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Genetic incompatibilities in reciprocal hybrids between populations of *Tigriopus californicus* with low to moderate mitochondrial sequence divergence.

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Publication Date

2023-07-05

DOI

10.1093/evolut/qpad122

Peer reviewed

1 Genetic incompatibilities in reciprocal hybrids between populations of *Tigriopus californicus*
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17 **Abstract**

18 All mitochondrial-encoded proteins and RNAs function through interactions with
19 nuclear-encoded proteins, which are critical for mitochondrial function and eukaryotic fitness.
20 Coevolution maintains inter-genomic (i.e., mitonuclear) compatibility within a taxon, but
21 hybridization can disrupt coevolved interactions, resulting in hybrid breakdown. Thus,
22 mitonuclear incompatibilities may be important mechanisms underlying reproductive isolation
23 and, potentially, speciation. Here we utilize Pool-seq to assess the effects of mitochondrial
24 genotype on nuclear allele frequencies in fast- and slow-developing reciprocal inter-population
25 F₂ hybrids between relatively low-divergence populations of the intertidal copepod *Tigriopus*
26 *californicus*. We show that mitonuclear interactions lead to elevated frequencies of coevolved
27 (i.e., maternal) nuclear alleles on two chromosomes in crosses between populations with 1.5% or
28 9.6% fixed differences in mitochondrial DNA nucleotide sequence. However, we also find
29 evidence of excess mismatched (i.e., non-coevolved) alleles on three or four chromosomes per
30 cross, respectively, and of allele frequency differences consistent with effects involving only
31 nuclear loci (i.e., unaffected by mitochondrial genotype). Thus, despite substantial effects of
32 mitonuclear coevolution on individual chromosomes, our results for low-divergence crosses

33 suggest an underlying role for mitonuclear interactions in variation in hybrid developmental rate
34 without a clear bias for coevolved interactions.

35

36 **Keywords:** copepod, mitonuclear, mitochondria, coevolution, Pool-seq, inter-genomic

37

38 **Introduction**

39 Mitochondrial functions are highly reliant on interactions between proteins and RNAs
40 encoded in the mitochondrial and nuclear genomes, and compatibility between gene products of
41 the two genomes is maintained by coevolution within independent taxa (Rand et al. 2004; Burton
42 et al. 2013; Hill 2015). Hybridization between populations or species disrupts inter-genomic
43 coevolution, because in second- and higher-generation hybrids, mitochondrial genotypes are
44 found on nuclear backgrounds with regions that are homozygous for foreign nuclear alleles
45 (Burton et al. 2006). Thus, given likely link between mitochondrial performance and fitness
46 (e.g., Lane 2005), mitonuclear incompatibilities may be key mechanisms underlying hybrid
47 breakdown, reproductive isolation and speciation (Gershoni et al. 2009; Burton and Barreto
48 2012; Hill 2016; Hill et al. 2019), particularly at early stages of isolation due to the relatively
49 high evolutionary rate of the mitochondrial genome (Lynch 1997; Wallace 2010; Burton and
50 Barreto 2012).

51 For mitonuclear compatibility to play a substantial role in reproductive isolation, there
52 would have to be strong selection favouring compatible genotypes among hybrid organisms
53 (Sloan et al. 2017; Hill et al. 2019). Yet, despite known fitness consequences of mitonuclear
54 incompatibilities in several taxa (e.g., Ellison and Burton, 2008b; Meiklejohn et al. 2013),
55 introgression of mitochondrial genotypes across species or population boundaries has been
56 observed in many cases (Chan and Levin 2005; Toews and Brelsford 2012; Sloan et al. 2017).
57 As a result, the extent to which selection for inter-genomic compatibility presents a substantial
58 barrier for gene flow among taxa in general remains largely unresolved (Hill 2016, 2019; Sloan
59 et al. 2017; Burton 2022).

60 Perhaps the most direct demonstration of strong selection for mitonuclear compatibility
61 comes from studies on inter-population hybrids of the intertidal copepod *Tigriopus californicus*
62 (Healy and Burton 2020; Han and Barreto 2021). Adults of this species reach ~1.0 mm in length
63 and inhabit splash pools along the Pacific coast of North America from Baja California, Mexico
64 to Alaska, USA. There is essentially no gene flow among populations from different rocky
65 outcrops, resulting in substantial genetic divergence with populations frequently fixed for
66 different alleles and sharing few polymorphisms (e.g., Burton and Lee 1994; Burton 1997;
67 Edmands 2001; Burton et al. 2007; Pereira et al. 2016; Barreto et al. 2018). However, inter-
68 population hybrids are viable in the laboratory, and loss of performance in fitness-related traits
69 (i.e., survivorship, fecundity, developmental rate) as a result of mitonuclear incompatibilities has
70 been observed across many *T. californicus* studies (Edmands and Burton 1999; Harrison and
71 Burton 2006; Ellison and Burton 2006, 2008b; Healy and Burton 2020; Han and Barreto 2021;
72 Pereira et al. 2021). The effects of incompatibilities on performance are generally similar among
73 these traits (Ellison and Burton 2006), and recent studies have utilized developmental rate as a
74 proxy for fitness that can be scored for high numbers of individual hybrids (Healy and Burton
75 2020; Han and Barreto 2021). Links between fitness and developmental rate in this species are
76 supported by at least two lines of evidence. First, the ephemeral nature of *T. californicus*
77 habitats leads to cycles of extirpation and recolonization of individual pools suggesting rapid
78 developing offspring may be more successful in repopulating available habitat (Dybdahl 1994;
79 Powlik 1998), and mature individuals (i.e., late-stage copepodids and adults) may be more
80 resilient to scouring events by achieving a better grip on substrates (Park 2019; Ligouri 2022).
81 Second, developmental rate displays countergradient variation among populations relative to the

82 latitudinal thermal gradient (Hong and Shurin 2015), which is putatively adaptive, counteracting
83 the slowing thermodynamic effects of low.

84 Variation in developmental rate has been associated with differences in mitochondrial
85 performance and in the degree of mitonuclear compatibility in F₂ *T. californicus* hybrids between
86 a population from San Diego, California and populations from either Santa Cruz, California
87 (Healy and Burton 2020) or Strawberry Hill Wayside, Oregon (Han and Barreto 2021). In both
88 of these cases, there are extremely high levels of nucleotide sequence divergence between the
89 populations contributing to these crosses. Approximately 21% of the nucleotide sites in
90 mitochondrial DNA (mtDNA) display fixed differences between San Diego and Santa Cruz or
91 between San Diego and Strawberry Hill Wayside (Barreto et al. 2018; Han and Barreto 2021).
92 Additionally, median percentages of fixed nucleotide sequence differences across nuclear-
93 encoded genes between copepods from San Diego and Santa Cruz or Strawberry Hill Wayside
94 are 2.5% or 2.8%, respectively (Han and Barreto 2021). As a result, the extent to which the
95 evidence for strong effects of mitonuclear interactions in *T. californicus* can be generalized to
96 other species is unclear. For instance, in *Drosophila sp.*, which are another well-established
97 model for the study for mitonuclear interactions (Dowling et al. 2007; Hoekstra et al. 2013;
98 Meiklejohn et al. 2013; Carnegie et al. 2021; Rand et al. 2022), the percentages of fixed
99 differences in mtDNA nucleotide sequences even among species are much lower than among *T.*
100 *californicus* populations (e.g., ~4.5% between *D. melanogaster* and *D. simulans*; Ballard 2000).
101 *T. californicus* populations offer an ideal opportunity to address this issue, because the amount of
102 genetic differentiation among populations is variable across the species range (Edmands 2001;
103 Peterson et al. 2013; Pereira et al. 2016) and the phenotypic effects of mitonuclear interactions
104 demonstrate wide ranges of impact among crosses between different populations (Burton 1990).

105 In the current study, we assess variation in nuclear allele frequencies associated with
106 differences in developmental rate among reciprocal F₂ hybrids between two pairs of *T.*
107 *californicus* populations: San Diego and Bird Rock in southern California, USA, and Santa Cruz
108 and Pescadero Beach in central California. There is little evidence of significant variation in
109 developmental rate among populations across this narrow latitudinal range (e.g., Hong and
110 Shurin 2015; Healy et al. 2019; Healy and Burton 2020), and these population pairs have
111 relatively low levels of inter-population genetic divergence (Pereira et al. 2016). We use Pool-
112 seq to examine allele frequency biases at population-diagnostic SNPs (1) in fast- compared to
113 slow-developing hybrids within each pair of reciprocal crosses, and (2) in either fast developers
114 or slow developers between the pairs of reciprocal crosses. These complementary comparisons
115 resolve genomic regions where nuclear allele frequencies depend on mtDNA genotype or
116 developmental rate; elevated frequencies of maternal (i.e., coevolved) alleles in fast-developing
117 hybrids are consistent with a positive relationship between mitonuclear compatibility and
118 developmental rate. We also examine potential genetic effects involving only sites in the nuclear
119 genome by identifying loci at which the allele originating from one of the two populations
120 contributing to a pair of reciprocal crosses is at higher frequency regardless of mtDNA genotype.

121

122 **Materials and methods**

123 *Population sampling and copepod husbandry*

124 Adult *T. californicus* were collected from supralittoral tidepools at four locations along
125 the coast of California, USA in the spring of 2018: San Diego (SD: 32° 44' 45" N, 117° 15' 18"
126 W), Bird Rock (BR: 32° 48' 51" N, 117° 16' 24" W), Santa Cruz (SC: 36° 56' 58" N, 122° 02'
127 49" W) and Pescadero Beach (PE: 37° 15' 35" N, 122° 24' 51" W). Copepods were transported

128 to Scripps Institution of Oceanography in 1 L plastic bottles within 24 h. 250 mL laboratory
129 cultures were established with filtered seawater (0.44 μm pore size; 35 psu) in 400 mL glass
130 beakers that were placed inside incubators set to 20 °C and 12h:12h light:dark. Copepods
131 consumed natural algal growth, and were also fed weekly with powdered spirulina (Salt Creek,
132 Inc., South Salt Lake City, UT, USA) and ground TetraMin® Tropical Flakes (Spectrum Brands
133 Pet LLC, Blacksburg, VA, USA). These cultures and conditions were maintained for at least one
134 month (~1 generation) prior to initiating experiments.

135 *Experimental crosses and classification of fast- or slow-developing F₂ hybrids*

136 Virgin *T. californicus* females from each population were obtained by splitting mate-
137 guarding pairs (Burton 1985), and were used in two sets of reciprocal inter-population hybrid
138 crosses (4 crosses total): SD♀ x BR♂ (SDxBR), BR♀ x SD♂ (BRxSD), SC♀ x PE♂ (SCxPE)
139 and PE♀ x SC♂ (PExSC). Crosses were performed similarly to the protocols of Healy and
140 Burton (2020; see Supplemental Methods for details). At the F₂ generation, variation in
141 developmental rate among hybrids was scored by time to stage-1 copepodid (C1) metamorphosis
142 with fast- and slow-developing hybrids classified as those that metamorphosed 8-12 or ≥ 22 days
143 post hatch (dph), respectively, as in Healy and Burton (2020). To establish the fast- and slow-
144 developing groups, at 12 dph all copepodids present were transferred to separate cross-specific
145 petri dishes (“fast developers”), and at 21 dph all individuals at naupliar developmental stages
146 (i.e., before C1 metamorphosis) were also transferred to separate cross-specific dishes (“slow
147 developers”). Between 83 and 221 F₂ egg sacs per cross were assessed in the developmental rates
148 trials, and the numbers of egg sacs scored and of fast- or slow-developing copepodids for each
149 cross can be found in Table S1.

150 *DNA isolation and sequencing*

151 Approximately 160 haphazardly selected adults from each developmental rate group (see
152 Table S1) were pooled and preserved at -80 °C. Note that high numbers of *T. californicus* are
153 required to obtain sufficient DNA for Pool-seq analyses, and as a result one pool of fast
154 developers and one pool of slow developers was produced per reciprocal cross in our study.
155 However, to maintain acceptably low densities of copepods in our F₁ cultures, three groups of
156 40♀ x 40♂ contributed to each reciprocal cross, creating the possibility for group-level genetic
157 effects. This potential limitation is unlikely to be a major factor influencing allele frequencies in
158 our study, because the majority of genetic variation between these populations is fixed between
159 populations (Pereira et al. 2016); this is likely particularly true for variation contributing to
160 mitonuclear coevolution given almost all mtDNA sequence variation in *T. californicus* is also
161 fixed between populations (e.g., Willett and Ladner 2009; Peterson et al. 2013). Additionally,
162 each group made approximately equal contributions to the fast- and slow-developing pools.
163 DNA was isolated from the frozen pools of individuals following the methods of Healy and
164 Burton (2020), and whole-genome sequencing was conducted on an Illumina NovaSeq 6000
165 (Illumina Inc., San Diego, CA, USA; 150 base pair paired-end reads) by Novogene Co., Ltd.
166 (Sacramento, CA, USA).

167 *Data analysis and statistics*

168 Mapping pooled sequencing data from hybrid organisms to the reference genome for one
169 of the two populations contributing to the cross creates the potential to overestimate the
170 frequencies of the reference alleles due to biased read mapping (e.g., Lima and Willett 2018).
171 We accounted this potential bias by mapping reads to “hybrid reference” genomes, which are
172 duplicated reference genomes with the first copy of each homologous chromosome containing
173 genetic variants from one population in a cross and the second copy containing genetic variants

174 from the other population. We validated this approach for the current *T. californicus* reference
175 genome (Barreto et al. 2018) using the sequencing data for a pool of unhatched F₂ hybrids from
176 Lima and Willett (2018; see Dataset S1; Fig. S1; Table S2). The final hybrid reference genomes
177 used in the current study were prepared as described in Healy and Burton (2023; see
178 Supplemental Methods for details).

179 Between 58,681,492 and 137,851,793 paired-end reads were obtained for our F₂ hybrid
180 pools, and after filtering as in Healy and Burton (2020) the reads were mapped to the
181 corresponding hybrid genome with *BWA MEM* v0.7.12 (Li 2013; 83.7-93.6% mapping).
182 Alignments were filtered to remove those with MAPQ scores less than 20, and allele-specific
183 read counts at single-nucleotide polymorphisms (SNPs) with fixed differences between the SD
184 and BR or SC and PE populations were determined using *SAMtools* v1.14 (Danecek et al. 2021)
185 and *PoPoolation2* v1.201 (Kofler et al. 2011) similar to Lima and Willett (2018), Lima et al.
186 (2019) and Healy and Burton (2020). At the ‘pileup’ step, the population-specific identifiers for
187 the homologous chromosomes in the hybrid reference were removed from the alignments, and
188 the pileup file was created against either the SD or SC reference genome as appropriate for the
189 cross. Nuclear allele frequencies were calculated for the common set of SNPs with a minimum
190 coverage of 50X and a maximum coverage of 400X across all the pools for each pair of
191 reciprocal crosses.

192 Statistical analyses were conducted in *R* v4.2.0 (R Core Team 2022) with $\alpha = 0.05$ unless
193 otherwise noted, and tests relying on count data utilized effective counts (Wiberg et al. 2017). A
194 limitation of our Pool-seq design with a single pool of fast- or slow-developing hybrids for each
195 reciprocal cross is that we cannot estimate the variation associated with the average allele
196 frequency estimates in our study. Thus, we followed the general analytical protocols of Lima and

197 Willett (2018) who validated the robustness of Pool-seq allele frequencies in *T. californicus* by
198 comparison to PCR-based genotyping of specific loci. First, allele frequencies were averaged
199 across large chromosomal regions (1.5 Mb). Second, differences between sequencing pools were
200 assessed using Kolmogorov-Smirnov (KS) tests (Lima and Willett 2018; Lima et al. 2019)
201 followed by Bonferroni correction of $\alpha = 4.17 \times 10^{-3}$. We also performed site-by-site Fisher's
202 tests, which are subject to highly variable Pool-seq allele frequency estimates for individual
203 SNPs (Kofler et al. 2011); these tests generally support similar patterns of variation as those
204 detected by KS tests at the chromosomal level in our study (reported in Supplementary Tables S3
205 and S4 for comparison). Third, we examined allele frequency variation involving only nuclear
206 loci as in Lima et al. (2019) by evaluating differences from neutral expectations (i.e., 0.5) biased
207 towards the allele from a single source population regardless of the direction of the cross (i.e.,
208 regardless of mtDNA genotype). Lima et al. (2019) determined the distribution of allele
209 frequencies for each chromosome in a pool of unhatched F₂ nauplii which reflect experimental
210 error around an expected value of 0.5. These authors established that a nuclear-only effect could
211 be resolved if the 10th frequency percentile of the allele from one of the populations in the cross
212 exceeded 0.52 in both reciprocal crosses (i.e., a threshold of ± 0.02 relative to 0.5). This threshold
213 was re-defined slightly in the current study to 0.521 (i.e., ± 0.021) after re-assessment using a
214 hybrid reference genome for read mapping (Dataset S1).

215

216 **Results**

217 Allele frequencies were scored for 261,237 population-specific nuclear SNPs in our
218 SDxBR and BRxSD F₂ hybrids (average coverage 73X; Table S6, S7), and for 52,202
219 population-specific SNPs in the SCxPE and PExSC hybrids (average coverage 118X; Table S8,

220 S9). Across all pools, a minimum of 96.4% of the mtDNA reads mapped to the sequence from
221 the maternal population in the cross ($99.0 \pm 0.5\%$, $\mu \pm \text{SEM}$; Table S5), confirming the almost
222 exclusive maternal inheritance of mtDNA in these crosses.

223 In hybrids with the SD mitochondrial genotype (i.e., SDxBR), differences in allele
224 frequencies between the fast- and slow-developing copepodids were detected for chromosomes
225 2, 5, 6 and 7 ($p \leq 6.5 \times 10^{-4}$; Fig. 1a) with higher frequencies of SD alleles in fast developers on
226 chromosomes 2 and 6, and in slow developers on chromosomes 5 and 7. In the BRxSD hybrids,
227 chromosome 6 displayed a significant difference between the BRxSD fast and slow developers
228 ($p = 1.1 \times 10^{-5}$; Fig. 1b) with higher BR allele frequencies in slow-developing hybrids. There
229 were significant differences in allele frequencies between fast and slow developers for
230 chromosome 9 in SCxPE hybrids ($p = 6.5 \times 10^{-4}$; Fig. 2a) with higher frequencies of SC alleles
231 in slow-developing hybrids. In contrast, in hybrids with the PE mitochondrial genotypes (i.e.,
232 PExSC) there were higher frequencies of PE nuclear alleles on chromosomes 3 and 5 in slow
233 developers than in fast developers ($p \leq 2.2 \times 10^{-4}$), whereas there were higher PE allele
234 frequencies on chromosome 5 in fast- compared to slow-developing hybrids ($p = 2.8 \times 10^{-6}$; Fig.
235 2b).

236 In comparisons between reciprocal fast-developing hybrids, differences between SDxBR
237 and BRxSD were found for three chromosomes with higher maternal allele frequencies on
238 chromosomes 2 and 6, and higher paternal allele frequencies on chromosome 11 ($p \leq 2.1 \times 10^{-3}$;
239 Fig. 3a). Only two chromosomes displayed differences in fast developers between SCxPE and
240 PExSC with higher maternal or paternal allele frequencies on chromosome 2 or 8, respectively (p
241 $\leq 2.2 \times 10^{-4}$; Fig. 3b). Variation in allele frequencies consistent with nuclear-only effects were

242 observed in fast developers, but only in the SDxBR and BRxSD crosses with biases for SD
243 alleles on chromosomes 6 and 7.

244 In slow-developing SDxBR and BRxSD hybrids there were higher frequencies of
245 paternal alleles on chromosomes 2 and 11 ($p \leq 1.1 \times 10^{-5}$; Fig. S2a), whereas in slow-developing
246 SCxPE and PExSC hybrids there were higher frequencies of maternal alleles on chromosomes 2
247 and 5 ($p \leq 2.1 \times 10^{-3}$; Fig. S2b). No allele frequency patterns consistent with effects involving
248 only nuclear loci were observed for slow developers from crosses between the SC and PE
249 populations, whereas chromosomes 7 and 9 displayed nuclear-only effects in slow-developing
250 SDxBR and BRxSD hybrids with biases for SD alleles.

251

252 **Discussion**

253 In the absence of selection, allele frequencies at population-specific diagnostic markers
254 are expected to be 0.5 in hybrids, reflecting simple bi-parental inheritance. When asymmetries in
255 allele frequencies are observed between reciprocal crosses, one explanation is that certain alleles
256 are favored via interactions with the maternally inherited mtDNA. For example, after stratifying
257 hybrids by variation in a fitness-related trait such as developmental rate, signatures of
258 mitonuclear coevolution can be detected in two ways: (1) significantly higher maternal allele
259 frequencies in fast-developing hybrids compared to slow-developing hybrids in either one or
260 both reciprocal cross directions, or (2) significant differences in allele frequencies between
261 reciprocal crosses such that maternal alleles are favoured. The former of these patterns directly
262 associates mitonuclear coevolution with variation in developmental rate, whereas the relationship
263 between coevolution and developmental rate based on variation between reciprocal crosses

264 depends on the differences observed in fast developers compared those observed in slow
265 developers.

266 In reciprocal crosses between populations with either 1.5% (SC and PE; the current
267 study) or 9.6% (SD and BR; Barreto et al. 2018) fixed differences in mtDNA nucleotide
268 sequence (compared to ~21% in Healy and Burton [2020] and in Han and Barreto [2021]), no
269 consistent maternal versus paternal allele frequency biases between fast and slow developers
270 were detected on the same chromosome in both directions of the cross. There were significant
271 differences in allele frequencies between fast- and slow-developing hybrids on chromosome 6 in
272 both the SDxBR and BRxSD crosses; however, in both cases, SD alleles were at higher in fast
273 developers suggesting a possible effect of nuclear variation independently of mtDNA genotype.
274 In contrast, several chromosomes demonstrated significant allele frequency differences between
275 fast- and slow-developing hybrids in one cross of each reciprocal pair. As the initial nuclear
276 contributions from each of the populations in the reciprocal crosses are equivalent in both
277 directions, these patterns are most likely consistent with asymmetrical mitonuclear interactions
278 (i.e., a GxG interaction), which are also often observed in *Drosophila sp.* However, relatively
279 few of these differences support higher frequencies of coevolved nuclear alleles in fast
280 developers in our study: higher maternal alleles on chromosome 2 in SDxBR hybrids and
281 chromosome 7 in PExSC hybrids versus higher paternal alleles on chromosomes 5 and 7 in
282 SDxBR hybrids, chromosome 9 in SCxPE hybrids, and chromosomes 3 and 5 in PExSC hybrids.

283 Evidence for a disproportionate role of coevolved interactions underlying developmental
284 rate was also generally absent in comparisons between the pairs of reciprocal crosses. In the
285 SDxBR and BRxSD crosses, higher frequencies of maternal alleles were detected on
286 chromosomes 2 and 6, particularly in fast-developing hybrids, consistent with effects of

287 mitonuclear coevolution on hybrid developmental rate. In contrast, maternal alleles were at
288 higher frequencies on chromosome 5 in the SCxPE and PExSC crosses, but only in slow
289 developers, and paternal alleles were at higher frequencies in fast developers on chromosome 8.
290 Interestingly, higher frequencies of coevolved alleles were detected on chromosome 2 in both
291 fast- and slow-developing SCxPE and PExSC hybrids, suggesting a bias for coevolved
292 mitonuclear genotypes independently of developmental rate. However, the opposite pattern of
293 higher frequencies of paternal alleles in both fast and slow developers was observed in the
294 SDxBR and BRxSD hybrids on chromosome 11. Additionally, there were also effects of nuclear
295 genes independently of mtDNA genotype in the SDxBR and BRxSD crosses with SD allele
296 frequencies above neutral expectations on two chromosomes in fast developers (6 and 7) and two
297 chromosomes in slow developers (7 and 8). Taken together, the results of the current study
298 indicate that mitonuclear interactions play a key role underlying hybrid breakdown in these
299 relatively low-divergence crosses, but that positive effects associated with mitonuclear
300 coevolution are not more common than positive effects of novel mitonuclear genotypes
301 involving foreign (i.e., paternal) nuclear alleles. This suggests that the effects of mitonuclear
302 incompatibilities at low levels of genetic divergence between populations are reduced relative to
303 the substantial effect these incompatibilities have on hybrid performance and fitness in high-
304 divergence crosses (Healy and Burton 2020; Han and Barreto 2021).

305 Despite the reduced effects of mitonuclear evolution on F₂ allele frequencies in the
306 current study, differences consistent with mitonuclear coevolution were detected in both pairs of
307 reciprocal crosses. For example, maternal alleles were higher on chromosome 2 in at least one
308 comparison in the crosses between the SD and BR populations, and the SC and PE populations,
309 and this is the only chromosome for which consistent effects of mitonuclear coevolution were

310 also detected in high-divergence *T. californicus* crosses (Healy and Burton 2020; Han and
311 Barreto 2021). The allele frequency deviations on chromosome 2 detected here appear modest
312 compared to neutral expectations for F₂ hybrids (i.e., 0.5), reaching a maximum of 0.583 or
313 0.539 in the fast developers from crosses between the SD and BR populations or the SC and PE
314 populations, respectively. However, there is little evidence of selection against heterozygous
315 nuclear genotypes in F₂ hybrids in *T. californicus* (Pritchard et al. 2011; Foley et al. 2013),
316 suggesting the allele frequency deviations in the current study are consistent with up to 57% or
317 29% under-representations of homozygous mismatched genotypes on chromosome 2,
318 respectively. Although these signatures of mitonuclear coevolution imply strong selection for
319 compatibility on this chromosome, the magnitudes of allele frequency deviations in these low-
320 divergence crosses are still lower than those previously resolved in high-divergence crosses
321 between *T. californicus* populations (e.g., 87%; Healy and Burton 2020). The relatively low
322 numbers of inter-population crosses with available data precludes drawing firm conclusions
323 regarding the relative role of mitonuclear interactions in hybrid breakdown as genetic divergence
324 increases. However, the data available to date suggest that mitonuclear incompatibilities may
325 play a proportionally greater role in high-divergence crosses than in low-divergence crosses.
326 Thus, it is possible that multiple loci on the same chromosome may develop coevolved
327 interactions as divergence progresses (see Willett et al. 2016 for evidence for effects of multiple
328 loci on chromosome 3), or that divergence may strengthen the effects of incompatibilities over
329 time despite little evidence for direct selection on incompatibilities among allopatric populations
330 of *T. californicus* (Burton and Barreto 2012). Regardless, our current results suggest that effects
331 of mitonuclear coevolution can be substantial for some chromosomes even at low levels of
332 mtDNA sequence divergence between populations.

333 The majority of mitonuclear interactions in the current study were observed consistently
334 across entire chromosomes, which is expected as large regions of chromosomes are inherited
335 together in F₂ hybrids due to only one opportunity for inter-population recombination (Lima and
336 Willett 2018; Lima et al. 2019), and to a lack of recombination in female *T. californicus* (Burton
337 et al. 1981). As a result, there is limited resolution of specific chromosomal regions or nuclear
338 genes potentially involved in the mitonuclear interactions detected here. Yet, 599 nuclear-
339 encoded mitochondrial (N-mt) genes have been annotated in the *T. californicus* genome (Barreto
340 et al. 2018; see Fig. 3 and Table S10). Given the consistent signatures of mitonuclear coevolution
341 on chromosome 2 across studies examining allele frequency biases in fast-developing F₂ *T.*
342 *californicus* hybrids (the current study; Healy and Burton 2020; Han and Barreto 2021), the 107
343 N-mt genes on this chromosome may represent the best candidate mechanisms for involvement
344 in coevolution among populations of this species.

345 Physiological research in hybrid *T. californicus* has demonstrated negative effects of
346 mitonuclear incompatibilities on the mitochondrial electron transport system (ETS; Ellison and
347 Burton 2006, 2008b; Barreto and Burton 2013; Healy and Burton 2020; Han and Barreto 2021)
348 and on mitochondrial transcription (Ellison and Burton 2008a). In addition, elevated rates of
349 sequence evolution among populations of *T. californicus* have been detected for nuclear-encoded
350 ETS subunits, mitochondrial ribosomal proteins and aminoacyl-tRNA synthetases consistent
351 with interactions between the products of these genes and fast-evolving mitochondrial-encoded
352 proteins and rRNAs (Barreto and Burton 2012; Barreto et al. 2018). These findings suggest
353 mitonuclear interactions affect at least three major mitochondrial functions in *T. californicus*:
354 oxidative phosphorylation, mitochondrial translation and mitochondrial transcription (Burton and
355 Barreto 2012; Hill 2015, 2017; Hill et al. 2019). No mitochondrial DNA or RNA polymerases

356 are encoded on chromosome 2 in the nuclear genome of *T. californicus*, whereas three ETS
357 subunits (complex I: *ndufb7* and *ndufv2*; complex III: *uqcr10*), twelve mitochondrial ribosomal
358 proteins and three aminoacyl-tRNA synthetases are transcribed from this chromosome.

359 The current study demonstrates that genetic interactions between the mitochondrial and
360 nuclear genomes have substantial effects on variation in fitness-related traits and hybrid
361 breakdown in F₂ hybrids. However, widespread genome-level effects consistent with mitonuclear
362 coevolution were generally absent in the crosses in the current study, indicating that positive
363 effects of epistatic interactions between the genomes do not always reflect inter-genomic
364 coevolution at relatively low levels of genetic divergence. Yet, effects of mitonuclear
365 coevolution were evident on a small number of chromosomes, resulting in under-representations
366 of homozygous mismatched mitonuclear genotypes on these chromosomes in fast-developing
367 hybrids. Thus, selection to maintain mitonuclear compatibility in hybrids may not limit gene
368 flow across the entire genome, but instead has the potential to have substantial impacts in
369 specific genomic regions even before high levels of mtDNA divergence are achieved.

370

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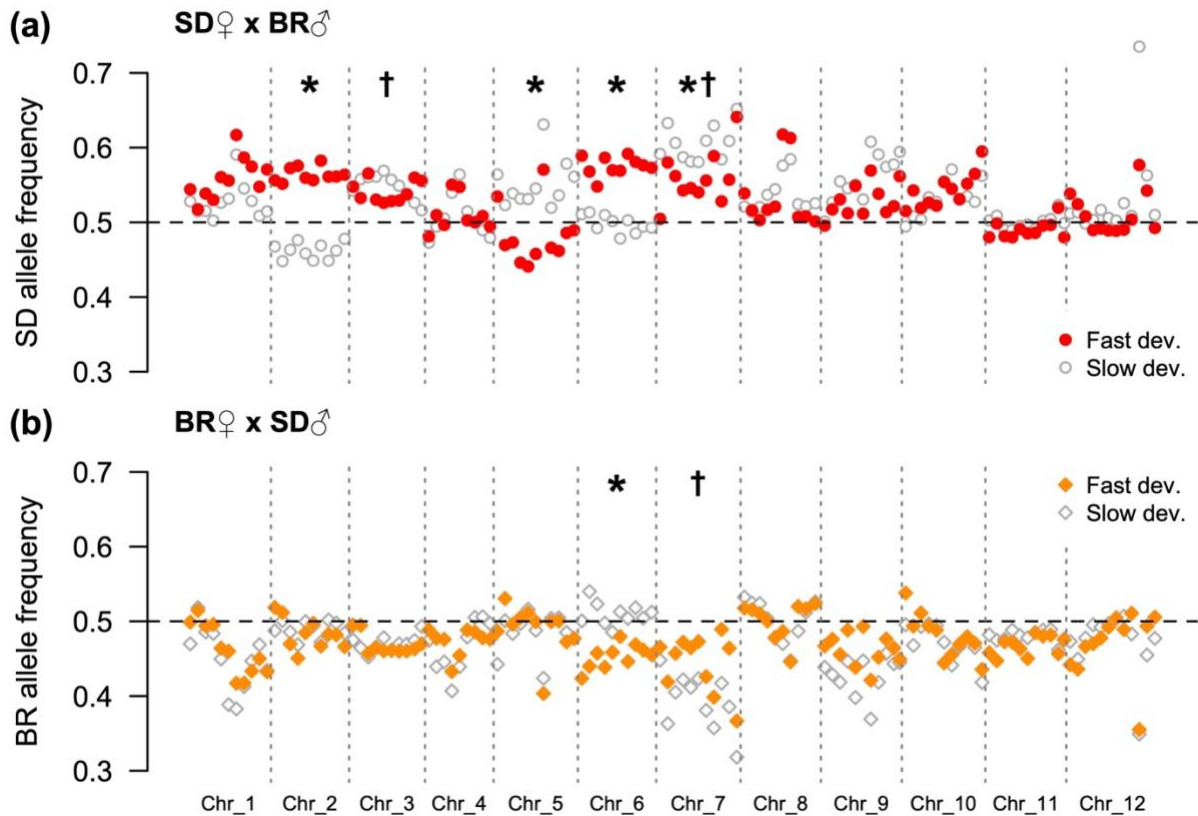
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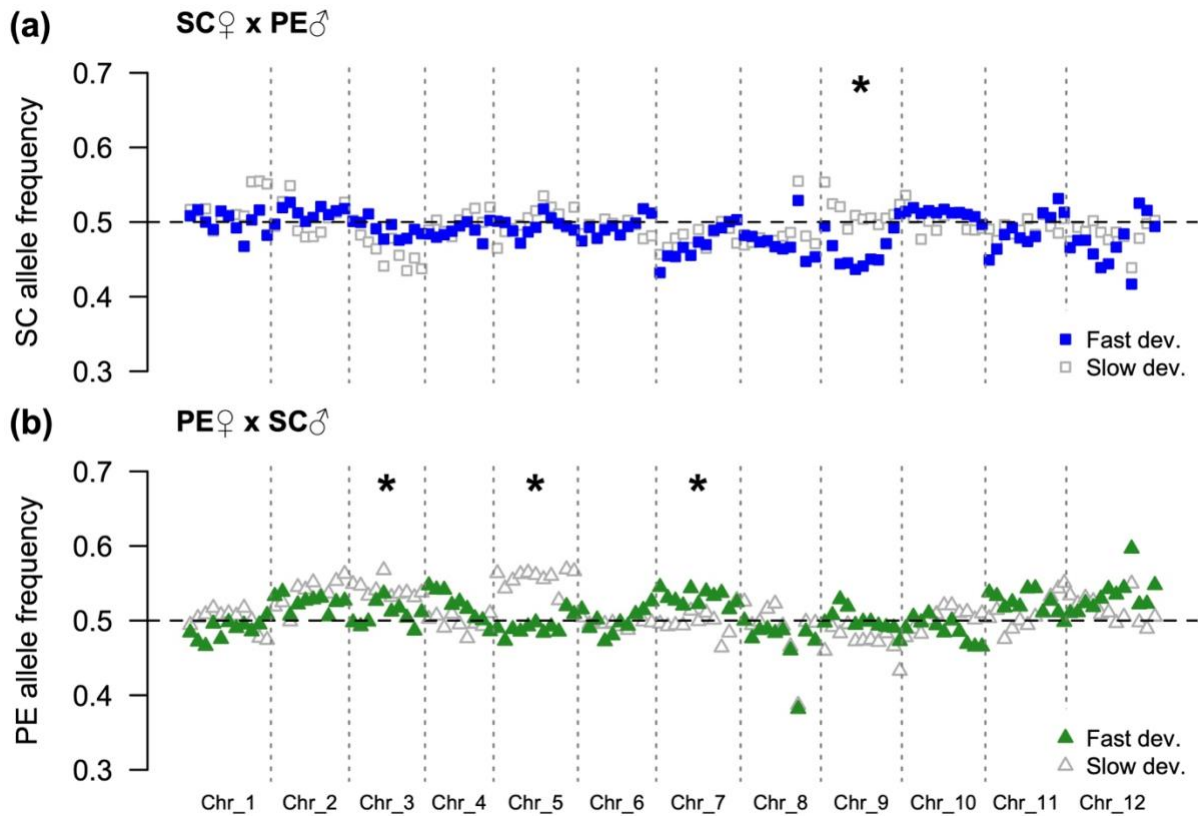
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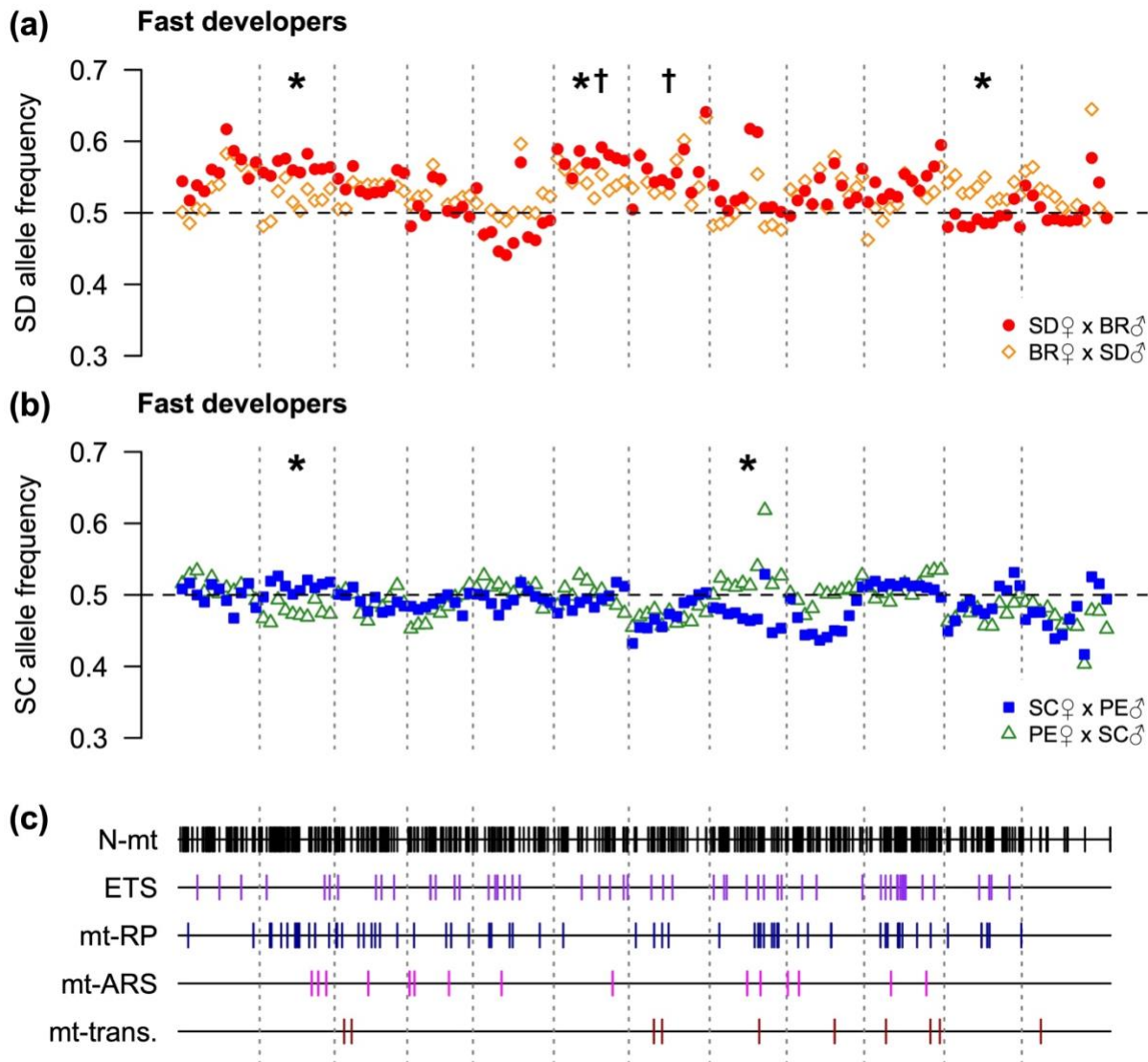
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599 **Figure 1.** Nuclear allele frequencies for 1.5 Mb chromosomal windows in fast- and slow-
 600 developing F₂ hybrids between the SD and BR populations (a: SDxBR, SD allele frequencies,
 601 fast developers – filled red circles, slow developers – empty grey circles; b: BRxSD, BR allele
 602 frequencies, fast developers – filled orange diamonds, slow developers – empty grey diamonds).
 603 Asterisks indicate chromosomes with significant differences between reciprocal crosses based on
 604 KS tests.
 605



606 **Figure 2.** Nuclear allele frequencies for 1.5 Mb chromosomal windows in fast- and slow-
 607 developing F₂ hybrids between the SC and PE populations (a: SCxPE, SC allele frequencies, fast
 608 developers – filled blue squares, slow developers – empty grey squares; b: PExSC, PE allele
 609 frequencies, fast developers – filled green triangles, slow developers – empty grey triangles).
 610 Asterisks indicate chromosomes with significant differences between reciprocal crosses based on
 611 KS tests.
 612



613 **Figure 3.** Nuclear allele frequencies for 1.5 Mb chromosomal windows in fast-developing F₂
 614 hybrids (a: SD allele frequencies, SDxBR – filled red circles, BRxSD – empty orange diamonds;
 615 b: SC allele frequencies, SCxPE – filled blue squares, PExSC – empty green triangles). Asterisks
 616 indicate chromosomes with significant differences between reciprocal crosses based on KS tests,
 617 and daggers indicate chromosomes with patterns consistent with nuclear-only effects. Genomic
 618 locations of 599 nuclear-encoded mitochondrial genes (c: N-mt genes – black, electron transport
 619 system [ETS] genes – purple, mitochondrial ribosomal proteins [mt-RP] – navy, mitochondrial
 620 aminoacyl tRNA synthetases [mt-ARS] – magenta, and mitochondrial transcription and DNA
 621 replication [mt-trans.] – dark red).