Molecular evolution of a sex-linked inversion polymorphism in zebra finches

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* joint contribution

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Significance Statement

Inversion polymorphisms are often found associated with complex traits, acting as supergenes. Here, we explore the molecular evolution and gene expression of a large, Z-linked inversion polymorphism in a passerine bird, the zebra finch (*Taeniopygia guttata*). We find patterns of sequence divergence consistent with relaxed purifying selection and mutation accumulation. This result illustrates the molecular costs of reduced recombination in inversion polymorphisms.

ABSTRACT

Recent years have seen an explosion in examples of supergenes, where recombination is suppressed between haplotypes, often via inversion polymorphisms, to control complex traits. However, an enduring problem in evolutionary biology is understanding the molecular consequences of recombination suppression, especially when it has been present for long periods. Here, we explore the molecular evolution of the A and B haplotypes of a large, sex-linked inversion polymorphism in a passerine bird, the zebra finch (*Taeniopygia guttata,*) responsible for most of the genetic variation in sperm morphology. We find evidence for reduced efficacy of purifying selection acting on the coding sequence of the Z-linked haplotypes and an increase in mutational load. However, we fail to find a positive association between expression and sequence divergence, consistent with an absence of any compensatory expression evolution for the accumulation of deleterious alleles.

INTRODUCTION

In recent years, numerous studies have shown that chromosomal inversions can be a genetic cause of phenotypic polymorphisms (Charlesworth 2023). Prominent examples have been reported in plants (Lowry and Willis 2010), invertebrates (Koch et al. 2021; Wang et al. 2013), fish (Meyer et al. 2024; Pearse et al. 2019), mammals (Harringmeyer and Hoekstra 2022), and birds (Tuttle et al. 2016; Küpper et al. 2016; Lamichhaney et al. 2016; Huynh et al. 2011; Thomas et al. 2008). Often, these inversions are large, spanning 10s of megabases and containing many (tens-to-hundreds) of genes. Because meiotic recombination is usually suppressed between inversion haplotypes, linked alleles are co-inherited and segregate as if they are a single Mendelian locus; i.e. they behave as a supergene (Berdan et al. 2022; Charlesworth 2024). Supergenes are physically linked sets of co-adapted alleles, which in the absence of recombination become increasingly divergent through the effects of both drift and selection. If a supergene contains many genes, it can be responsible for variation in complex phenotypes comprising multiple traits (Schwander et al. 2014; Thompson and Jiggins 2014). It has long been recognised that heterozygotes at supergenes often have a fitness advantage as they do not express deleterious recessive alleles fixed on either haplotype (Sturtevant and Mather 1938; Charlesworth 2024; Durmaz et al. 2020). Inversion polymorphisms can be regarded as a specific, perhaps the most common, form of supergene (Thompson and Jiggins 2014).

An ongoing problem in evolutionary biology is understanding how inversion polymorphisms persist in populations. Their evolution is often described as having two distinct stages (Faria et al. 2019; Durmaz et al. 2020; Berdan et al. 2023). Firstly, a newly arisen mutation must become established (i.e. increase in frequency) in the population (Durmaz et al. 2020). Many inversion mutations are likely to cause problems during meiosis, resulting in aneuploidy (Anton et al. 2005). These mutations will typically have low fitness and are likely to be eliminated by selection. For a new inversion mutation to become established, it must either confer a fitness benefit and be under positive selection or, if the effective population size is low, be selectively neutral and increase in frequency by genetic drift (Durmaz et al. 2020; Faria et al. 2019; Berdan et al. 2023). Perhaps the best-known model is the 'epistatic coadaptation' model (Charlesworth and Charlesworth 1973) whereby a new inversion captures a high-fitness combination of two alleles at a pair of loci with epistasis. In heterokaryotypes, the lack of recombination ensures that the high fitness haplotype is not broken up, and so it increases in frequency. Similar two-locus models rely on dominance rather than epistatic effects. For example, if deleterious recessive alleles are found on different haplotypes, then an inversion could mean heterokaryotypes are masked from homozygosity of these recessive alleles and have a greater fitness than homokaryotypes (Sturtevant and Mather 1938). However, it has been suggested that it is harder for dominance-based models to meet the conditions required for initial positive selection (Charlesworth 2024).

Recent attention has shifted to the molecular evolution of inversions, in particular, the consequences of recombination suppression. Among these studies, some consistencies have been found. For example, an increase in the number of structural variants, transposable elements or repetitive DNA sequences on derived, rare inversion haplotypes has been interpreted as a build-up of mutational load in the butterfly *Heliconius numate* (Jeong et al. 2022a), in white-throated sparrows (Jeong et al. 2022a) and in fire ants *Solenopsis invicta (Stolle et al. 2019)*. However, this pattern is not ubiquitous; see, for example, the inversion that causes alternative mating strategies in the ruff *Calidris pugnax* (Hill et al. 2023). It is also common to observe lower sequence diversity within derived, inverted haplotypes relative to ancestral haplotypes (Pracana et al. 2017), sometimes strikingly so (Jeong et al. 2022a). This presumably reflects a low effective population size, a lack of gene flux and a low frequency of inversion homozygotes within which recombination can occur. It should be noted that a low haplotype diversity on an inversion haplotype could also be a consequence of positive selection or the haplotype being recently introgressed from a different species (Hill et al. 2023). Ratios of nonsynonymous and synonymous coding differences between ancestral and derived haplotypes typically show an elevation of nonsynonymous divergence, relative to patterns seen elsewhere in the genome (Jay et al. 2021; Jeong

et al. 2022a; Pracana et al. 2017), which is typically taken as a sign of mutation accumulation and the degeneration of the derived haplotype. Again, the ruff inversion polymorphism appears to be an exception, with the rate of nonsynonymous and synonymous divergence being very similar (Hill et al. 2023).

If an inversion haplotype has undergone mutation accumulation, then it might be expected that gene expression evolves adaptively to compensate for the degeneration of coding sequences. One way to test for this is within individuals that are heterozygous for an inversion polymorphism; the more degenerated allele should have reduced expression. In both fire ants (Martinez-Ruiz et al. 2020) and white-throated sparrows (Jeong et al. 2022a) a negative relationship between the number of nonsynonymous substitutions and allele-biased expression on genes within an inversion has been observed. Typically, the derived inversion haplotype is the one with reduced gene expression and more nonsynonymous changes. i.e. the putatively degenerated allele has lower expression.

In this paper, we explore the molecular evolution and gene expression of a large, Z-linked inversion polymorphism in a passerine bird, the zebra finch (*Taeniopygia guttata*). The Z chromosome harbours a large (~60Mbp) pericentric inversion polymorphism (Itoh and Arnold 2011; Knief et al. 2016) which encompasses the majority (~85%) of the Z chromosome, and completely suppresses crossing over in heterozygotes. There are at least three distinct inversion haplotypes, A, B and C, where A is the ancestral form, which are found in populations that are thought to have diverged 1.2 to 2.8 million years ago (Balakrishnan and Edwards 2009; Knief et al. 2016; Kim et al. 2017). Despite representing just 7% of the genome, these karyotypes are responsible for 67-90% of the additive genetic variance in sperm morphology in male finches of both wild and captive populations (Knief et al. 2017; Kim et al. 2017). The sperm of heterozygous males, specifically AB and AC, have the greatest motility, determined by an intermediate midpiece-to-tail ratio relative to homozygotes (Kim et al. 2017; Knief et al. 2017; Bennison et al. 2016), and harbour more copies of mitochondrial DNA (Knief et al. 2021). This greater sperm motility results in increased fertilisation success in within-pair matings and greater paternity success in extra-pair matings (Fisher 2017; Knief et al. 2017; Kim et al. 2017; Knief et al. 2016; Birkhead et al. 1988), which seems likely to have helped maintain the inversion as a balanced polymorphism through heterozygote advantage (Fisher 2017; Knief et al. 2017; Kim et al. 2017). The greater reproductive success of heterozygous males during sperm competition may have further helped maintain the polymorphism. No relationships between inversion haplotype and other life history or morphological traits have been identified (Assersohn et al. 2024; Knief et al. 2016), despite the large number of genes (greater than 800) within the inversion. Thus, heterozygote advantage seems to mostly operate through sperm traits, rather than a suite of other phenotypes.

The sex-linkage of the zebra finch inversion polymorphism also complicates predictions about its molecular evolution. First, the effective population size is reduced relative to an autosomal inversion, meaning new deleterious alleles could become even more readily fixed by genetic drift (Mank et al. 2010; Wright et al. 2015; Vicoso and Charlesworth 2009). This could accelerate the rate of mutation accumulation on each different haplotype. Second, if there are sexually-antagonistic mutations arising on the Z chromosome, male-beneficial dominant alleles and female-beneficial recessive alleles are the most likely to go to fixation (Rice 1984; Charlesworth et al. 1987). This could increase the rate at which new mutations go to fixation on the inversion haplotype they first appear on, but the lack of recombination means they will be restricted to that haplotype. Thus, over time, each Z-linked haplotype will accumulate different beneficial alleles, but only heterozygous males reap the benefit of carrying all of those alleles. Under both scenarios, which are not mutually exclusive, heterozygote advantage is likely to increase over time, resulting in maintenance of the inversion polymorphism.

The aim of this paper is to explore the molecular evolution of the A and B haplotypes of the zebra finch Z chromosome inversion polymorphism. In particular, we explored patterns of selection following recombination suppression by examining the level of genetic diversity within each inversion haplotype and the divergence between them. A further aim was to compare rates of nonsynonymous and synonymous substitution rates, and to explore whether this would yield further patterns consistent with reduced purifying selection, background selection, or mutation accumulation.. Finally, we measured gene expression in the testes of AA and AB males to explore whether there was evidence of allele-specific biased gene expression in a pattern consistent with mutation accumulation.

RESULTS AND DISCUSSION

Using a high-density genotyping dataset of 477 homokaryotype individuals that we had previously generated for the zebra finch (Kim et al. 2017) (Table S1), we identified 2,123 SNPs to karyotype the Z-linked inversion and distinguish three haplotypes, A, B and C. We karyotyped 24 individuals for which whole genome sequencing data were available using these diagnostic SNPs (Singhal et al. 2015) (Table S2). In total, we scored 8 birds as inversion haplotype A or AA (4 females, 4 males), 7 birds as inversion haplotype B or BB (6 females, 1 male), 1 female bird as inversion haplotype C, 4 male birds as AB heterozygotes, and another 4 males as AC heterozygotes (Figure S1). Additionally, we generated a separate gene expression dataset with another 10 birds, sequenced using Illumina paired-end RNA-seq and, using diagnostic SNPs within these data, categorised

three as female B birds, two as female A birds, three as AB heterozygous males, and two as AA homozygous males (Table S3).

Characterising the structure of the Z-linked inversion

To first characterise patterns of Z chromosome diversity, we calculated π for non-coding sites, using a combination of intronic and intergenic sites, across the bTG1.4 reference genome for the 15 AA/A and BB/B individuals (Singhal et al. 2015), and compared patterns of diversity within and between haplotypes. Previous work showed that the inversion regions encompass the majority of the ~75Mb Z chromosome, apart from approximately 5 Mbp at either end (Knief et al. 2016, 2017; Kim et al. 2017). The regions outside of the inversion can act as a useful control to test for deviation from patterns of Z chromosome evolution under neutrality. Linkage mapping studies show that most of the Z chromosome recombination occurs at the end of the chromosome, even in homokaryomorphs (AA or BB males). Thus, given the well-documented relationship between recombination rate and diversity (Begun and Aquadro 1992), the expectation is that diversity will be greater outside of the inversion than within it. In accordance with patterns expected from ongoing recombination, we found that nucleotide diversity was markedly higher at the ends of the Z (0-6.50Mb and 70.10-75.39Mb) (Figure 1a) - more than triple the mean across the inversion region and only slightly reduced relative to the genome-wide average (Table 1). We also found that mean d_{XY} and F_{ST} between the A and B haplotype at the ends of the Z outside of the inversion were similar to autosomal values (Table 1) (Figure 1 a & b). Whilst we might expect that diversity should be reduced on the Z relative to the autosomes given its lower effective population size (Irwin 2018), there are multiple processes that may account for the patterns we observe at the ends. First, as the Z exists 2/3rds of the time in males, it will be exposed to an elevated male-biased mutation rate, somewhat compensating for the reduced Ne (Bergeron et al. 2023). Second, the elevated diversity outside of the inversion may be attributed to the very high recombination rate at each end of the Z chromosome, where it exceeds recombination rates observed across most autosomal regions (Backström et al. 2010). A similar pattern is also described in the great reed warbler *Acrocephalus arundinaceus*, where male-recombining regions of the Z-chromosome exhibit elevated neutral diversity relative to autosomes, attributed to elevated recombination rates at the ends of the Z chromosome (Ponnikas et al. 2022).

Next, within the inversion, when diversity was measured in the A and B birds separately it was lower still, as expected due to their reduced rates of recombination. Diversity was especially reduced in birds with the B haplotype (Table 1), in keeping with its lower frequency, both in the wild, and in captive populations (Knief et al. 2017), and the fact that it is presumably younger than the A haplotype. Mean F_{ST} between A and B haplotypes within the inversion region was exceptionally elevated relative to the autosomes or the Z regions flanking the inversion. Additionally, d_{XY}, an absolute

measure of divergence, was not elevated between the two haplotypes - in fact it was ~6 times lower than on the autosomes (Table 1). This is indicative of a region of reduced within-population diversity, especially in the B haplotype, suggesting either its recent origin, a selective sweep or very effective background selection (although data at nonsynonymous sites, see below, would seem to rule out this last explanation). These results are not a consequence of unequal numbers of A and B haplotypes; the pattern holds when the A haplotypes are down-sampled to be equal to the number of B haplotypes (Figure S2).

Next, we explored the sequence differences that have accumulated between the A and B inversion. We identified a total of 363 fixed differences (Table S4) within the coding regions of 203 of the 753 protein-coding genes on the Z chromosome. All of the fixed coding region differences are found in genes within the inversion polymorphism. Because there are no fixed differences outside of the inversion, it is not possible to perform a chi-square test to compare the numbers outside and inside the inversion. However, there are a total of 628 protein-coding genes inside and 125 protein-coding genes outside the inversion. There is no difference in coding sequence length within or outside of the inversion (protein-coding genes; within inversion mean CDS length = 1899, outside inversion mean CDS length = 1915, $t = 0.084$, $P = 0.93$, d.f. = 604). If the rate of nonsynonymous and synonymous fixed differences outside of the inversion was the same as the observed rate inside, then we would expect to see 27-45 (95% confidence interval) nonsynonymous and 27-44 synonymous fixed differences outside of the inversion. Unsurprisingly, the observed number (zero of each) is significantly lower than this null expectation ($P = 2.6*10^{-19}$, nonsynonymous; $P = 3.5*10^{-19}$, synonymous). Taken together, the patterns of diversity and divergence on the Z suggest that the region outside of the inversion is similar to the autosomes and that the inversion region is very different. Thus, the patterns within the inversion cannot be regarded as typical of general features of the Z chromosome (Vicoso and Charlesworth 2009; Oyler-McCance et al. 2015).

Tests of selection in the inversion region

Sequence differentiation as pronounced as we observe between A and B haplotypes might be interpreted as the consequence of several processes. First, positive selection may be acting on advantageous alleles on one or both haplotypes. Second, background selection, whereby diversity is reduced at neutral sites linked to sites under purifying selection, is expected to be more efficient in regions with low recombination rates, which could result in a lack of diversity within each haplotype and elevated divergence between haplotypes. Given we observe low nucleotide diversity (π), elevated relative divergence (F_{ST}), but reduced absolute divergence (d_{XY}) in the inversion region, which is

known to have a low recombination rate, background (purifying) selection may have been acting independently on the haplotypes (Cruickshank and Hahn 2014; Wolf and Ellegren 2016). However, given the very low recombination rate following an inversion event, we might also expect a relaxed response to purifying selection due to Hill-Robertson interference and an increase in mutational load.

To test these hypotheses, first, we calculated a series of population genetic site frequency selection (SFS) statistics including Fay and Wu's H, Tajima's D, and Zeng's E. D and H identify regions under positive selection by respectively searching for regions with a depletion or enrichment of rare variants, and by comparing the level of high frequency to intermediate frequency variants. E overcomes D and H's lack of sensitivity to other factors affecting the site frequency spectrum by looking at intermediate frequency variants (Zeng et al. 2006). These SFS statistics were estimated for A and B haplotypes independently then compared between the inversion and outside of the inversion as well as to those autosomes of a similar size and sharing a similar recombination landscape (Chromosomes 1, 1a, 2, 4, 5, and 6) (Backström et al. 2010). Notably, for all statistics, we found less deviation from 0 (neutrality) within the inversion relative to either the autosomes or the Z chromosome outside of the inversion (Figure 2 & S3). Thus, there is no compelling evidence for recent selective sweeps on the A or B haplotypes.

Next, we compared patterns of polymorphic sites and fixed differences within and across the A and B haplotypes (Table 2). First, we found that patterns of coding region polymorphism were significantly different between the inversion and non-inversion region for both A and B haplotypes (χ^2=275.01, d.f. =1, P=<0.00001 ; χ^2=217.68, d.f. =1, P=<0.00001 respectively). Specifically, in the inversion region of A and B haplotypes, nonsynonymous polymorphisms were more prevalent than synonymous ones, whereas the opposite was observed outside of the inversion breakpoints (Table 2). Nonsynonymous mutations are usually selected against by purifying selection, and so this excess in the inversion region could be caused by a relaxation of purifying selection or, if these changes are adaptive, by ongoing positive selection acting on each haplotype.

To distinguish between these two alternate models, we employed the McDonald Kreitman test (McDonald and Kreitman 1991). If positive selection were acting, we would expect a greater proportion of nonsynonymous fixed differences relative to nonsynonymous polymorphisms. However, we found no significant relationship between the ratio of nonsynonymous to synonymous polymorphisms in A haplotypes relative to fixed differences between A and B haplotypes (χ^2=0.84, d.f.=1, P=0.360). Whilst we did find a significant relationship when comparing polymorphism in B haplotypes to A vs B fixed differences (χ^2=8.06, d.f.=1, P=0.005), it was opposite to that expected under positive selection, with more nonsynonymous polymorphisms than fixed differences.

Notably, this inversion is pericentric (Itoh and Arnold 2011) and evidence from various species indicates that the suppression of crossing over in pericentromeric regions results in reduced variability and reduced efficacy of natural selection, due to various types of Hill-Robertson interference processes (Charlesworth and Jensen 2021).

Taken together, these independent lines of evidence point towards an increased mutational load on the Z-linked inversion haplotypes in zebra finches. None of the population genetic tests we ran gave strong evidence for recent or ongoing positive selection and instead suggest either effective background selection or relaxed purifying selection acting on the A and B haplotypes. Consistent with this, whilst the relative number of Z-linked nonsynonymous polymorphisms outside of the inversion boundary does not differ between A and B haplotypes (χ^2=0.43, d.f. = 1, P=0.510), B haplotypes exhibit relatively more nonsynonymous polymorphisms than A within the inversion region (χ^2=9.19, d.f. =1, P=0.002). This is consistent with the expectation that the rarer inversion haplotype will experience greater mutation accumulation (Charlesworth 2024).

Interestingly, in Arctic cod (Matschiner et al. 2022), genetic exchange between haplotypes of inversions responsible for migratory lifestyle has been shown to counter the accumulation of deleterious mutations, leading to long term polymorphism maintenance. However, the zebra finch supergene is located in a region of low recombination, with linkage mapping studies showing low recombination in this part of the Z chromosome (Stapley et al. 2008; Backström et al. 2010; Stapley et al. 2010). Instead, our data are consistent with Jay et al.'s (Jay et al. 2021) interpretation of inversion polymorphisms in Heliconius, where initially advantageous structural variants increase in mutational load as a result of recombination suppression. This may lead to patterns of heterozygous advantage with contributions from both the initially overdominant loci and the masking of subsequent deleterious recessive alleles. Recent work in the fire ant may exhibit the extreme end of this process (Pracana et al. 2017), where the supergene appears to be maintained as a result of an extreme relaxation of purifying selection, the accumulation of deleterious alleles (in fire ants Sb homozygotes are usually lethal), and associative overdominance protecting heterozygotes from recessive lethals.

Gene expression is conserved between Z-linked haplotypes

Differential expression of supergene haplotypes has been observed in several species (Berdan et al. 2021; Jeong et al. 2022b; Martinez-Ruiz et al. 2020; Sun et al. 2018) as well as cis- and trans-regulatory effects of `the inversion across the whole genome (Arsenault et al. 2023). We might expect selection to result in the downregulation of expression of A and B alleles that have independently accumulated deleterious nonsynonymous mutations. Indeed, in fire ants, alleles of the SB haplotype tend to be more highly expressed than the Sb allele mirroring patterns of sequence degeneration

(Martinez-Ruiz et al. 2020). Similarly, sequence degeneration is greater for differentially expressed than unbiased genes on mating-type chromosomes of the anther-smut fungus *Microbotryum* (Ma et al. 2020), and reduced gene expression is associated with the early stages of sex chromosome decay (Pucholt et al. 2017). Given that we find an increase in mutational load on the coding sequence of the Z-linked haplotypes, we tested whether expression is evolving to compensate for the accumulation of deleterious mutations or is likewise susceptible to drift.

We find a handful of genes with significant differential gene expression between the testes of AB and AA males (2.51%) or ovaries of A and B females (1.35%) within the inversion (Table S5, Figure S4), consistent with previous expression studies in the zebra finch (Viitaniemi et al. 2023; Kim et al. 2017). However, this proportion, relative to the autosomes, was only weakly significant for the comparison between AB and AA males ($\chi^2 = 7.55$, $P_{MCMC} = 0.010$) and was borderline significant for the A and B female comparison ($\chi^2 = 4.36$, $P_{MCMC} = 0.049$). Together, this is consistent with a lack of large-scale expression divergence on the Z.

To next test whether gene expression buffers the potentially deleterious impacts of higher mutational load in genes that have accumulated greater numbers of nonsynonymous mutations, we investigated patterns of allele bias expression within heterozygous AB individuals. Importantly, this analysis also mitigates issues arising from comparisons of bulk expression values between samples across heterogeneous tissues, such as the gonad. Such analyses can be problematic due to differences in cellular composition (Price et al. 2022; Montgomery and Mank 2016), and either mask differential expression or produce false signatures of expression change. This is particularly relevant for our male AB and AA comparisons, where we might expect differences in the cellular composition of the testes (Lüpold et al. 2020). As we now compare expression of different alleles within the same sample, this goes some way to minimise biases arising from variation in tissue structure between individuals. However, an enrichment of allele-specific expression of the Z could be a product of the inactivation of a single Z chromosome due to dosage compensation, although there is limited evidence that this occurs in birds (Mank and Ellegren 2009; Segami et al. 2022). To test this, we examined allele-specific expression in AA males. We did find a weak significant enrichment of genes exhibiting allele-specific expression on the Z relative to the autosomes 1-10 (Table S6) at sites polymorphic within the A inversion. This pattern held when analysing expression within each individual independently or when only including genes exhibiting allele-specific expression in both the two AA males (Table S6 & S7). However, the proportion of Z-linked genes with allele-specific expression was very small, where only 17 genes had allele-specific expression in both AA birds. This is consistent with previous results in the chicken gonad (Zimmer et al. 2016) and supports a lack of Z chromosome inactivation in zebra finches.

Having shown that Z chromosome inactivation is unlikely, we then tested for allele-specific expression between A and B haplotypes in heterozygous males (Table S6). Of the 200 genes on the Z we tested, only 8 exhibited significant allele-specific expression in all three AB males, none of which had known functions consistent with sperm morphology or fertility (Table S7 & S8), and only one of which (*MRPL50*), overlapped with the differentially expressed genes associated with sperm morphology identified by Kim et al. (Kim et al. 2017). However, our power to identify significant allele-specific expression may be limited by our low sample size of heterozygous males. A recent study identified 3 candidate genes with differential expression between A and B haplotypes in the testes of young zebra finches (Viitaniemi et al. 2023). However, two of these genes were not expressed in our adult testes dataset and the other mapped to the W chromosome in our reference genome. Together, this suggests that the expression of the supergene may vary through development. Importantly, in our study, the proportion of expressed genes with significant allele-specific expression on the Z inversion did not differ between homozygous and heterozygous males ($\chi^2 = 3.51$, $P_{MCMC} = 0.07$).

To test whether expression is evolving to compensate for the accumulation of deleterious mutations in coding sequences, as has been observed in other species (Martinez-Ruiz et al. 2020; Jeong et al. 2022a), we compared patterns of allele-specific expression with sequence divergence between A and B haplotypes. In contrast to the fire ant (Martinez-Ruiz et al. 2020), we found no association between the magnitude of allele-specific expression and synonymous (p=0.58) or nonsynonymous (p=0.24) sequence divergence. It is important to note that unlike the Sb of the fire ant and ZAL2m of the white throated sparrow, the A and B haplotypes both occur in homozygous states, suggesting that the need for buffering of regions of high mutational load may not be yet necessary due to ongoing recombination in homokaryotypes. Together, these results suggest that regions of high sequence divergence do not necessarily overlap with regions of expression divergence and indicate an absence of any evidence for compensatory evolution of expression.

CONCLUSION

Our analyses highlight the impact of inversion polymorphisms on the evolution of the coding and regulatory sequence of the Z chromosome. Importantly, we show an excess of nonsynonymous differences in the inversion, consistent with an increase in mutational load, but an absence of any compensatory expression evolution for the accumulation of potentially deleterious alleles.

MATERIALS & METHODS

Identifying diagnostic SNPs for the Z-linked inversion genotype

We previously genotyped male zebra finches (n=1202) from a population that was previously maintained at the University of Sheffield (Kim et al. 2017). Genotypes included 3056 SNPs spanning the Z chromosome (Kim et al. 2017). With these data, we identified markers with the most significant difference in genotype frequency between the haplotypes and were therefore highly diagnostic of the inversion karyotype. Specifically, each marker was given a weighted chi-square score ranging from 0 to 1; any SNPs with a weighted chi-square score greater than 0.9 (90% of maximum score) were included in a list of 2123 “diagnostic SNPs” (Table S1).

Variant calling

We downloaded publicly available sequence reads (www.ebi.ac.uk/ena/data/view/PRJEB10586) from 24 zebra finches (11 female, 13 male), and one female long-tailed finch (*Poephila acuticauda acuticauda*) (Table S2). All birds were originally sequenced using Illumina HiSeq 2000 paired end sequencing (read length=100bp) in 2012 and 2013 (Singhal et al. 2015).

These reads were aligned to a male zebra finch reference genome (bTG1.4) (Rhie et al. 2021) with Bowtie v2.3.4.3, using the default settings (Langmead and Salzberg, 2012). This reference genome was constructed using DNA from a male zebra finch known as “Black17”, which has been shown to be an AB heterokaryotype for the Z chromosome inversion polymorphism (Pepper 2022).

Variants were called using genome analysis toolkit (GATK) version 4.2.5.0 (van der Auwera and O’Connor 2020). Specifically, duplicate reads were removed for each individual, using GATK MarkDuplicates, before GATK HaplotypeCaller was run using aligned reads. Genotypes were then called using GATK GenotypeGVCFs. Indels were discarded whilst called SNPs were retained and then filtered for quality using GATK VariantFiltration. Parameters that were used in variant quality filtering were: variant confidence (QD) <2.0, which is intended to normalise the variant quality in order to avoid inflation caused when there is deep coverage; phred-scaled probability of strand bias (FS) >60.0 and symmetric odds ratio test for strand bias (SOR) >4.0, which both describe whether the alternate allele is seen more or less often on the forward or reverse strand than the reference allele; mapping quality of reads (MQ) <40.0, and the compared mapping qualities of reads supporting the reference and alternate allele (MQRankSum) <-12.5; and a comparison of positions of the reference and alternate alleles within different reads (ReadPosRankSum) <-8.0.

Calculation of population genetic statistics

We calculated summary statistics using R v4.2.1 and the package "PopGenomeR" v2.7.5 (Pfeifer et al. 2014). Specifically, we calculated the fixation index (F_{ST}) from minor allele frequencies (Hudson et al. 1992), as well as between haplotype diversity (d_{XY}) between the 8 zebra finches of inversion haplotype A or AA and the 7 zebra finches of inversion haplotype B or BB. Nucleotide diversity (π), Tajima's D, Fay and Wu's H and Zeng's E (a composite statistic of D and H) were calculated for birds of each haplotype. In each case, the statistic was calculated for sliding windows of 100Kb of sequence, with each window overlapping by 10Kb, across the Z chromosome. For the three neutrality test statistics (D, H & E), sliding windows were also calculated for the 30 largest autosomes (Chromosomes 1-30). For calculating Fay and Wu's H and Zeng's E, a sequence from a female long-tailed finch was used as a closely related outgroup (~6MY) (Hooper and Price 2015) to distinguish the derived and ancestral alleles at sites which are polymorphic in zebra finches. Each statistic was independently calculated with four-fold degenerate sites, zero-fold degenerate sites and non-coding silent sites (from introns or outside of genic regions).

Since there were an unequal number of A or AA birds and B or BB birds, nucleotide diversity, Tajima's D, Fay and Wu's H and Zeng's E were also all calculated using just 8 chromosomes from A or AA birds so that the number of A haplotype Z chromosome sequences was equal to the number of B haplotype Z chromosome sequences (n=8).

Identifying synonymous and nonsynonymous SNPs

Genome annotations from the NCBI *Taeniopygia guttata* annotation release 106 (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Taeniopygia_guttata/106/) were used to determine synonymous and nonsynonymous sites within coding regions on the zebra finch (bTG1.4) Z chromosome. Using PopGenomeR v2.7.5 (Pfeifer et al. 2014), SNPs found through variant calling with GATK v4.2.6.1 were sorted into those falling

within and those outside of coding sequence. Then, SNPs within coding regions were sorted into those which were fixed between A and B and at either a nonsynonymous ($N_{Dnonsyn}$) or a synonymous (N_{Dsyn}) position, and those which were polymorphic within either the A or B haplotype and at either a nonsynonymous ($N_{Pnonsyn}$) or synonymous (N_{Psyn}) position. The number of SNPs within each of these categories was then counted, and these counts were used to perform a series of chi-square tests to test for nonsynonymous and synonymous variation between the A and B haplotypes inside and outside of the inversion region. Chi-square tests of independence (df=1) were performed to examine differences in proportions of $N_{Dnonsyn}$ and N_{Psyn} polymorphisms: (a) between the two haplotypes, and (b) from the proportion of N_{Dnonsy} and N_{Psyn} fixed differences between the two haplotypes. Tests were performed within the inversion, outside of the inversion and across the entire Z chromosome (Table 2). These tests are analogous to McDonald Kreitman tests (McDonald and Kreitman 1991) except they compare divergence between the A and B haplotypes rather than between two related species.

Expression data

RNA-seq data were obtained from a captive population of zebra finches at Queen Mary University of London. Individuals were in their first breeding season, having formed breeding pairs and produced fertile eggs. Samples were collected in accordance with national guidelines. The left gonad was dissected from five males and five females, homogenised and stored in RNAlater until preparation. We used the Animal Tissue RNA Kit (Qiagen) to extract RNA. Dual-indexed, strand-specific RNASeq libraries were prepared at the NERC Environmental Omics Facility (NEOF) Liverpool using the NEBNext polyA selection and Ultra II Directional RNA library preparation kits. RNA was sequenced on the Illumina NovaSeq using S4 chemistry, resulting in an average of 74 million 150bp paired-end reads per sample.

The data were quality assessed using FastQC 0.38 and filtered using Trimmomatic v0.38. Specifically, we removed reads containing adaptor sequences and trimmed reads if the sliding window average Phred score over four bases was <15 or if the leading/trailing bases had a Phred score <3. Reads were removed post filtering if either read pair was <95 bases in length.

Genotyping individuals with RNA-seq data

Trimmed reads were aligned against the indexed bTG1.4 reference (Rhie et al. 2021) using HISAT2 v2.1.0. Using GATK v4.1.4 (van der Auwera and O'Connor 2020), reads in each BAM file were then assigned a new shared readgroup and duplicated reads were marked. Reads were then split if they contained Ns in their cigar strings. Variants were called for chromosomes 1-10 and the Z chromosome, using GATK HaplotypeCaller and filtered for a minimum depth (DP) of 10 reads, and genotyping

quality (GQ) of 30. Then samples were genotyped using the set of diagnostic SNPs we previously identified.

Quantifying differential gene expression

Salmon v1.8.0 (Patro et al. 2017) was used to quantify expression for all samples. Briefly, trimmed reads were pseudo-aligned against the bTG1.4 transcriptome, creating read count matrices. Outputs were analysed using EdgeR v3.34.1 (Robinson et al. 2010). Gene-level counts were generated and any gene with log rpkm < 2 in less than half of the individuals in each genotype was removed, following our previously described approaches (Harrison et al. 2015; Wright et al. 2018).

Next, we quantified allele-specific expression (ASE) in all samples. WASP v0.3.4 (van de Geijn et al. 2015) was used to identify reads overlapping differentiating sites, following the WASP documentation. Upon read remapping, mapping stringency was reduced to 10 mismatches (MX=10) to reduce mapping bias in regions of high divergence between haplotypes. Variants were then recalled using HaplotypeCaller and genotyped before being filtered for a minimum depth (DP) of 10 reads, and genotyping quality (GQ) of 30. Reads with more than 4 SNPs in a 95bp window were also removed (Stevenson et al. 2013) to reduce further mapping bias to the reference allele. Allele-specific expression was then quantified at the genic level using phASER v1.1.1 (Castel et al. 2016). phASER advances on SNP-based ASE detection approaches by phasing variants across an entire gene, leveraging the linkage between variants, giving a single value for the gene as opposed to for each individual SNP. For final analyses, any heterozygous gene in with log rpkm < 2, from Salmon, or with a total read count from phASER < 20 was removed. Genes were defined as having allele-specific expression if the log fold change of the expression value (plus one to handle zero count data for the A or B allele) between alleles >=1 with an FDR-adjusted p-value < 0.05. To evaluate whether the absolute level of ASE was associated with the number of synonymous or nonsynonymous differences, we fitted generalized linear mixed models using glmmTMB (Brooks et al. 2017). The model used was:

AbsASE ~scaled(no_syn or nonsyn)/exon_length) + (1|gene_name) + (1|sample)

where *no_syn/nonsyn* represents the count of synonymous or nonsynonymous differences controlled by total exon length. Random intercepts for gene identity and sample were included to account for non-independence within genes and across samples. Because absolute ASE values were right-skewed, we specified a Gamma distribution.

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DATA AVAILABILITY

Code to reproduce analyses can be found:
https://github.com/petedprice/SupergeneEvolutionZebraFinch

Gene expression data is deposited at: DOI: 10.5061/dryad.7pvmcvf1p

TABLES

Table 1: Population genomic statistics for the autosomes and Z chromosome

Measure	Variable Site	Autosomes Mean (±SD)	Z outside inversion Mean (±SD)	Z within inversion Mean (±SD)	A birds within inversion Mean (±SD)	B birds within inversion Mean (±SD)
π ($*10^{-3}$)	0-fold	1.318 (0.00134)	1.177 (0.00172)	0.366 (0.000576)	0.218 (0.000376)	0.057 (0.000175)
	4-fold	8.274 (0.00495)	6.914 (0.00465)	1.144 (0.00236)	0.651 (0.00133)	0.139 (0.000894)
	Non-Coding	5.671 (0.00165)	4.695 (0.00202)	0.907 (0.000566)	0.601 (0.000554)	0.110 (0.00013)
dxy ($*10^{-3}$): A vs B	0-fold	1.318 (0.00134)	1.177 (0.00172)	0.366 (0.000576)		
	4-fold	8.274 (0.00495)	6.914 (0.00465)	1.144 (0.00236)		
	Non-Coding	5.671 (0.00165)	4.695 (0.00201)	0.907 (0.000566)		
FST: A vs B	0-fold	$-3*10^{-4}$ (0.0310)	-0.0012 (0.0450)	0.388 (0.385)		
	4-fold	$-8*10^{-4}$ (0.0306)	0.0035 (0.0514)	0.388 (0.401)		
	Non-Coding	$5*10^{-4}$ (0.0114)	0.0119 (0.0433)	0.609 (0.200764)		

Table 2: Comparison of coding region polymorphism and fixed differences between A and B haplotypes

	Inside Inversion		Outside Inversion		Whole Z Chromosome		Inversion vs Outside Inversion
	N	S	N	S	N	S	
A polymorphisms	1150	1024	1584	3351	2734	4375	χ^2 = 275.01, p <0.00001
B polymorphisms	463	318	1220	2663	1683	2981	χ^2 = 217.68, p <0.00001
A vs B fixed differences	182	181	0	0	182	181	
Polymorphism in A compared to A vs B fixed differences	χ^2 = 0.84, p = 0.3600						
Polymorphism in B compared to A vs B fixed differences	χ^2 = 8.06, p = 0.0045						
Polymorphism in A compared to polymorphism in B	χ^2 = 9.19, p = 0.0024		χ^2 = 0.43, p = 0.5100		χ^2 = 6.67, p = 0.0098		

N = nonsynonymous changes, S = synonymous changes

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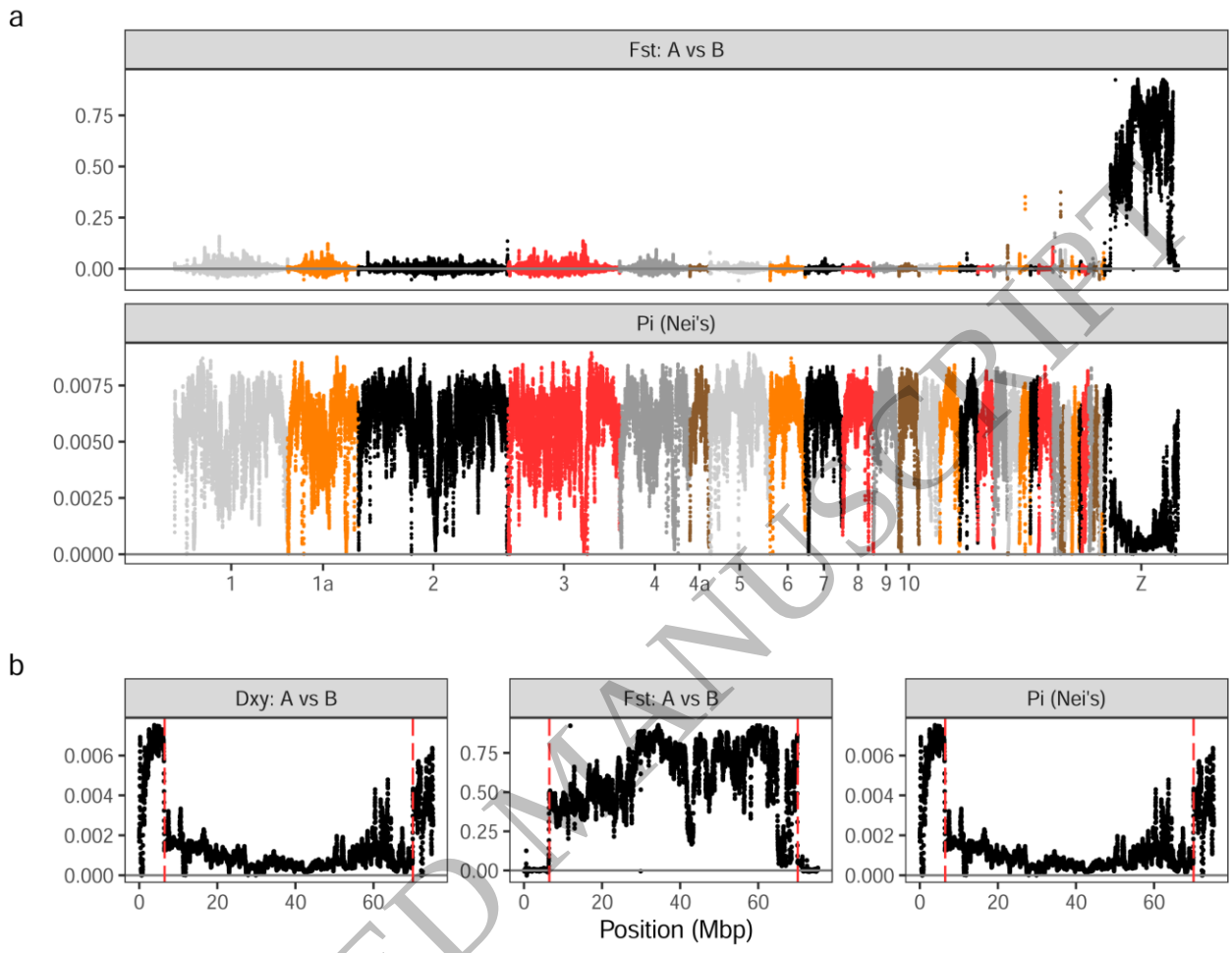

Figure 1
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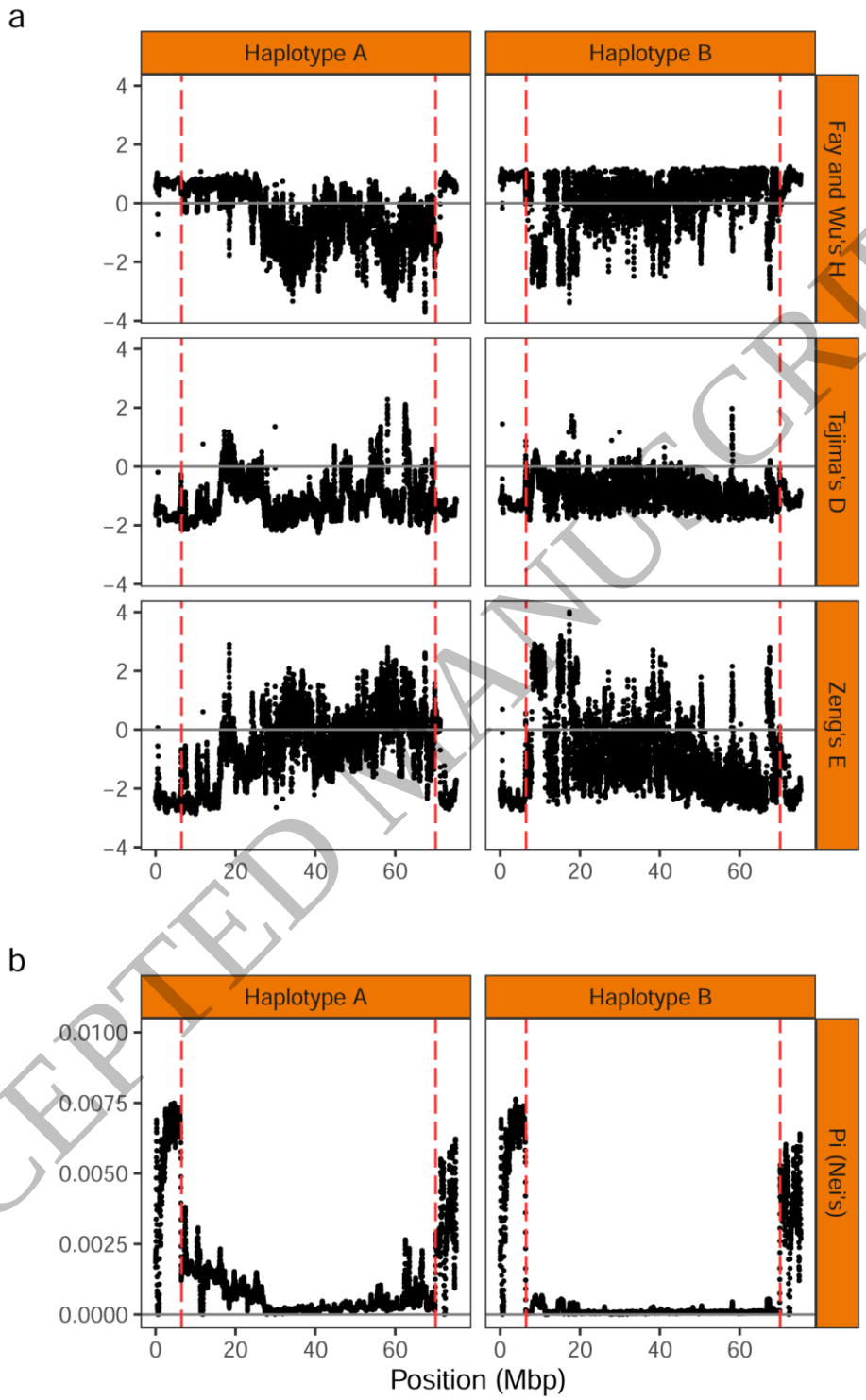

Figure 2
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