

1 **ARTICLE**

2 **Methods**

3
4 **Secondary evolve and re-sequencing: an experimental confirmation of**
5 **putative selection targets without phenotyping**

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14 *****KEYWORDS**

15 experimental evolution, *Drosophila simulans*, repeatability of evolution, evolve and re-
16 sequence

17
18 **ABSTRACT**

19 Evolve and re-sequencing (E&R) studies investigate the genomic responses of adaptation
20 during experimental evolution. Because replicate populations evolve in the same controlled
21 environment, consistent responses to selection across replicates are frequently used to identify
22 reliable candidate regions that underlie adaptation to a new environment. However, recent work
23 demonstrated that selection signatures can be restricted to one or a few replicate(s) only. These
24 selection signatures frequently have a weak statistical support, and given the difficulties of
25 functional validation, additional evidence is needed before considering them as candidates for
26 functional analysis. Here, we introduce an experimental procedure to validate candidate loci
27 with weak or replicate-specific selection signature(s). Crossing an evolved population from a
28 primary E&R experiment to the ancestral founder population reduces the frequency of
29 candidate alleles that have reached a high frequency. We hypothesize that genuine selection
30 targets will experience a repeatable frequency increase after the mixing with the ancestral
31 founders if they are exposed to the same environment (secondary E&R experiment). Using this
32 approach, we successfully validate two overlapping selection targets, which showed a mutually
33 exclusive selection signature in a primary E&R experiment of *Drosophila simulans* adapting

34 to a novel temperature regime. We conclude that secondary E&R experiments provide a
35 reliable confirmation of selection signatures that are either not replicated or show only a low
36 statistical significance in a primary E&R experiment. Such experiments are particularly helpful
37 to prioritize candidate loci for time-consuming functional follow-up investigations.

38

39 INTRODUCTION

40 Experimental evolution provides the opportunity to study evolutionary processes over time
41 scales short enough to be followed experimentally (Garland and Rose 2009; Kawecki et al.
42 2012). The combination of high-throughput sequencing with experimental evolution (Evolve
43 and Re-sequence, E&R) has been widely used to identify adaptive alleles across multiple
44 replicates starting from the same reservoir of standing variation in highly similar, well-
45 controlled environments (Turner et al. 2011; Long et al. 2015; Schlötterer et al. 2015). E&R
46 studies successfully characterized the genomic responses during adaptation to novel selective
47 pressures usually on organisms with short generation times (e.g.: Turner and Miller 2012;
48 Burke et al. 2014; Lenski 2017; Papkou et al. 2019; Remigi et al. 2019). Laboratory natural
49 selection experiments using the E&R framework studied responses to thermal (Orozco-
50 terWengel et al. 2012; Tobler et al. 2014; Michalak et al. 2019) or desiccation stress (Schou et
51 al. 2014), starvation (Michalak et al. 2019) and salt- and cadmium- enriched environments
52 (Huang et al, 2014). The advantage of E&R studies starting from natural variation is that
53 adaptation is possible without de novo mutations (Teotónio et al. 2009). Hence, even organisms
54 with moderate experimental population sizes, such as *Drosophila*, are able to adapt to novel
55 conditions within experimentally feasible time scales. Furthermore, when the starting variation
56 is sampled from a natural population, E&R studies provide direct information about the
57 frequency of the selected alleles in the wild (Barghi et al. 2019).

58

59 Standard statistical tests applied to E&R data (e. g. Cochran Mantel Haenszel (CMH) test
60 (Agresti, 2002; Spitzer et al, unpublished data) or Generalized Linear Modeling (Phillips et al.
61 2018)) require parallel selection responses across replicates. Two different, not mutually
62 exclusive, factors can severely compromise the detection of selection targets based on these
63 approaches. Polygenic adaptation to a new trait optimum results in reduced genomic
64 parallelism across replicates (Franssen et al. 2017; Barghi et al. 2019). Furthermore, selected
65 alleles with low starting frequencies are not only less likely to reach a detectable selection
66 signature, but genetic drift, i.e. chance, also results in lower repeatability across replicates
67 (Lenormand et al. 2016). One further complication for the identification of selection targets

68 with low starting frequencies arises from hitchhiking of SNPs shared with haplotypes carrying
69 the favorable allele (Nuzhdin and Turner 2013; Tobler et al. 2014; Franssen et al. 2015). In this
70 case, the limited number of recombination events during the experiment results in large
71 genomic regions with selection signatures when selection operates on low frequency alleles,
72 that make the identification of individual candidate genes impossible.

73

74 The functional characterization of selected alleles in E&R studies is an important next step for
75 a better understanding of adaptation processes, but despite the recent advances based on the
76 CRISPR/Cas9 technology (Bassett et al. 2013), the functional characterization of different
77 alleles in a standardized genetic background is still a challenging and time-consuming task.
78 This implies that investigators are well-advised to have high confidence in alleles that are going
79 to be functionally tested.

80

81 We propose a simple experimental procedure to validate candidate regions with weak statistical
82 support, either due to a weak selection signature across replicates or replicate-specific selection
83 signatures. The basic idea of this approach is that an evolved population is “diluted” with
84 ancestral genotypes. This reduces the frequency of putatively selected alleles and the
85 reproducible increase in frequency of selected alleles in multiple replicates evolving under the
86 same selection regime (secondary E&R) serves as a validation of candidate regions. Because
87 secondary E&R experiments provide the opportunity for additional recombination events, we
88 also evaluated whether this approach increases the mapping resolution, which is particularly
89 important for low frequency beneficial alleles.

90 Applying secondary E&R to a candidate region identified in *D. simulans* populations that have
91 been exposed to a novel constant hot environment at 23°C for 70 generations, we demonstrate
92 that candidate selection targets can be experimentally confirmed.

93

94 NEW APPROACHES

95 Previously, experimental evolution studies exposed laboratory evolved populations to selection
96 regimes in the opposite direction (reverse evolution) (Teotónio and Rose 2001; Porter and
97 Crandall 2003; Teotónio et al. 2009). The secondary E&R design introduced here, also relies
98 on already laboratory selected populations, but rather than changing the selection regime, the
99 same selection regime is applied after manipulating the evolved population. Secondary E&R
100 is designed to provide researchers additional confidence about selection targets by repeating a
101 selection signature in replicate populations after adding genotypes from the founder population,

102 which reduces the frequency of selected alleles. The repeated, parallel frequency increase of
103 candidate regions provides a reliable confirmation of selection targets that were either having
104 a weak selection signal or were only detected in a single replicate.

105

106 RESULTS

107 **Discovery of candidate SNPs: primary E&R**

108 Three replicates of a *D. simulans* founder population were maintained in a constant hot
109 environment (23°C) for 70 non-overlapping generations. Sequencing pools of 1,250
110 individuals (Pool-Seq, Kofler and Schlötterer 2013; Schlötterer et al. 2014; Table SI 1) resulted
111 in a catalogue of 2,560,538 polymorphic SNPs (see Methods, Table SI 2). We identified
112 candidate SNPs by contrasting allele frequency changes (AFC) between ancestral and evolved
113 populations with a CMH test after accounting for drift using a 1% empirical FDR threshold
114 (see Methods). Since p-values obtained from contingency tables tests are affected by coverage,
115 we also accounted for coverage heterogeneity among samples (56x – 261x, Table SI 3) by
116 weighting p-values following the Iterative Hypothesis Weighting procedure (IHW, Ignatiadis
117 et al. 2016) (see Methods). The genome-wide analysis identified a candidate region of 1.628Mb
118 on chromosome arm 3R with a pronounced AFC between ancestral and evolved populations
119 (Fig. 1., top left, the full genomic analysis will be published elsewhere).

120 The power of the CMH test relies on the experimental replicates to detect putative targets of
121 selection. However, its power is limited when candidates are not shared across replicates.
122 Analyzing this genomic region separately for each of the replicates using a Fisher's Exact Test
123 (FET) indicated considerable heterogeneity among them: among the SNPs with the most
124 significant CMH p-values across all three replicates, the top 20 SNPs in the FET of replicate x
125 were only significant in replicate x (FET_x, Fig. 1., bottom left, top center, top right, red), with
126 16 SNPs being close to fixation. Removing replicate x from the CMH analysis and using only
127 replicates y and z, we obtained a much weaker selection signature in the CMH test (CMH_{y,z},
128 Fig. 1., bottom right). Only three of the 20 most significant SNPs of this analysis (CMH_{y,z})
129 were overlapping with the most significant SNPs of the analysis including x (CMH_{x,y,z}).
130 Instead, the 20 most significant SNPs of CMH_{y,z} changed in both replicates y and z with a mean
131 AFC of 0.55. This AFC is less pronounced than the one observed for the significant SNPs of
132 replicate x (0.96). This heterogeneity among replicates suggested that at least two distinct

133 classes of haplotypes were selected.

134 We further scrutinized the hypothesis of at least two distinct selected haplotypes and plotted
135 the AFC of the two sets of top 20 SNPs in the candidate region on chromosome arm 3R (Fig.
136 3.): 20 SNPs from FET_x and 20 SNPs from the joint analysis of replicates y and z, i.e. CMH_{y,z}.
137 The two sets of candidate SNPs displayed group-specific AFC; one set showed a pronounced
138 AFC in replicate x and the other one in replicate z, but almost no change in the other (Fig. 3.,
139 Fig. SI 1.).

140 **Validation of candidate SNPs: secondary E&R**

141 The primary E&R study provided two sets of candidate SNPs. One set of candidates increased
142 strongly in replicate x only, while the other set of candidates increased weakly in the two
143 replicates y and z. To demonstrate that both sets of SNPs are associated with a selection target,
144 we aimed to validate both selection signatures experimentally. Reasoning that fewer replicates
145 are needed to confirm strong selection, only two diluted replicates were generated from evolved
146 replicate x (x.1 and x.2), while three diluted replicates were generated from evolved replicate
147 z (z.1, z.2 and z.3) which showed the weakest response in the initial E&R experiment. For both
148 secondary E&R experiments we added flies from a reconstituted founder population (Nouhaud
149 et al. 2016) aiming for a starting frequency around 0.5 for the most prominent candidate SNPs
150 (see Fig. SI 2). This starting frequency of the candidate SNPs in the secondary E&R ensures a
151 deterministic selection response and still provides sufficient opportunity for frequency
152 increase.

153

154 After 30 generations of evolution at the same culture conditions, we sequenced the founders
155 (D0) and evolved replicates (D30) of the secondary E&R experiments (see Fig. 2. for an
156 overview). We contrasted the dynamics of the two groups of top candidate SNPs in each of the
157 replicates in the primary and secondary E&R experiments over four time points (F0, F70, D0,
158 D30). A very pronounced frequency increase can be noted in both the primary and secondary
159 E&R experiments in the focal replicate from which the candidates were obtained (Fig. 3., Fig.
160 SI 3). From an average starting allele frequency of 0.52 and 0.31 the candidate SNPs reach a
161 mean final frequency of 0.98 (x) and 0.73 (z) in the replicates of the secondary E&R. The
162 consistent AFC in the primary and secondary E&R experiments confirms a high repeatability
163 of selection. Also, the candidate SNPs from the non-focal replicate consistently failed to show
164 selection signatures (Fig. 3., Fig. SI 3). The only exception are 4 SNPs from the candidate set

165 of replicate z, which also increased in frequency in the primary and secondary E&R of replicate
166 x (Fig. 3., Fig. SI 3, Fi. SI 6). Because the AFC was less pronounced than the one of the focal
167 candidate SNPs of replicate x, we conclude that these SNPs may be shared between the two
168 alternatively selected haplotype classes.

169

170 For a more complete picture we expanded our analysis of the 20 most significant SNPs to all
171 significant SNPs ($FDR < 0.01$) of the primary E&R. We jointly plotted the distribution of
172 selection coefficients obtained from the primary and secondary E&R experiments (see
173 Methods). Consistent with the previous analyses, all candidate SNPs had a selection coefficient
174 larger than zero in their focal replicate - independently of whether primary or secondary E&R
175 experiments were analyzed (Fig. 4.a., Fig. SI 4). The inferred selection coefficients for replicate
176 x are about twice as high as the ones for replicate y. The mean selection coefficients from the
177 20 candidate SNPs are 0.26 and 0.27 for diluted replicates from x (0.26 in the primary E&R)
178 and 0.08, 0.09, 0.12 for diluted replicates from z (0.09 in the primary E&R). As expected the
179 selection coefficients of the non-focal top 20 candidate SNPs were distributed around zero.

180

181 Finally, to evaluate the influence of genetic drift, we simulated the dynamics of the significant
182 SNPs ($FDR < 0.01$) in the primary E&R under neutrality and compared them to their observed
183 dynamics (Fig. 4.b., Fig. SI 5 and Methods). Plotting the pairwise observed and simulated
184 neutral AFC of the primary E&R against the AFC of the secondary E&R experiment, we find
185 that the simulated data are clearly distinct from the experimental ones. The significant SNPs of
186 the experimental data cluster together in the upper right quadrant and do not overlap with
187 neutral simulations, showing that genetic drift cannot explain the concordant signatures of the
188 significant SNPs. As expected the separation of neutral and selected SNPs was clearer for the
189 replicate x, where selection was stronger (Fig. 4.a.).

190

191 **No increased mapping resolution for the selection target**

192 Given that the dilution reduced the frequency of the selection target, we anticipated that
193 additional recombination events occurring during the repeated spread of the selection targets
194 would also increase the mapping resolution. Nevertheless, we noted that the selection signature
195 was broader in the secondary E&R experiment than in the primary one (Fig. SI 6). Hence,
196 despite the highly repeatable selection signature of candidate SNPs, the secondary E&R
197 experiment did not yield more confidence about the focal target of selection than the primary
198 E&R experiment.

199 DISCUSSION

200 One of the undisputed advantages of experimental evolution is that the precise experimental
201 conditions are known, which allows to impose the same selection pressure on different
202 populations and time points in a replicated manner. Hence, unless strong epistatic interactions
203 dominate, it should be possible to confirm selected variants by experimentally manipulating
204 allele frequencies in the population in which a favorable variant spread.

205 In this report we introduce a simple manipulation of the evolved populations. By adding
206 unevolved genotypes, we reduce the frequency of the selection target, which provides the
207 opportunity to monitor a repeatable frequency increase of selected alleles in replicated
208 populations. Our results demonstrate that this novel approach accurately recovers the selection
209 signature of candidate SNPs. Despite the mapping resolution of the primary E&R experiment
210 could not be improved, it is striking how consistent the selection coefficients of the top
211 candidate SNPs were replicated in the secondary E&R experiments, in particular because no
212 phenotypes were measured and the actual selective force is not yet characterized.

213

214 We propose that secondary E&R experiments with unevolved genotypes provide an attractive
215 approach to experimentally validate selection signatures. This is particularly important for
216 either non-replicated or small allele frequency changes - both signatures of polygenic
217 adaptation. The power of secondary E&R experiments is well-illustrated in our proof of
218 principle study, in which no single SNP passed the genome-wide significance threshold in this
219 genomic region in the primary E&R experiment in replicate z. Only by combining two
220 replicates, y and z, we identified significant candidates, which could be confirmed in the
221 secondary E&R experiment. Thus, we demonstrated that even populations with weak selection
222 signatures can be used to confirm the presence of selection, which could not be recognized
223 before.

224 Secondary E&R experiments are not fast, the 30 generations of this experiment took about 14
225 months, but the maintenance of replicate populations does not require many resources and
226 provides therefore a very good approach to experimentally validate genomic regions
227 experiencing selection. Mapping of causative variants could not be achieved in this pilot study
228 and requires alternative approaches to do so. Nevertheless, the dynamics of selected genomic
229 regions are highly informative of the underlying genetic architecture of beneficial mutations.
230 Polygenic adaptation to a novel trait optimum displays characteristic dynamics (Franssen et al,
231 2017), which are best detected in multiple replicates. We anticipate that the analysis of multiple
232 replicates in secondary E&R experiments will provide an unprecedented opportunity to study

233 replicated dynamics of selection targets in order to understand the architecture of adaptation.
234 It is also conceivable to use this experimental setup to study the dynamics of a given selected
235 region in an alternative selection regime.

236

237 A particularly interesting pattern could be confirmed in this study: two different haplotype
238 classes are carrying adaptive variants that increase fitness of the populations in a novel hot
239 environment. It is particularly remarkable that the two groups of haplotypes seem to be
240 mutually exclusive - we see either one or the other increasing in frequency in the primary E&R
241 experiment. Also in the secondary E&R experiments we see no evidence of parallel selection
242 of both haplotype classes, but their different starting frequencies in the secondary E&R
243 considerably decrease the opportunity for a strong frequency increase of the haplotype with the
244 lower starting frequency. The mapping resolution is not high enough to determine whether the
245 same gene is carrying a beneficial mutation in both haplotype classes or different genes are
246 selected. Thus, similar to many other E&R studies, a good strategy for fine mapping is needed
247 to answer these questions.

248

249 MATERIALS AND METHODS

250 **The Primary E&R Experiment**

251 **Experimental Population and Selection Regime**

252 We collected a natural *D. simulans* population 10 km North of Stellenbosch, South Africa, in
253 February and March 2013 and established isofemale lines that were maintained in the
254 laboratory for approximately eight generations. For starting the primary E&R experiment, three
255 mated females from each of 426 isofemale lines were combined three times to generate three
256 replicates of the ancestral population (replicates x, y and z) in F0. They were subsequently
257 maintained as independent populations with a census population size of 1,250 and non-
258 overlapping generations under a constant 23°C temperature regime with a 12 hour light/12 hour
259 dark cycle (LD 12:12) for 70 generations (F70). The 426 lines used for constituting the
260 ancestral population were maintained as isofemale lines.

261

262 **Creation of a Bona Fide SNP Catalogue for the Primary E&R study**

263 We generated Pool-Seq data for the 3 replicates of F0 from females only and for the 3 replicates
264 in F70 (sex ratio ~ 50:50). DNA extraction, barcoded library preparation and sequencing
265 followed standard procedures and are given in Supplementary Table I. We followed standard
266 approaches for quality control, read mapping, read filtering, trimming as well as SNP calling

267 and SNP filtering.

268 We used libraries with different insert sizes, which can result in false positives (Kofler et al,
269 2016). To account for this, we expanded the double-mapping procedure suggested by Kofler et
270 al, 2016, and used three different mappers (NovoAlign (<http://novocraft.com>), Bowtie2
271 (Langmead and Salzberg, 2012) and BWA-MEM (Li and Durbin, 2009)). We filtered for bi-
272 allelic SNPs outside of repeat regions, and removed SNPs from positions outside the 99%
273 quantile in terms of genome wide coverage. From this set of pre-filtered SNPs we keep only
274 those for which the SNP frequency did not differ between all three mappers ($p > 0.01$, after FDR
275 correction). We call this procedure triple-mapping. This resulted in a set of 2,560,538 high
276 quality SNPs. Details are given in Supplementary Material I.

277

278 **Identifying Regions under Selection in the Primary E&R**

279 We performed Fisher's exact tests (FET) between the ancestral F0 and the evolved F70
280 generation within each replicate and Cochran-Mantel-Haenszel tests (CMH) (Agresti, 2002)
281 across replicates. As coverage variability (see Supplementary Table II) affects the power of
282 FET and CMH tests, we used the independent hypothesis weighting (IHW) procedure
283 (Ignatiadis et al, 2016) to weight the empirical p-values using the mean coverage at each SNP
284 calculated from all replicates included in any particular test, as a covariate.

285 To determine the list of candidate SNPs, we ran neutral forward Wright-Fisher simulations for
286 each replicate based on N_e estimates (Table 1) that we obtained for autosomes and the X
287 chromosome using the poolSeq package (Taus et al, 2017). Neutral p-values were also
288 submitted to the IHW procedure. Candidate SNPs were declared at a 1% FDR cut off, applying
289 a conservative nonparametric empirical FDR estimator (Strimmer, 2008) using the weighted
290 p-values from our simulations and the weighted p-values from our observed data. This was
291 done separately for FET and CMH tests for each replicate.

292 Selection coefficients were determined for each SNP in each replicate on pseudo-count data
293 (detailed in Supplementary Material I) using the poolSeq package assuming a dominance
294 coefficient of 0.5.

295

296 **The Secondary E&R Experiment**

297 **Experimental Population, Selection Regime and Sequencing**

298 Based on the primary E&R selection signature screen, we picked a candidate region on 3R
299 (region details in Supplementary Figure 1) for further investigation. This region showed a very
300 strong signal of positive selection in a CMH test across replicates x, y and z. We used evolved

301 flies from replicates x and z after 77 generations of evolution in the primary E&R experiment
302 (F77) to set up a secondary E&R experiment in which the evolved flies were mixed with flies
303 from a reconstituted ancestral population (Nouhaud et al, 2016, Supplementary Figure 2). We
304 call this generation D0. Selection targets are expected to increase in frequency again in the
305 secondary E&R experiment, which used the same culturing conditions as the primary E&R
306 experiment.

307 Mixing proportions of ancestral and evolved populations to create D0 were chosen such that
308 selected SNPs in our candidate region had allele frequencies of approximately 0.5 in D0: for
309 replicate x, a 30:70 ratio between evolved and reconstituted ancestral flies, and for replicate z,
310 a 50:50 ratio, respectively. We created two replicates for D0 for replicate x (x.1 and x.2), and
311 three replicates for the diluted replicate z (z.1, z.2 and z.3). Replicates for D0 and D30 were
312 subjected to Pool-Seq.

313

314 **Validation of Signatures of Selection in the Secondary E&R**

315 Selection coefficients and neutrality tests were performed exactly as described for the primary
316 E&R experiment.

317

318 **ACKNOWLEDGMENTS**

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320 especially Neda Barghi, Rui Borges, Lukas Endler, Andreas Futschik, Anna Maria Langmüller,
321 Sonja Lečić, Kathrin Otte. Thomas Taus first proposed the secondary E&R. This work was
322 supported by the Austrian Science Fund (FWF, grant W1225) and an European Research
323 Council (ERC) grant (ArchAdapt).

324 Data availability. Raw Pool-Seq data will be uploaded to SRA XXX and available upon
325 publication.

326

327 **AUTHORS CONTRIBUTION**

328 V.N. performed experiments. P.N. did the first preliminary analysis of the primary E&R.
329 C.B. analyzed the data. M.D. provided statistical support. C.B., C.S. wrote the paper.

330 FIGURES LEGENDS

331 **Figure 1** Replicate-specific selection signatures in the primary E&R study.

332 Manhattan plots of chromosome arm 3R displaying the negative log₁₀-transformed weighted
333 p-values of 680,937 SNPs for different statistical tests. A) CMH_{x,y,z} (175/443), B) FET_y (0/122),
334 C) FET_z (0/0), D) FET_x (660/1,776) and E) CMH_{y,z} (9/85). The number of candidates at 1%/5%
335 empirical FDR thresholds for each test are given in parenthesis. The gray dotted line shows the
336 1% (upper) and 5% (lower) empirical FDR thresholds of the corresponding test, computed over
337 the autosomes from neutral simulations assuming no linkage. At the 1% empirical FDR
338 threshold, CMH_{x,y,z} and FET_x identify a candidate peak region of 169 (9,042,023-
339 10,670,451bp, 1.628Mbp) and 660 (9,000,008-10,384,933bp, 1.385Mbp) SNPs. The overlap
340 between these two tests is 92 significant SNPs spanning 1.343Mb (see SI Fig. 1. for a close up
341 of this genomic region). In all panels the top 20 SNPs from FET_x and CMH_{y,z} are highlighted
342 in red and in blue.

343

344 **Figure 2** Schematic outline of the experimental design.

345 Three replicated populations of flies starting from the same founders evolved in parallel during
346 70 generations (primary E&R, indicated in blue). The darkness of the flies symbolizes the level
347 of adaptation to the new environment. For a given evolved replicate, the evolved flies are
348 “diluted” with ancestral genotypes and independent replicates evolving for an additional 30
349 generations under the identical environmental conditions as in the primary E&R (secondary
350 E&R, indicated in black). The bottom panel indicates the allele frequency changes of candidate
351 SNPs during the experiments. In the primary E&R the allele frequency increases (blue). By
352 adding ancestral genotypes, the frequency of the candidate SNPs is decreased (black dotted
353 lines). 30 generations of the secondary E&R result in a repeated frequency increase of the
354 candidate SNPs, confirming non-neutral evolution.

355

356 **Figure 3** Allele frequency changes of the 20 most significant SNPs from FET_x (red) and
357 CMH_{y,z} (blue) for the primary E&R (generation F0-F70) and secondary E&R (D0-D30).

358 The left panel shows experiment x and the right panel experiment z. Only first replicate the
359 secondary E&R is shown for each experiment, for the other replicates, see supplement.

360

361 **Figure 4** Repeatability of selection signatures in primary and secondary E&R.

362 A) Selection coefficients are very similar. Symmetrical violin plots of the selection coefficients
363 from primary E&R (dark gray) and the first replicate of the secondary E&R experiment (light

364 gray) for candidates in the region of interest. Black segments represent the median per sample.
365 Ticks indicate SNPs. Left: experiment x. Right: experiment z. The 20 most significant SNPs
366 from FET_x (red) and $CMH_{y,z}$ (blue) are shown in color. B) Parallel changes in allele
367 frequencies. Observed allele frequency changes for candidate SNPs (empirical FDR <1%) in
368 replicate x (left) are shown in gray. For comparison, the expected neutral allele frequency
369 changes based on the same starting frequency and coverage and a single simulation run are
370 shown in orange. The 20 most significant SNPs from FET_x and $CMH_{y,z}$ are shown in red and
371 blue. Since, for replicate z no SNP exceeded the empirical FDR of 1% in the primary E&R,
372 only the top 20 SNPs are shown (right panel). Ellipses around the empirical focal SNPs indicate
373 the 99% probability range to visualize the bivariate densities.

374 TABLE

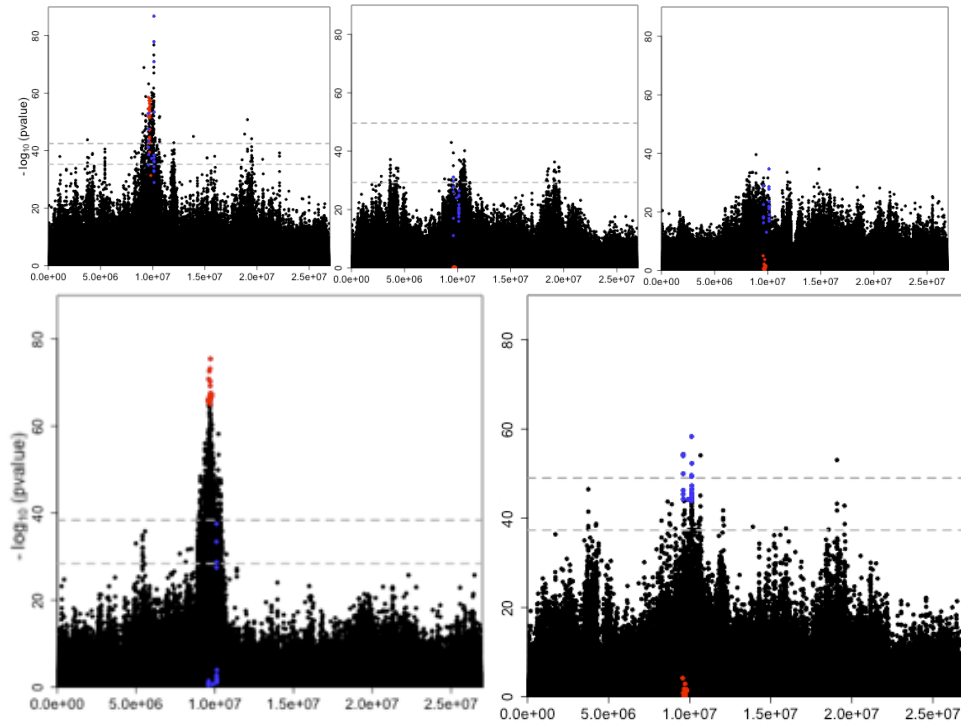
375 **Table 1** Autosomal N_e estimates of the primary and secondary E&R experiments.

	Replicate x	Replicate y	Replicate z
Primary E&R	206	263	226
Secondary E&R	134, 144	-	216, 193, 167

376

377 FIGURES

378

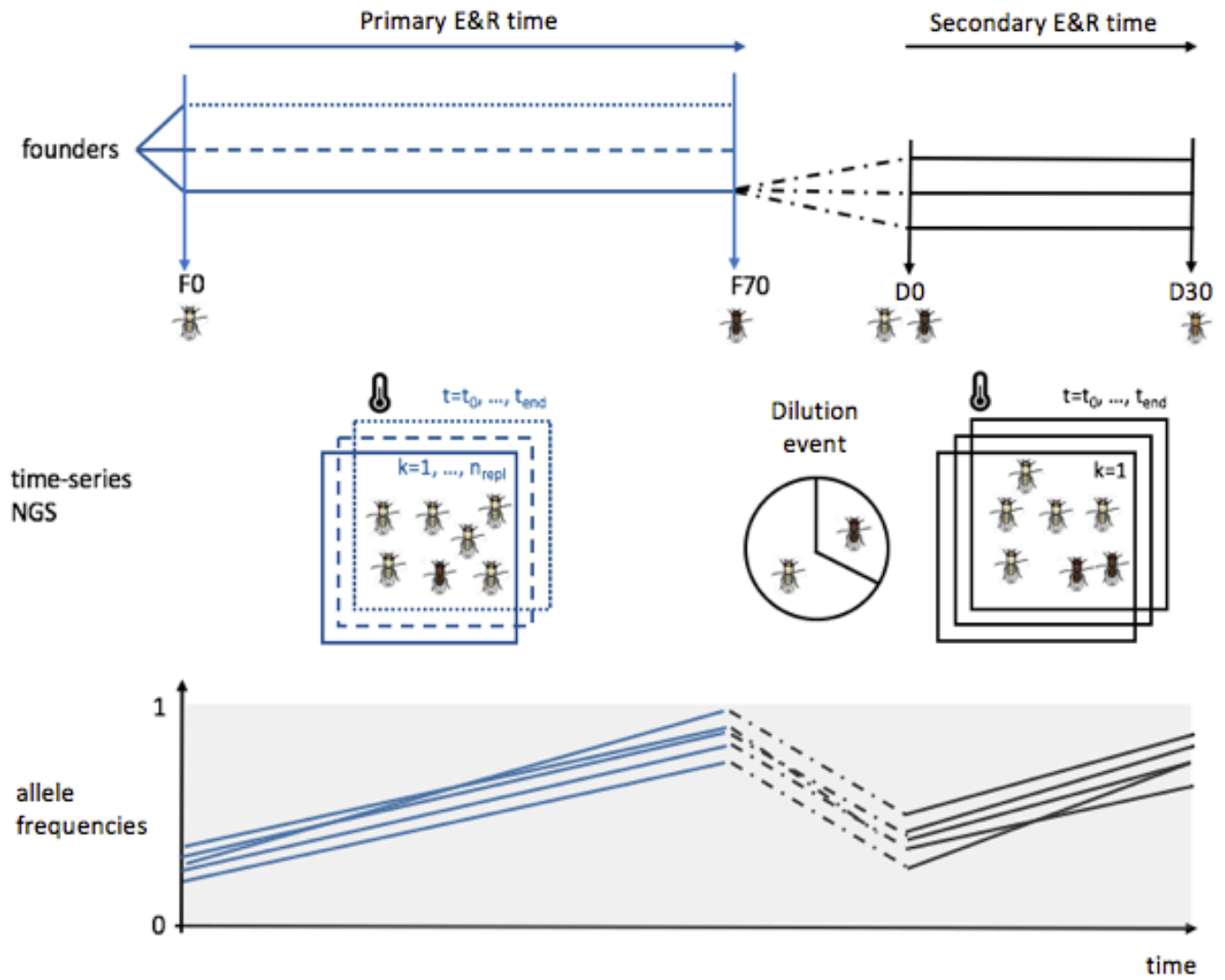


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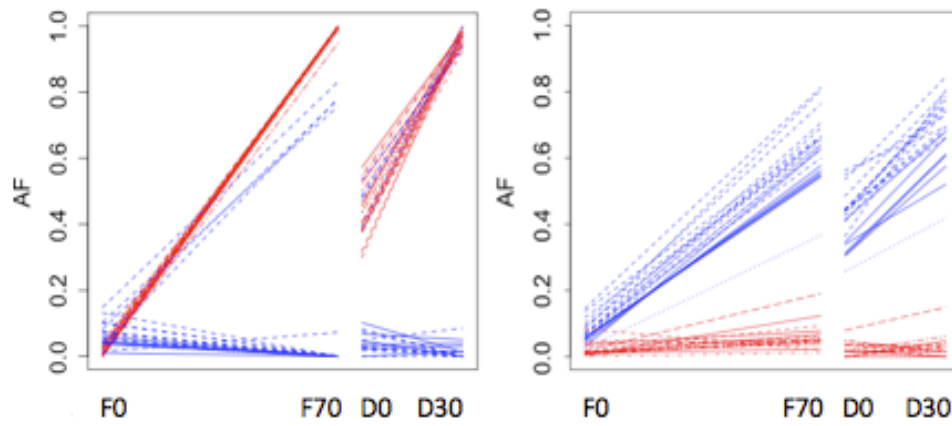
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Figure 1 Replicate-specific selection signatures in the primary E&R study.



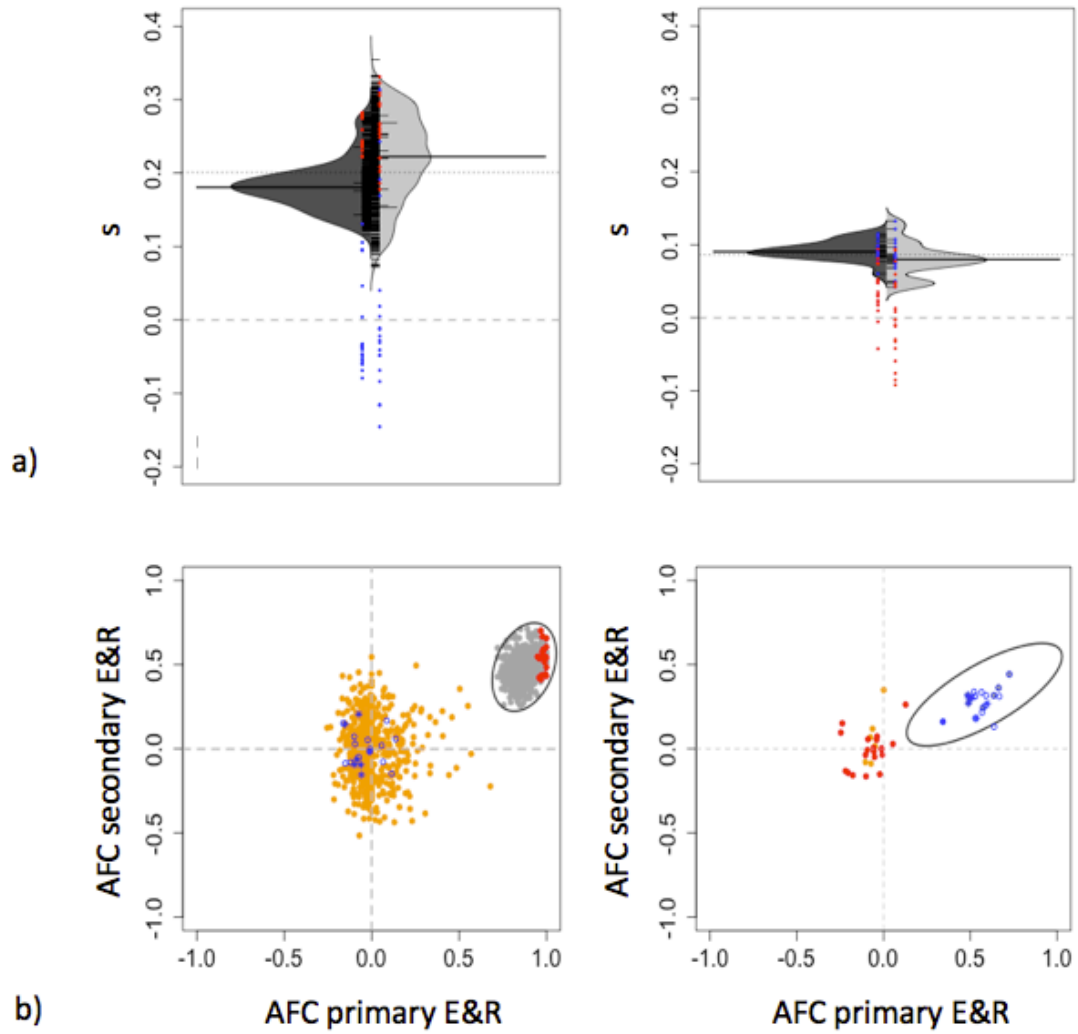
382

383 **Figure 2** Schematic outline of the experimental design.



384

385 **Figure 3** Allele frequency changes of the 20 most significant SNPs from FET_x (red) and
386 CMH_{y,z} (blue) for the primary E&R (generation F0-F70) and secondary E&R (D0-D30).



387

388 **Figure 4** Repeatability of selection signatures in primary and secondary E&R.

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