0427
NCU02633	Hypothetical protein similar to nuclear mRNA splicing factor	>0.0001	0.0001	$\omega_{mat\ a} > \omega_{mat\ A}$	0.0010
NCU02675	Hypothetical protein similar to RNA-3'-phosphate cyclase family protein	0.0001	0.13	$\omega_{mat\ A} > \omega_{mat\ a}$	0.0558
NCU02761	Hypothetical protein	>0.0001	0.0001	$\omega_{mat\ a} > \omega_{mat\ A}$	0.0534
NCU09382	Predicted protein	0.0001	0.6752	$\omega_{mat\ A} > \omega_{mat\ a}$	0.0496
NCU09317	Predicted protein	>0.0001	0.0001	$\omega_{mat\ a} > \omega_{mat\ A}$	0.067
NCU09310	Conserved hypothetical protein	>0.1311	0.1311	$\omega_{mat\ a} > \omega_{mat\ A}$	0.0254
NCU03152	Conserved hypothetical protein	0.3087	0.0001	$\omega_{mat\ a} > \omega_{mat\ A}$	0.0978
NCU09142	Hypothetical protein similar to cytoplasmic dynein intermediate chain	0.1328	0.0001	$\omega_{mat\ a} > \omega_{mat\ A}$	0.0496
NCU08348	Conserved hypothetical protein	0.0001	0.5494	$\omega_{mat\ A} > \omega_{mat\ a}$	0.0338
NCU07362	Hypothetical protein similar to mitochondrial cytochrome b2	0.3567	0.0001	$\omega_{mat\ a} > \omega_{mat\ A}$	0.0025
NCU00921	Conserved hypothetical protein	0.4264	>0.4264	$\omega_{mat\ A} > \omega_{mat\ a}$	0.0936
NCU00771	Conserved hypothetical protein	0.0001	0.3085	$\omega_{mat\ A} > \omega_{mat\ a}$	0.0111
NCU00499	All development altered-1	>0.0001	0.0001	$\omega_{mat\ a} > \omega_{mat\ A}$	0.0964
NCU03013	Anchored cell wall protein-10	0.0001	>0.0001	$\omega_{mat\ A} > \omega_{mat\ a}$	0.0592

Abbreviations: LRT, likelihood ratio test; PAML, phylogenetic analysis by maximum likelihood.

P-values of likelihood ratio tests (LRT) are provided.

^aHypothetical proteins are predicted from genomic data, but have no evidence of expression to date; predicted proteins have evidence of expression from ESTs or other mRNA analysis.

^bCases with a value beginning with > indicates that the dS level was very low (<0.0001) or equal to zero while dN was markedly >0 , wherein PAML assigns no estimate of ω (that is, a default value of 999), but only indicates whether a statistically significant difference is present and the direction of the difference.

non-synonymous substitutions in the region of suppressed recombination *N. tetrasperma* *mat* chromosomes (Figure 1, Table 2) are best explained by genomic degeneration in this young system.

The marked accumulation of allele-specific substitutions reported herein (Figure 1 and Table 2) combined with the variation in ω among the *N. tetrasperma* *mat* chromosomes, suggest that relaxed purifying selection is inherent to the region of suppressed recombination. Hill-Robertson effects, background selection and/or Muller's Ratchet may have a role driving these genomic changes in the region of suppressed recombination (Hill and Robertson, 1966; Charlesworth and Charlesworth, 2000; Gordo and Charlesworth, 2001). Rare events of positive selection could also substantially contribute to these genomic alterations (Bachtrog, 2004). In particular, positive selection (that is, selective sweeps) for strongly beneficial mutations might lead to the fixation of linked deleterious mutations in recombinationally suppressed regions (Charlesworth and Charlesworth, 2000; Bachtrog, 2004). Although we did not find evidence of positive selection herein, adaptive evolution likely affects only a few sites and is often not detectable from branch-model analysis (Yang *et al.*, 2000; Yang and Nielsen, 2002; Yang, 2007). Thus, as more genomic data becomes available across a broader range of species, future studies may reveal whether site-specific positive events are associated with the region of suppressed recombination (Yang and Nielsen, 2002; Yang, 2007). Altogether, it is evident that both relaxed purifying selection (and/or background selection, Muller's Ratchet) and positive selection models could each have a role in shaping the genomic alterations in the region of suppressed recombination in *N. tetrasperma* *mat* chromosomes.

Pattern of genomic changes

In contrast to ancient dimorphic X/Y and W/Z sex chromosome systems, wherein only one chromosome is recombinationally suppressed and degenerates, our results reveal that each of the *N. tetrasperma* *mat* chromosomes has independently undergone changes consistent with degeneration. This is evidenced by the prevalence of allele-specific non-synonymous substitutions on each of the two *mat* chromosomes, and by the fact that the allele with higher ω value could occur on either the *mat a* or *mat A* chromosome (Figure 1 Table 4). The most readily evident explanation for our observations of genomic alterations on each of the *mat* chromosomes is the fact that both chromosomes are recombinationally suppressed in *N. tetrasperma*, which differs from ancient dimorphic sex chromosomes. Nonetheless, another possible contributing factor for the genomic changes on both *mat* chromosomes is the novel mating system inherent to *N. tetrasperma*. In *N. tetrasperma*, both of the *mat* chromosomes (*mat a* and *mat A*) are normally present in the sexual spores and in vegetative cells, consistent with a predominant heterokaryotic state (Jacobson, 1995). Thus, the evolution of the *mat* chromosomes in *N. tetrasperma* may parallel that expected for organisms with haploid mating systems, wherein the sexes are haploid and the zygote is diploid. In such systems, genomic degeneration is expected to occur at similar, and low, levels among sex chromosomes, because of extensive haploid selection and the lack of sheltering of mutations (Bull, 1983). Our data are consistent with degeneration on both *mat* chromosomes, but not necessarily at the very low levels predicted for haploid systems (Figure 1 and Table 2). Accordingly, it may be speculated that the recombinationally suppressed regions of the *N. tetrasperma* *mat* chromosomes

might evolve in a hybrid manner, sharing traits associated with ancient dimorphic sex chromosomes, such as a high level of degeneration, as well as those inherent to haploid systems, for example, degeneration on both chromosomes. Thus, even though the data presented here suggest that mating system does not alter evolution in the normally recombining regions of the *N. tetrasperma* *mat* chromosomes (as shown in Table 3), it could substantially contribute to the pattern of divergence among alleles in the recombinationally suppressed region. Further data from a range of very young sex chromosomes will be needed to ascertain whether a hybrid system of evolution is inherent to a range of organisms.

The role of sex-specific biases

It is worthwhile to note that most studies of sex chromosome evolution to date have been conducted in diecious species, wherein male and female gametes are confined to different individuals. In many of these organisms, sex-specific biases in mutation rates and sex-specific selection pressures have been widely reported for example, humans, mice, birds and plants (Filatov and Charlesworth, 2002; Bachtrog, 2004; Ellegren, 2007). These biases are believed to contribute to the degeneration of the permanently heterozygous chromosome, especially in systems with male heterogamety. For example, in *Drosophila*, male-driven mutation rates, resulting from greater number of cell divisions in male than female germlines, are believed to contribute to Y chromosome sequence divergence at synonymous sites, while positive selection events for male fitness in this taxon are believed to be at least partially responsible for degeneration at non-synonymous sites (Bachtrog, 2004, 2005, 2008). In contrast to these systems, however, sex-specific mutation and selection biases cannot contribute to our findings in *N. tetrasperma*, because each individual in this organism is able to produce both male and female reproductive structures, and the sexual spores contain both the *mat a* and *mat A* chromosomes (Raju and Perkins, 1994; Jacobson, 1995). Accordingly, our findings of changes consistent with degeneration on both *mat* chromosomes in *N. tetrasperma* suggest that sex-specific mutation rates and/or sex-specific selection pressures are not required for genomic alterations in sex-regulating chromosomes. Recombination suppression alone is sufficient to explain the observed genomic changes.

Conclusions

In totality, the present data provide important new insights into the evolution of the young region of recombination suppression in the *N. tetrasperma* *mat* chromosomes. These insights include findings that the region of recombination suppression in the *mat* chromosomes accumulates non-synonymous substitutions in a time-dependent manner, that each of the *mat* chromosomes are subjected to genomic changes consistent with degeneration, that the *mat* chromosomes evolve independently and have marked levels of allele-specific substitutions, and that these processes are likely driven by weakening of purifying selection (possibly because of Hill-Robertson effects, background selection and/or Muller's Ratchet) as well as by rare positive selection events. As more data becomes available, further studies across a greater number of species, and among populations, will be highly valuable in precisely defining the relative roles of relaxed purifying selection and positive

selection. In addition, studies of transpositions, insertions/deletions and other degenerative traits in the *N. tetrasperma* *mat* chromosomes are warranted to reveal whether these types of degenerative changes also accumulate in a manner similar to non-synonymous mutations.

Conflict of interest

The authors declare no conflict of interest.

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