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Recovery from hybrid breakdown reveals a complex genetic architecture of mitonuclear incompatibilities

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Abstract

Reproductive isolation is often achieved when genes that are neutral or beneficial in their genomic background become functionally incompatible in a foreign genomic background, causing inviability, sterility or other forms of low fitness in hybrids. Recent studies suggest that mitonuclear interactions are among the initial incompatibilities to evolve at early stages of population divergence across taxa. Yet, the genomic architecture of mitonuclear incompatibilities has rarely been elucidated. We employ an experimental evolution approach starting with low-fitness F_2 interpopulation hybrids of the copepod *Tigriopus californicus*, in which frequencies of compatible and incompatible nuclear alleles change in response to an alternative mitochondrial background. After about nine generations, we observe a generalized increase in population size and in survivorship, suggesting efficiency of selection against maladaptive phenotypes. Whole genome sequencing of evolved populations showed some consistent allele frequency changes across three replicates of each reciprocal cross, but markedly different patterns between mitochondrial backgrounds. In only a few regions (~6.5% of the genome), the same parental allele was overrepresented irrespective of the mitochondrial background. About 33% of the genome showed allele frequency changes consistent with divergent selection, with the location of these genomic regions strongly differing between mitochondrial backgrounds. In 87% and 89% of these genomic regions, the dominant nuclear allele matched the associated mitochondrial background, consistent with mitonuclear co-adaptation. These results suggest that mitonuclear incompatibilities have a complex polygenic architecture that differs between populations, potentially generating genome-wide barriers to gene flow between closely related taxa.

KEYWORDS
co-evolution, experimental evolution, hybridization, incompatibilities, speciation

Pereira and Lima are contributed equally to this work.

1 | INTRODUCTION

Speciation occurs when populations accumulate genetic differences that cause reproductive incompatibilities in hybrids, leading to maladaptive phenotypes such as inviability, sterility or other forms of reduced fitness relative to parentals. Bateson (1909), Dobzhansky (1937) and Muller (1942) have proposed that such incompatibilities would involve two or more loci, so that substitutions that are adaptive or neutral in their own genomic background can be functionally incompatible with alleles that are present in a foreign genomic background, the so-called Dobzhansky–Muller incompatibilities or DMIs. Although this model has guided research on the genomic basis of speciation for more than 70 years, only within the last two decades have genomic regions (and in few cases specific genes) causing full sterility or inviability been identified in taxa as diverse as yeast, flies, mice and plants (Presgraves, 2010). Such genes are found to evolve rapidly, as expected for genes caught up in open-ended molecular evolutionary arms races, and are often involved in co-evolution between host and pathogen, or between selfish genes and suppressors (Presgraves, 2010). Particularly in studies with highly divergent species pairs, DMIs seem to have a complex genomic architecture that involves tens of genes spread throughout the genome (Schumer et al., 2018; Tang & Presgraves, 2009). Yet, full sterility or inviability evolve typically later during the speciation process, and thus it is unclear whether genes underlying such phenotypes reflect initial barriers to gene flow or whether they reflect other evolutionary forces that arose after strong reproductive isolation was established (Coughlan & Matute, 2020). Therefore, understanding the genetic architecture of incompatibilities causing partial reproductive isolation, such as reduced fertility, remains an important task in evolutionary biology (Corbett-Detig et al., 2013; Rafati et al., 2018; Turner & Harr, 2014).

Recent studies in taxa as diverse as arthropods, nematodes, vertebrates, yeast and angiosperms (reviewed in Burton et al., 2013; Hill et al., 2018; Sloan et al., 2017) have shown that the co-evolution between the mitochondrial and the nuclear genome often results in partially reduced fitness in hybrids between closely related taxa (i.e., species, subspecies or even populations). This observation has led several authors to suggest that mitonuclear DMIs play a disproportional role at early stages of reproductive isolation relative to the more widely studied nuclear–nuclear DMIs (Burton & Barreto, 2012; Hill, 2016). To understand why such a general pattern would arise across species, one needs to consider the origin and evolution of the mitochondrion in eukaryotes. Some 1.5 billion years ago, when a proteobacterial heterotroph (proto-mitochondrion; Martijn et al., 2018) became the obligatory endosymbiont of an archaebacterial methanogen (proto-nucleus), this symbiosis resulted in a strong functional partition (Rivera et al., 1998), where the proto-mitochondrion became responsible for metabolic functions and the proto-nucleus for transcription and translation. Because intracellular clonal replication favours smaller genomes (Clark et al., 2012; Taylor et al., 2002), a race for replication of the proto-mitochondrion led to the reduction in the endosymbiont genome size, both through the loss of redundant genes and through gene transfer to the host genome (Selosse et al., 2001; Timmis et al., 2004). Such selective pressure is still observed today in the genome evolution of intracellular endosymbionts in insects (Wernegreen, 2002), in the recent transposition of mitochondrial genes into the nuclear genome (Hazkani-Covo et al., 2010), or even in the deletion of essential mitochondrial genes with significant fitness costs for the organism (Taylor et al., 2002). Currently, in most metazoans, over 1000 proteins are housed in the mitochondrion (Calvo & Mootha, 2010), but only 13 of those remain coded in the mitochondrial genome. Hence, mitochondrial function is dependent upon nuclear-encoded proteins, many of which interact closely with mitochondrial DNA (mtDNA)-encoded proteins, RNAs or DNA-binding sites. This mitonuclear cooperation sets the stage for a tight evolutionary arms race between the nuclear and mitochondrial genomes to preserve functionality within eukaryotic evolutionary lineages in essential cell functions, such as respiration and mitochondrial protein synthesis, that have large impacts on organismal fitness. Such mitonuclear co-adaptation becomes exposed in F_2 hybrids, where independently evolving mitochondrial and nuclear alleles are forced to interact, often being functionally incompatible, disrupting organelle function and, consequently, causing hybrid breakdown (Burton et al., 2013).

Contrary to nuclear–nuclear DMIs, mitonuclear incompatibilities are expected to be led by one of the coevolving genes for a variety reasons, as described in the model of compensatory co-evolution (Rand et al., 2004). First, because of the mode of replication of its circular genome, in animals, mutation rate (μ) is typically higher in the mitochondrial relative to the nuclear genome (Allio et al., 2017). Second, due to its lack of recombination and matrilineal inheritance, mitochondrial genes have an effective population size (N_e) that is four times smaller than that of nuclear genes, resulting in higher rates of fixation via genetic drift and conversely a reduced efficiency of selection. These two processes result in the consistent observation of faster evolution rates of mitochondrial relative to nuclear genes, which are two-fold in drosophilids, 20-fold in ungulates and up to 40-fold in primates (Osada & Akashi, 2012). Even though most de novo mutations are partially deleterious, the relatively higher levels of genetic drift in mitochondrial genomes more often allow the fixation of these new mutations. This difference in μ and N_e leads to a higher fixation rate for weakly deleterious mutations in the mitochondrial genomes relative to the nuclear genome, making mitochondrial genomes more prone to the process known as "Muller's ratchet" (Lynch & Blanchard, 1998). Such accumulation of deleterious mutations in the mitochondrial genome elicits compensatory mutations in the interacting nuclear genes (Barreto & Burton, 2013a; Osada & Akashi, 2012; Sloan et al., 2014) but not in the rest of the nuclear genome, maintaining the stability and function of mitonuclear protein complexes. Although such mitochondrial deleterious mutations are effectively silenced within a population, they become exposed in interpopulation crosses where co-adapted mitonuclear complexes become mismatched in hybrids, contributing to the establishment of genetic barriers between recently diverged taxa (Sloan et al., 2017). The magnitude of such early barriers to gene

flow directly depends on the genetic architecture of mitonuclear DMIs, which remains unknown across species.

Although mitonuclear incompatibilities are now recognized to play a general role in establishing reproductive isolation between emerging species (Reinhardt et al., 2013), their effect is most visible in taxa presenting relatively high mitochondrial evolution rates, such as the copepod *Tigriopus californicus* (Willett, 2012). Allopatric divergence between populations of this species resulted in parallel patterns of genomic divergence that are consistent with mitonuclear co-evolution happening within independent populations. Protein coding genes from the mitochondria evolve 2–14 times faster than those from the nuclear genome (Pereira et al., 2016), suggesting that mitochondrial genes drive intragenomic co-evolution. Nuclear encoded proteins that functionally interact with the mitochondria evolve up to 6.5 times more rapidly than noninteracting nuclear encoded proteins (Barreto et al., 2018), suggesting that selection favouring compensatory mutations targets specific nuclear genes. Finally, experimental interpopulation F_2 hybrids show that fitness breakdown in multiple life history traits (such as fecundity, survivorship and developmental time) scales with mitochondrial divergence (Burton, 1990a; Edmands, 1999). Notably, F_2 fitness breakdown is rescued in maternal backcrosses (Ellison & Burton, 2008b), where mitochondrial and nuclear co-evolving units are rematched, demonstrating that fitness breakdown is caused by interactions between the mitochondria and unknown nuclear loci. Although intrinsic selection will lead to parallel mitonuclear co-evolution within each population, evolutionary processes conditioned by local habitat are decidedly unique to each population. For example, ecological tradeoffs along latitudinal gradients have resulted in differential adaptation to temperature (Hong & Shurin, 2015; Pereira et al., 2017; Willett, 2010) and to salinity (Leong et al., 2017). Moreover, smaller N_e at southern populations has resulted in stronger genetic drift relative to northern populations (Pereira et al., 2016), and potentially in a faster accumulation of deleterious mutations.

Recent studies with interpopulation hybrids of *T. californicus* have established that mitonuclear incompatibilities result in fitness breakdown at various organizational levels. While heterozygous F_1s are vigorous, mismatched mitonuclear complexes in F_2 and inbred lines results in: reduced mitochondrial function (Ellison & Burton, 2008a), reduced ATP production (Ellison & Burton, 2006), elevated oxidative damage to DNA (Barreto & Burton, 2013b), upregulation of pathways involved in physiological stress (Barreto et al., 2015) and breakdown at multiple life history traits (fecundity, survivorship and developmental time; Burton, 1990a; Edmands, 1999). It is still unclear whether such generalized fitness breakdown cascades from mitonuclear DMIs involving few nuclear genes with generalized effect, or involving multiple regions, spread throughout the nuclear genome. It is worth noting that this species lacks sex chromosomes, and instead has polygenic sex determination (Alexander et al., 2015; Voordouw & Anholt, 2002). Therefore, factors leading to the disproportionate role of sex chromosomes in the evolution of DMIs reported in many species (Presgraves, 2018) do not apply in this system.

Previous efforts to understand the genetic architecture of mitonuclear DMIs in *T. californicus* have focused on deviations from the expected Mendelian inheritance in F_2 hybrids surviving to adulthood (Foley et al., 2013; Lima et al., 2019; Lima & Willett, 2018), in hybrid swarms with recovered fitness (Pritchard & Edmands, 2012) or among high- and low-fitness hybrids within an F_2 cohort (Healy & Burton, 2020). Although those studies generally confirm a prominent role of mitonuclear incompatibilities relative to nuclear-nuclear DMIs (but see Pritchard et al., 2011), they differ strongly in the estimated number, location and effect size of nuclear loci interacting with the mitochondria (ranging from a few loci to all 12 chromosomes). Among several reasons, such discrepancies can be explained by population-specific accumulation of deleterious mutations in the mitochondria and hence by the nuclear genes compensating for deleterious mutations within each specific population. Understanding this potential asymmetry of mitonuclear co-evolution between populations requires testing the interactions of parental alleles with reciprocal mitochondrial backgrounds. Moreover, the length of the experiment is also a source of discrepancy between previous studies, as in long-term recombination in hybrid swarms will break down linkage between nuclear loci that remain associated in F_2 hybrids.

Here, we employ a new strategy in an effort to reveal the genomic architecture of mitonuclear incompatibilities. Starting with replicate populations of low-fitness F_2 interpopulation hybrids, we employ an experimental evolution approach where parental nuclear alleles competed in alternative mitochondrial backgrounds (established by reciprocal crosses), for about nine generations (Figure 1). To assess the efficiency of selection in small experimental populations we tested for an increase in population size and for fitness recovery in female fecundity and nauplii survivorship, which are known to be associated with mitonuclear incompatibilities. Next, to find regions probably responding to selection (uniform or divergent between treatments), we identified genomic regions with significant allelic frequency change during the experiment. Finally, to tease apart nuclear genomic regions involved in mitonuclear incompatibilities we identified regions that responded differently between reciprocal mitochondrial backgrounds. We found genomic regions probably involved in mitonuclear DMIs on multiple chromosomes and that these regions differ between mitochondrial backgrounds, suggesting that mitonuclear incompatibilities have a complex and asymmetric genetic architecture.

2 | MATERIAL AND METHODS

2.1 | Sampling

To understand the genetic architecture of mitonuclear incompatibilities we focused on two well-studied populations of *Tigriopus californicus*: San Diego (SD) and Santa Cruz (SC). These populations were suitable for a competition experiment between divergent nuclear alleles under fixed mitochondrial backgrounds because previous studies have shown that: (i) marked mitochondrial divergence (22.17%;

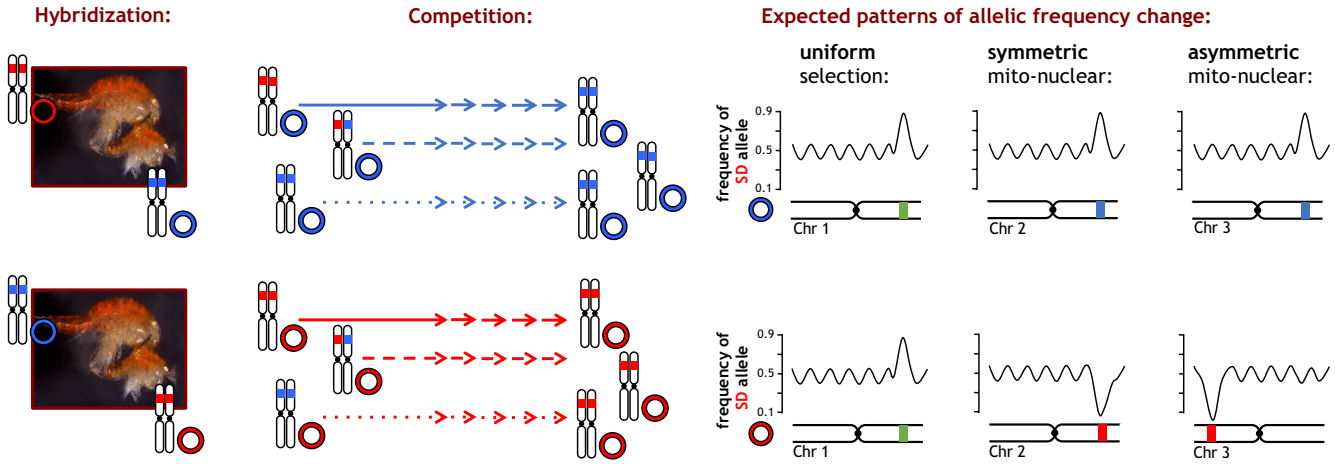

FIGURE 1 Competition experiment to reveal genomic regions involved in mitonuclear incompatibilities and their genomic architecture. Experimental hybridization results in F_1 hybrids with the same nuclear composition but fixed alternative mitochondrial backgrounds (SD in red and SC in blue). Experimental evolution starts with F_2 hybrids where recombination generated matched and mismatched mitonuclear complexes, leading to a decrease in average fitness of the starting population. Variation of individual fitness at multiple life history traits favours matched mitonuclear genotypes, leading to a recovery of life history traits and census size of the evolved populations. Comparison of allelic frequency change between lines evolving under alternative mitochondrial backgrounds allows distinguishing between genomic regions affected by uniform selection, where the favoured nuclear allele is the same irrespective of the mitochondrial background, from those affected by mitonuclear incompatibilities, where the favoured nuclear allele matched the mitochondrial background. Mitonuclear incompatibilities were labelled as "symmetric" when the same nuclear regions interact with both mitochondria, or as "asymmetric" when different nuclear regions interact with each mitochondrial lineage. In a fourth less likely scenario ("antagonistic selection," not pictured here) the favoured nuclear allele is mismatched from the mitochondrial background

Pereira et al., 2016) has driven adaptive evolution targeting genes functionally interacting with the mitochondria (Barreto & Burton, 2013a), (ii) 91% of the nuclear polymorphisms are fixed among parental populations (Pereira et al., 2016) allowing an accurate estimation of allelic frequencies in hybrids (Lima et al., 2019), (iii) F_2 hybrids show breakdown at all physiological and life history traits known to be associated with mitonuclear incompatibilities (Barreto & Burton, 2013b; Barreto et al., 2015; Ellison & Burton, 2006), and (iv) strong selection during F_2 development results in significant deviations from the expected Mendelian inheritance (Foley et al., 2013; Healy & Burton, 2020).

Stock populations were collected in San Diego (32°44′41.17″N, 117°15′19.43″W) and Santa Cruz (36°56′58.32″N, 122°2′48.98″W). Multiple cultures from each site were maintained in 400-ml beakers with large census sizes (>1000 adults) for 1 month before the beginning of the crossing design. All cultures were maintained at common garden at 20°C with a 12:12-hr light:dark photoperiod, in filtered seawater mixed with ground "Algae Wafers" (Kyorin Co.). The medium was renewed monthly.

2.2 | Experimental evolution

Tigriopus californicus is sexually dimorphic. An adult male clasps an immature female until her terminal moult, when she is then inseminated and released. Females can mate only once and use stored sperm to fertilize sequential clutches of eggs that can add to several hundred progeny (Vittor, 1971). This species lacks heteromorphic sex chromosomes and recombination occurs only in males (Burton et al., 1981). We obtained virgin females by separating clasped pairs, and produced F_1s that are heterozygous for the nuclear genome but that have fixed mitochondrial backgrounds; that is SD mitochondrial background on SD♀ × SC♂ cross, and SC mitochondrial background on SC♀ × SD♂. For each reciprocal cross, we outcrossed F_1s to produce recombinant F_2 hybrids that were allowed to mate randomly.

Each experimental line started with 100 outbred F_2 gravid females. Lines evolved under these conditions for 9 months with overlapping generations, replenishing the growing medium monthly. Since on average females reach adulthood in 2–3 weeks and produce multiple egg clutches until they are 4–6 weeks old, this experimental design corresponds to approximately nine generations of experimental evolution up to F_{11}. This procedure was replicated 10 and seven times for the SC and SD mitochondrial backgrounds, respectively. We followed the same procedure to generate one control line with fully matched nuclear and mitochondrial genomes for each parental population (i.e., SC♀ × SC♂ and SD♀ × SD♂).

2.3 | Fitness recovery

Relatively small experimental populations may lead to strong genetic drift, and conversely to limited response to selection imposed by the fixed mitochondrial backgrounds. If selection is strong relative to drift in evolved lines we expect an increase in productivity and associated recovery in one or multiple fitness traits associated with mitonuclear incompatibilities (Ellison & Burton, 2008b). To test these

hypotheses, at the end of the experimental evolution, we measured: census size (as the number of adults), fecundity (as the number of nauplii larvae hatching from the first clutch of a female) and survivorship (as the fraction of nauplii surviving to 14 days). Fecundity measurements were replicated 4–12 times, depending on the number of available virgin females, and survivorship measurements were replicated 10–28 times, depending on the number of available gravid females. To monitor how average fitness varied along the course of the experiment, we also measured survivorship 3–8 additional times, using 4–12 replicates. Additionally, we measured these two fitness traits for the initial reciprocal F_2 hybrids and for the pure parental populations, as a reference for fitness breakdown and recovery respectively. We estimated mean ± 1 *SE*. We tested for significant hybrid breakdown by comparing fitness of the F_2 hybrid with its maternal population, using a Mann–Whitney *U*-test and an alpha of 0.05 in R 2.15.1 (R Development Core Team). We tested for significant recovery in lines for which the mean reached or passed the reference parental fitness, adjusting the *p*-value when multiple comparisons occur at the same time.

2.4 | Allelic frequency change

To provide insights into the genetic architecture of mitonuclear incompatibilities we examined allelic frequency change across the whole genome in lines evolving under each mitochondrial background, relative to the initial F_2 females. We used the pool-seq approach, which has recently been established for evolve-and-resequencing studies (Schlotterer et al., 2015), including in *T. californicus* (Griffiths et al., 2020; Healy & Burton, 2020; Lima et al., 2019; Lima & Willett, 2018).

For lines evolving under each mitochondrial background, we selected three replicates that showed larger census size, no further decrease in fecundity and some recovery in survivorship. We pooled 200 adult individuals, or the maximum available, and extracted genomic DNA using the phenol chloroform protocol and digestion with RNAse (Sambrook & Russell, 2001). For the initial F_2 females, we also extracted DNA from pools of 100 adult outcross F_2 females that gave rise to the experimental populations, after removing their egg sacs. All samples were sequenced in the Illumina HiSeq 2500 platform with 100-bp paired-end libraries. Reads were trimmed for quality using POPOOLATION2 (Kofler et al., 2011), discarding bases with Phred quality scores lower than 25 and keeping reads of at least 50 bp after trimming.

We only considered single nucleotide polymorphisms (SNPs) that were fixed between natural parental populations (Barreto et al., 2018) and therefore that can be used to determine the ancestry of the nuclear alleles favoured in either mitochondrial background. We used the bioinformatics pipeline established by Lima and Willett (2018; Lima et al., 2019) (see references for details), and the syntenic reference genomes of SD and SC, where more than 90% of the genome is anchored to the 12 homologous chromosomes (Barreto et al., 2018). In short, first, we made the two reference genomes equivalent in length and accuracy by adding N's to any position where either parental reference had an "N." Second, we established a list of fixed SNPs by (i) performing reciprocal mapping of parental reads from one population (Barreto et al., 2018) against the reference of the other, (ii) considering only "fixed" positions where all mapped parental reads showed an alternative nucleotide to the reference of the other parental population (coverage ≥15) and (iii) comparing reciprocal mapping to keep only SNPs that were "fixed" in both mappings. Third, we mapped our reads for each hybrid data set to both parental references using BWA-MEM with default parameters (Li & Durbin, 2009) and keeping reads that mapped with MAPQ score >20. When mapping reads from hybrids to one of the parental references, there is a known bias towards an over-representation of reads of the reference population (Lima & Willett, 2018). By averaging the read counts for each SNP, from the alignments to each parental population, this reference-population bias is overcome. Allele counts for the fixed SD and SC alleles and respective frequencies were determined using POPOOLATION2 (Kofler et al., 2011) (minimum coverage of minor allele ≥4). Finally, data for all cross data sets were compared and only SNP that passed all filters up to this point, for both reciprocal crosses, were kept for statistical analysis of allele frequency change. These SNPs are expected to show high levels of variance, due to the variation on sequencing coverage and sampling alleles from the pool of individuals (Lima & Willett, 2018). We averaged allele counts (and frequencies) in nonoverlapping windows of 500 consecutive SNPs (average size of 54 kbp; *SD* of 30 kbp). We refer to these as "genomic windows." We choose to use a fixed number of SNPs per window, instead of fixed number of base pairs, in order to maintain statistical power across windows.

In each mitochondrial background separately, we determined which of these genomic windows show a significant allelic frequency change by comparing the initial F_2 hybrids to the evolved hybrids, using the Cochran–Mantel–Haenszel (CMH) test as implemented in the mantelhaen.test function in the statistical R package (R Development Core Team). This test operates on 2 × 2 contingency tables (times the number of replicates), comparing the counts for SD and SC alleles at the beginning and at end of the experiment, and thus it is highly sensitive to variation in sequencing coverage. To account for differences in coverage between the initial and evolved hybrids, we estimated initial counts of SD and SC alleles by multiplying the allelic frequency estimated in the initial F_2 hybrid pool by the total coverage observed at each evolved hybrid separately (i.e., counts for SD + SC). This approach results in a *p*-value per genomic window, which reflects the probability of rejecting the null hypothesis of similar allelic frequency during experimental evolution in a given mitochondrial background.

We used a *z*-score of 2 to identify genomic windows showing higher *p*-values relative to the average values observed genome wide, consistent with stronger selection driving allelic frequency change. We have estimated three *z*-score thresholds based on three distributions of *p*-values: (i) from both crosses taken together (corresponding to a $-\log_{10}$ (*p*-value) of 3.24), which allows identifying differences in the strength of selection between the

two mitochondrial backgrounds; (ii) only from crosses evolving under the SC mitochondria (corresponding to a $-\log_{10}$ (*p*-value) of 4.17), which allows identifying targets of selection within the SC mitochondrial background; and (iii) only from crosses evolving under the SD mitochondria (corresponding to a $-\log_{10}$ (*p*-value) of 1.73), which allows identifying targets of selection within the SD mitochondrial background. We considered the lower threshold to compare windows across mitochondrial backgrounds. Since adjacent genomic windows are physically linked, we looked for consecutive genome windows that are under selection. We refer to these as genomic regions. Genomic regions were classified as under selection through the following criteria: (i) at least 10 genomic windows with $-\log_{10}$ (*p*-value) ≥ 1; (ii) with at least one window above a *z*-score of 2; and (iii) ending with one window $-\log_{10}$ (*p*-value) <1. To represent which parental allele dominates during the experimental evolution, we classified genomic regions as blue for overrepresentation of the SC nuclear allele and red for overrepresentation of SD allele. By comparing allelic frequency change in reciprocal crosses, we identified genomic regions fitting three categories: "uniform selection" where the same allele dominates irrespective of the mitochondrial background; "divergent selection" where the dominant nuclear allele matches the mitochondria; and "antagonistic selection" where the dominant allele does not match the mitochondria. While we chose to use *z*-scores as threshold for selection, the results were qualitatively similar when considering a threshold based on the top 5% of the distribution of *p*-values.

3 | RESULTS

3.1 | Fitness recovery

Both control parental lines went extinct after two generations of experimental evolution. In contrast, all hybrid lines survived the 9 months of experimental evolution, with the exception of SC3, which went extinct in the 6th month. Most of the surviving lines showed an increase in population size (6/9 of the SC lines, and 6/7 of the SD lines), with census size showing an increase between 1.1-fold in SD4 to 9-fold in SD6 (Figure 2a).

Regarding fecundity (Figure 2b), F_2 hybrids had lower fitness than the parental population with the respective mitochondrial lineage, confirming F_2 hybrid breakdown in this life history trait. Hybrid breakdown was more extreme in the comparison with the SC mitochondria (p = .005), relative to that with the SD mitochondria (p = .017), consistent with stronger breakdown in the SC mitochondrial background. Of the hybrid lines that survived experimental evolution, seven SC and six SD lines had sufficient virgin females to assess fecundity. Mean fecundity of all hybrid lines was within the range observed in the respective F_2 hybrids (mean ± 1 *SE*), consistent with no or little recovery from initial hybrid breakdown.

Regarding survivorship (Figure 2c), both reciprocal F_2 hybrids had lower fitness than parental populations with the respective mitochondrial lineage, yet this was nonsignificant (p > .125), further supporting breakdown in this trait. The ± 1 *SE* intervals overlapped in SD but not in the SC comparison, again suggesting that F_2 hybrid breakdown is stronger in the SC mitochondrial background. A total of eight SC and six SD lines had sufficient gravid females to assess survivorship of nauplii. Of these, four SC lines and four SD lines had survivorship (mean ± 1 *SE*) at or above the fitness of the respective parental, suggesting recovery from the initial hybrid breakdown. These differences were significant for the SC4, SC7, SC8, SD3 and SD7 lines (respective corrected p = .001, .009, .017, .043, .043; Figure 2c).

Based on fewer replicates measured during the experiment, we observed a large temporal variability on survivorship (Figure 2d; Figure S1). The increase of survivorship was more noticeable among SC lines, where F_2 breakdown was stronger, and lines stayed for a few generations at the fitness level of their parental population.

Based on these results, for the lines evolving under the SC mitochondria, we chose to sequence SC4, SC8 and SC10 because, from lines with the highest increase of population size, these were the ones with highest survivorship at the end of the experiment. For the lines evolving under the SD mitochondria, we chose to sequence SD3, SD4 and SD6 because they showed some increase in survivorship, without a noticeable decrease in fecundity (contrary to SD7). We note that although the choice of lines is necessarily subjective, given that this experimental design did not employ direct selection on a specific phenotype, surviving lines showing population growth would be good representatives of recovery from initial F_2 breakdown.

3.2 | Allelic frequency change

We sequenced between 53 and 74 million reads per pool (BioProject PRJNA716105, BioSamples SAMN18394772-9). After quality trimming and mapping, this sequencing effort resulted in an average genome coverage of 31.9× (SC mtDNA) and 41.1× (SD mtDNA) for the initial F_2 hybrids, 33.9×, 43.1× and 35.4× for the lines evolving under the SC mitochondria, and 33.5×, 33.5× and 34.5× for the lines evolving under the SD mitochondria. After reciprocal mapping to the reference genomes of both parental populations and filtering for fixed SNPs, our approach allowed us to estimate read counts for 1,658,000 ancestry-informative SNPs, corresponding to 3316 genomic windows, and covering all 12 chromosomes of *T. californicus*.

The allelic frequencies in adult F_2 were generally close to 50% (Figure S2), varying between 38% and 64% in the F_2s with the SC mitochondria, and between 34% and 61% in the F_2s with the SD mitochondria. In contrast, allelic frequencies in hybrid lines after experimental evolution showed strong deviations from 50% (Figure S3).

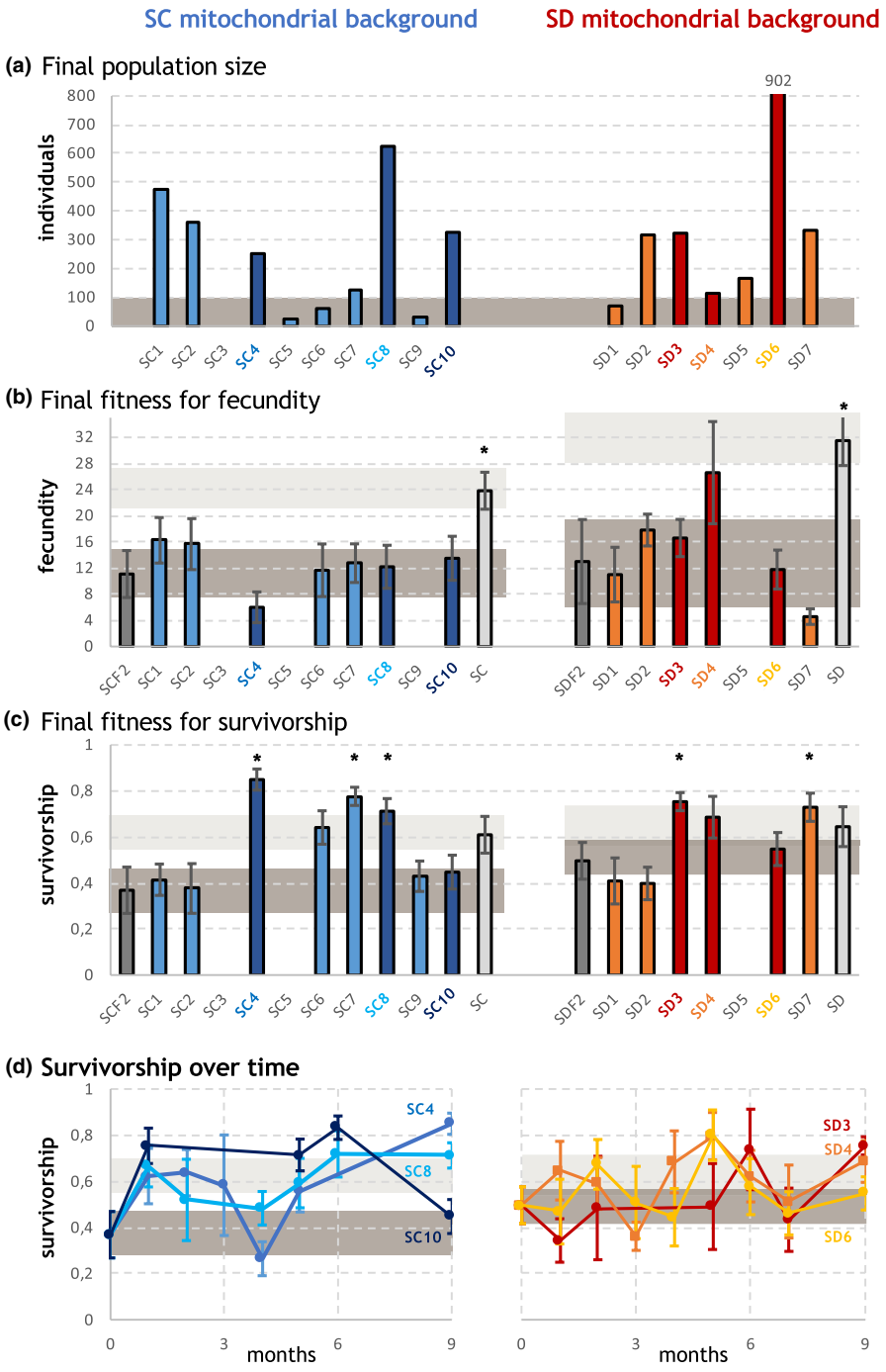

FIGURE 2 Variation of proxies for fitness during experimental evolution of hybrid lines under two alternative mitochondrial backgrounds (SC in blue, SD in red). (a) Population size expressed as the number of adult individuals. (b) Female fecundity as the number of nauplii in the first clutch. (c) Nauplii survivorship during 14 days of development. (d) Temporal change in nauplii survivorship in the lines selected for sequencing (see Figure S1 for other lines). Dark grey bars and rectangles refer to initial F_2 hybrids (mean ± 1 *SE*), and light grey refers to the reference parental population. Lines selected for sequencing are marked in darker colours. Significant differences ($p < .05$) are marked with an asterisk

This deviation was most pronounced in the lines evolving under the SC mitochondrial background, where the frequency of the SD allele ranged between 5% and 90%. The lines evolving under the SD mitochondrial background showed a more modest allele frequency change, ranging from 20% to 76% for the SD allele. Contrary to F_2s, changes in allelic frequency were variable along each of the 12 chromosomes, showing that the effective population recombination rate was enough to reduce linkage disequilibria during the 9 months of experimental evolution.

The SC lines showed the most extreme *p*-values, rejecting the null hypothesis of no allelic frequency change (Figure 3), with genomic windows reaching the threshold of $-\log_{10}$ (*p*-value) =1.73 on all 12 chromosomes. Larger genomic regions were identified in eight chromosomes, seven of which favoured the nuclear allele matching the mitochondrial background (i.e., SC allele). The only region in which the opposite allele was favoured (i.e., SD allele in chromosome 11) did not show a similar pattern in lines evolving under the SD mitochondria, consistent with the pattern expected for mitochondrial "antagonistic selection." The SD lines showed less extreme *p*-values supporting allelic frequency change (Figure 3), with genomic windows above the threshold located in six chromosomes. Larger genomic regions were identified in five chromosomes, three of which favoured the nuclear allele matching the mitochondrial background (i.e., SD allele). Notably, the

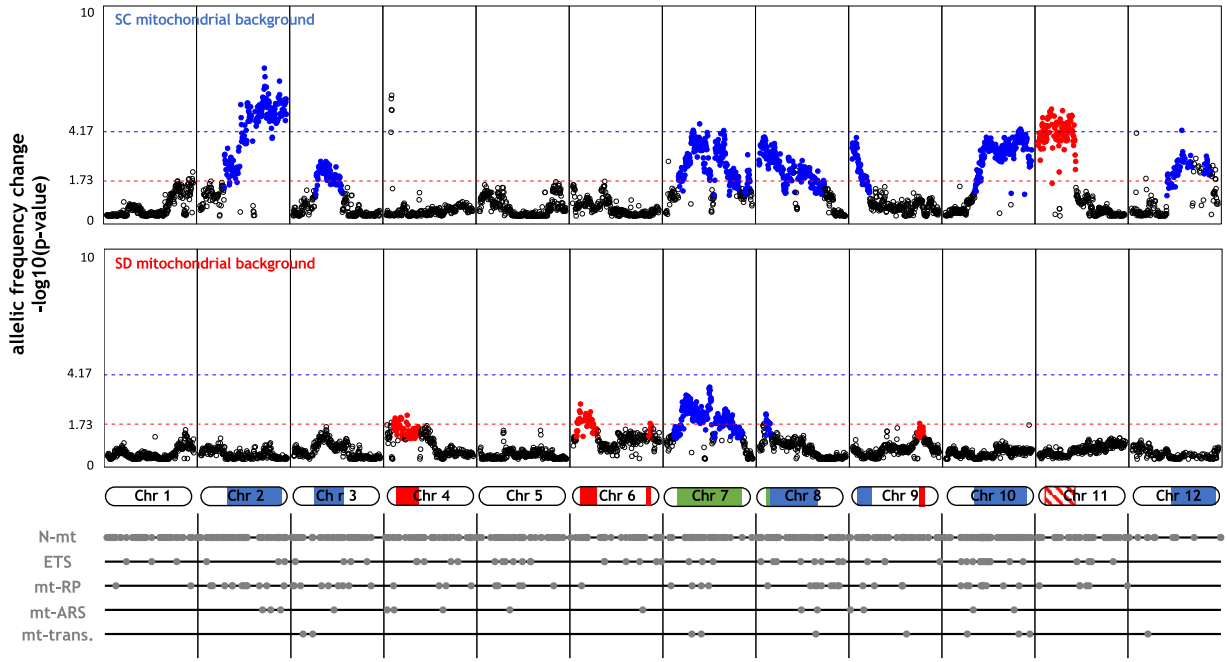

FIGURE 3 Consistent allele frequency changes identify genomic regions involved in mitonuclear incompatibilities. Genomic windows skewed towards the SC allele are demarked in blue, while windows skewed towards the SD allele are in red. The dotted lines mark *p*-value thresholds assuming a *z*-score of 2, based on the variation observed within the SC lines (blue) and within the SD lines (red). Coloured squares on the chromosomes mark genomic regions consistent with: "uniform selection" (green), "mitonuclear incompatibilities favouring co-adaptation" (blue for SC and red for SD) and "mitonuclear antagonistic selection" (dashed). Below are the locations of 599 nuclear-encoded mitochondrial genes and their function (all mitonuclear [N-mt], electron transport system [ETS], mitochondrial ribosomal proteins [mt- RP], mitochondrial aminoacyl tRNA synthetases [mt-ARS], and mitochondrial transcription and DNA replication [mt-trans.])

chromosomal regions in which the opposite parental allele was favoured (i.e., SC allele in chromosomes 7 and 8) show a similar pattern in the SC lines, consistent with the pattern expected for "uniform selection." Genomic regions responding to selection varied between 62 kbp and 6.2 Mb for the SC lines and 512 kbp and 3.2 Mb for the SD lines.

From the 3,316 genomic windows analysed here, 60.5% were not skewed in both lines and occurred in all 12 chromosomes, consistent with reduced selection and a prevalent role of genetic drift. About 6.5% of the windows showed a consistent skew towards the SC allele on both mitochondrial backgrounds, mostly in chromosome 7 and in part of chromosome 8, consistent with uniform selection favouring SC alleles. The SD alleles were never consistently favoured in both mitochondrial backgrounds. The remaining 33% of the genome show skews dependent on the mitochondrial background and are therefore consistent with divergent selection due to mitonuclear interactions.

Of the 909 genomic windows that are skewed only in the SC mitochondrial background, 88.6% show allele frequencies favouring the matching SC nuclear allele, in chromosomes 2, 3, 8,10 and 12. The remaining 11.4%, all in chromosome 11, show a skew towards the mismatched SD nuclear allele. Of the 187 genomic windows that are skewed only in the SD mitochondrial background, 87% show a skew towards the matching SD nuclear allele, in chromosomes 4, 6 and 9. The remaining 13% show a skew towards the mismatching SC nuclear allele, but notably all these windows are adjacent to windows probably under uniform selection favouring the same allele in chromosome 7.

4 | DISCUSSION

A mounting body of work has suggested that mitonuclear incompatibilities are among the first genetic barriers to evolve at early stages of population divergence across taxa (Burton & Barreto, 2012; Burton et al., 2013; Hill, 2016; Hill et al., 2018; Sloan et al., 2017, 2018), affecting multiple life history traits that render hybrids unfit. Yet, mitonuclear incompatibilities can strongly vary in their genomic architecture across species, from two genes, such as between the mt-tRNA and one nuclear tRNA synthase in *Drosophila* (Meiklejohn et al., 2013), to a network of genes, such as that between mt-rRNA and several nuclear ribosomal proteins dispersed across the genome of *Tigriopus californicus* (Barreto & Burton, 2013a). Additionally, it seems likely that the genetic architecture of mitonuclear incompatibilities will also differ between populations of the same species, since the accumulation of deleterious mutations in the mitochondria is a stochastic process, and thus would be expected to elicit compensatory evolution in different nuclear genes. Understanding the genetic architecture of mitonuclear incompatibilities at early stages of divergence can

benefit from the "evolve-and-resequence" approach developed for model organisms (Burke et al., 2014; Schlotterer et al., 2015), where adaptation to divergent environments stems from standing genetic variation generated from an initial hybrid population. In this study, we evolved low-fitness F_2 hybrids of the copepod *T. californicus*, where parental nuclear alleles are represented at similar frequencies, and monitor change in these allele frequencies after experimental evolution under two divergent mitochondrial backgrounds. We hypothesize that recovery of hybrid fitness will primarily result from selection favouring mitonuclear compatibility and that allelic frequency changes across the genome will reveal locations where such selection has acted.

4.1 | Increased productivity in hybrid lines suggests recovery from fitness breakdown

Previous long-term evolution experiments in *T. californicus* have found varying degrees of fitness recovery from F_2 breakdown (Hwang et al., 2011, 2016; Pritchard et al., 2012), presumably because hybrids with matching mitonuclear complexes would have higher generalized fitness than their siblings with unmatched mitonuclear complexes. Thus, matched (co-adapted) nuclear alleles would outcompete unmatched (incompatible) alleles over multiple generations (Figure 1). However, those experimental lines started with a mix of pure parental individuals, so that unmixed nuclear genotypes and the two mitochondrial lineages could persist well into the experiment. This design made it challenging to distinguish between fitness recovery caused by the exclusion of incompatible nuclear alleles in a hybrid background, from fitness recovery caused by competition and drift of pure parental genotypes. In contrast, we initiated experimental lines with F_2 hybrids and in fixed mitochondrial backgrounds, so that differences in fitness and allele frequency can only be explained by competition between parental nuclear alleles.

Our results show that control parental populations went extinct within 2 months of experimental evolution, probably due to inbreeding depression described in this species (Palmer & Edmands, 2000). In contrast, almost all hybrid lines survived the 9 months of experimental evolution, with most showing an increase in population size from 1.1- to 9-fold (Figure 2a). During this time, average survivorship of the hybrid lines has typically reached that of parental populations (Figure 2d; Figure S2). Yet, these results must be interpreted with caution, as hybrid lines were genetically highly heterogeneous, as evidenced by the wide confidence intervals and by temporal fluctuations of these estimates based in a small number of replicates. From the many traits known to be involved in hybrid breakdown in *T. californicus* (Burton, 1990a, b; Edmands, 1999; Edmands & Burton, 1999; Ellison & Burton, 2006, 2008a, 2010), we have assessed recovery in two using a large number of technical replicates: female fecundity and nauplii survivorship. With respect to fecundity, we observed a strong F_2 breakdown ($p < .017$), and no recovery in evolved hybrid lines, irrespective of the mitochondrial background (Figure 2b). This suggests that this is a complex trait that could not evolve in these experimental conditions of population size, number of generations and selective regime. In contrast, with respect to survivorship, we observed less severe breakdown in F_2 generation ($p > .125$). Notably, survivorship breakdown was stronger in the F_2 hybrids with the SC mitochondria, suggesting that selection imposed by mitonuclear incompatibilities is stronger in the SC relative to SD mitochondrial background. In both backgrounds, several hybrid lines recovered up to or beyond parental fitness levels ($p < .043$; Figure 2c), suggesting that recombination and competition over 9 months were sufficient to cause evolution at this trait, even though selection was not directed to this specific trait. Rescue in survivorship up to or beyond parental fitness levels has also been reported in F_9 recombinant hybrid lines of *T. californicus* (Pereira et al., 2014), suggesting that the genetic architecture of this specific trait might be relatively simple. Nevertheless, we note that the line showing the largest increase in productivity (9-fold increase in SD6) did not show recovery in survivorship, and therefore selection imposed by our experimental design is probably multifarious and responding with phenotypes beyond those measured here. Future studies employing directed selection on a specific trait (Healy & Burton, 2020) over many generations of experimental evolution may provide insights into this question.

4.2 | Allele frequency change is consistent with asymmetric mitonuclear incompatibilities

By measuring allelic frequency change from the initial F_2 hybrids to the replicated hybrid lines that evolved under each mitochondrial background, we can scan the genome for regions probably under selection, which should show a consistent change across replicates, from regions under genetic drift, which should show no or inconsistent change across replicates. Although the choice of sequenced lines is debatable, we assume that the three chosen replicates were adequate for testing our hypothesis because they all show increased in population size, and survivorship has recovered to parental fitness for multiple generations (Figure 2d). By comparing the allelic frequencies across the three replicated lines evolving under each mitochondrial background (Figure S3), we observe that few genomic regions have remained in their initial allelic frequency (Figure S2) and that most regions have changed in a different direction. This is consistent with a pervasive contribution of genetic drift across most of the genome, as expected given the relatively small size of the experimental populations. Nevertheless, we found some genomic regions with consistent allelic frequency change in two or the three replicates of each reciprocal cross (Figure S3). Such pattern suggests that in some genomic regions directional selection is stronger than drift. Yet, given the initial relatively small size of the experimental populations (100 outbred gravid females) and the low number of replicates sequenced here (three per mitochondrial background) these results need to be interpreted with caution, as genetic drift may lead both to the random exclusion of slightly beneficial alleles, and to the random increase in the frequency of neutral variants. Considering the variation across replicates with the CMH test, we found significant allelic

frequency change in lines evolving under both mitochondrial backgrounds (Figure 3), consistent with strong selection. This was particularly pronounced on the SC lines, where we found more extreme *p*-values rejecting the null hypothesis of genetic drift (Figure 3), also reflected by the higher lineage-specific thresholds based on a *z*-score of 2 ($-\log_{10}$ (*p*-value) of 4.17 for SC and 1.73 for SD). Together with our observation that F_2 breakdown in survivorship is stronger in the SC background (Figure 2c), this suggests that the strength of selection acting against mitonuclear incompatibilities was stronger in this mitochondrial background relative to SD. Such asymmetries in the strength of mitonuclear incompatibilities are also supported by reciprocal crosses of multiple populations of *T. californicus* (Ganz & Burton, 1995; Peterson et al., 2013). Such differences in the strength of selection might result from several nonexclusive factors such as: the number of nuclear genes compensating for the accumulation of deleterious mutation on each mitochondrial lineage, their genomic location, the effect size of each interacting nuclear gene or on dominance effects among parental alleles. As most of the relative contributions of these factors are difficult to disentangle, below we discuss the number and location of genomic regions involved in mitonuclear incompatibilities.

By comparing the genomic regions responding to selection in lines evolving under alternative mitochondrial backgrounds, we can distinguish regions responding to uniform selection (i.e., where the same nuclear allele dominates) from regions responding to divergent selection (i.e., where different alleles dominate). Consistent with uniform selection in 6.5% of the genome (Table 1), we find that the SC allele dominates in much of chromosome 7 and a small segment of chromosome 8. Uniform selection could be explained by a relative contribution of purging of slightly deleterious alleles that have been fixed in one of the parental populations, by adaptation to laboratory conditions favouring alleles of one of the populations, or by DMIs between two or more nuclear factors (nuclear–nuclear instead of mitonuclear incompatibilities). These possibilities are also not mutually exclusive, and in fact are all likely to be contributing to the observed partners. Previous genomic studies of natural populations found that effective population size in SD is less than half that of SC (Pereira et al., 2016), and thus this population is expected to accumulate deleterious mutations twice as fast. Moreover, experimental studies have revealed that these populations are differentially adapted to temperature (Hong & Shurin, 2015; Pereira et al., 2017; Willett, 2010) and to salinity (Leong et al., 2017), and that rearing temperature used in this study favours SC alleles, relative to SD (Willett, 2010). Studies with reciprocal F_2 hybrids from multiple population crosses have found skews towards the same allele in one or two chromosomes (Foley et al., 2013; Lima et al., 2019), suggesting a role of nuclear–nuclear incompatibilities in *T. californicus*. While these three selective regimes are impossible to distinguish under our experimental design, future evolve-and-resequencing experiments varying environmental factors (such as in Griffiths et al. (2020), but with reciprocal mitochondrial backgrounds) will shed light on the relative role of ecological adaptation driving this signature of uniform selection.

TABLE 1 Comparison of allelic frequency change in the genome of *Tigriopus californicus*, after 9 months of experimental evolution under divergent mitochondrial backgrounds

	Windows	Proportion
Consistent with genetic drift		
No skew in both	2005	60.5%
Consistent with uniform selection		
SC allele is favoured in both	215	6.5%
SD allele is favoured in both	0	0%
Consistent with directional selection in both lines (symmetric selection)		
Matched allele in both	0	0%
Mismatched allele in both	0	0%
Consistent with directional selection only in SC (asymmetric selection)		
SC allele is favoured in SC (matched)	806	24.3%
SD allele is favoured in SC (mismatched)	103	3.1%
Consistent with directional selection only in SD (asymmetric selection)		
SD allele is favoured in SD (matched)	163	4.9%
SC allele is favoured in SD (mismatched)	24	0.7%

Abbreviations: SC, Santa Cruz; SD, San Diego.

Consistent with divergent selection, we find that allele frequencies differ in 33% of the genome, depending on the mitochondrial background. Yet, concerning the targets of divergent selection, there is no region where opposite alleles dominate, as would be expected if the same genomic region was under divergent selection in both mitochondrial backgrounds (i.e., if mitonuclear incompatibilities had a symmetric genetic architecture). Instead, the location of genomic regions consistent with divergent selection differs strongly among mitochondrial backgrounds. For example, the SC mitochondria seem to interact strongly with regions in chromosomes 2, 3, 8, 9, 11 and 12, while the SD mitochondria seem to interact with regions in chromosomes 4, 6 and 9 (Figure 3). In agreement with this, previous studies using F_2 hybrids from reciprocal crosses have reported allele frequency changes during a single generation in multiple chromosomes, both in response to selection for rapid development (five chromosomes, in Healy & Burton, 2020) or simply as a consequence of differential survivorship during development (four or five chromosomes, in Lima et al., 2019). In contrast to those studies, where skews consistent with divergent selection were small in magnitude (up to ~15%) and were consistent across entire chromosomes, by evolving hybrids over multiple generations, we were able to identify genomic regions that span between half to small portions of the chromosomes (62 kbp to 6.2 Mb; Figure 3). Nevertheless, as is apparent from Figure 3, our regions under divergent selection each still contain numerous candidate genes known to interact with proteins, RNAs or regulatory binding sites encoded in the mtDNA, thus

precluding identification of the causal genes underlying mitonuclear incompatibilities. Indeed, the function of each mtDNA gene requires intimate interactions with one or more nuclear gene partners. Given the extensive divergence of the mitochondrial genomes of SC and SD (22.17%; Pereira et al., 2016), it is not surprising that multiple genomic regions would be involved in compensatory evolution. Future "evolve-and-resequence" studies increasing the effective recombination rate, either by increasing the size of experimental populations or the number of generations under experimental evolution, will provide higher resolution into the asymmetric and polygenic genomic architecture of mitonuclear incompatibilities described here.

Consistent with our hypothesis, the direction of divergent selection favouring mitonuclear adaptation is strongly supported, as nuclear alleles matched the mitochondrial background in 88.6% and 87% of the cases (Table 1). Some of the remaining windows with skews towards the mismatched allele can be explained by linked selection. For example, in SD lines, all windows showing skews towards the mismatched SC allele are physically linked to a large region of chromosome 7 where the SC allele is favoured in both mitochondrial backgrounds, and thus may be explained by the stochasticity of recombination events during this experiment. In contrast, in SC lines, windows with the mismatched allele can only be explained by antagonistic selection, as these are located in half of chromosome 11, which shows no change in the reciprocal cross. Although this pattern is usually not expected for mitonuclear incompatibilities, empirical studies in F_2 hybrids show that it is not uncommon. In studies with F_2 hybrids, Lima et al. (2019) found patterns consistent with antagonistic selection in one to three chromosomes, depending on the population cross, and Healy and Burton (2020) found such a pattern in F_2 hybrids between SD and SC also in chromosome 11.

Together, our results offer new insights into the genomic architecture of mitonuclear incompatibilities between allopatric populations of the same species. We find that mitonuclear incompatibilities in *T. californicus* are highly asymmetric, both in the magnitude of selection and in the location of targeted nuclear regions, supporting theoretical predictions of DMIs involving uniparentally inherited genes (Turelli & Moyle, 2007). Periods of geographical isolation are common across species, often leading to fixation and deep phylogenetic divergence of mitochondrial lineages between populations that are otherwise similar (Avise, 2000; Wake, 2009). Given the stochasticity of the location of the deleterious mutations, and the vast network of interacting nuclear genes that can restore mitonuclear dysfunction within each population independently (Figure 3), it is perhaps unsurprising that such mitonuclear incompatibilities can have a polygenic genetic architecture that differs among populations. Our findings in *T. californicus* imply that, despite the small size and limited gene content of the mitochondrial genome, mitonuclear co-adaptation can result in barriers to gene flow that are widespread across the genome, even at early stages of species formation. The rapid evolution of the mitochondria and co-evolving nuclear genes during periods of geographical isolation may thus constitute important reproductive barriers that persist after taxa establish secondary contact or sympatry.

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CONFLICTS OF INTEREST

No conflict of interest has been declared by the authors.

AUTHORS CONTRIBUTIONS

R.J.P. conceived the project with direct input from L.C. and R.S.B. R.J.P. performed the research. R.J.P., T.G.L. and N.T.P. analysed the data. R.J.P., T.G.L. and R.S.B. wrote the manuscript, and all authors approved the final version.

DATA AVAILABILITY STATEMENT

Raw reads were deposited in the NCBI Sequence Read Archive (BioProject PRJNA716105; BioSample accession SAMN18394772-9). The dryad archive (https://doi.org/10.5061/dryad.bg79cnpb0) contains the reference genomes, estimated allelic frequencies, *p*-values for allelic frequency change, list of genomic windows under selection and fitness data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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