Title: A Lethal Genetic Incompatibility between Naturally Hybridizing Species in Mitochondrial Complex I

One sentence summary: Accelerated evolution of three interacting nuclear and mitochondrial genes underlies a lethal incompatibility in swordtail hybrids.

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Abstract

The evolution of reproductive barriers is fundamental to the formation of new species and can help us understand the diversification of life on Earth. These reproductive barriers often take the form of "hybrid incompatibilities," where genes derived from two different species no longer interact properly. Theory predicts that incompatibilities involving multiple genes should be common and that rapidly evolving genes will be more likely to cause incompatibilities, but empirical evidence has lagged behind these predictions. Here, we describe a mitonuclear incompatibility involving three genes within respiratory Complex I in naturally hybridizing swordtail fish. Individuals with specific mismatched protein combinations fail to complete embryonic development while those heterozygous for the incompatibility have reduced function of Complex I and unbalanced representation of parental alleles in the mitochondrial proteome. We localize the protein-protein interactions that underlie the incompatibility and document accelerated evolution and introgression in the genes involved. This work thus provides a precise characterization of the genetic architecture, physiological impacts, and evolutionary origin of a multi-gene incompatibility impacting naturally hybridizing species.

41 **Main text**

42

43 Biologists have long been fascinated by the question of how new species are formed and
44 what mechanisms maintain isolation between them. One key factor in the formation and
45 maintenance of new species is the emergence of genetic incompatibilities that reduce viability or
46 fertility in hybrids. When species diverge from each other, they accumulate unique sets of
47 mutations (*1*). As originally described by the Dobzhansky-Müller model of hybrid
48 incompatibility (DMI model; *2*, *3*), when these mutations are brought together in hybrids, they
49 may interact poorly, given that they have never been tested against one another by selection. Due
50 to the technical challenges of identifying these interactions (*4*), only a handful of genes involved
51 in hybrid incompatibilities have been precisely mapped (*5*) and exploration of the functional and
52 evolutionary causes of hybrid incompatibilities has been limited to a small number of cases in
53 model organisms (*4*).

54 As a consequence of this knowledge gap, key predictions about the genetic architecture
55 of hybrid incompatibilities and the evolutionary processes that drive their emergence remain
56 untested. For one, theory suggests that incompatibilities should be more common within dense
57 gene networks, both because the number of potentially incompatible genotypes explodes as the
58 complexity of the genetic interaction increases and because genes involved in such interactions
59 are expected to be tightly co-evolving (*6*, *7*). Consistent with this prediction, mutagenesis
60 experiments have highlighted the sensitivity of multi-protein interactions to changes in any of
61 their components (*6*). However, genetic interactions are notoriously difficult to detect
62 empirically except in systems with especially powerful genetic tools (*8*), and this problem is
63 exacerbated with complex genetic interactions (*9*, *10*). Such technical challenges may explain

64 their rarity in the empirical literature (*6*, but see *11–14*). Another largely untested prediction is
65 that rapid molecular evolution will increase the rate at which incompatibilities accumulate
66 between species (*4*, *5*, *15*). While several incompatibilities identified to date show signatures of
67 positive selection, it is unclear how unusual rates of protein evolution are in genes involved in
68 hybrid incompatibilities relative to the genomic background (*5*, *15*).

69 Another open question is the degree to which the genes involved in hybrid
70 incompatibilities may be predictable from their molecular or evolutionary properties. The
71 mitochondrial genome, in particular, has been proposed as a hotspot for the accumulation of
72 genetic incompatibilities (*16*, *17*). Mitochondria are essential for energy production in nearly all
73 eukaryotic organisms (*18*). In addition to this critical role, the particularities of mitochondrial
74 inheritance and function might drive the rapid evolution of hybrid incompatibilities between
75 species. Uniparental inheritance of mitochondria is predominant in animals, plants, and many
76 fungi (*19*), creating the potential for sexually antagonistic selection (*20*, *21*). In many animals,
77 mitochondrial genomes also experience elevated mutation rates relative to the nuclear genome
78 which, combined with reduced effective population size and a lack of recombination, results in
79 up to ~25X higher mitochondrial substitution rates in some species (*22–24*). At the same time,
80 nuclear and mitochondrial gene products must directly interact with each other in key steps of
81 ATP synthesis, increasing the likelihood of coevolution between these genomes (*25*, *26*). These
82 molecular and evolutionary factors suggest that interactions between mitochondrial- and nuclear-
83 encoded proteins could play an outsized role in the emergence of hybrid incompatibilities (*16*).

84 Although few studies have successfully identified the individual genes underlying hybrid
85 incompatibilities (*4*, *5*), crosses in numerous species have provided indirect evidence for the
86 prevalence of mitonuclear incompatibilities, since hybrid viability often depends on the identity

of the maternal species (*27–30*). However, the field has struggled to move beyond these coarse-scale patterns, especially in non-model systems where large mapping experiments can be infeasible. Despite predictions that mitonuclear incompatibilities play a disproportionate role in the evolution of reproductive isolation, few studies have mapped mitonuclear incompatibilities to the single gene level (*31–34*) and none of those identified to date have been studied in species that naturally hybridize.

As we make progress identifying the individual genes underlying hybrid incompatibilities, the next frontier is evaluating the processes that drive their evolution. Here, we use an integrative approach to precisely map the genetic basis of a lethal mitonuclear hybrid incompatibility in swordtail fish and to uncover its evolutionary history. Sister species *Xiphophorus birchmanni* and *X. malinche* began hybridizing in the last ~100 generations in multiple river systems (*35*) after premating barriers were disrupted by habitat disturbance (*36*), and are a powerful system to study the emergence of hybrid incompatibilities in young species. Given their recent divergence (~250,000 generations; 0.5% divergence per basepair; *37*), some hybrids between *X. birchmanni* and *X. malinche* are viable and fertile, while others experience strong selection against incompatibilities (*37*, *38*). Leveraging data from controlled laboratory crosses and natural hybrid zones, we pinpoint three genes that generate a lethal mitonuclear hybrid incompatibility, characterize its developmental and physiological effects, and trace its evolutionary history.

110 **Admixture Mapping Reveals a Lethal Mitonuclear Incompatibility**

111

112 To identify loci under selection in *X. birchmanni* × *X. malinche* hybrids, we generated
113 ~1X low-coverage whole-genome sequence data for 943 individuals from controlled laboratory
114 crosses and 281 wild-caught hybrid adults, and applied a hidden Markov model to data at
115 629,524 ancestry-informative sites in order to infer local ancestry (~1 per kb; *39*, *40*;
116 Supplementary Materials 1.1.1-1.1.4). Using these results, we found evidence for a previously
117 unknown incompatibility between the nuclear and mitochondrial genomes of *X. birchmanni* and
118 *X. malinche* (Supplementary Materials 1.1.4-1.1.7). Our first direct evidence came from
119 controlled laboratory crosses (Supplementary Materials 1.1.1, 1.1.2). Because the cross is largely
120 unsuccessful in the opposite direction, all lab-bred hybrids were the offspring of F_1 hybrids
121 generated between *X. malinche* females and *X. birchmanni* males and harbored a mitochondrial
122 haplotype derived from the *X. malinche* parent species. Offspring of F_1 intercrosses are expected
123 to derive on average 50% of their genome from each parent species. This expectation is satisfied
124 genome-wide and locally along most chromosomes in F_2 hybrids (on average 50.3% *X. malinche*
125 ancestry; Fig. S1). However, we detected severe segregation distortion along a 6.5 Mb block of
126 chromosome 13, where average ancestry among surviving individuals was 67% *X. malinche*.
127 Closer examination of genotypes in a 3.75 Mb subregion showed that almost none of the
128 surviving individuals harbored homozygous *X. birchmanni* ancestry in this region of
129 chromosome 13 (Fig. 1A; Fig. S2; 0.5% observed vs 25% expected). This pattern is unexpected
130 even in the case of a lethal nuclear-nuclear incompatibility (where we should recover
131 homozygous *X. birchmanni* ancestry in 6% of individuals; Supplementary Materials 1.1.2), but is
132 consistent with a lethal mitonuclear incompatibility. Using approximate Bayesian computation

133 (ABC) approaches we asked what strength of selection against *X. birchmanni* ancestry in this
134 region was consistent with the genotypes and ancestry deviations observed. We estimated
135 posterior distributions of selection and dominance coefficients and found that inferred selection
136 on this genotype in F_2s is largely recessive and essentially lethal (maximum a posteriori estimate:
137 $h = 0.12$ and $s = 0.996$, 95% credible interval $h = 0.010$-0.194 and $s = 0.986$-0.999; Fig. 1D,
138 Supplementary Materials 1.2.1-1.2.2).

139 To directly evaluate evidence for a mitonuclear incompatibility and pinpoint the region
140 involved on chromosome 13, we leveraged data from natural hybrid populations. Most naturally
141 occurring *X. birchmanni* × *malinche* hybrid populations are fixed for mitochondrial haplotypes
142 from one parental species (Supplementary Materials 1.1.6). However, a few segregate for the
143 mitochondrial genomes of both parental types, and we focused on one such population (the
144 "Calnali Low" population, hereafter the admixture mapping population). Admixture mapping of
145 mitochondrial ancestry (controlling for genome-wide ancestry) revealed a sharp peak of
146 association that spanned approximately 380 kb within the region of chromosome 13 identified
147 using F_2 crosses (Fig. 1B; Fig. S3; Supplementary Materials 1.1.5). We replicated this signal in
148 another hybrid population (Fig. S4).

149 Of the 32 genes in this region (Table S1), two had known mitochondrial functions, but
150 only one, the NADH dehydrogenase ubiquinone iron-sulfur protein 5 (*ndufs5*), directly interacts
151 with mitochondrially encoded proteins. Using three natural hybrid populations that had fixed the
152 mitochondrial haplotype of one of the parental species (Fig. S5), we fine-mapped the
153 incompatible mitochondrial interactor within the 380 kb admixture mapping peak. All
154 populations showed a depletion of non-mitochondrial parent ancestry at *ndufs5* (Supplementary

155 Materials 1.1.6), implicating it as the nuclear component of the mitonuclear incompatibility (Fig.
156 1C).

157 Given that hybrids with *X. malinche* mitochondria and homozygous *X. birchmanni*
158 ancestry at *ndufs5* suffer hybrid lethality, we investigated evidence for selection on the opposite
159 genotype combination (*X. birchmanni* mitochondria and homozygous *X. malinche* ancestry at
160 *ndufs5*) using hybrids from the admixture mapping population (Supplementary Materials 1.2.1;
161 Fig. S6). We found that this genotype combination was also observed at unexpectedly low
162 frequencies (Fig. 1D, 1G; $p<10^{-4}$ by simulation, Supplementary Materials 1.2.1) and inferred to
163 be under strong selection, albeit much weaker than the opposite ancestry combination (maximum
164 a posteriori estimate: 0.17, 95% credible intervals 0.08-0.55; Fig. 1D, Supplementary Materials
165 1.2.2). We focus primarily on the incompatibility involving the *X. malinche* mitochondria in our
166 results below (Fig. 1G) but discuss this reciprocal incompatibility in Supplementary Materials
167 1.2.3.

168

169 **Mitonuclear incompatibility in *X. birchmanni* × *X. malinche* hybrids involves three genes**

170

171 Mitochondrial protein respiratory Complex I is the first component of the electron transport
172 chain that ultimately allows the cell to generate ATP. In vertebrates, this complex has a total of
173 47 subunits and assembly factors, including *ndufs5* (Table S2). While the genome-wide scan that
174 we used to identify *ndufs5* is an unbiased method to search for mitochondrially interacting loci, it
175 has low power given our sample size. After determining that genes in Complex I were involved
176 in the incompatibility, we performed targeted analyses to determine if other genes in this
177 complex appear to be under selection in hybrids (Fig. S7-S8; Supplementary Materials 1.1.7).

178 This analysis identified a significant interaction between mitochondrial ancestry and another
179 member of Complex I, the *ndufa13* gene on chromosome 6 (Supplementary Materials 1.1.7;
180 Bonferroni corrected p-value = 0.02). This indicates that the *X. malinche* mitochondria is actually
181 incompatible with at least two other genes (Fig. 1G), although we discuss uncertainty about the
182 exact architecture of the interaction in Supplementary Materials 1.1.8.
183 Depletion of non-mitochondrial parent ancestry at *ndufa13* was unidirectional (Fig. 1E),
184 consistent with selection acting only against the combination of the *X. malinche* mitochondria
185 with homozygous *X. birchmanni* ancestry at *ndufa13* (see Supplementary Materials 1.2.3 for
186 more detail). Notably, we also detect selection on *ndufa13* in our dataset of lab-bred hybrids that
187 harbor *X. malinche* mitochondria. The proportion of individuals with homozygous *X. birchmanni*
188 ancestry at *ndufa13* is 3% (compared to 0.5% at *ndufs5* and 25% expected genome-wide).
189 However, ABC approaches indicate that selection against the mismatch between *X. malinche*
190 mitochondria and homozygous *X. birchmanni* ancestry at *ndufa13* is also severe (maximum a
191 posteriori estimate: 0.91, 95% credible interval 0.87-0.94; Fig. 1E, Supplementary Materials
192 1.2.2).
193
194 **Lethal Effect of Incompatibility in Early Development**
195
196 The incompatibility involving the *X. malinche* mitochondria appears to be lethal by the
197 time individuals reach adulthood. To pinpoint the developmental timing of the incompatibility,
198 we genotyped pregnant females from the admixture mapping population and recorded the
199 developmental stages of their embryos (*41*; swordtails are livebearing fish). We focused on the
200 interaction between *X. malinche* mitochondria and homozygous *X. birchmanni* ancestry at

201 *ndufs5*, given that we did not detect an effect of ancestry at *ndufa13* on developmental stage (Fig.
202 S9-10; Supplementary Materials 1.3.1). While developmental asynchrony is typically on the
203 scale of 0-2 days in pure species (*42*; Supplementary Materials 1.3.1), we observed much greater
204 variation in broods collected from the admixture mapping population where the mitochondrial
205 incompatibility is segregating (e.g. stages normally separated by 12 days of development found
206 in the same brood; Supplementary Materials 1.3.1; Fig. 2A-B). Genotyping results revealed that
207 lethal mitonuclear genotype combinations were present in embryos at early developmental
208 stages, but that these embryos failed to reach a phenotype beyond that typical of the first seven
209 days of gestation (the full length of gestation is ~21 days in *Xiphophorus*; Fig. 2A). Comparing
210 siblings with incompatible and compatible genotypes revealed a nearly universal lag in
211 development between individuals with incompatible genotypes and the most fully developed
212 individual in their brood (Fig. 2B, 2C). This developmental lag could itself cause mortality, since
213 *Xiphophorus* embryos that do not complete embryonic development within the mother fail to
214 survive more than a few days after birth (Supplementary Materials 1.3.1).
215
216 **Mitochondrial Biology in Hybrids**
217
218 The phenotypes of developing embryos suggest that the incompatibility may inhibit
219 somatic growth and development, likely through mitochondrial dysfunction. Given that
220 individuals with the incompatibility involving the *X. malinche* mitochondria generally do not
221 complete embryonic development, we turned to F_1 hybrids between *X. birchmanni* and *X.*
222 *malinche* to further explore the hybrid incompatibility *in vivo*. Since F_1 hybrids, which derive
223 their mitochondria from *X. malinche* and are heterozygous for ancestry at *ndufs5* and *ndufa13*,

224 are largely viable, we asked whether there was evidence for compensatory nuclear or
225 mitochondrial regulation that might be protective in F_1 hybrids. We found no evidence for allele-
226 specific expression of *ndufs5* or *ndufa13* in F_1 hybrids (Supplementary Materials 1.3.2; Fig. S11-
227 12) or significant differences in mitochondrial copy number in F_1 hybrids (Supplementary
228 Materials 1.3.3; Fig. S13).

229 With no clear indication of a compensatory regulatory response, we reasoned that we
230 might be able to detect reduced mitochondrial function, particularly in respiratory Complex I, in
231 hybrids heterozygous for the incompatibility. To examine mitochondrial function in *X.*
232 *birchmanni*, *X. malinche*, and hybrids harboring the *X. malinche* mitochondria and heterozygous
233 ancestry at *ndufs5* and *ndufa13,* we quantified respiratory phenotypes in isolated mitochondria
234 using a multiple substrate, uncoupler, and inhibitor titration protocol with the Oroboros O2K
235 respirometer (Fig. S14; Supplementary Materials 1.3.4). We found that Complex I efficiency was
236 significantly lower in hybrids compared to the two parental species (Fig. 2D; Fig. S15, P =
237 0.023, n = 7 per genotype), although overall levels of mitochondrial respiration were unchanged
238 (Fig. 2E, P = 0.97; Supplementary Materials 1.3.4). This points to reduced function of Complex I
239 in heterozygous individuals, as well as possible physiological compensation by other
240 components in the oxidative phosphorylation pathway.

241 Given physiological evidence for reduced Complex I function in hybrids heterozygous at
242 *ndufs5* and *ndufa13*, we predicted that there might be an altered frequency of protein complexes
243 incorporating both *X. malinche* mitochondrial proteins and *X. birchmanni* proteins at *ndufs5* and
244 *ndufa13* in F_1 hybrids. To test this prediction, we took a mass spectrometry based quantitative
245 proteomics approach. We used stable isotope-labeled peptides to distinguish between the *X.*
246 *birchmanni* and *X. malinche ndufs5* and *ndufa13* peptides in mitochondrial proteomes extracted

247 from F_1 hybrids (see Supplementary Materials 1.4.1-1.4.4). While native *ndufa13* peptides were
248 too rare to quantify accurately, we found consistent deviations from the expected 50-50 ratio of
249 *X. birchmanni* to *X. malinche* peptides for *ndufs5* in F_1 hybrids, with a significant
250 overrepresentation of *ndufs5* derived from *X. malinche* in the mitochondrial proteome (Fig. 2G-
251 H; Supplementary Materials 1.4.5). Since we did not observe allele-specific expression of *ndufs5*
252 (Fig 2F; Supplementary Materials 1.3.2; Fig. S11-12), this result is consistent with
253 disproportionate degradation of *X. birchmanni*-derived *ndufs5* peptides in the mitochondrial
254 proteome or differences in translation of *ndufs5* peptides from the two species.
255
256 **Substitutions in two mitochondrial proteins contact *ndufs5* and *ndufa13***
257
258 While we can leverage the independent recombination histories of natural hybrid
259 populations to pinpoint the nuclear components of the hybrid incompatibility, we cannot take this
260 approach to distinguish among the 37 genes in the swordtail mitochondrial genome, which do
261 not undergo meiotic recombination. To investigate the possible mitochondrial partners of *ndufs5*
262 and *ndufa13*, we therefore turned to protein modeling, relying on high quality cryo-EM based
263 structures (*43–45*).
264 Barring a hybrid incompatibility generated by regulatory divergence, which we address in
265 Supplementary Materials 1.3.2, we expect hybrid incompatibilities to be driven by amino acid
266 changes in the interacting proteins (*46*). We used the program RaptorX (*47*) to generate predicted
267 structures of *X. birchmanni* and *X. malinche ndufs5, ndufa13*, and nearby Complex I
268 mitochondrial and nuclear genes, which we aligned to a mouse cryo-EM Complex I structure
269 (Fig. 3A; Fig. S16; *43*). Using this structure, we detected amino acid substitutions between *X.*

270 *birchmanni* and *X. malinche* at the interfaces of *ndufs5, ndufa13* and four mitochondrially
271 encoded genes: *nd2, nd3, nd4l,* and *nd6* (Fig. 3A). Notably, one *ndufs5* substitution directly
272 contacts a substitution in *nd2* (Fig. 3B, Fig. S16). An unstructured loop in *nd6* that passes
273 between *ndufs5* and *ndufa13* harbors substitutions between *X. birchmanni* and *X. malinche* in 4
274 out of 22 amino acids that make up the loop, including one that is predicted to be in close
275 proximity to a substitution in *ndufs5* (Fig 3B, Fig. S16; Supplementary Materials 1.4.6). Results
276 of these structural predictions were robust to a number of technical variables (Supplementary
277 Materials 1.4.6). The direct contact between species-specific mitochondrial and nuclear
278 substitutions point to *nd2* and *nd6* as the genes most likely to be involved in the mitochondrial
279 component of the hybrid incompatibility (Fig. 3A, 3B; Fig. S17).
280
281 **Rapid evolution of Complex I proteins**
282
283 Theory predicts that hybrid incompatibilities are more likely to arise in rapidly evolving
284 genes (*4*, *5*, *15*). Consistent with this hypothesis, *ndufs5* is among the most rapidly evolving
285 genes genome-wide between *X. birchmanni* and *X. malinche* (Fig. 3C, 3D). Aligning the *ndufs5*
286 coding sequences of *X. birchmanni, X. malinche*, and twelve other swordtail species revealed
287 that all four amino acid substitutions that differentiate *X. birchmanni* and *X. malinche* at *ndufs5*
288 were derived on the *X. birchmanni* branch (Fig. 3C). Phylogenetic tests indicate that there has
289 been accelerated evolution of *ndufs5* on this branch (dN/dS > 99, N = 4, S = 0, CodeML branch
290 test $P = 0.005$, Fig. 3C). Similar patterns of rapid evolution are observed at *ndufa13*, which also
291 showed evidence for accelerated evolution in *X. birchmanni* (Fig. 3E, dN/dS = 1.2, N = 3, S = 1,
292 CodeML branch test $P = 0.002$).

293 Rapid evolution of *ndufs5* and *ndufa13* may be the result of coevolution with
294 mitochondrial substitutions, a mechanism that has been proposed to explain the outsized role of
295 the mitochondria in hybrid incompatibilities (*16*, *48*). Indeed, there is an excess of derived
296 substitutions in the *X. birchmanni* mitochondrial protein *nd6*, one of the proteins that interacts
297 with *ndufs5* and *ndufa13* (Table S3, Fig. S18; CodeML branch test $P = 0.005$). Moreover, a
298 number of the substitutions in both mitochondrial and nuclear genes are predicted to have
299 functional consequences (Supplementary Materials 1.5.1; Table S4, Fig. S19-20) and occur in
300 regions of direct protein-protein interaction between *ndufs5, ndufa13, nd2*, and *nd6* (Fig. 3A,
301 3B). Such colocalization of substitutions predicted to affect protein structure is precisely what
302 would be expected under classic models of hybrid incompatibility.
303
304 **Introgression of genes underlying mitonuclear incompatibility**
305
306 The presence of a mitonuclear incompatibility in *Xiphophorus* is especially intriguing, given
307 previous reports that mitochondrial genomes may have introgressed between species (*49*). While
308 *X. malinche* and *X. birchmanni* are sister species based on the nuclear genome, they are
309 mitochondrially divergent, with *X. malinche* and *X. cortezi* grouped as sister species based on the
310 mitochondrial phylogeny (Fig. 4A; *49*). As we show, all *X. cortezi* mitochondria sequenced to
311 date are nested within *X. malinche* mitochondrial diversity (Fig. 4B; Supplementary Materials
312 1.5.2; Fig. S19-S21), including the interacting mitochondrial genes *nd2* and *nd6* (Fig. 3A).
313 Simulations indicate that gene flow, rather than incomplete lineage sorting, likely drove
314 replacement of the *X. cortezi* mitochondria with the *X. malinche* sequence ($P < 0.002$ by
315 simulation; Fig. 4C; Supplementary Materials 1.5.3-1.5.4).

316 The introgression of the mitochondrial genome from *X. malinche* into *X. cortezi* suggests the
317 possibility that other Complex I genes may have co-introgressed (*50*). Indeed, the nucleotide
318 sequence for *ndufs5* is identical between *X. malinche* and *X. cortezi*, and the sequence of *ndufa13*
319 differs by a single synonymous mutation (although conservation of both genes is high throughout
320 *Xiphophorus*; Fig. S22-23). Identical amino acid sequences at the genes underlying the hybrid
321 incompatibility between *X. malinche* and *X. birchmanni* suggest that *X. cortezi* and *X.*
322 *birchmanni* are likely to harbor the same mitonuclear incompatibility, as a result of ancient
323 introgression between *X. malinche* and *X. cortezi* (Supplementary Materials 1.5.3-1.5.4). This
324 inference is supported by analysis of ancestry in two contemporary *X. birchmanni* × *X. cortezi*
325 hybrid zones, which reveals a significant depletion of non-mitochondrial parent ancestry at
326 *ndufs5* and *ndufa13* (Fig. 4D, Supplementary Materials 1.5.5; *51*). These results are consistent
327 with the mitonuclear incompatibility observed in *X. birchmanni* × *X. malinche* being active in *X.*
328 *birchmanni* × *X. cortezi* populations (see also *51*). This exciting finding hints that genes
329 underlying hybrid incompatibilities can introgress together, transferring incompatibilities
330 between related species.
331
332 **Implications**
333
334 An open question in the field is what genetic and evolutionary forces drive the emergence of
335 hybrid incompatibilities, especially between closely related species. We identified a mitonuclear
336 incompatibility that involves at least three genes and causes hybrid lethality in lab and wild
337 populations. Theory predicts that hybrid incompatibilities involving multiple genes should be
338 common (*6*, *7*), but with few exceptions (*8*, *12–14*), they remain virtually uncharacterized at the

genic level (*6*). Moreover, we show that there has been exceptionally rapid evolution in both mitochondrial and interacting nuclear genes in *X. birchmanni*, which may have introduced mutations that are incompatible in hybrids (Fig. 4). Whether driven by rapid adaptation or some other mechanism, this is consistent with the hypothesis that the coevolution of mitochondrial and nuclear genes can drive the overrepresentation of mitonuclear interactions in hybrid incompatibilities (*25*, *26*, *48*).

Characterizing the incompatibility across multiple scales of organization allowed us to identify the mechanisms through which it acts (*52–54*). Our results suggest that the lethal form of the hybrid incompatibility is driven by dysfunctional protein-protein interactions in Complex I of the mitochondrial electron transport chain that cause breakdown in bioenergetic pathways. These physiological impacts are observed in hybrids heterozygous for the incompatibility, even though these individuals escape its lethal effects.

Finally, this mitonuclear incompatibility provides a new case in which the same genes are involved in incompatibilities across multiple species (*55–57*). Surprisingly, we found that introgression has resulted in the transfer of genes underlying the incompatibility from *X. malinche* to *X. cortezi*, and evidence from *X. birchmanni* × *X. cortezi* hybrid populations indicates that the incompatibility is likely under selection in these populations as well. The possibility that hybridization could transfer incompatibilities between species has not been previously recognized, perhaps due to an underappreciation of the frequency of hybridization. The importance of past hybridization in the structure of present reproductive barriers is a promising area for future inquiry.

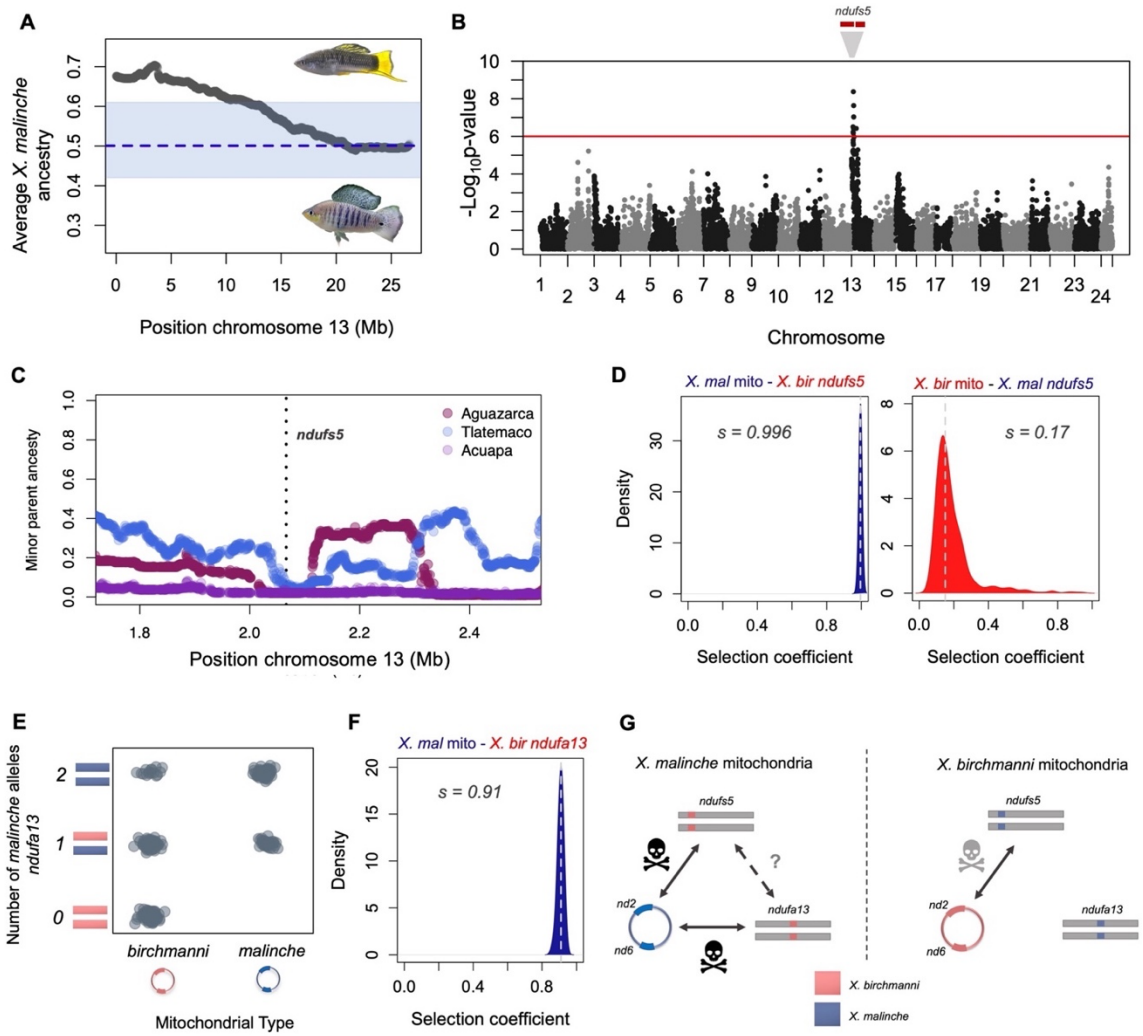

Figure 1. Admixture mapping pinpoints a mitonuclear incompatibility in *Xiphophorus*. (**A**) F_2 hybrid offspring of a laboratory cross between *X. malinche* (top) and *X. birchmanni* (bottom) have segregation distortion towards *X. malinche* ancestry across a large region of chromosome 13. Blue envelop shows the 99% quantiles of *X. malinche* ancestry at all ancestry informative sites genome wide. Dashed line shows the expected *X. malinche* ancestry for this cross. (**B**) Admixture mapping results for association between nuclear ancestry and mitochondrial haplotype in natural hybrids, controlling for genome-wide ancestry. Red line indicates genome-wide significance threshold determined by simulations. (**C**) Non-mitochondrial parent ancestry in natural hybrid populations fixed for *X. birchmanni* (Aguazarca, Acuapa) or *X. malinche* (Tlatemaco) mitochondrial haplotypes. Vertical dashed line represents the position of *ndufs5*, one of three genes in a shared ancestry desert at the center of the admixture mapping region. (**D**)

373 Results of approximate Bayesian computation (ABC) simulations estimating selection coefficients against
374 heterospecific ancestry combinations at *ndufs5* and the *X. birchmanni* or *X. malinche* mitochondrial
375 haplotypes. Shown here are posterior distributions from accepted simulations; vertical lines indicate the
376 maximum a posteriori estimates for selection coefficient in each direction. (**E**) Observed genotype
377 frequencies at *ndufa13* in the admixture mapping population. (**F**) Results of ABC simulations estimating
378 the strength of selection against the combination of *X. malinche* mitochondria with *X. birchmanni*
379 ancestry at *ndufa13*. Shown here is the posterior distribution of accepted simulations; the vertical line
380 indicates the maximum a posteriori estimate for the selection coefficient. (**G**) Schematic of identified
381 interactions with both the *X. malinche* and *X. birchmanni* mitochondrial genomes from our mapping data
382 and strength of selection underlying each interaction in hybrids (gray – moderate, black – near lethal). We
383 discuss evidence for possible interactions between *ndufs5* and *ndufa13* (indicated by the dashed line) in
384 Supplementary Materials 1.1.8.

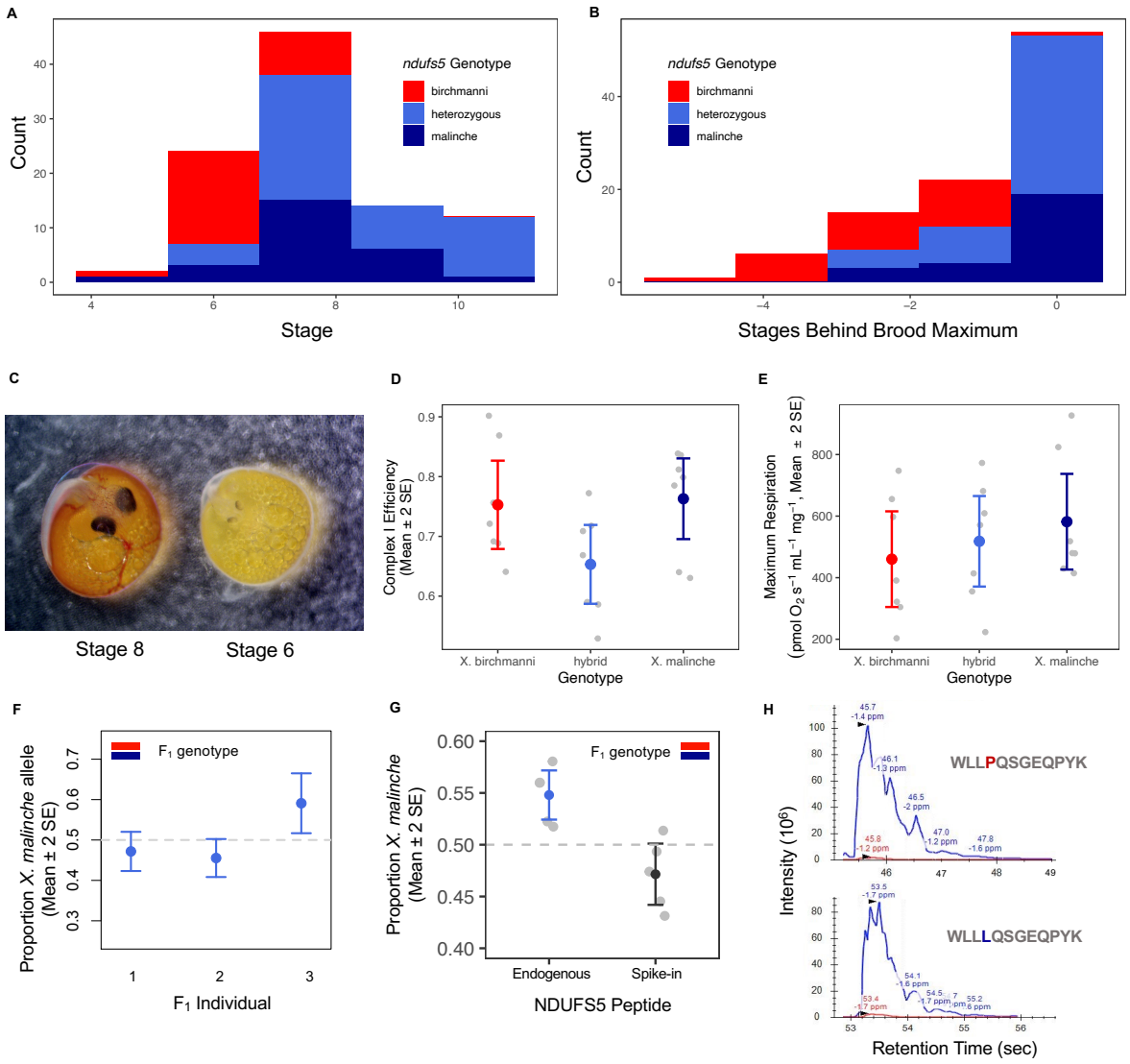

Figure 2. Developmental, physiological, and proteomic phenotypes associated with the hybrid incompatibility. (**A**) Developmental stage and *ndufs5* genotypes of hybrid embryos with *X. malinche* mitochondria. (**B**) Lag in development of hybrid embryos with *X. malinche* mitochondria compared to the most developed embryo in the brood as a function of *ndufs5* genotype. (**C**) Siblings from the admixture mapping population at different developmental stages. (**D**) Results of Oroboros O2K respirometer Complex I efficiency assay for adult *X. birchmanni*, *X. malinche*, and hybrid individuals with *X. malinche* mitochondria and heterozygous ancestry at *ndufs5* and *ndufa13* (n=7 per genotype). Colored points show the mean, whiskers show two standard errors, and gray points show individual data. (**E**) Despite differences in Complex I efficiency, maximum respiration rates do not differ between groups. (**F**) Allele-

395 specific expression of *ndufs5* in three adult F_1 hybrids. Points show the proportion of reads in F_1 hybrids
396 supporting the *X. malinche* allele, bars show two standard errors. (**G**) Results of quantitative mass
397 spectrometry analysis of *ndufs5* peptides in mitochondrial proteomes derived from five adult F_1 hybrids.
398 Grey points show the proportion of area under the spectral curves contributed by the *X. malinche* allele in
399 a given individual, colored points show the mean, and bars show two standard errors. The left column
400 shows results for endogenous peptides present in F_1s, the right column shows results for the control where
401 heavy-labeled standards of each peptide were spiked in. (**H**) Representative Skyline traces of the *X.*
402 *malinche* and *X. birchmanni* versions of the *ndufs5* peptide from parallel reaction monitoring. Blue lines
403 represent the spectral intensity from heavy-labeled peptides, red lines represent endogenous peptides.
404 Inset shows the sequence of the *X. birchmanni* and *X. malinche* peptides being detected.
405

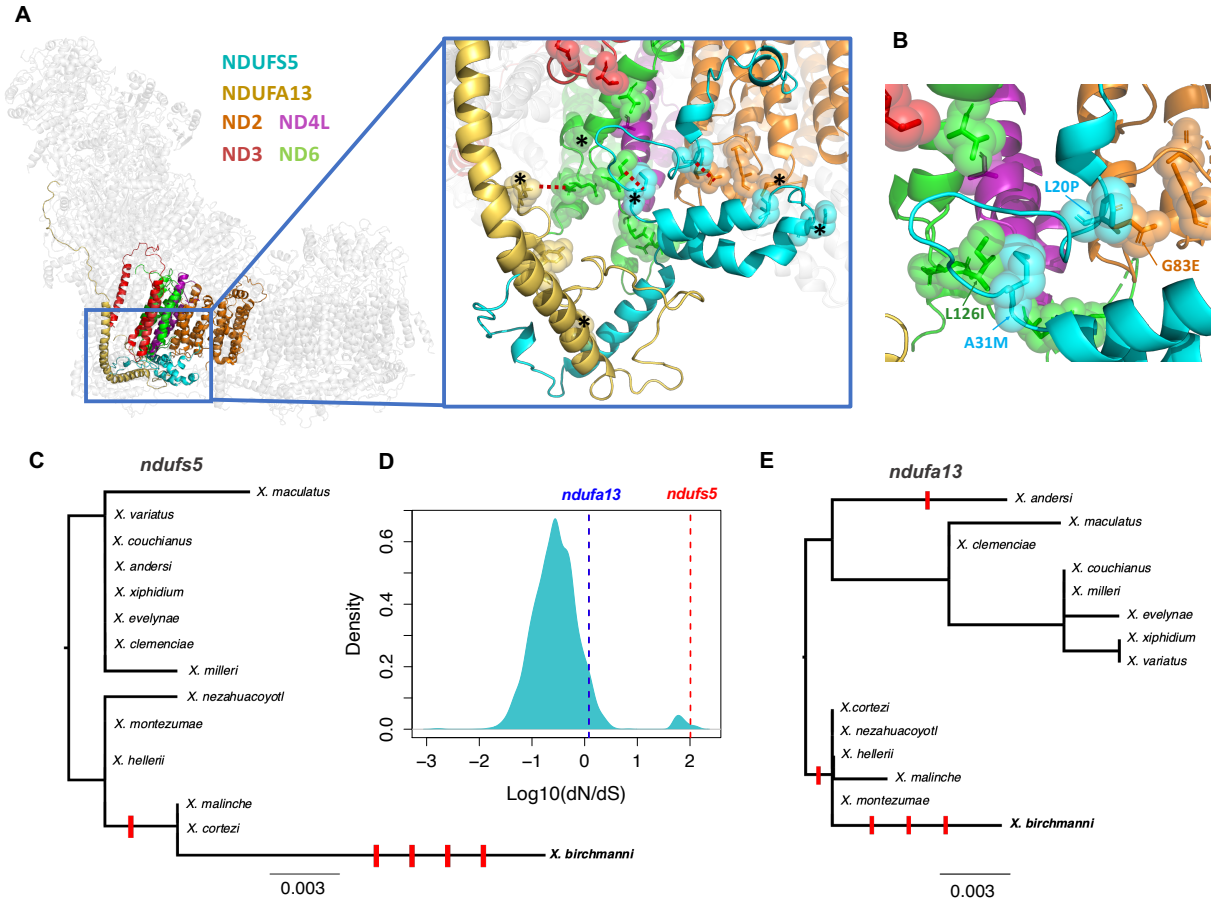

Figure 3. Predicted structures of *Xiphophorus* respiratory Complex I reveal interacting substitutions at protein interfaces. (**A**) *Xiphophorus* respiratory Complex I structures generated by RaptorX using alignment to a template mouse cryo-EM structure. Colored protein structures include *ndufs5, ndufa13*, and the four mitochondrially encoded *nd* genes in contact with *ndufs5* or *ndufa13*. Inset shows interaction surface between these genes. Asterisks denote residues with substitutions in *X. birchmanni* predicted to affect protein function (Table S4). Dotted red lines highlight substitutions in *ndufs5, ndufa13, nd2,* and *nd6* in close proximity. (**B**) Detailed view of interaction interface between *ndufs5*, *nd2*, and *nd6.* Arrows highlight substitutions in direct contact (represented as spheres), with letters denoting the *X. malinche* allele, the residue number, and the *X. birchmanni* allele, respectively. (**C**) Gene tree for *ndufs5* generated with RAxML, highlighting an excess of substitutions along the *X. birchmanni* branch. Scale bar represents number of nucleotide substitutions per site. Derived non-synonymous substitutions are indicated by red ticks along the phylogeny. Note that spacing between ticks

419 is arbitrary. (**D**) Distribution of Log_{10} dN/dS between *X. birchmanni* and *X. malinche* across all nuclear

420 genes in the genome with values for *ndufs5* and *ndufa13* highlighted. (**E**) Gene tree for *ndufa13* generated

421 with RAxML, highlighting an excess of substitutions along the *X. birchmanni* branch (as in C).

422

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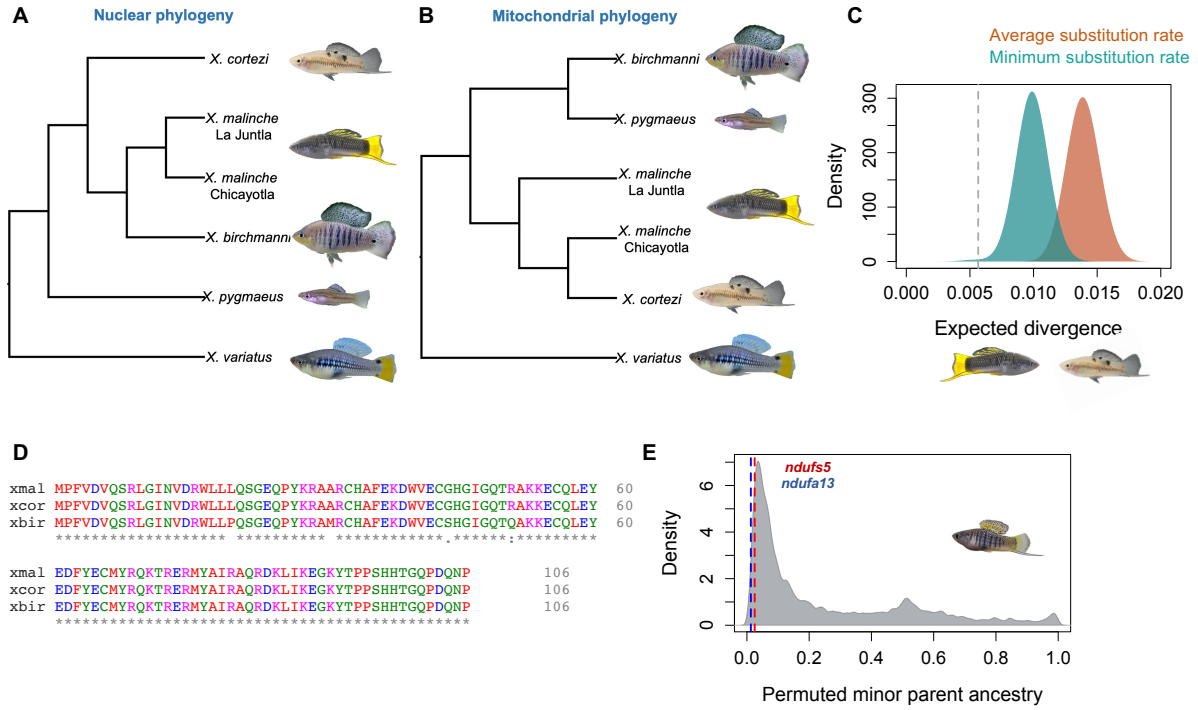

Figure 4. Phylogenetic analysis and ancestry mapping suggest that genes underlying the mitonuclear incompatibility have introgressed from *X. malinche* into *X. cortezi*. (**A**) Nuclear phylogeny of *Xiphophorus* species, showing that *X. birchmanni* and *X. malinche* are sister species (*49*). (**B**) Phylogeny constructed from whole mitochondrial genome sequences showing that *X. cortezi* mitochondria are nested within *X. malinche* mitochondrial diversity. (**C**) Results of simulations modeling expected mitochondrial divergence between *X. malinche* and *X. cortezi* in a scenario with no gene flow. Distributions represent pairwise sequence divergence in two sets of simulations. The first set used the average mitochondrial substitution rate observed between *Xiphophorus* species (red), and the second used the minimum mitochondrial substitution rate observed (blue). The dotted line shows observed divergence between mitochondrial haplotypes in *X. malinche* and *X. cortezi*, indicating that past mitochondrial introgression is more consistent with the observed data than incomplete lineage sorting (Supplementary Materials 1.5.3). (**D**) Clustal alignment of *ndufs5* sequences. *X. malinche* and *X. cortezi* have identical amino acid sequences at *ndufs5*, while *X. birchmanni* is separated from them by four substitutions. Colors indicate properties of the amino acid, asterisks indicate locations where the amino acid sequences are

439 identical. (**E**) Non-mitochondrial parent ancestry is lower than expected by chance in two natural *X.*
440 *cortezi* × *X. birchmanni* hybrid populations fixed for the *X. cortezi* mitochondrial haplotype (Fig. S24) at
441 *ndufs5* (red line) and *ndufa13* (blue line). Gray distribution shows permutations randomly drawing 0.1
442 centimorgan windows from the two *X. cortezi* × *X. birchmanni* hybrid populations. Inset shows a *X.*
443 *cortezi* × *X. birchmanni* hybrid.
444

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Supplementary Materials

Materials and Methods

Tables S1 – S11

Figs. S1 – S27