Title: Pre- and post-mating reproductive isolation evolve independently during rapid adaptation to high temperature

Authors: Sheng-Kai Hsu[1,2], Wei-Yun Lai[1,2], Johannes Novak[3], Felix Lehner[1], Ana Marija Jakšić[1,2,4], Elisabetta Versace[5], Christian Schlötterer[1*]

Affiliations:

[1] Institut für Populationsgenetik, Vetmeduni Vienna, Vienna, Austria.

[2] Vienna Graduate School of Population Genetics, Vetmeduni Vienna, Vienna, Austria.

[3] Institute of Animal Nutrition and Functional Plant Compounds, Vetmeduni Vienna, Vienna, Austria.

[4] Current affiliation: École polytechnique fédérale de Lausanne, Lausanne, Switzerland.

[5] Department of Biological and Experimental Psychology, Queen Mary University of London, London, UK.

*****Correspondence: christian.schloetterer@vetmeduni.ac.at; Tel.: +43-1-25077-4300.

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Abstract

Ambient temperature is one major ecological factor driving adaptation in natural populations, but its impact on the emergence of new species is not yet clear. Here, we explored the evolution of reproductive isolation during temperature adaptation by exposing 10 replicate *Drosophila simulans* populations to a hot temperature regime. Within less than 200 generations, both pre- and post-mating reproductive isolation evolved. The altered lipid metabolism of evolved flies also affected the cuticular hydrocarbon (CHCs) profiles. Different CHC profiles could explain the emerged assortative mating between ancestral and evolved populations. Hence, we identified the hallmark of ecological speciation driven by temperature adaptation. While this pre-mating isolation occurred only between ancestral and evolved replicate populations, post-mating reproductive isolation was observed among evolved replicate populations. We propose that epistatic interactions of reproduction-related genes between males and females resulted in adaptive co-evolution. Incompatibilities between different gene combinations favored in each replicate could explain the observed post-mating reproductive isolation. We anticipate that this "mutation-order-like speciation" from standing genetic variation, a new speciation process, is widespread in nature when highly polygenic traits are involved in adaptation.

Main text:

Introduction

The emergence of new species is one of the most fundamental processes in biology (Coyne and Orr, 2004; Darwin, 1859; Dobzhansky, 1940). Several speciation models highlight the importance of genetic adaptation as a driving force for the evolution of reproductive isolation (Nosil, 2012; Schluter, 2009, 2001). Ecological speciation is one of the most prominent adaptation-related speciation mechanisms. It occurs as by-product of local adaptation where selection modifies a “magic trait” that affects both survival and reproduction or two genetically linked traits (Mayr, 1947; Nosil and Schluter, 2011; Rundle and Nosil, 2005; Schluter, 2009, 2001). Ecological speciation has not only been empirically demonstrated (Dettman et al., 2008, 2007; Dodd, 1989; Funk et al., 2006; Nosil et al., 2009; Rundle et al., 2000) but also the pleiotropic role of the selected traits has been shown (Boughman et al., 2005; Jiggins, 2008; Mallet and Barton, 1989; Nagel and Schluter, 1998; Rundle et al., 2005).

Ambient temperature is one of the major ecological factors driving adaptation in natural populations. Nevertheless, it is not yet clear to what extent adaptation to temperature resulted in reproductive isolation (Keller and Seehausen, 2012). Various studies on *Drosophila* populations adapted to different temperature regimes provided mixed results. While studies on Vetukhiv's experimental populations failed to find thermal adaptation-associated reproductive isolation (Ehrman, 1964), Kilias et al. (1980) demonstrated assortative mating for individuals adapted to the same temperature regime. The prezygotic isolation in Kilias and colleagues’ work was independent of the temperature in which it was assessed (Kilias et al., 1980). This is in contrast to the documented temperature-dependent increase in mating

success of males in their native temperature regimes in other *Drosophila* populations (Dolgin et al., 2006), which failed to be reproduced (Correia et al., 2010).

In this study, we tackle this open question of temperature adaptation-driven reproductive isolation using a highly replicated experimental evolution study (Barghi et al., 2019), where 10 replicate populations derived from a single polymorphic *Drosophila simulans* population have been independently adapting to a novel hot temperature regime for more than 100 generations. The genomic selection signature of these replicate populations indicated a high level of genetic redundancy for temperature adaptation (Barghi et al., 2019). Genetic redundancy refers to the situation that more genetic variants are segregating than needed for adaptation (Barghi et al., 2020; Láruson et al., 2020). Since these high temperature-adapted populations achieved convergent fitness increase during temperature adaptation by different genetic paths (Barghi et al., 2019), they serve as a very interesting system to study thermal adaptation-associated speciation. First, we can address the question whether adaptation to high temperature results in reproductive isolation between evolved and ancestral populations. Second, the use of different genetic paths in individual replicates provides the opportunity to test whether reproductive isolation also occurred between replicate populations adapting to the same environment. Our experiments uncovered different mechanisms of reproductive isolation. First, all evolved replicates developed pre-mating isolation to the ancestral populations but not among evolved ones. The latter showed, however, significant post-mating reproductive isolation.

Results

Adapting for less than 200 generations to a novel hot environment, replicated populations evolved pre-mating reproductive isolation from their common ancestors. Male flies discriminated between ancestral and evolved females in temperature-controlled courtship arenas. While evolved males preferentially chased evolved females of the same population, ancestral males preferred ancestral females (Figure 1a, Kruskal-Wallis test, $p < 0.05$). This pre-mating isolation was also confirmed by significant positive assortative mating in multiple-choice mating assays (Figure 1b, $Y_{avg.} = 0.23$, Fisher's exact test, $p < 0.01$). Males and females from the same population preferentially mated with each other. This pattern is highly consistent across evolved replicate populations (Figure 1b). No assortative mating was observed between flies from two independently replicate evolved populations (Figure 1b), suggesting that they have either adapted using the same genetic path or different paths that did not build assortative mating among them.

Cuticular hydrocarbons (CHCs) serve central roles in both mate recognition (Coyne, 1996; Pardy et al., 2019; Seeholzer et al., 2018) and desiccation resistance (Chung and Carroll, 2015; Gibbs, 1998), and are therefore well-established candidates for a magic trait in *Drosophila* (Rundle et al., 2005). We tested the divergence in CHC composition between ancestral and evolved populations by measuring all hydrophobic compounds coated on the outer skeleton of virgin flies of each sex with gas chromatography/MASS spectrometry (GC/MS). We detected 18 major CHC compounds across both sexes, which differ in length of carbon chain or numbers of double bonds (Dembeck et al., 2015; Pardy et al., 2019) (Table 1 and Figure 2 — Figure Supplement 1). The abundance of most CHCs differed significantly between sexes as well as between evolved and ancestral populations. No significant interactions between sexual dimorphism and evolution were observed for these traits (Table

1). This pattern is visualized by a principal component analysis (PCA) on the relative abundance of 12 CHCs that are detected in all samples (Figure 2a). The first PC, which explains 68.43% of total variance, reflects the differences between the two sexes – males synthesize more unsaturated CHCs than females (Figure 2b). The variation caused by adaptation to a hot laboratory environment is reflected by PC2 which accounts for 16.38% of the total variance (Figure 2a). As already shown by the lack of significant interaction of CHC abundance (Table 1), the PCA also indicates parallel evolutionary changes in CHC composition between the two sexes. Evolved flies synthesize more unsaturated CHCs with longer carbon chains (Figure 2b and Figure 2c). Interestingly, similar modification in CHC compositions has been documented for multiple *Drosophila* species in latitudinal clines (Frentiu and Chenoweth, 2010), suggesting a causal link with temperature adaptation.

We used gene expression data of the focal populations (Hsu et al., 2020) to elucidate the basis of the CHC composition changes. Among the 469 genes with a significant increase in expression across replicate evolved populations, we found a prominent enrichment for genes involved in "very long-chain fatty acid biosynthetic process" in both sexes. 27 out of the 36 (33 are detected in our data) previously reported CHC synthesis-related genes in *Drosophila melanogaster* (Dembeck et al., 2015) showed significant expression changes in at least one sex during adaptation in the replicated *D. simulans* populations (Fisher's exact test, odds ratio = 3.33, p-value < 0.01; Figure 2 — Figure Supplement 2). 16 of these genes changed their expression in the same direction in both sexes. *Desaturases* (*desat 1* and *desat 2*) and *elongases* (*CG9458*, *CG18609* and *CG30008*) were consistently up-regulated in all replicate evolved populations (Figure 2c and Figure 2 — Figure Supplement 2). This consistent transcriptional modification of long-chain fatty acid/CHC biosynthesis during the evolution can explain the significant changes in CHC compositions in the evolved flies.

Adaptation to high temperature in the studied populations does not only affect the fatty acid metabolism, CHC profiles and mating preference, but also changes in other organismal phenotypes such as neuronal signaling (Jakšić et al., 2020), male reproductive activity (Hsu et al., 2020) and female fecundity (Barghi et al., 2019). Based on the heterogeneous genetic response among the 10 replicate evolved populations (Barghi et al., 2019), we reasoned that these parallel phenotypic changes may have been achieved by different genetic paths. Hence, it is possible that alternative adaptive paths generated post-mating incompatibilities among replicate evolved populations – even without pre-mating isolation. Testing for post-mating reproductive isolation among three replicates of evolved populations (Figure 3a), we found that F1 individuals from the same replicate evolved population produced more viable offspring than F1 from crosses between replicate evolved population (Figure 3b, Wilcoxon's test, $p < 0.05$). This 8.3% reduction in fitness indicates a significant but moderate reproductive isolation between different replicate evolved populations adapting to the same new environment. Considering the absence of pre-mating isolation (Figure 1b) and no fecundity difference (Barghi et al., 2019) among replicate evolved populations, the reduced hybrid fitness indicates that another (post-mating; fertilization- and/or viability-affecting) process leading to reproductive isolation occurs along with pre-mating isolation between the ancestral and evolved populations.

We used RNA-Seq to explore the genetic basis of this post-mating reproductive isolation. Reasoning that putative causal genes may diverge in gene expression, we searched for genes differentially expressed among replicate evolved populations. In total, we identified 3,062 genes which differed in at least one evolved population from the others (Figure 4 — Figure Supplement 1and Supplementary file 1). Functional enrichment analysis revealed that a

substantial portion of these divergently expressed genes is highly expressed in testis and is involved in reproduction-related processes (e.g. multicellular organism reproduction (GO:0032504); Supplementary file 2). This provides a connection between the divergence in reproduction-related genes and the observed incompatibilities among replicates. Such transcriptomic divergence and potentially related incompatibilities can be a consequence of genetic drift or replicate-specific adaptive responses to common selection pressure. Focusing on the 255 reproduction-associated genes that diverged in expression across replicate evolved populations (see Materials and methods), replicate-specific selection responses were detected (Figure 4a) with a significantly higher heterogeneity compared to a random set of genes (Figure 4b, Wilcoxon's test, $p < 0.001$). Most of these reproduction-associated genes changed their expression in only one or two replicate population(s) towards the same direction (mostly up-regulation) during the adaptation (Figure 4a and Figure 4 — Figure Supplement 1and Supplementary file 2). The higher expression of reproduction-related genes is consistent with the increased reproductive activity of the evolved populations (Figure 1A and (Hsu et al., 2020)). Genetic drift is unlikely to cause such unidirectional changes of different sets of functionally similar genes in independent replicate populations. In particular, this upregulation pattern was specific for reproduction-related genes and could not found for other divergently expressed genes (Figure 4 — Figure Supplement 1and Supplementary file 3). It is conceivable that the polygenic and epistatic control of post-mating reproductive processes (Cabot et al., 1994; Hollocher et al., 1997; Palopoli and Wu, 1994) results in a heterogeneous response to common selection (possibly for higher competitive ability of male). With the limitation of only three data points, the stronger post-mating isolation of replicate evolved population pairs with more diverged gene expression further supports alternative selection responses in replicate populations as underlying genetic mechanism (Figure 4 — Figure Supplement 1and Supplementary file 4).

Discussion

The analysis of replicate populations adapting to the same environment demonstrated that reproductive isolation could rapidly evolve during adaptation to a novel temperature regime. We found evidence for pre-mating isolation between the ancestral and evolved populations as well as post-mating isolation among the replicated evolved populations (Figure 5a). The effects ($Y_{avg.}$ = 0.23 for pre-mating isolation; 8.3% reduction in fitness for post-mating isolation) are very similar to the ~10% of reproductive isolation between *D. simulans* and *D. mauritiana*, which diverged already 0.25 million year ago (Carracedo et al., 2000). Only for species pairs which diverged for longer time-scales, stronger reproductive isolation has been shown. For instance, *D. melanogaster* and *D. simulans*, which diverged 5.4 million years ago show more than 50% reproductive isolation (Carracedo et al., 2000).

The assortative mating between the ancestral and each evolved population clearly reflects a by-product of temperature adaptation. Consistent with several experimental speciation studies in *Drosophila* (Davis et al., 2021; Rundle et al., 2005), which showed that a modified CHC profile can result in assortative mating, we also found that the CHC profile in all evolved populations changed in the same direction. Further support for environmental factors, such as temperature, shaping CHC composition comes from latitudinal clines of multiple *Drosophila* species (Frentiu and Chenoweth, 2010). Hence, we propose that the assortative mating between the ancestral and each replicate of the evolved population is a hallmark of incipient ecological speciation mediated by CHC modification during the adaptation to a new hot environment.

While no pre-mating isolation was observed among the replicated populations adapting to the same environment, significant post-mating incompatibilities evolved. This reproductive isolation among replicate evolved populations is particularly interesting as it implies that incompatibilities were established within 200 generations of parallel adaptation from standing genetic variation. De novo mutations are unlikely to contribute to such short-term adaptation in experiments with polymorphic ancestral populations (Burke et al., 2010). This contrasts the mutation-order speciation process during which incompatibilities arise from new beneficial mutations that arise independently in isolated populations adapting to the same environments (Mani and Clarke, 1990; Nosil, 2012; Schluter, 2009, 2001) (Figure 5 — Figure Supplement 1). This mutation-order speciation process has been empirically demonstrated in yeast adapting to a stressful environment. Different beneficial mutations were independently acquired from an isogenic progenitor and resulted in incompatibilities among replicates (Dettman et al., 2007; Ono et al., 2017). Unlike our experiment, the absence of standing genetic variation and the large effective population size facilitated the emergence and spread of novel mutations. Hence, the post-mating reproductive isolation in our experiment probably differs from mutation-order speciation (Figure 5 — Figure Supplement 1), which implies that we uncovered a hitherto uncharacterized speciation process during parallel adaptation from standing genetic variation.

We propose the term “mutation-order-like speciation” to explain the reproductive isolation among the evolved replicates because of its conceptual similarity to the mutation-order speciation model. Genetic redundancy and particularly epistasis are crucial in the proposed model. Genetic redundancy allows alternative genetic solutions to common selection on a polygenic trait. If alternative adaptive paths for the same magic trait comprise stochastic sets of independent and redundant variants, we expect classic ecological speciation with

independently adapted populations, but no reproductive incompatibilities are predicted as the selected variants are interchangeable (Figure 5b: left panel). In contrast, when epistatic interactions are involved on top of genetic redundancy, replicate populations will take alternative paths that require compatibility between the involved genes (i.e. interacting genes are prone to be co-selected). This requirement of the compatibility among involved genes can lead to incompatibilities not only between the ancestral and adapted populations but also between replicate populations (Figure 5b: right panel). The evolution in reproductive isolation between the replicate populations in this case is termed “mutation-order-like speciation”.

Genes involved in male reproduction are not only relevant for males, but also serve important functions in females, either synergistically or antagonistically (Avila et al., 2010; Chapman, 2006; Friberg and Arnqvist, 2003; LaFlamme et al., 2012; Liddle et al., 1995; Tram and Wolfner, 1998; Wolfner, 1997). Hence, we hypothesized that the distinct combinations of reproduction-related genes being favored in response to the common selection may drive the observed post-mating incompatibilities (Figure 4). Selection for a reproduction-related gene in males may trigger an evolutionary response in females, which in turn could affect another reproduction-related gene in males and so on and so forth. A nice demonstration of this co-evolutionary cascade comes from an experimental evolution study during which the evolution of males and females has been uncoupled by preventing the evolution of females (Rice, 1996). Such a run-away process (Brockhurst et al., 2014) may not have a single solution given the complexity in post-mating reproductive processes (Cabot et al., 1994; Hollocher et al., 1997; Palopoli and Wu, 1994), but rather multiple different paths. This will not only lead to an accelerated divergence of multiple genes among replicate populations but also builds incompatibilities among different co-evolutionary cascades established separately in each

replicate (Haerty et al., 2007). Support for our hypothesis that co-evolutionary processes for reproduction-related genes could drive post-mating incompatibilities in our experiment comes from the negative association between the similarity in the co-evolutionary cascades (i.e. expression of reproduction related genes) and the incompatibilities between evolved replicates (Figure 4 — Figure Supplement 4). Thus, sexual selection-induced co-evolution is a likely mechanism that caused the epistatic interaction leading to incipient mutation-order-like speciation (Figure 4c).

While it would be clearly interesting to test the proposed mutation-order-like speciation model functionally, we would like to add a cautionary note that this may not be addressed with gene knockout/knockdown experiment by mutants or RNAi. Since the putative transcriptional responses involved quantitative rather than qualitative changes, gene knockouts are unlikely to mirror the phenotypic changes observed in our study (Zhang et al., 2021). Allelic replacements are probably more suitable, but given the numbers of candidate genes involved and the potential epistatic interaction among them, this becomes a daunting task, which requires the development of high-throughput methodologies to functionally validate hundreds of candidate variants/genes behind a polygenic trait.

This study highlights the possibility that reproductive isolation could evolve among replicate populations adapting to the same environment due to selection on different sets of standing genetic variants. This implies that mutation-order-like speciation proposed here may frequently occur among repeated ecological speciation events, but remains uninvestigated in natural populations. Polygenic control and heterogeneous genomic divergence have been documented in some iconic cases of replicated ecological speciation in nature such as the

convergent morphological radiation of cichlid species in East African lakes (Kautt et al., 2020; Kocher et al., 1993) as well as the repeated colonization to fresh water systems of oceanic stickleback species (Conte et al., 2015; Lackey and Boughman, 2017). As suggested by the proposed mutation-order-like speciation model, potential incompatibilities may evolve even among sister species with the same adaptive phenotypes, but alternative genetic pathways with interacting genes. Hence, to more thoroughly understand the genetics of speciation, it is important to investigate also such non-parallel adaptive divergence and potentially associated incompatibilities in the natural systems with extra caution to the effects of drift and niche dimensionality (Chevin et al., 2014; Thompson et al., 2019).

Table 1. Abundance of cuticular hydrocarbons (CHCs) in relation to sex and evolution

Peak No.	Retention index	Name	% in anc. female	% in anc. male	% in evo. female	% in evo. male	ANOVA (p-value)		
							Sex	Evolution	Sex:Evolution
1	2187	x-Docosene (x-C22:1)	0.96±0.02	0.69±0.02	0.88±0.02	0.66±0.01	1.18E-18	1.88E-09	9.04E-01
2	2195	cis-Vaccenyl Acetate (cVA)	-	-	-	-	-	-	-
3	2200	n-Docosane (n-C22)	1.35±0.04	0.58±0.01	1.26±0.03	0.67±0.03	1.37E-31	1.38E-02	3.40E-01
4	2279	9-Tricosene (9-T)	1.67±0.07	2.77±0.09	2.16±0.11	3.78±0.09	1.99E-27	1.23E-04	6.95E-01
5	2285	7-Tricosene (7-T)	75.48±0.42	75.29±0.44	73.64±0.44	71.70±0.52	2.64E-01	1.01E-06	6.02E-01
6	2294	5-Tricosene (5-T)	0.68±0.03	0.83±0.04	0.64±0.04	0.79±0.02	1.04E-03	1.47E-03	5.59E-01
7	2300	n-Tricosane (n-C23)	9.64±0.20	8.39±0.14	9.08±0.15	8.14±0.17	1.39E-05	1.65E-07	9.04E-01
8	2383	6-Tetracosene (6-C24:1)	0.64±0.01	0.58±0.02	0.87±0.03	0.86±0.03	1.43E-01	1.97E-12	5.49E-01
9	2391	5-Tetracosene (5-C24:1)	0.25±0.01	0.27±0.01	0.40±0.02	0.42±0.02	1.46E-01	1.33E-11	5.59E-01
10	2400	n-Tetracosane (n-C24)	0.34±0.02	0.13±0.02	0.39±0.02	0.15±0.02	1.52E-21	7.32E-01	6.27E-01
11	2465	7,11-Pentacosadiene (7,11-PD)	0.35±0.03	0.82±0.03	0.28±0.02	0.86±0.05	1.12E-29	1.53E-04	3.40E-01
12	2478	9-Pentacosene (9-P)	0.95±0.05	0.75±0.04	1.28±0.12	1.08±0.05	1.79E-06	9.40E-06	6.08E-01
13	2486	7-Pentacosene (7-P)	1.59±0.06	1.98±0.05	2.93±0.18	2.90±0.12	4.26E-02	8.37E-12	3.71E-02
14	2500	n-Pentacosane (n-C25)	1.44±0.05	1.00±0.04	1.31±0.03	0.85±0.03	2.45E-14	8.43E-07	5.59E-01
15	2665	7,11-Heptacosadiene (7,11-HD)	1.85±0.07	4.04±0.10	2.18±0.08	5.28±0.20	2.24E-45	1.38E-02	5.59E-01
16	2700	n-Heptacosane (n-C27)	1.56±0.08	0.68±0.05	1.28±0.09	0.45±0.05	1.22E-26	3.25E-05	7.45E-01
17	2863	7,11-Nonacosadiene (7,11-ND)	1.11±0.06	1.20±0.05	1.31±0.06	1.41±0.08	8.30E-02	7.21E-01	6.08E-01
18	3000	n-Triacotane (n-C30)	-	-	-	-			

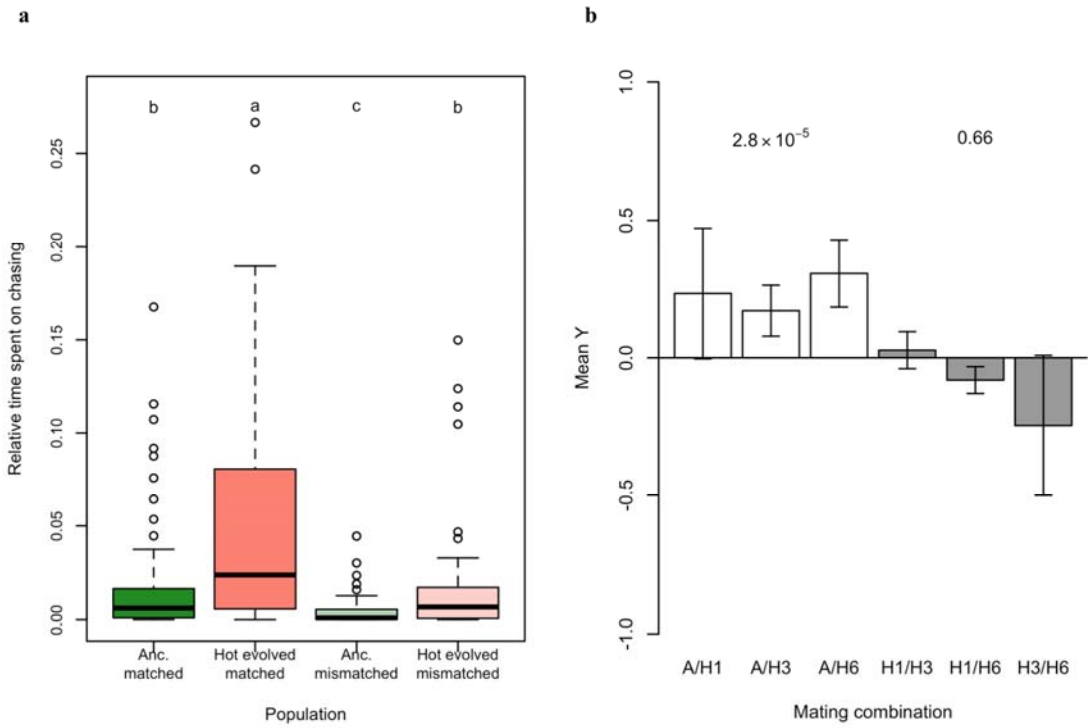

Figure 1. Evolution of mating preference and pre-mating reproductive isolation. a. Male reproductive activity in the presence of the females from the same evolved population or the ancestral one. The reproductive activity was measured as the time spent on chasing. Males spent significantly more time chasing females from the same populations (Kruskal-Wallis test, $p < 0.001$; post-hoc groups were indicated as the small-case letter above the boxes). **b.** Yule's index for assortative mating. There is significantly positive assortative mating between flies from the same populations (white bars) while there's no significant effect among the replicate evolved populations (p-values for Fisher's exact test).

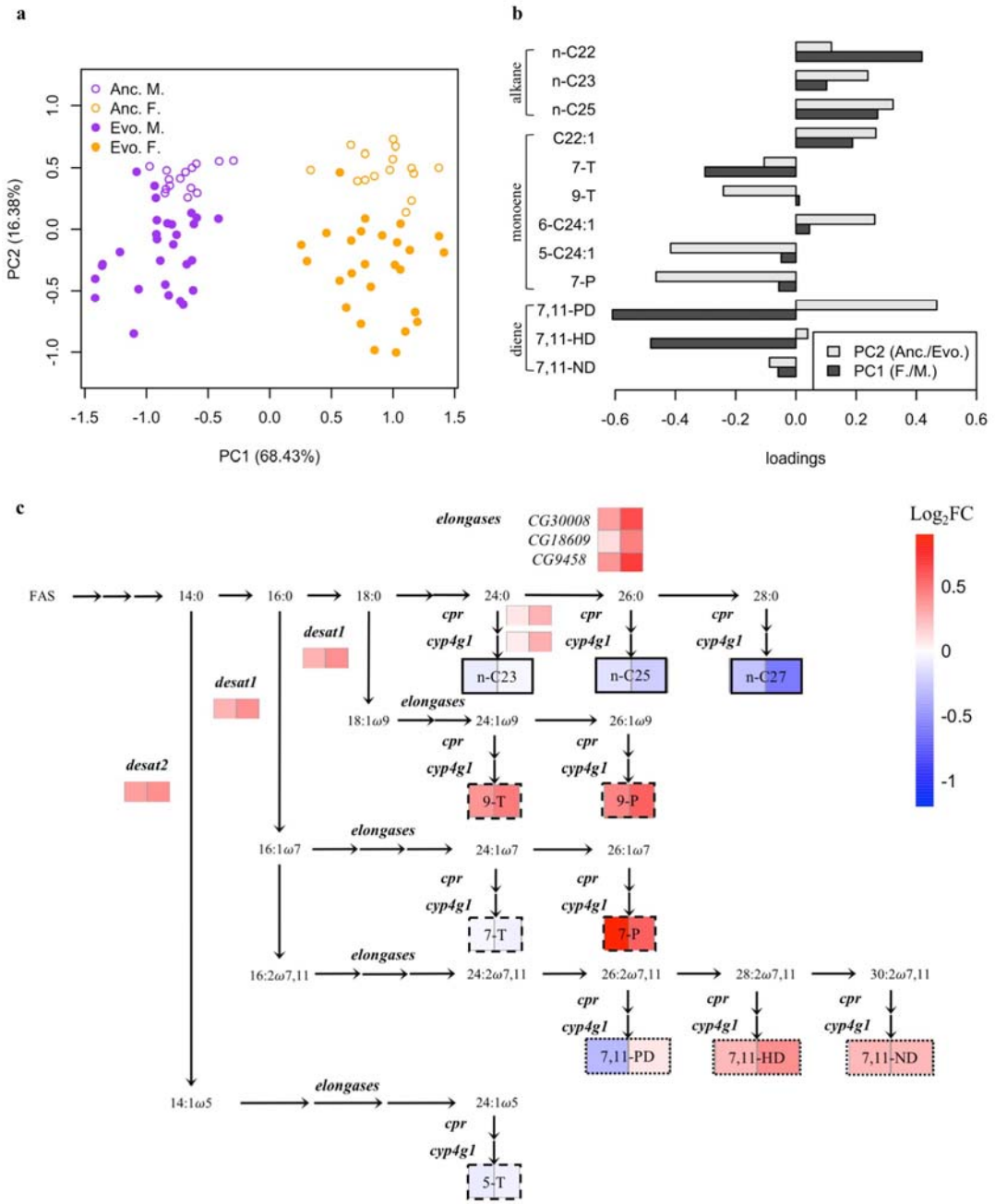

Figure 2. Evolution of cuticle hydrocarbons (CHCs) metabolism. a. Principal component analysis on the center-log transformed relative abundance of 12 CHCs detected in all samples. (orange: females; purple: males). Solid dots are evolved samples and empty ones are ancestral samples. **b.** Loading of each compound on the first and second principal components (PCs). Dark grey bars indicate the loadings for PC1. Positive loading suggests higher abundance in females. Light grey bars indicate the loadings for PC2 and positive values reflect higher abundance in the ancestral samples. **c.** Evolutionary changes in gene expression of the biosynthetic pathway for *Drosophila* cuticular hydrocarbons (CHCs). The heat maps underneath each compound (boxes with regular fonts) or genes (boxes with italic fonts) denote the changes in abundance during adaptation (log2-scale). The left cell denotes

the changes in females and right cell for males. Red indicates increase and blue indicates decrease in abundance.

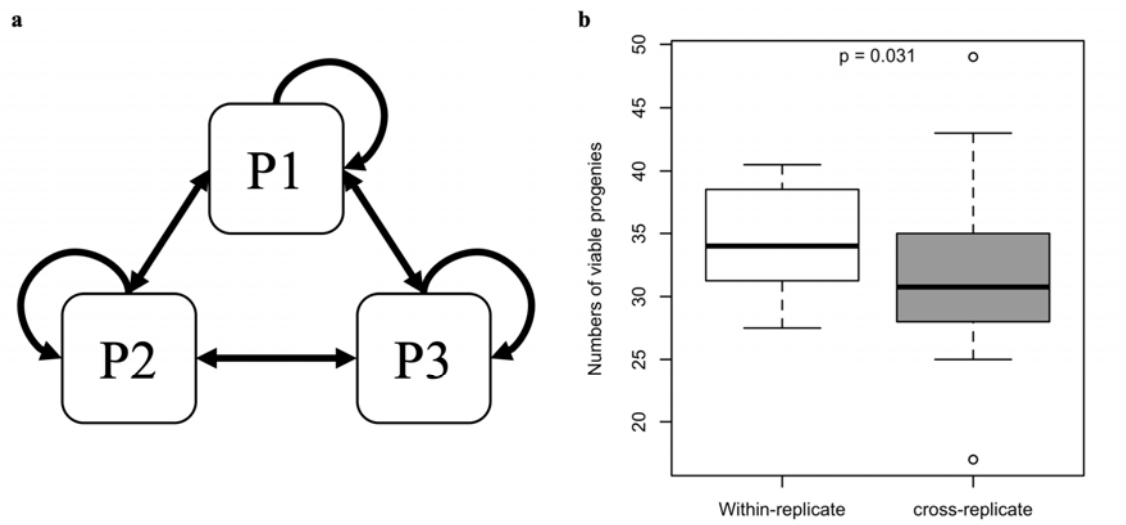

Figure 3. Evolution of post-mating incompatibility among independently evolved populations. a. Diallel cross design among three replicate evolved populations. Flies from each population were crossed with flies from the same population and the other populations reciprocally. **b.** Fitness difference between within-replicate crosses and cross-replicate ones. The fitness was measured as the numbers of viable adults in each cross. Fitness was significantly higher in within-replicate crosses ($p = 0.031$, Wilcoxon's test), indicating post-mating incompatibility between independently evolved replicates.

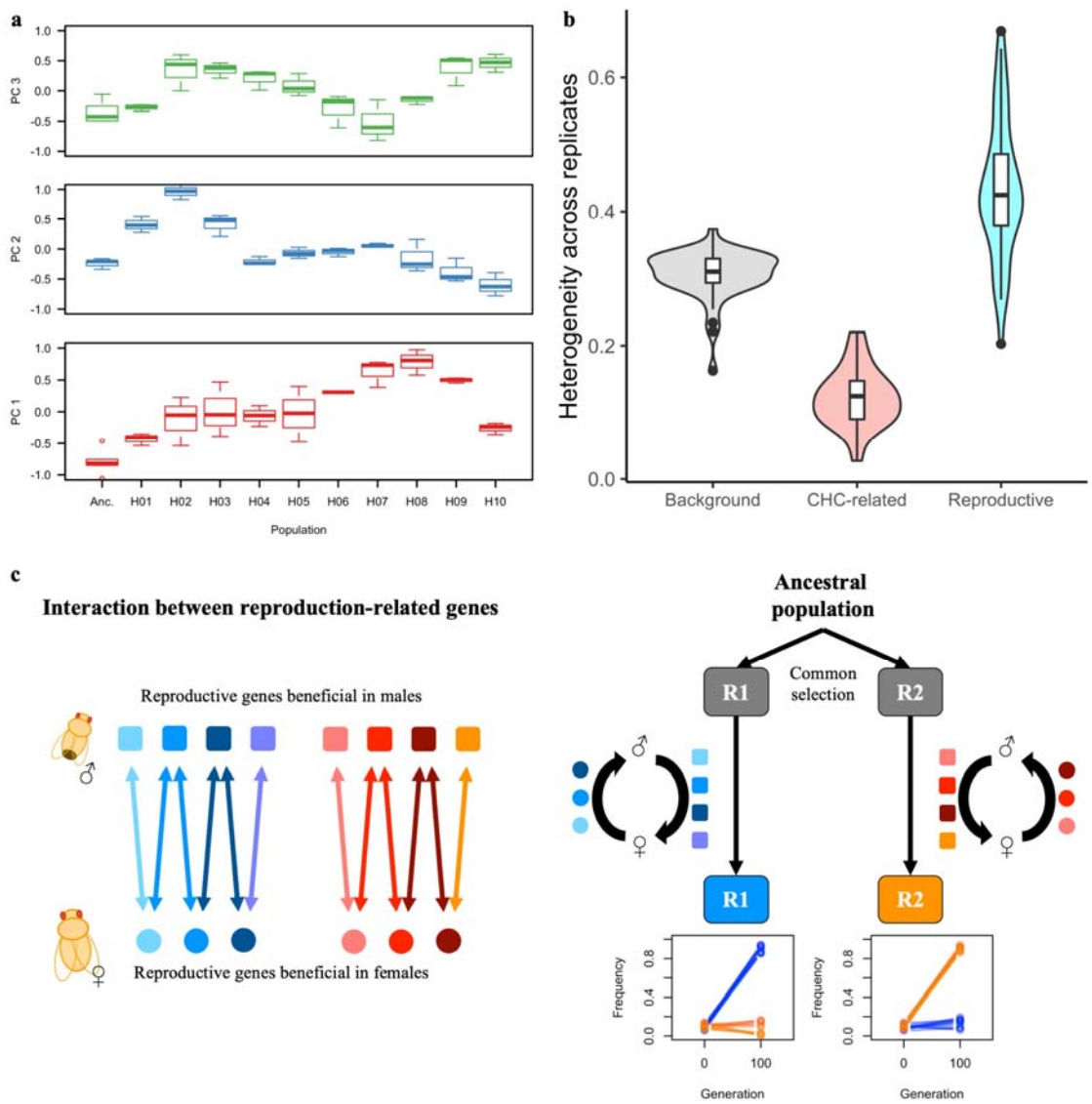

Figure 4. Heterogeneous adaptive changes in reproduction-related genes a. Replicate-specific evolution caused the expression divergence of reproduction-related genes. The expression variance among 255 reproduction-related genes (see materials and methods) is summarized by the leading principal components (PCs). The first three PCs suggest that different replicates evolved different sets of genes during adaptation. **b.** The heterogeneity in expression evolution differs among different sets of genes: background (all expressed genes), CHC-related (33 genes reported in (Dembeck et al., 2015)), reproduction-related (255 genes from GO term (GO:0032504)). The heterogeneity is measured as $\sum_{i,j}(1 - cor(x_i, x_j))/N$, where x is a vector consisting of the expression of each gene in each evolution replicate; $i, j \in [1,10], i \neq j$ and N is the total number of pairwise combination of 10 replicates. Reproduction-related genes evolved with significantly higher heterogeneity than background genes or CHC-related genes (Kruskal-Wallis test, $p < 0.001$). **c.** An illustration for how intersexual co-evolution could lead to heterogeneous and incompatible evolution in isolated populations: Genes involved in male reproduction also serve important functions in females via either synergistic or antagonistic interaction with female reproduction-related genes. Multiple epistatic modules with different genes involved (red- and blue-colors) may co-exist in the ancestral population. A cascade of intersexual co-evolution for a certain group of genes

is expected if selection pressure is imposed on either sex. As such co-evolutionary cascades (modules) are not unique, but redundant, replicate populations may evolve for high divergence and the alternative co-evolutionary paths may not be fully compatible with each other, resulting in fitness costs for between-replicate crosses.

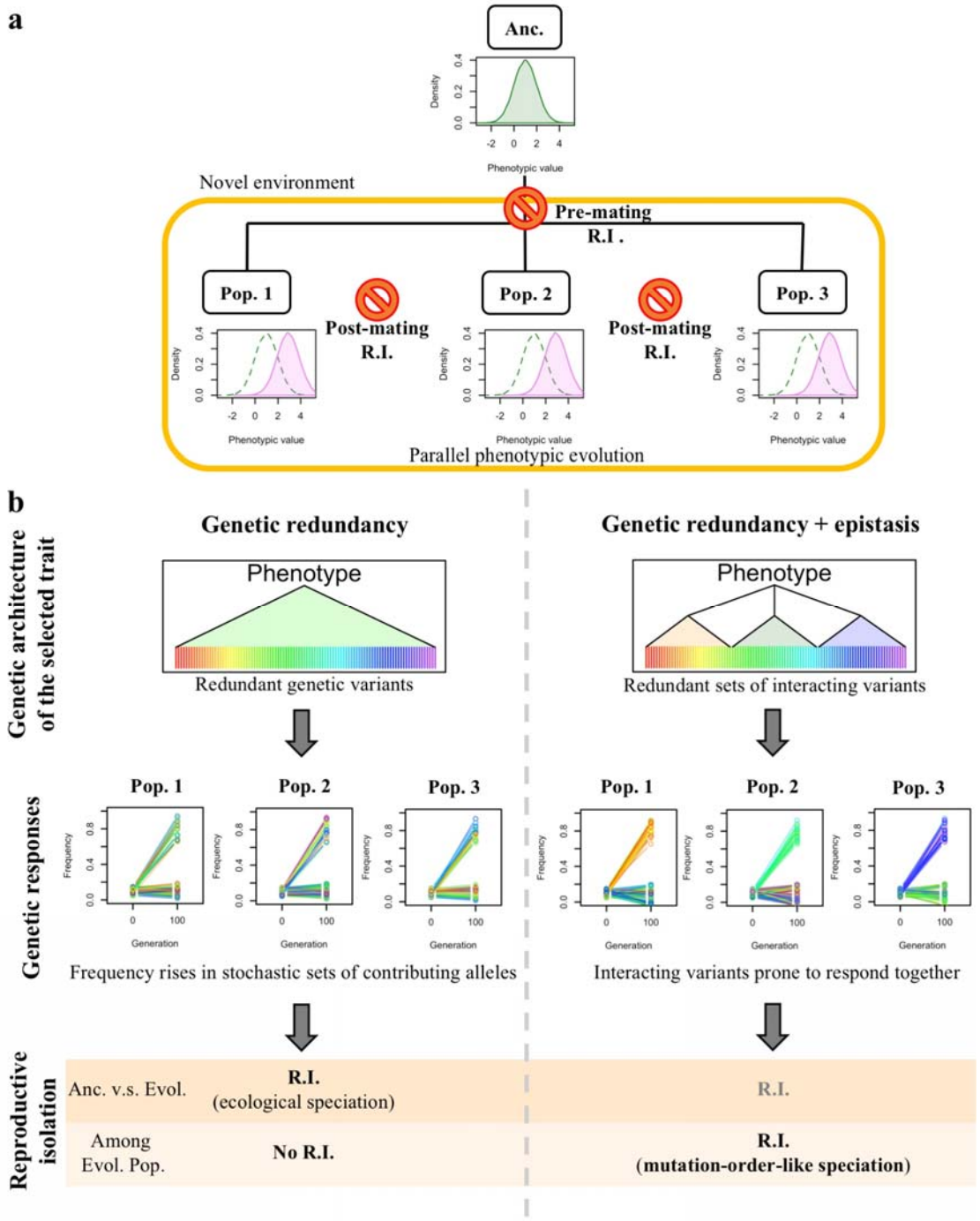

Figure 5. Speciation driven by adaptation. a. Schematic summary of this study: Temperature adaptation leads to the evolution of pre-mating reproductive isolation (R.I.) between ancestral and evolved populations, and post-mating R.I. among evolved replicates. **b.** Depending on the genetic architecture of the selected trait, different forms of reproductive isolation are expected. Left: For a polygenic trait simply controlled by many redundant small effect loci (represented by segments in rainbow colors), heterogeneous adaptive responses are expected in different replicates (frequency rises at random loci in each replicate), leading to reproductive isolation between ancestral and evolved populations but no incompatibility among evolved replicates. Right: In the case of adaptation building on interacting loci, different sets of genes are responding but their interaction causes incompatibilities among

evolved replicates which use different sets of interdependent genes to adapt (The grouping of loci into three groups with similar color reflects that interacting loci are changing their frequencies in a replicate specific manner). We call this form of reproductive isolation "mutation-order-like speciation" to highlight its similarity to the mutation-order speciation model, which relies on de novo mutations rather than standing genetic variation (see Figure 5 – Figure Supplement 1). Note that we predict that this mechanism should also result in R.I. between evolved and ancestral populations, but we could not demonstrate this given the confounding effects of ecological speciation. Hence, it is only shown in light gray in the table.

Material and Methods

Experimental evolution

The setup and maintenance of the experimental populations are detailed (Barghi et al., 2019). Briefly, 10 replicated outbred populations were constituted from the same 202 isofemale lines derived from natural *Drosophila simulans* populations collected in Florida, USA in 2010. Five females of each line were pooled to create one replicate of a reconstituted ancestral population (henceforth called ancestral population). Replicated populations independently adapted to a laboratory environment at 18/28°C with 12hr dark/12hr light photoperiod with the census population size of 1250 adult individuals per population per generation Based on genomic allele frequency changes, an effective population size of ~300 was inferred (Barghi et al., 2019). During the evolution experiment, all replicate populations were reared in the same incubator with randomization orientation/position across generations. Small environmental

Each founder isofemale lines was maintained at small population size (typically less than 50 individuals) at 18 °C for >50 generations on standard laboratory food before the reconstitution of the ancestral populations for common garden experiments. Potential adaptation to the lab environment with the residual heterogeneity or de novo mutation is unlikely, as discussed (Barghi et al., 2019). The small effective population size during the maintenance of each line prevents adaptation to the lab environment. This has also been experimentally tested by the lack of significant difference in allele frequencies in populations which were reconstituted shortly after the establishment of the lines and after 50 generations (Nouhaud et al., 2016). Furthermore, mutations occurring in the laboratory, if any, are mostly recessive (ref: Charlesworth & Charlesworth, 2010), and the effects are likely to be masked because after two generations of random mating during common garden maintenance, most individuals will be heterozygous for isofemale line-specific SNPs.

Common garden experiment

We performed multiple common garden experiments with the evolved populations and reconstituted ancestral populations for the transcriptomic, metabolomic and phenotypic assays in this study. The common garden procedures have been detailed in (Hsu et al., 2020; Jakšić et al., 2020). Briefly, replicates of ancestral populations were reconstituted by pooling five females each from the founder isofemale lines. These reconstituted ancestral populations and evolved replicates were reared at 18/28°C cycling with 12hr dark/12hr light photoperiod for at least two generations to minimize transgenerational and/or environmental effects before subjected to different phenotypic assays.

Assay for male reproductive activity

We measured the reproductive activity for evolved and reconstituted ancestral populations at generation 140. After four generations at common garden condition (18/28°C cycling), 10 pairs of 5-day-old mated males and females were placed together in an agar-based arena (4% agar, 4% sugar) and filmed for 15 minutes at 20 FPS (frame-per-second) at 28°C using FlyCapture2 system (PointGrey, Version 2.13.3.31). In total, for matched-setup (flies from the same population are assayed together), eight trials within the evolved populations and six trials within the ancestral populations were assayed. In addition, five and six trials of reciprocal mismatched-setup (ancestral males were assayed together with evolved females and vice versa) were included in the experiment. Each fly was tracked using flytracker (Eyjolfsdottir et al., 2014) for movement and behavior analysis. Janelia Automatic Animal Behavior Annotator (JAABA) was used to annotate and recognize the chasing behaviors (Kabra et al., 2012). The time a male fly spent on chasing females was quantified as its

reproductive activity and the difference in the different setups was tested with a Kruskal-Wallis test in R.

Multiple-choice mating assay for assortative mating

We performed a multiple-choice mating assay for assortative mating between 1) three ancestral populations and three selected evolved populations (repl. 1, 3 and 6, generation 194) 2) across the three independently evolved populations. We modified the experimental protocol from (Kwan and Rundle, 2010). After two generations of common garden condition to which the evolved populations have been exposed, 400 unmated males and females were collected from each population in six hours after eclosure. The collected flies were reared on corn meal in vials with moderate density (50 flies per vial, 8 replicates per sex per population) for four days and transferred onto yeast paste saturated with food coloring dyes (red or blue) one day before assaying. During the mating assay, 50 flies of each sex from each population in a combination of interest (one ancestral and one evolved populations/two evolved populations) were placed together in a cylindrical cage with a sealable entrance for aspirator. Eight cages (four for each color) for each population combination were setup and run simultaneously and each cage was checked sequentially. Mating pairs were captured with aspirator and identified under microscope based on the color of abdomen. The examination was terminated when the first 25 mating pairs in a cage were identified or when two hours elapsed. Based on a two-by-two contingency table from the assay for each population combination, Yule's index (Y) (Bishop et al., 2007) was calculated to quantify the strength of assortative mating and Fisher's exact test was applied for hypothesis testing.

Quantification and identification of cuticle hydrocarbons

We measured the cuticle hydrocarbons (CHCs) for five replicates of the ancestral population and 10 independently evolved populations at generation 158. After two generations in a common garden, three replicates of 20 virgin flies of each sex were collected two hours after eclosure from each population. The collected flies were aged for three additional days before CHC extraction. We used 100 μl of heptane containing an internal standard (IS, 10 μg n-C30) to wash the CHCs from each sample. The extracts were stored at -20°C until gas chromatography. GC/MS analysis was performed to isolate and quantify each compound. The analysis of chromatograph was performed with an Agilent 7890C gas chromatography system coupled to an Agilent 5975C MSD. Injection volume was 1 µl in an inlet at a temperature of 320°C, a split of 10:1. He was used as carrier gas at a velocity of 1 ml/min and the compounds separated in a HP-5MS column (30 m x 250 µm ID x 0.25 µm film thickness) with a temperature program 180°C to 240°C with 6°C/min. and then with 20°C/min. to 320°C. Transfer line temperature to the MSD was 280 °C, mass range of the MSD was 40-400 m/z and electromagnetic voltage was set at 70 eV.

Identification of the constituents were done based on retention index (RI), determined with reference to homologues series of n-alkanes (C8-C30) and mass spectra with the databases NIST 05. Each identified chemical compounds were validated with published *Drosophila* CHCs chromatograph (Dembeck et al., 2015; Pardy et al., 2019). For integration, identification and quantification of compounds, Automated Mass spectral Deconvolution and Identification System (AMDIS) (Mallard and Reed, 1997) and openChrom (Wenig and Odermatt, 2010) were used. The relative concentration of each CHC compound (excluding cis-Vaccenyl acetate) was calculated and subjected to centered-log ratio transformation (Pardy et al., 2019). Two-way analysis of variance (ANOVA) and principal component analysis (PCA) were performed to dissect the effect of evolution and sex on different CHCs.

Cross-replicate compatibility assay

At generation 194 of the evolved populations, we performed a diallel cross among three selected evolved replicates (repl. 1, 3 and 6) and measured the number of progenies that survived until adulthood to approximate the fitness of each cross. Since we did not find assortative mating (Figure 1a) nor fecundity difference among evolved replicates (Barghi et al., 2019), this assay was designed to identify fitness differences caused by viability or fertilization success. The ancestral populations were not included because of the potential impact of assortative mating. After three generations of common garden rearing (18/28°C cycling), 75 unmated males and females were collected from each evolved replicate population and aged to four days old. For each cross, five males and five females from the same/different population were placed together in a vial (with food surface area of 3.14cm^2; no crowding effect is expected) at the same common garden condition and two transfers were made for all vials with a 48-hour interval. The measurements of viable progeny numbers after 14 days from both transfers were averaged and treated as one replicate for the data analysis. Five replicates were generated for each cross and there were in total nine types of cross in a full diallel cross design among three populations.

RNA-Seq data analysis for divergently expressed genes

RNA-Seq data for the evolution experiment at generation 103 were obtained from previous studies where contrasts between ancestral and evolved populations were made (Hsu et al., 2020; Jakšić et al., 2020). In this study, we reanalyzed the data for the 5-day-old whole-body male samples of the evolved populations to identify the genes that were divergent across replicates. Only two replicates are available from each evolution replicates for females, preventing similar analysis in females (Hsu et al., 2020, 2019). Genes with count per million (CPM) > 0.1 across all samples were retained and we modeled their expression as: $Y =$

$repl + \varepsilon$, where Y is the normalized expression values; $repl$ indicates the effect among evolution replicates and ε is the random error. Likelihood ratio tests implemented in edgeR(McCarthy et al., 2012) were used to perform differential expression analysis on the effect of $repl$. Benjamini and Horchberg's FDR correction (Benjamini and Hochberg, 1995) was applied with a FDR cutoff of 0.05. Genes differentially expressed in at least one replicate were identified for further analysis.

Gene set enrichment analysis

In order to explore the functional implication of the parallel changes and random divergence in transcriptome, we tested for an enrichment of gene ontology (GO), tissue-specific expression and reproductive functions among the genes evolving for a parallel response (Hsu et al., 2020) or those evolved for significant divergence across the 10 evolution replicates (this study). GO enrichment was performed using default "weight01" algorithm implemented in topGO (version 2.32.0) (Alexa et al., 2006). Genes highly expressed in each tissue were identified based on flyatlas2 expression dataset (Leader et al., 2018) (required > 2 fold higher expression in a certain tissue than whole-body). Genes involved in the cuticle hydrocarbon (CHC) metabolism were obtained from (Dembeck et al., 2015). Fisher's exact tests were applied for the enrichment analysis. Except for the GO enrichment analysis which already accounts for multiple testing(Kushwaha and Salis, 2015), Benjamini and Horchberg's FDR correction (Benjamini and Hochberg, 1995) was applied to account for multiple testing.

Replicate-specific evolution in divergently evolving reproductive genes

In order to understand the driving force underlying the divergent evolution in the expression of genes involved in "multicellular organism reproduction" (GO:0032504), we investigated the replicate-specific gene expression evolution of 255 reproductive genes in this term that

were significantly diverged across independent replicates. Using the expression matrix of these genes in all samples (Figure 4 — Figure Supplement 2), we summarized the variation in expression of the 255 genes by the principal components (PCs). The PC scores of each sample reflect the expression difference across the sample. Thus, comparing different evolved replicates to the ancestral samples could inform us about replicate-specific evolution in a certain subset of genes. Additionally, we investigated the heterogeneity in the expression changes across replicates as:

$$Heterogeneity = \sum_{i,j} (1 - cor(x_i, x_j))\ /N$$

Where x is a vector consist of the expression of each gene in each evolution replicate, $i, j \in [1,10], i \neq j$ and N is the total number of pairwise combination of 10 replicates.

Data and materials availability

Sequence reads from this study is available from the European Sequence Read Archive (http://www.ebi.ac.uk/ena/) under the study accession number PRJEB35504 and PRJEB35506. Additional script and raw data are available on Github upon publication.

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Competing interests: The authors declare no competing interests.

Figure supplements

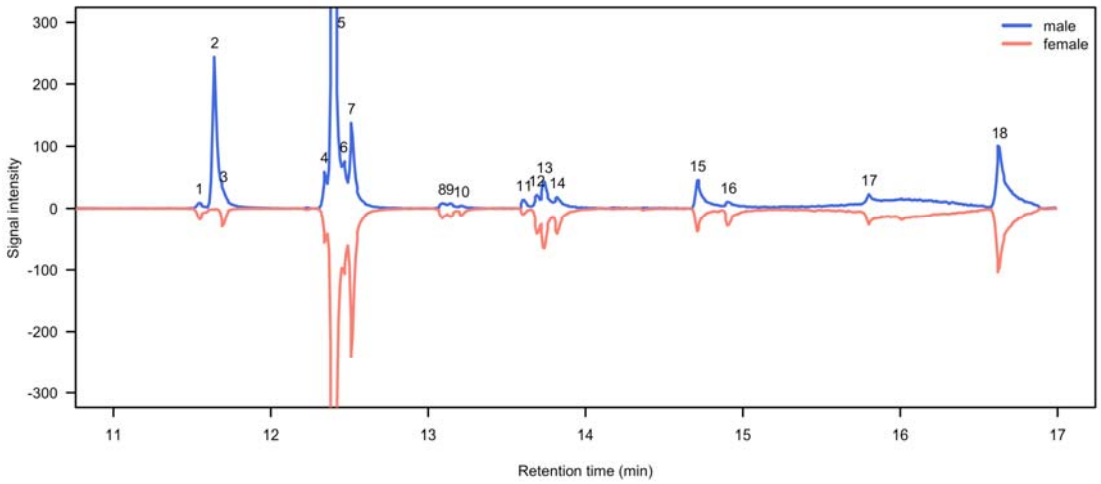

Figure 2 — Figure Supplement 1. Gas chromatography of the cuticular hydrocarbons (CHCs) in both sexes of *Drosophila simulans*.

The x axis denotes the retention time and the y axis indicates the signal intensity. Blue curve is the chromatography for male and red curve is for female. In total, 18 peaks can be detected. The major sex difference is the presence and absence of peak 2 which is identified as cis-vaccenyl acetate (c-VA).

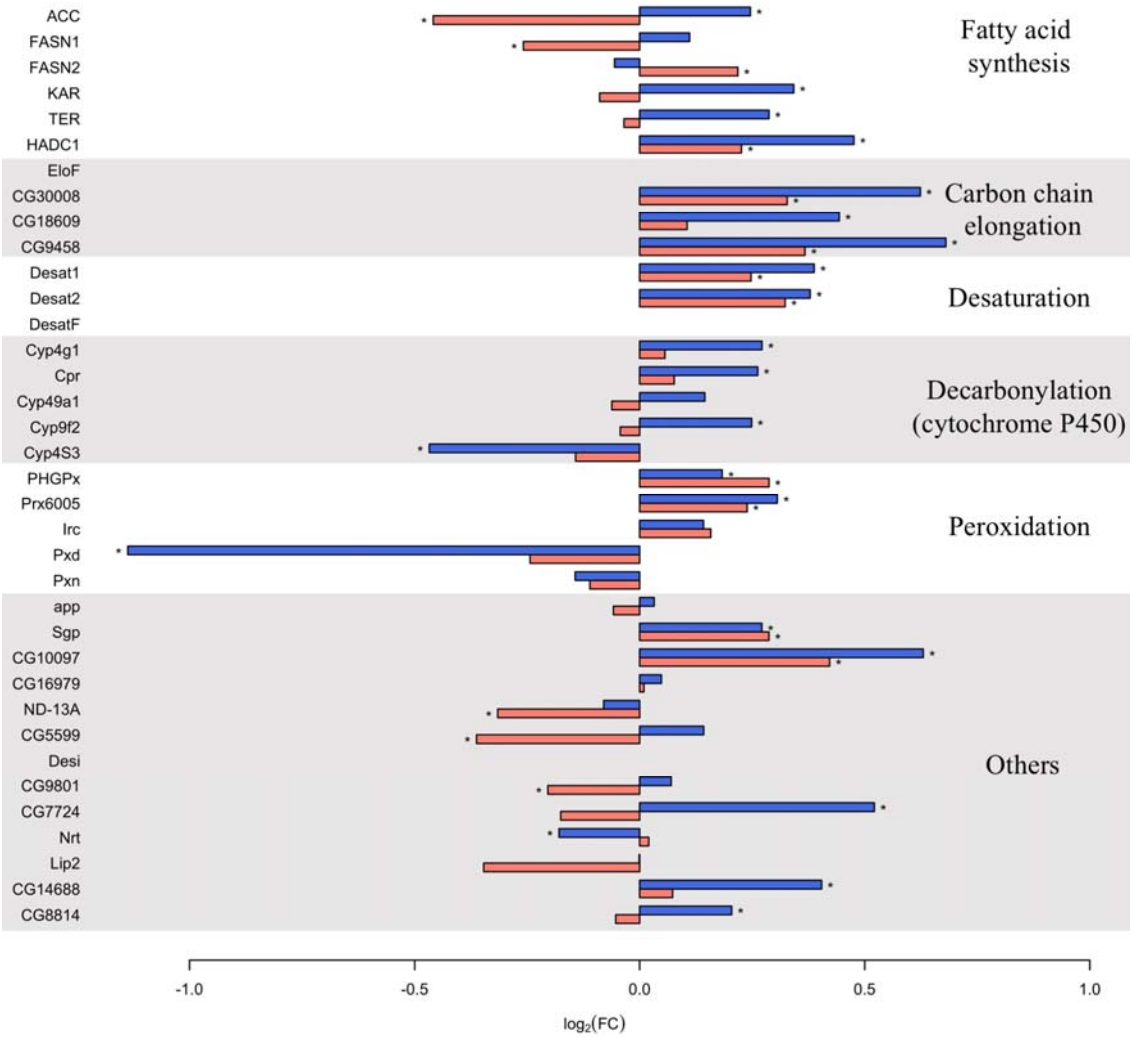

Figure 2 — Figure Supplement 2. Expression evolution of genes associated with cuticular hydrocarbons (CHCs) metabolism in both sexes of *Drosophila simulans*.

A substantial number of genes reported to be associated with CHC metabolism (Dembeck et al., 2015) evolved for expression changes during the adaptation to a novel hot environment. The expression changes are represented by $\log_2$-fold change before and after the adaptation. Red bars denote female changes and blue bars for male. The asterisks indicate statistical significance (FDR < 0.05).

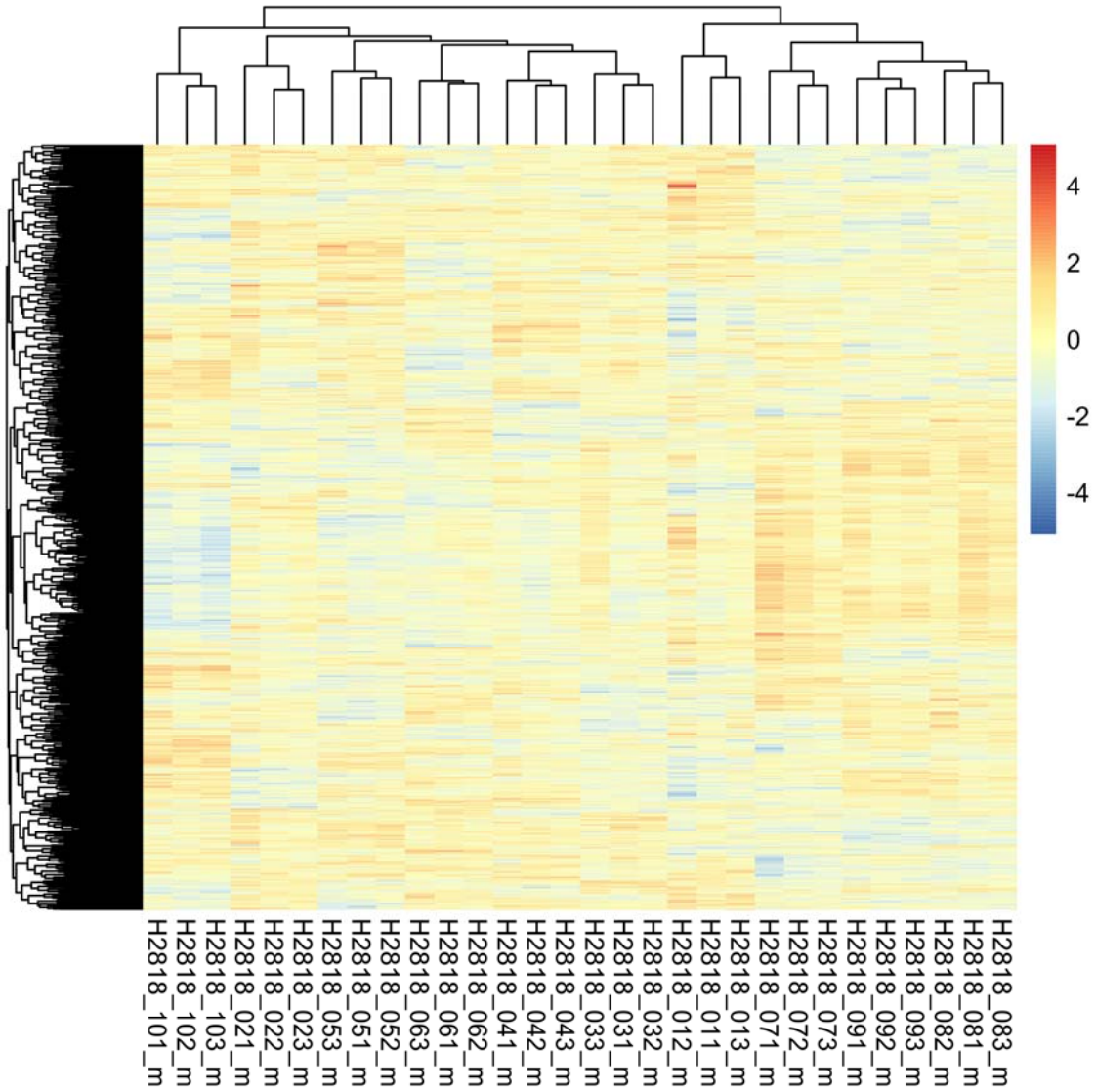

Figure 4 — Figure Supplement 1. Transcriptomic divergence among replicate populations adapting to the same environment.

Normalized expression values of the 3,062 genes exhibiting significant expression changes across replicates are shown (heat color in each cell). Each row indicates a gene and each column is one biological samples. Based on the expression of these divergently expressed genes, the samples from the same replicate populations are clustered together.

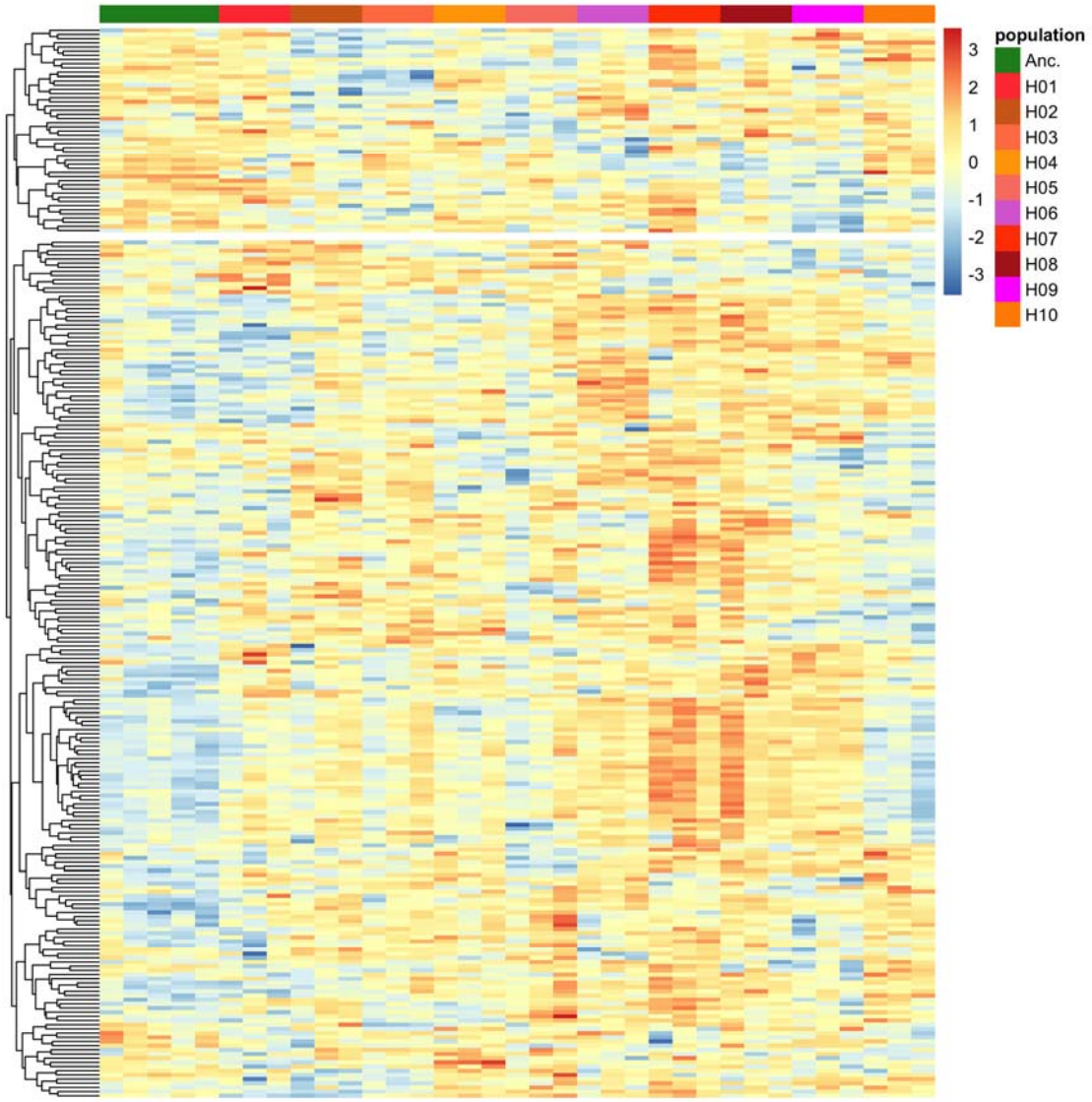

Figure 4 — Figure Supplement 2. Transcriptomic divergence of reproduction-related genes among replicate populations.

Normalized expression values of the 255 reproduction-related genes exhibiting significant expression divergence across replicates are shown (heat color in each cell). Each row indicates a gene and each column is one biological samples. The column annotation indicates the identities of each biological samples. Most of the reproduction-related genes evolved for up-regulation but distinct sets of genes evolved in different replicates.

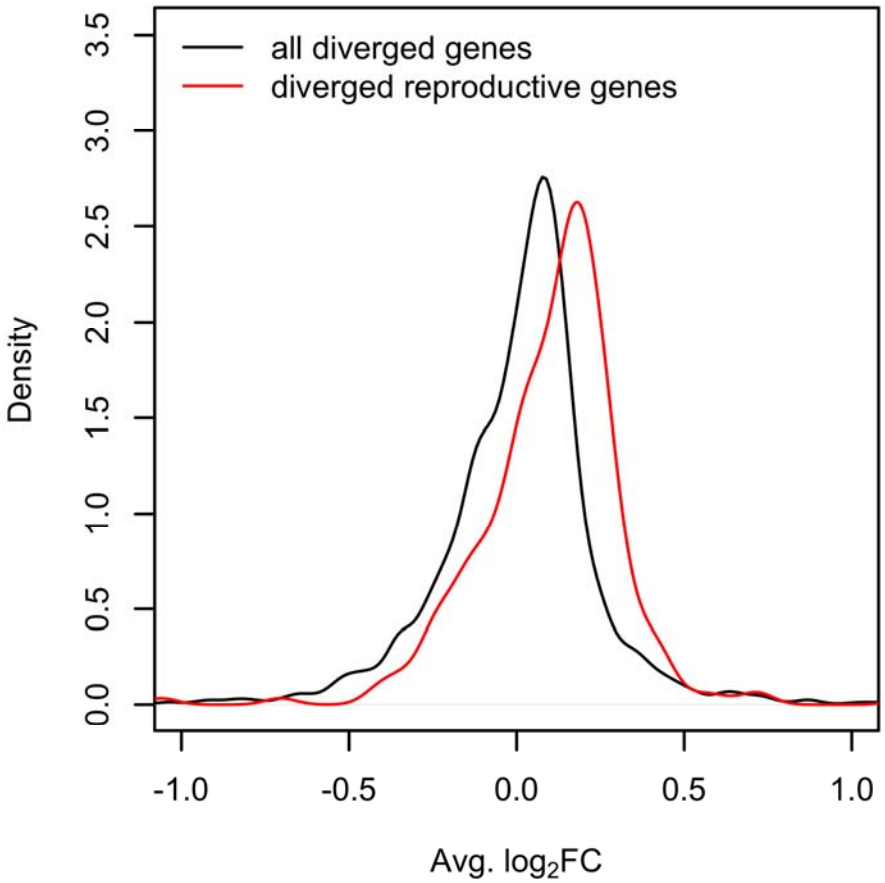

Figure 4 — Figure Supplement 3. The predominance of up-regulation is specific for reproduction-related genes.

Average expression difference between the ancestral and evolved populations (log_2-scaled fold change) for each of the 255 reproduction-associated genes were compared to all divergently expressed genes. The systematic shift toward higher expression is statistically significant (Wilcoxon's test, $p < 0.001$).

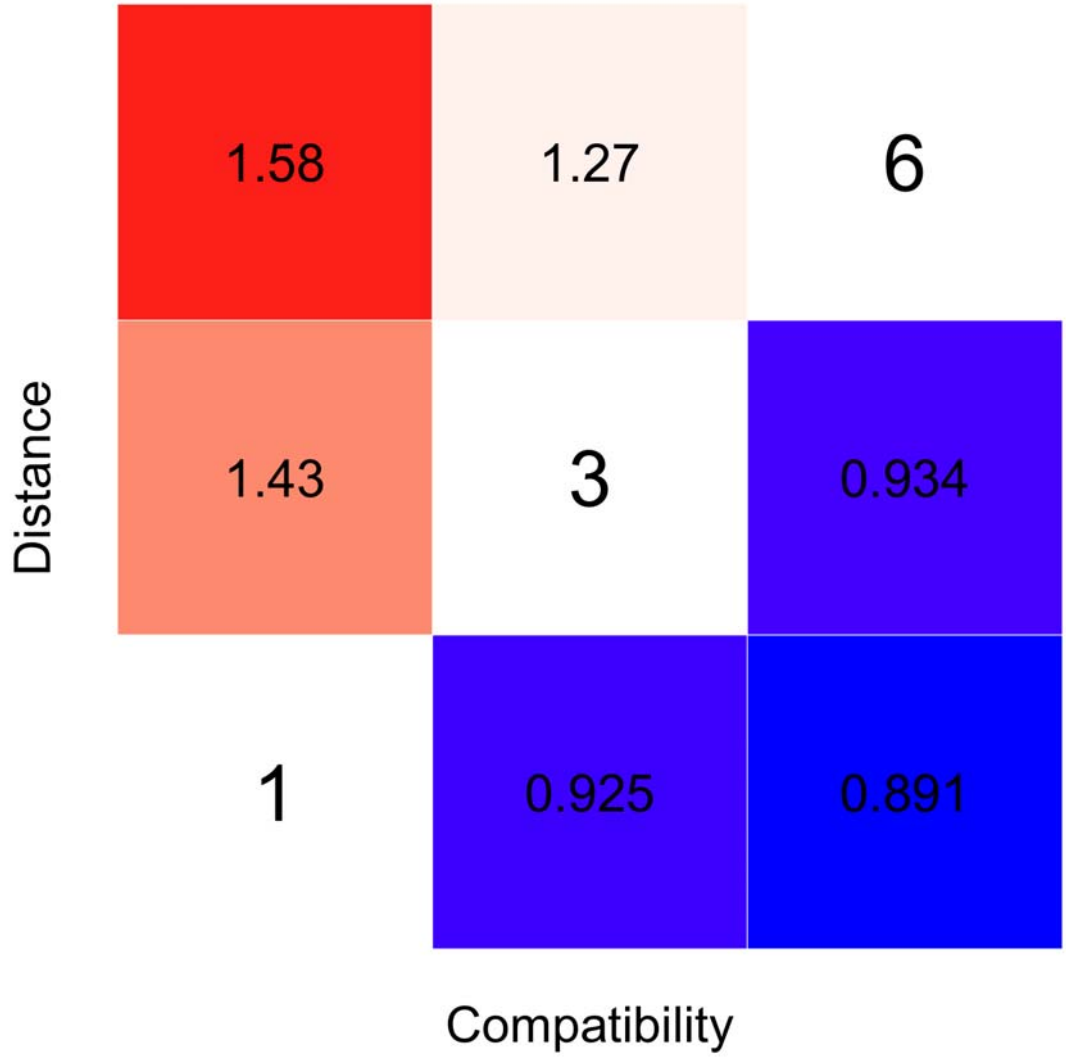

Figure 4 — Figure Supplement 4. The correlation between the genetic distances and the post-mating compatibilities.

Among the three replicate populations subjected to the cross-replicate compatibility assay (repl. 1, 3 and 6), the genetic distances (Euclidean distance) were estimated based on the expression of the 255 reproduction-related genes with significant expression divergence across replicates (upper triangle) and correlated to the observed compatibilities ( ). A negative correlation is observed. The further the two populations are, the less compatible they are.

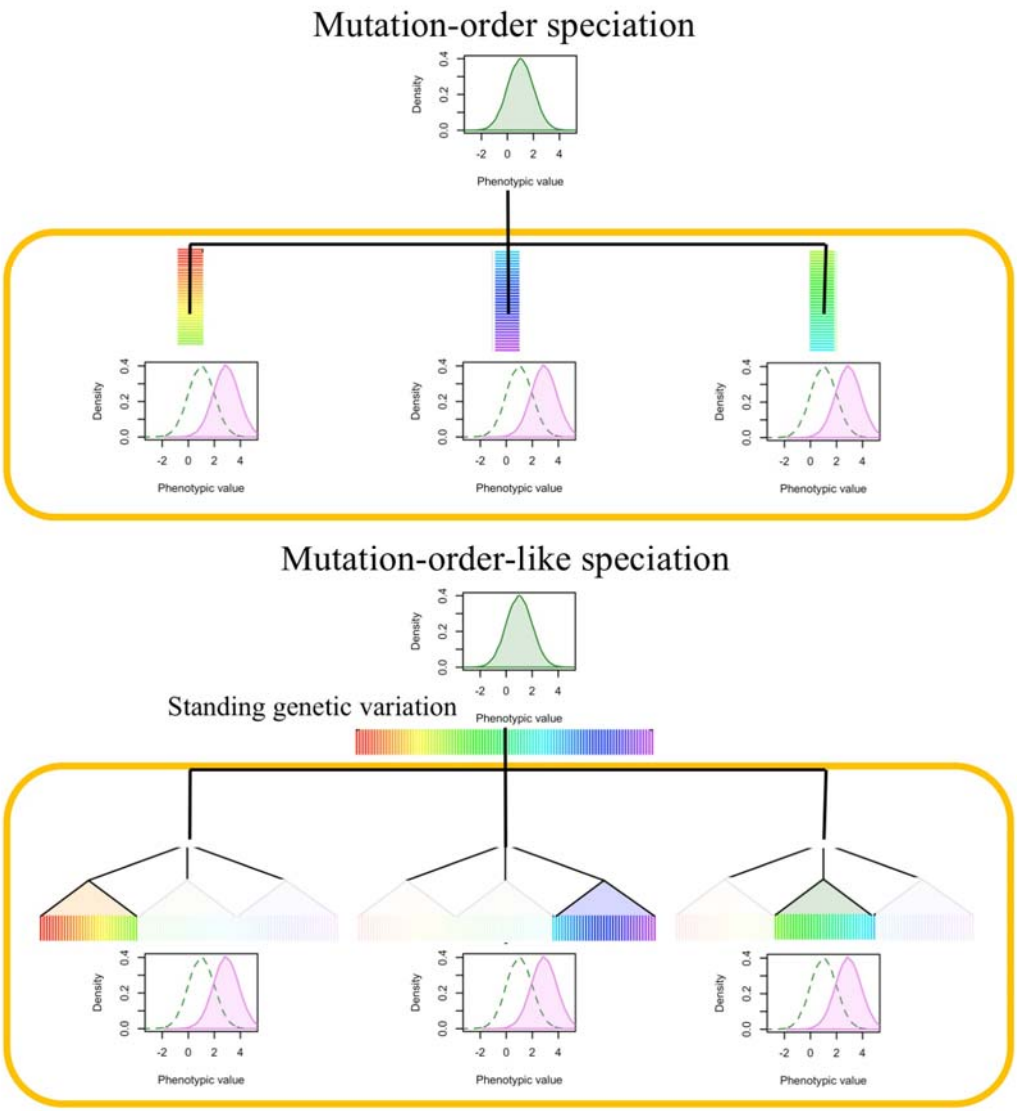

Figure 5 — Figure Supplement 1. Mutation and mutation-order-like speciation models. These are two conceptually similar but different speciation mechanisms. For both cases, reproductive isolation evolves between populations adapting to the same environment due to the epistatic incompatibility between different sets of beneficial variants. While the mutation-order speciation occurs alongside the sequential accumulation of distinct sets of interacting novel mutations, the mutation-order-like model proposed in this study is operated on the weak but many epistatic interactions among subsets of the segregating genetic variants.

Titles and legends for supplementary Files

Supplementary File 1. Likelihood ratio test for differential expression among evolved replicates.

The file records the results of likelihood ratio test for the expression divergence among evolved replicates for each gene.

Supplementary File 2. Gene ontology (GO) enrichment analysis on genes of interest.

Results of gene ontology enrichment analysis using topGO among the genes showing significant divergence in expression among evolved replicates were documented in this file.